New Phytologist

Research

Copper speciation and isotopic fractionation in plants: uptake and translocation mechanisms

Brooke M. Ryan[1], Jason K. Kirby[2], Fien Degryse[1], Hugh Harris[3], Mike J. McLaughlin[1,2] and Kathleen Scheiderich[2]

[1]Soil Sciences, University of Adelaide, Adelaide, SA, 5064, Australia; [2]CSIRO Land and Water, Contaminant Chemistry and Ecotoxicology Program, Waite Campus, Adelaide, SA, 5064, Australia; [3]School of Chemistry and Physics, University of Adelaide, Adelaide, SA, 5000, Australia

Author for correspondence:
Brooke Ryan
Tel: +61 8 83036787
Email: brooke.ryan@adelaide.edu.au

Received: *12 December 2012*
Accepted: *12 March 2013*

New Phytologist (2013) **199:** 367–378
doi: 10.1111/nph.12276

Key words: copper, fractionation, isotopes, plant, speciation, spectroscopy, translocation, uptake.

Summary

- The fractionation of stable copper (Cu) isotopes during uptake into plant roots and translocation to shoots can provide information on Cu acquisition mechanisms.
- Isotope fractionation ($^{65}Cu/^{63}Cu$) and intact tissue speciation techniques (X-ray absorption spectroscopy, XAS) were used to examine the uptake, translocation and speciation of Cu in strategy I (tomato–*Solanum lycopersicum*) and strategy II (oat–*Avena sativa*) plant species. Plants were grown in controlled solution cultures, under varied iron (Fe) conditions, to test whether the stimulation of Fe-acquiring mechanisms can affect Cu uptake in plants.
- Isotopically light Cu was preferentially incorporated into tomatoes ($\Delta^{65}Cu_{\text{whole plant-solution}}$ = *c.* $-1‰$), whereas oats showed minimal isotopic fractionation, with no effect of Fe supply in either species. The heavier isotope was preferentially translocated to shoots in tomato, whereas oat plants showed no significant fractionation during translocation. The majority of Cu in the roots and leaves of both species existed as sulfur-coordinated Cu(I) species resembling glutathione/cysteine-rich proteins.
- The presence of isotopically light Cu in tomatoes is attributed to a reductive uptake mechanism, and the isotopic shifts within various tissues are attributed to redox cycling during translocation. The lack of isotopic discrimination in oat plants suggests that Cu uptake and translocation are not redox selective.

Introduction

Copper (Cu) is an essential micronutrient for plant growth, playing a key role in the function of many proteins. Cu is involved in electron transport during photosynthesis, lignin formation and cell wall metabolism (Burkhead *et al.*, 2009). Cu-deficient plants (< 5 mg kg^{-1} in vegetative tissue) exhibit reduced growth and development, with reproductive organs and youngest leaves displaying the most severe symptoms (Burkhead *et al.*, 2009; Broadley *et al.*, 2012). At above optimal concentrations (usually > 10 μM in solution), Cu can act as a toxin to plants, causing nutrient loss and oxidative stress (Martins & Mourato, 2006; Reichman *et al.*, 2006). Under toxic conditions, damaging hydroxyl radicals are formed that attack the cell structure, and inhibit photosynthesis (Fernandes & Henriques, 1991; Yruela, 2009).

The mechanisms of Cu mobilization and uptake by roots from soil solutions remain unclear. Cu in soils is strongly associated with organic matter, as well as iron (Fe) and aluminium (Al) oxides (Flemming & Trevors, 1989). Plants have developed two uptake mechanisms for the acquisition of highly insoluble Fe(III) in soils: strategy I plants (dicotyledons and nongraminaceous monocotyledons) mobilize Fe through acidification of the rhizosphere, causing the dissolution of Fe/Al oxides, and the upregulation of Fe reductases, which reduce Fe(III) to Fe(II), whereas strategy II plants (graminaceous monocotyledons) can complex Fe(III) through the release of high-affinity Fe(III) root exudates, phytosiderophores (Guerinot & Yi, 1994; Marschner & Romheld, 1994). These Fe reduction and complexation mechanisms have been shown to affect Cu speciation in controlled laboratory environments. For instance, ferric reductase oxidase 2 (FRO2) is capable of reducing Cu(II) and FRO3 is upregulated during Cu deficiency (Robinson *et al.*, 1999; Burkhead *et al.*, 2009; Palmer & Guerinot, 2009; Bernal *et al.*, 2012). In addition, phytosiderophores, or similar exudates, are capable of Cu complexation (Welch *et al.*, 1993; Georgatsou *et al.*, 1997; Schmidt *et al.*, 1997; Yruela, 2009). The importance of these mechanisms in Cu mobilization and uptake in the soil environment remains unclear. Although no studies have confirmed the mobilization and uptake mechanism of Cu, there appears to be a strong overlap between Fe and Cu uptake, with Cu shown to competitively inhibit Fe uptake (Schmidt *et al.*, 1997; Michaud *et al.*, 2008; Yruela, 2009).

Stable isotope fractionation can be an effective method of monitoring Cu biogeochemical cycling, in particular, nutrient uptake. Bigalke *et al.* (2010a) found that organic top soil horizons of three hydromorphic soils were isotopically enriched in light Cu, relative to mineral fractions, suggesting a biologically induced enrichment from soil–plant Cu cycling. A study of two

soil-grown monocots (rye, *Elymus virginicus*, and hairy leaved sedge, *Carex hirsutella*) found preferential incorporation of the light Cu isotope into vegetative tissues, relative to the soil, of between −0.94‰ and −0.33‰ (Weinstein *et al.*, 2011). Similarly, a recent study of hydroponically grown tomato and wheat plants showed enrichment of the light Cu isotope in roots relative to the growth medium ($\Delta^{65}Cu_{root\text{-}solution}$) of between −0.84‰ and −0.47‰ for strategy I species and −0.48‰ and −0.11‰ for strategy II species (Jouvin *et al.*, 2012). The authors attributed this isotopic shift to the reduction of Cu(II) to Cu(I) before uptake into the root symplasm. Their study used chelator-buffered solutions which complicate the interpretation of isotope data, as fractionation during Cu-chelate dissociation and uptake of chelated Cu through the apoplastic pathway need to be considered. A study in which the speciation of Cu provided to the plant is held constant is required to gain a more complete understanding of the mechanisms involved during uptake and translocation.

Although the magnitude and direction of isotope fractionation will be influenced by the binding environment and redox state of Cu, it is not possible to confirm the speciation and bonding environment without other techniques. X-Ray absorption spectroscopy (XAS) provides an *in situ*, nondestructive method of assessing Cu speciation in plant tissues. Previous work has found that Cu exists as both Cu(I) and Cu(II) in the vegetative tissue of *Larrea tridentate* (creosote bush), despite the fact that Cu absorbed from the soil is Cu(II), indicating redox changes within the plant or during uptake (Polette *et al.*, 2000). The Cu accumulator *Crassula helmsii* was found to bind Cu almost exclusively through oxygen (O)-rich ligands (Kuepper *et al.*, 2009), whereas *Thlaspi caerulescens*, a hyperaccumulator for Cd and Zn but not Cu, stored excess Cu with strong ligands rich in sulfur (S), possibly metallothioneins (Mijovilovich *et al.*, 2009).

The aim of this study was to use highly precise isotope fractionation and intact tissue speciation techniques to examine the uptake and translocation of Cu in plants. Tomato (*Solanum lycopersicum*), a strategy I plant species, and oat (*Avena sativa*), a strategy II species, were grown hydroponically for 30 d, with an Fe deficiency treatment imposed during the last week of growth, to test the hypothesis that the stimulation of Fe-acquiring mechanisms can affect Cu uptake and translocation in plants. The speciation of Cu in the nutrient solution was closely controlled without using chelator buffering, so that Cu(II) activities in Fe-sufficient and Fe-deficient nutrient solutions were similar and isotope signatures in plants were not affected by isotopic fractionation in the solution or the possible uptake of intact Cu chelates from the hydroponic solutions.

Materials and Methods

Hydroponic plant growth

Six tomato (*Solanum lycopersicum* L.) and six oat (*Avena sativa* L.) plants were grown for a total of 30 d under controlled conditions in a nutritionally complete solution at pH 4.5. To maintain Fe in a soluble form for plant growth, Fe-specific chelators were used: *N,N′*-bis(2-hydroxybenzyl)ethylenediamine-*N,N′*-dipropionic acid (HBED) (12 μM) for tomatoes and ethylenediamine-*N,N′*-bis (2-hydroxyphenylacetic acid) (EDDHA) (12 μM) for oats. The EDDHA chelate was used for oats, given the inefficiency of strategy II plants to extract Fe from the HBED complex (Parker & Norvell, 1999). These chelates were selected on the basis of GEOCHEM modelling of nutrient solutions at pH 4.5, as they yielded the highest possible 'free' Cu(II) concentrations, with HBED having no Cu complexation and EDDHA only complexing 6% of the total Cu (Supporting Information Table S1 for GEOCHEM modelling). The Cu concentration in the solution was 1 μM and the nutrient solution was kept at pH 4.5 in order to avoid significant complexation, maintaining > 85% of Cu in solution as 'free' Cu^{2+} (Table S1); this was confirmed using a Cu ion-selective electrode. The Cu concentration selected for this hydroponic study was chosen to enable sufficient concentrations in plant tissues to allow for accurate and precise isotope ratio and speciation analysis. The maintenance of all Cu as free Cu^{2+} at lower concentrations would have been extremely difficult. On day 24, Fe deficiency was induced in three replicates from each species by placing the plants in a nutrient solution that contained no Fe or chelator, but was otherwise nutritionally identical. Nutrient solutions were changed every 4 d for the first week, every 2 d for the second week and daily for the final 2 wk of plant growth, to avoid significant Cu isotope fractionation as a result of the build up of Cu-complexing exudates. This allowed the assumption of a constant Cu isotope ratio. The frequency of solution changes in conjunction with pH buffering from 2-(*N*-morpholino)ethanesulfonate (MES; 2.5 mM) prevented pH changes in solution (Methods S1).

All plants were harvested on day 30 and separated into roots, stems and leaves. The leaves for Fe-deficient treatments were further separated into green and chlorotic leaves. The roots were washed to remove any apoplastically bound Cu by submerging in ice-cold ultrapure deionized water (Millipore) for 5 min followed by 1 mM $LaCl_3$ and 0.05 mM $CaCl_2$ for 5 min (Weiss *et al.*, 2005). Plant tissues (roots, stems and leaves) were frozen and freeze–dried for 7 d to a constant mass (ModulyoD; Thermo Scientific, Waltham, MA, USA). The plant tissues were homogenized using stainless steel scissors.

Digestion of plant tissues

Approximately 0.05–0.1 g of the freeze–dried plant tissues (roots, stems, leaves) were cold digested in 3 ml of nitric acid (HNO_3) and 1 ml of hydrogen peroxide (H_2O_2) in polytetrafluoroethylene (PTFE) vials, followed by a 2-h hotplate reflux at 140°C. Samples were then evaporated to dryness and redissolved in 7 ml HNO_3 and 3 ml H_2O_2, and transferred to closed PTFE vessels. The samples were microwave digested in sealed vessels (Ethos E; Milestone, Shelton, CT, USA; Methods S1). The digest extracts were then dried and redissolved in 7 M HCl for Cu purification, and Cu, macro- and micronutrients were measured on inductively coupled plasma mass spectrometry (ICP-MS) or ICP-optical emission spectroscopy (ICP-OES; Table S4).

The accuracy of the digestion and analysis procedures for the determination of total Cu concentrations in plants was assessed using National Institute of Standards and Technology (NIST)

1573a tomato leaves standard reference material. The total Cu concentrations determined in the tomato leaves standard reference material was in close agreement with the certified value (4.6 ± 0.2 mg kg^{-1} (mean $\pm$ SD) compared with a reference value of 4.7 ± 0.1 mg kg^{-1}; Table S2).

Cu purification

Cu for isotope analysis was purified using AG-MP-1 anion exchange resin (BioRad), following the procedure described by Marechal *et al.* (1999) (Table S3). High concentrations of matrix elements in the plant tissues required the column purification to be run twice. For stem and leaf tissues, a third column purification was needed to ensure that alkali metals were below the instrumental detection limits. The column purification procedure was optimized using NIST 1573a tomato leaves (Fig. S1, Methods S1). Digest and column purified Cu extracts were checked for Cu recoveries, and samples with $100 \pm 8\%$ were found to show no significant difference in δ^{65}Cu values.

The Cu fraction from the columns was collected in PTFE vials and evaporated to dryness on a hotplate. The samples were resuspended in 0.5 ml HNO_3 and refluxed for 2 h at 140°C to digest any organic compounds that may have been released from the column (Bigalke *et al.*, 2010a,b), before being evaporated to dryness again. Samples were redissolved in 2% HNO_3, and 0.1-ml aliquots of the sample solutions were taken for total Cu analysis to determine Cu recoveries from the column purification procedure.

Cu isotope measurements

Cu isotope ratios (^{65}Cu/^{63}Cu) of purified samples were determined using a multicollector-inductively coupled plasma-mass spectrometer (MC-ICP-MS; Thermo Scientific). The Cu isotope ratios were expressed as δ^{65}Cu values relative to NIST 976 Cu isotopic reference material according to Eqn 1:

$$\delta^{65}\mathrm{Cu} = 1000 \cdot \left[\frac{(^{65}\mathrm{Cu}/^{63}\mathrm{Cu})_{\mathrm{sample}}}{(^{65}\mathrm{Cu}/^{63}\mathrm{Cu})_{\mathrm{NIST976}}} - 1 \right] \quad \text{Eqn 1}$$

Cu isotope ratios were measured by methods outlined previously by Ehrlich *et al.* (2004) and Bigalke *et al.* (2010a,b), with internal mass bias correction with NIST 986 Ni, and are briefly described in Methods S1. The precision of each individual sample is constrained by multiple analyses of each sample, and reported as twice the standard deviation in Table 1. In addition, long-term external reproducibility for the entire method (i.e. digestion, purification and isotope analysis) is defined by the processing and analysis of NIST 1573a tomato leaves ($n = 5$), with each replicate sample measured at least three times. This standard has $\delta^{65}\mathrm{Cu} = 0.63 \pm 0.16‰$ (mean $\pm$ 2SD). The error afforded to each sample as a result of sample preparation is therefore assumed not to exceed $\pm 0.16‰$. Treatments have their own variations, reported in Table 1 as 2SD, generated from the inherent variability between replicate plants.

The samples and standards were measured in 2% HNO_3 at concentrations of 300 µg Cu l^{-1} (^{63}Cu = *c.* 7–10 V) and 1000 µg Ni l^{-1} (^{60}Ni = *c.* 10–12 V) in low-resolution mode. The samples were analysed using wet plasma conditions with a 100-µl PFA nebulizer and platinum sampler and skimmer cones.

All measured δ^{65}Cu values were normalized to the δ^{65}Cu value for the nutrient solution ($\Delta^{65}\mathrm{Cu}_{\mathrm{tissue\text{-}solution}}$) by subtracting the δ^{65}Cu value of the solution Cu (+0.25‰). Similarly, fractionation during Cu translocation, which was simplified to include only a

Table 1 Biomass (DW), copper (Cu) concentrations and isotopic measurements for tomato (*Solanum lycopersicum*) and oat (*Avena sativa*) plants grown in iron (Fe)-sufficient (+Fe) and Fe-deficient (−Fe) hydroponic conditions

Species	Treatment	Tissue	*n*	Biomass (DW) (g)	SD	Cu (mg kg^{-1})	SD	$\Delta^{65}\mathrm{Cu}_{\mathrm{tissue\text{-}solution}}$	2SD
Tomato	+Fe	Root	3	0.054	0.001	448	56	−1.43	0.17
		Stem	3	0.088	0.019	15	1.3	−0.08	0.33
		Leaves	3	0.214	0.030	60	14	−0.45	0.25
	−Fe	Root	3	0.061	0.010	766	170	−1.09	0.29
		Stem	3	0.086	0.037	14	1.5	−0.22	0.62
		Green leaves	3	0.098	0.035	50	11	−0.56	0.51
		Chlorotic leaves	3	0.053	0.014	32	8.3	−0.26	0.71
Oat	+Fe	Root	3	0.089	0.024	611	80	−0.20	0.12
		Stem	3	0.094	0.034	15	4.4	−0.20	0.11
		Leaves	3	0.163	0.030	17	2.6	−0.26	0.23
	−Fe	Root	3	0.085	0.022	1093	189	−0.12	0.02
		Stem	3	0.092	0.028	13	1.5	0.00	0.44
		Green leaves	3	0.068	0.069	21	2.2	0.00	0.13
		Chlorotic leaves	3	0.070	0.016	20	2.5	−0.27	0.20

Sample	*n*	Cu (mg kg^{-1})	SD	δ^{65}Cu	2SD
NIST 1573a tomato leaves	5	4.6	0.23	0.63	0.16
Nutrient solution	4			0.25	0.04

Data for National Institute of Standards and Technology (NIST) 1573a tomato leaf samples used for method development, as well as δ^{65}Cu value for hydroponic nutrient solution, are included in this table.

Cu-supplying tissue (or solution) (a) and a Cu-receiving tissue (b), is defined as $\Delta^{65}Cu_{b\text{-}a}$ (‰) according to the following equation:

$$\Delta^{65}Cu_{b-a} = \delta^{65}Cu_b - \delta^{65}Cu_a \quad \text{Eqn 2}$$

The whole-plant $\Delta^{65}Cu_{\text{whole plant-solution}}$ (‰) values were calculated from a mass balance equation (Eqn 3) to determine the uptake-induced fractionation:

$$\Delta^{65}Cu_{\text{whole plant}-\text{solution}} = \left(\sum_i F_i \times \delta^{65}Cu_i\right) - \delta^{65}Cu_{\text{nutrient solution}} \quad \text{Eqn 3}$$

(F_i, fraction of Cu in a given tissue i (e.g. root, stem or leaves); $\delta^{65}Cu_i$ (‰), isotope ratio of Cu in tissue i).

Similarly, to examine the fractionation between roots and shoots ($\Delta^{65}Cu_{shoot-root} = \delta^{65}Cu_{shoot} - \delta^{65}Cu_{root}$), a mass balance calculation was used to determine the $\delta^{65}Cu$ of shoots (‰) (i.e. total of stems and leaves), from the Cu amount (mg), calculated from the Cu concentration and total dry weight, and the $\delta^{65}Cu$ (‰) value of stems and leaves:

$$\delta^{65}Cu_{shoots} = \left[\frac{\delta^{65}Cu_{stem}\cdot Cu_{stem} + \delta^{65}Cu_{leaf}\cdot Cu_{leaf}}{Cu_{stem} + Cu_{leaf}}\right] \quad \text{Eqn 4}$$

X-Ray absorption spectroscopy

Cu K-edge XAS of frozen roots and leaves of replicate Fe-sufficient and Fe-deficient tomato and oat plants were recorded on the XAS beamline at the Australian Synchrotron (AS), Melbourne, Australia. Extended X-ray absorption fine structure (EXAFS) scans were run on one root tissue replicate from each of the Fe-sufficient and Fe-deficient oat plants, but only on the Fe-deficient tomato plant roots, because of the Cu concentration limitations in the Fe-sufficient roots. Only X-ray absorption near-edge structure (XANES) scans were run on the corresponding leaf tissues, given the significantly lower Cu concentrations. The Cu concentrations in stem tissues were below the detection limit of the beamline, precluding the collection of XAS data.

The X-ray beam was monochromated by diffraction from a pair of Si(111) crystals. Plant tissues were cut to *c.* $1 \times 1\ cm^2$ in size, secured between Kapton tape and stored in a −70°C freezer and cooled to *c.* 10 K in a Cryo Industries (Manchester, NH, USA) cryostat for analysis. Spectra were recorded in fluorescence mode on a 100-pixel Ge detector array at 90° to the incident beam, with Ni absorption filters and Soller slits placed between the sample and fluorescence detector to improve the fluorescence to background ratio. The energy ranges used for XANES data collection were as follows: pre-edge region, 8779–8959 eV (10-eV steps); XANES region, 8959–9029 eV (0.25-eV steps); post-edge region, 9029–9167 eV (0.2-$Å^{-1}$ steps in k space). EXAFS spectra for roots were collected at the following energy ranges: pre-edge region, 8779–8959 eV (10-eV steps); XANES region, 8959–9029 eV (0.25-eV steps); EXAFS region, 9029–9623 eV (0.035-$Å^{-1}$ steps in k space to 13 $Å^{-1}$). A Cu foil standard was recorded simultaneously in transmission mode downstream of the sample to calibrate the energy scale to the first peak of the first derivative of the Cu edge (8980.3 eV).

Data reduction and analysis, including calibration, averaging and background subtraction of all spectra and principal component analysis (PCA), target transformation and linear regression analyses of XANES spectra, were performed using the EXAFSPAK software package (G. N. George, Stanford Synchrotron Radiation Lightsource, Menlo Park, CA, USA). Linear combination fits of XANES spectra were performed over the region 8950–9070 eV. Spectra of model Cu compounds for target and linear regression analyses were collected during the same beamtime, employing the same conditions as outlined above (Fig. S2; additional information on XAS analysis and model Cu compounds can be found in Methods S2).

Statistical analysis

Cu concentration and isotope data were analysed using ANOVA (GenStat, 14th edn; VSN International Ltd, Hemel Hempstead, UK) to determine significant differences between treatments (i.e. Fe-sufficient and Fe-deficient treatments), plant species and plant tissues (roots, stems, leaves). A 5% level of significance was used to compare treatment means.

Results

Plant biomass

Total concentrations of micro- and macro-elements in tomato and oat plants were found to be above critical deficiency concentrations suggested to limit plant growth and survival (Huett *et al.*, 1997; Reuter *et al.*, 1997). Fe deficiency in this study was visually observed in Fe-deficient treatments as chlorosis in leaves and measured by ICP-OES. Deficiency of Fe was found to have no significant effect on the dry biomass of roots, stems or leaves for either test species (Tables 1, S4).

Cu concentrations in roots of Fe-sufficient tomatoes ($448 \pm 56\ mg\ kg^{-1}$) were significantly lower than those in Fe-deficient roots ($766 \pm 170\ mg\ kg^{-1}$; Table 1). Similarly, oat plants showed significantly less Cu in Fe-sufficient roots ($611 \pm 80\ mg\ kg^{-1}$) than in Fe-deficient roots ($1093 \pm 189\ mg\ kg^{-1}$; Table 1). Increased Cu uptake in Fe-deficient plants had no observable effect on root growth (visually and root dry weight).

The absence of a significant difference in total Cu concentrations in stem and leaves between Fe treatments of tomato and oat plants, despite the very different root Cu concentrations (Table 1), aligns with the known regulation of Cu translocation between roots and shoots (e.g. Alaoui-Sossé *et al.*, 2004; Chen *et al.*, 2011). Concentrations in the range 2–50 $mg\ kg^{-1}$ (DW) are considered to be sufficient for tomatoes (Mills & Jones, 1996). The Cu concentrations in tomato leaves of both treatments were slightly above this range (Table 1); however, no visible signs of toxicity were observed.

Cu isotope fractionation during uptake

The whole-plant δ^{65}Cu for tomatoes and oats indicated a preferential incorporation of the light isotope into the plants from solution, but the magnitude of enrichment was much greater for tomatoes than for oats (Fig. 1). There was no significant difference between Fe treatments for tomatoes, with $\Delta^{65}Cu_{\text{whole plant-solution}}$ values of $-1.05 \pm 0.36‰$ and $-0.99 \pm 0.37‰$ for Fe-sufficient and Fe-deficient plants, respectively (Fig. 1). Such a large negative fractionation indicates the presence of a dominant reductive uptake mechanism, that is, the reduction of free Cu(II) to Cu(I) during uptake into the symplasm, as Cu reduction is known to induce such significant light Cu isotopic enrichment (Criss, 1999; Zhu *et al.*, 2002; Kavner *et al.*, 2008; Jouvin *et al.*, 2012).

Oat plants showed a small enrichment in the light Cu isotope during uptake and no significant difference between Fe treatments, with $\Delta^{65}Cu_{\text{whole plant-solution}}$ values of $-0.20 \pm 0.11‰$ and $-0.11 \pm 0.03‰$ for Fe-sufficient and Fe-deficient plants, respectively (Fig. 1). This fractionation is too small to imply a dominant reductive uptake mechanism, suggesting that another uptake mechanism is at play. Cu-phytosiderophore complexation and uptake may have contributed to uptake, which may be expected to induce slight heavy Cu isotope enrichment in the plant, given the small preference for heavy isotopes in organic complexes (Guelke & Von Blanckenburg, 2007; Bigalke *et al.*, 2010a,b).

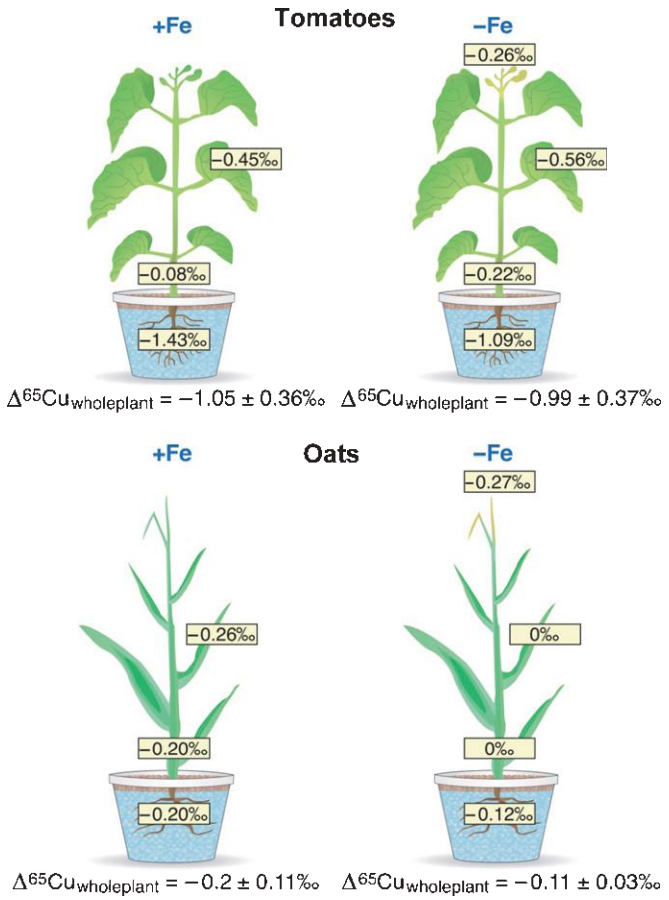

Fig. 1 Average $\Delta^{65}Cu_{\text{(tissue-nutrient solution)}}$ (mean ± 2SD, $n = 3$) of roots, stems and leaves for tomato (*Solanum lycopersicum*) and oat (*Avena sativa*) plants after 30 d of growth in either iron (Fe)-sufficient (+Fe) or Fe-deficient (−Fe) nutrient solutions.

Cu isotope fractionation during translocation

Tomato plants showed a large fractionation of Cu isotopes during translocation between different tissues, whereas oat plants maintained a fairly constant Cu isotope signature throughout all tissues (Fig. 1).

The δ^{65}Cu value of the whole plant results from the fractionation during uptake. δ^{65}Cu in plant tissues depends on both δ^{65}Cu of the whole plant and the isotopic fractionation during translocation. For instance, preferential translocation of a heavier isotope from a plant tissue will cause the Cu remaining in that tissue to be enriched with the light isotope. An overview of isotopic composition within the whole plant and the different tissues is given in Table 2.

For tomato, the value of $\Delta^{65}Cu_{\text{whole plant-solution}}$ is *c.* $-1.0‰$, suggesting a reductive uptake mechanism, as discussed above. The Cu translocated to shoots is more enriched in the heavy isotope, whereas Cu retained in roots is more enriched in the light isotope, compared with the whole plant (Table 2), indicating preferential translocation of the heavy Cu isotope to above-ground tissues from roots. Thus, the root will become more enriched in the light isotope as more Cu is translocated to the shoot (Rayleigh fractionation, see Notes S1). This agrees with the more negative $\Delta^{65}Cu_{\text{root-solution}}$ for the Fe-sufficient than for the Fe-deficient plants, as relatively more Cu is translocated to shoots in the Fe-sufficient plants (Notes S1). These data point towards a conserved, regulated mechanism of root to shoot Cu transport, which leads to a positive delta shift that is consistent across both Fe-sufficient and Fe-deficient treatments. The distribution of Cu in shoots between stems and leaves leads to heavier Cu isotope enrichment in the stems of both treatments, suggesting a preference for the light Cu isotope to be translocated to the leaves. The values of $\Delta^{65}Cu_{\text{leaves-solution}}$ and $\Delta^{65}Cu_{\text{shoot-solution}}$ are similar, because 91% and 84% of vegetative tissue Cu exists in the leaves in the Fe-sufficient and Fe-deficient plants, respectively. In contrast with the nonchlorotic leaves, the chlorotic leaves showed similar isotopic composition to the stems ($\Delta^{65}Cu_{\text{chlorotic leaves-stem}} = -0.04‰$; Table 2). The large Cu isotope fractionations between plant tissues throughout the tomato plants of both treatments suggest that redox-selective mechanisms are occurring during translocation.

The oats showed a constant Cu isotope signature (within error) throughout all tissues in the Fe-sufficient and Fe-deficient treatments, with the exception of the chlorotic leaves of the Fe-deficient treatment ($\Delta^{65}Cu_{\text{chlorotic leaves}} - \Delta^{65}Cu_{\text{stem}} = -0.27‰$; Table 2). The lack of any significant Cu isotope fractionation during translocation within the oat plants indicates that redox-selective transport is not a dominant translocation mechanism for Cu in this plant species.

New Phytologist

Table 2 Schematization of copper (Cu) isotope fractionation as Cu is translocated from roots to shoots, and then between stem and leaves, for iron (Fe)-sufficient (+Fe) and Fe-deficient (−Fe) conditions in tomato (*Solanum lycopersicum*) and oat (*Avena sativa*)

			Root to shoot translocation			Stem to leaf translocation		
Species	Treatment	$\Delta^{65}Cu_{whole\ plant\text{-}solution}$ (‰)	Tissue	*F*	$\Delta^{65}Cu_{tissue\text{-}solution}$ (‰)	Tissue	*F*	$\Delta^{65}Cu_{tissue\text{-}solution}$ (‰)
Tomato	+Fe	*−1.05*	Root	0.63	−1.43			
			Shoot	*0.37*	*−0.40*	Stem	0.03	−0.08
						Leaves	0.34	−0.45
	−Fe	*−0.99*	Root	0.86	−1.09			
			Shoot	*0.14*	*−0.43*	Stem	0.02	−0.22
						Green leaves	0.09	−0.56
						Chlorotic leaves	0.03	−0.26
Oat	+Fe	*−0.20*	Root	0.93	−0.20			
			Shoot	*0.07*	*−0.24*	Stem	0.02	−0.20
						Leaves	0.05	−0.26
	−Fe	*−0.11*	Root	0.96	−0.12			
			Shoot	*0.04*	*−0.08*	Stem	0.01	0.00
						Green leaves	0.03	0.00
						Chlorotic leaves	0.01	−0.27

The $\Delta^{65}Cu$ values are reported normalized to the nutrient solution value, and *F* is the fraction of total plant Cu contained within the given tissue. Mass balance calculated values shown in italics.

XAS analysis

The Cu K-edge XANES spectra of root and leaf samples of both species showed that Cu speciation was dominated by Cu(I), as identified by the low rising edge inflection energy of Cu(I) species at *c.* 8984 eV (Kau *et al.*, 1987; Fig. 2) and linear combination fitting of model compounds using EXAFSPAK (Fig. 3a,b; Table S5). PCA indicated that the samples had three primary Cu species present and, as such, the three model compounds with the lowest target transformation residuals were fitted to the sample spectra: Cu(I)-glutathione (GSH), Cu(I)-cysteine and Cu(II)-histidine (Figs S3, S4).

XANES fitting indicated that Fe-deficient tomato roots contained 86% Cu(I), whereas Fe-sufficient tomato roots contained 58% Cu(I) (Fig. 3a, Table S5). However, in the tomato plants grown in Fe-sufficient solution, the three model compounds accounted for only 85–88% of the spectral fit for roots and leaves, suggesting that a fourth, unidentified component may have been present (Table S5). Although having significantly different total Cu concentrations, tomato roots showed very similar Cu(II) concentrations of 120 and 108 mg kg^{-1} for Fe-sufficient and Fe-deficient plants, respectively (Fig. 3a), suggesting a highly controlled re-oxidation of the stored Cu(I) to Cu(II). Cu(I) dominated the speciation of tomato leaves in both treatments. Oat roots and leaves showed similar Cu speciation in both treatments, with the speciation strongly dominated by Cu(I) species (Fig. 3a, b). It should be noted that differentiating between Cu(I) and Cu(II) species in photosynthetic tissues/proteins can be difficult (Lee *et al.*, 2009) and, as such, the reported Cu(I) and Cu(II) proportions in the leaves should be taken as approximate.

EXAFS fitting of the tomato and oat root samples indicated that Cu was bound by S-donor ligands in a three-coordinate complex (e.g. Fig. 5; explaining why so much of the XANES data can be fitted with Cu(I)-GSH; Table S5). The fitting error in the EXAFS spectra was reduced significantly by including a Cu–Cu interaction at 2.66–2.71 Å (Tables 3, S6). This indicates that Cu in root tissues is being stored in small Cu clusters bridged by S ligands. The EXAFS spectra and corresponding Fourier transform plots of the observed and fitted data can be seen in Figs 5 and S5–S7. The most realistic Cu coordination number in the S-bridged cluster is three, forming a trigonal S-bridged poly-Cu cluster, as identified for the transcription factor proteins Mac1 and Ace1 (Brown *et al.*, 2002). However, the best fit of the EXAFS data varies with each sample from 2.5 to 3.5 S atoms (Table 3). This is probably the result of secondary Cu···Cu interaction which sits out of phase to the Cu···Cu interaction occurring at *c.* 2.7 Å (Brown *et al.* (2002). The speciation observed in these plants may have been quite different if plants had been grown at a lower free Cu concentration, as lower tissue Cu concentrations would see a decrease in excess root Cu, and hence less requirement for detoxification with metallothionein-type proteins.

Discussion

Tomatoes (strategy I)

Cu uptake A large enrichment of the light Cu isotope, relative to the growth medium, is seen in both Fe-sufficient and Fe-deficient tomato plants (Fig. 1). The uptake of Cu into tomato plants induces a fractionation of *c.* −1.00‰ in both treatments, in line with previously observed biologically induced Cu(II) to Cu(I) reduction during uptake ($\Delta^{65}Cu_{bacteria\text{-}solution}$ = −1.2‰ to −4.4‰; Navarrete *et al.*, 2011).

Jouvin *et al.* (2012) also found light Cu isotope enrichment in the roots of hydroponically grown strategy I plants (−0.84‰ to −0.63‰). These values were $\Delta^{65}Cu_{root\text{-}solution}$ values, for unwashed roots, relative to solution, and probably contained apoplastically sorbed, isotopically heavy Cu. Adsorbed, heavy Cu would have masked some of the light isotope enrichment present in the root symplasm, perhaps explaining why the light isotope

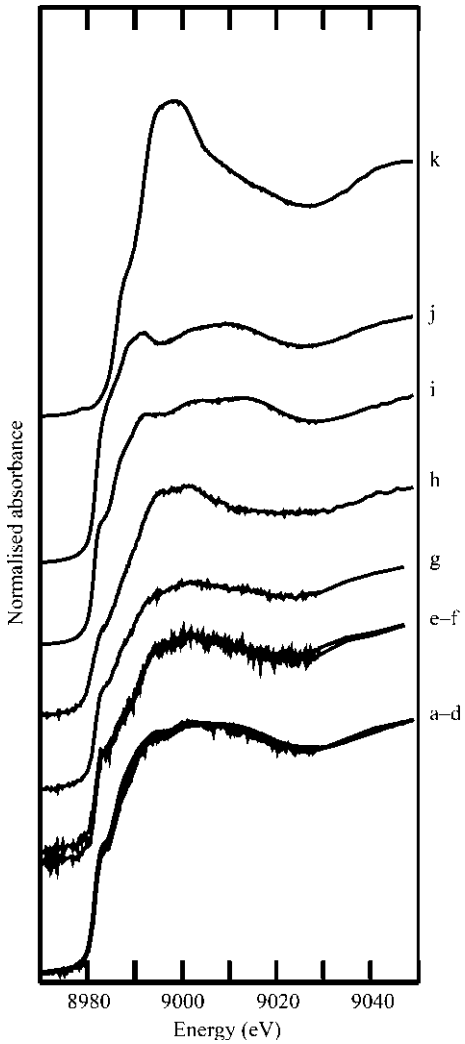

Fig. 2 X-Ray absorption near-edge structure (XANES) spectra of root and leaf samples of iron (Fe)-sufficient (+Fe) and Fe-deficient (−Fe) tomato (*Solanum lycopersicum*) and oat (*Avena sativa*) plants, with standard spectra used for linear combination fitting. Similar spectra have been grouped together as follows: tomato −Fe roots, tomato −Fe leaves, oat +Fe roots and oat −Fe roots (a–d); oat +Fe leaves and oat −Fe leaves (e–f); tomato +Fe roots (g); tomato +Fe leaves (h); Cu(I)-GSH (i); Cu(I)-cysteine (j); Cu(II)-histidine (k).

enrichment found by Jouvin *et al.* (2012) was less than that observed in this study. Moreover, Jouvin *et al.* (2012) used chelate buffering with nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA) or *N*-hydroxyethylenediaminetriacetic acid (HEDTA) as Cu-complexing ligands. There may have been direct, passive uptake of the Cu complexes from solution (Iwasaki & Takahashi, 1989; Laurie *et al.*, 1991; Collins *et al.*, 2002), which might also explain the less negative fractionation found by Jouvin *et al.* (2012). Regardless of these experimental differences, the large light Cu isotope enrichment in both studies is evident, and suggests that Cu(II) in hydroponic solutions is reduced to Cu(I) at the root membrane before it is taken up.

Reductive uptake of Cu in strategy I plants is reasonable, given the prevalence of the high-affinity Cu transporter protein (COPT) family in roots, which is a selective Cu(I) transporter (Sancenon *et al.*, 2003; Yruela, 2009). It is likely that Cu is being reduced by the Fe reductases FRO2 and FRO3, previously reported to be capable of reducing Cu(II) (Bernal *et al.*, 2012). The upregulation of Fe reductases may explain the significantly higher total Cu concentration in tomato roots in the Fe-deficient treatment (Fig. 3a), because of increased Cu reduction and uptake via COPT transporters.

The Cu EXAFS data for Fe-deficient roots indicate that the majority of Cu is being stored in small S-bridged Cu clusters (Fig. 5). These observed structures are probably reflections of strong metal complexes, such as metallothioneins and phytochelatins, both rich in cysteine, and glutathione as a cofactor, which are upregulated under excess Cu to buffer cell Cu concentrations (Cobbett & Goldsbrough, 2002). The root Cu EXAFS data in Fig. 5 (and Figs S5, S6) appear to be analogous to those presented for Cu-phytochelatin complexes by Polette *et al.* (2000), including the Cu–Cu interaction at 2.7 Å. The increase in Cu concentrations in roots coincides with an increase from 0% Cu (I)-cysteine species in Fe-sufficient plants to 20% in Fe-deficient plants (Fig. 3a, Table S5). This may indicate the stimulation of metallothionein/phytochelatin synthesis.

XAS analysis of Cu in cowpea roots after 24 h of exposure to 1.5 μM Cu found Cu to be bound predominantly to polygalacturonic acid, an O-rich ligand found in the cell wall of the rhizodermis and outer cortex (Kopittke *et al.*, 2011). Given that the cell wall is the site of apoplastic binding and transport, and the root washing procedure employed in the current study was designed to remove adsorbed apoplastic Cu, it is not surprising that little Cu binding to O-rich ligands was observed in the current study. Kuepper *et al.* (2009) noted that the cell wall and vacuoles, which are thought to be the site of Cu storage in Cu hyperaccumulator plants, lack strong S-rich ligands, such as metallothioneins, leaving only weak O-rich ligands. The current findings showed a limited amount of O-rich Cu binding, suggesting a limited role for the vacuole in Cu storage, and cell wall-bound Cu should have been removed during root washing. Co-precipitates of Fe and oxidized Cu may have formed on the root surface, known as Fe plaques, but, again, the lack of Cu(II) observed by XAS led to the conclusion that this was not a dominant Cu storage mechanism. The XAS data support the hypothesis that non-Cu accumulator plants store excess Cu in S-rich metallothionein-type structures, as suggested by Mijovilovich *et al.* (2009).

Cu translocation The $\Delta^{65}Cu_{shoot\text{-}solution}$ values were *c.* −0.40‰, compared with the whole-plant value of *c.* −1.0‰, for both the Fe-sufficient and Fe-deficient treatments (Table 2). The similar Cu(II) concentrations and $\Delta^{65}Cu_{shoot\text{-}solution}$ values across both treatments indicate a highly regulated mechanism of Cu transport between roots and stems, and, possibly, a highly regulated re-oxidation of Cu(I) to Cu(II), given the consistent amount of Cu(II) in roots, despite the very different total Cu concentrations (Fig. 3).

Root to shoot transport of Cu in tomato plants is dependent on the mugineic acid-derived metal chelate, nicotianamine (NA; Pich & Scholz, 1996; Curie *et al.*, 2009). The *chloronerva* tomato mutant is incapable of synthesizing NA and was found to accumulate Cu in its roots, whereas vegetative tissues suffered Cu

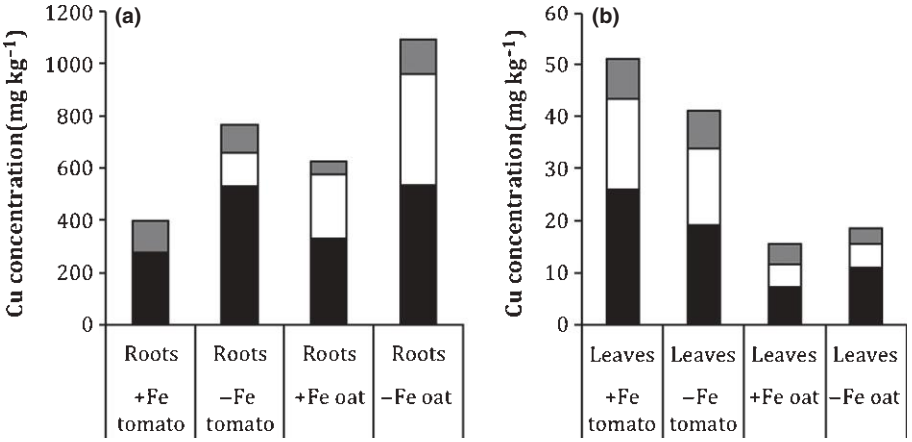

Fig. 3 Proportion of copper (Cu) species present in root tissues (a) and leaf tissues (b) for iron (Fe)-sufficient (+Fe) and Fe-deficient (−Fe) tomato (*Solanum lycopersicum*) and oat (*Avena sativa*) plants, as determined by linear combination fitting of X-ray absorption near-edge structure (XANES) Cu K-edge spectra (see Supporting Information Table S4). Cu(II)-histidine, grey bars; Cu(I)-cysteine, white bars; Cu(I)-glutathione, black bars. Note the different concentration scales on the two graphs.

Table 3 Best-fit parameters for extended X-ray absorption fine structure (EXAFS curve fitting of tomato (*Solanum lycopersicum*) and oat (*Avena sativa*) plant roots, grown in iron (Fe)-sufficient (+Fe) and Fe-deficient (−Fe) conditions, as determined by fitting error, physical reasonableness of the parameters and visual inspection of the fitted spectra

Sample	Shells	Interaction	*N*	*R* (Å)	σ^2	ΔE_o (eV)	Error
Tomato −Fe	2	Cu–S	3	2.254 (3)	0.0071 (2)	−14.2 (5)	0.340
		Cu···Cu	0.5	2.706 (3)	0.0022 (2)	−14.2 (5)	
Oat +Fe	2	Cu–S	2.5	2.261 (4)	0.0046 (2)	−13.5 (8)	0.511
		Cu···Cu	1	2.686 (5)	0.0051 (5)	−13.5 (8)	
Oat −Fe	2	Cu–S	3	2.251 (3)	0.0066 (2)	−15. 8 (6)	0.420
		Cu···Cu	0.5	2.661 (8)	0.0062 (8)		

A full set of trialled fitted parameters can be found in Supporting Information Table S5. (The *k* range was 1–13 Å^{-1} and a scale factor (S_0^2) of 0.9 was used for all fits. $\Delta E_0 = E_0 - 9000$ (eV), where E_0 is the threshold energy. Values in parentheses are the estimated standard deviations derived from the diagonal elements of the covariance matrix and are a measure of precision. The fitting error is defined as $[\Sigma k^6(\chi_{exp} - \chi_{calc})^2 / \Sigma k^6 \chi_{exp}{}^2]^{1/2}$.)

deficiency, as long-range Cu transport was severely impaired (Pich & Scholz, 1996). Liao *et al.* (2000) found that < 0.05% of Cu existed in tomato xylem as free Cu(II), with essentially all Cu present as Cu-NA. Given that NA strongly complexes Cu(II), oxidation of Cu(I) in roots would need to occur for NA complexation and subsequent translocation. Both oxidation and complexation products tend to be enriched in the heavier isotope, under equilibrium conditions, given the increased thermodynamic stability achieved with a heavier mass; however, oxidation induces much stronger heavier isotope enrichment than does complexation ($\Delta^{65}Cu_{Cu(II)\text{-}Cu(I)} = +1.9$ to $+5.3‰$ vs $\Delta^{65}Cu_{complex\text{-}free} = +0.27‰$; Mathur *et al.*, 2005; Asael *et al.*, 2007; Balistrieri *et al.*, 2008; Bigalke *et al.*, 2010b). Isotopically heavy Cu found in the shoots of this study may suggest selective transport of oxidized Cu(II) or Cu(II)-NA to the xylem for translocation. Further investigations are required to confirm this proposed mechanism of transport.

In contrast with our study, Jouvin *et al.* (2012) found isotopically lighter Cu ($\Delta^{65}Cu_{shoot} - \Delta^{65}Cu_{shoot\text{-}root} = -0.37$ to $-0.10‰$) in shoots compared with roots. As apoplastic Cu was not desorbed in their study, isotopically heavy Cu on the roots could have masked the extent of light Cu isotope enrichment in the roots. Investigations are currently underway to quantify the degree of heavy isotope enrichment caused by root adsorption of Cu. In addition, the translocation mechanisms used by the plant may be concentration specific and, given that our plants were exposed to, and contained, higher concentrations of Cu, this may have affected the translocation mechanism employed. However, both studies indicate the storage of Cu in roots and the strong regulation of Cu translocation to shoots.

The $\Delta^{65}Cu$ values of (green) leaves were more negative than those of the stem, indicating enrichment of the light isotope as Cu is translocated from stem to leaves (Table 2; $\Delta^{65}Cu_{leaf} - \Delta^{65}Cu_{leaf\text{-}stem} = -0.53‰$ and $-0.34‰$ for Fe-sufficient and Fe-deficient plants, respectively). This is supported by the presence of the Cu(I) COPT transporter protein in leaf tissues (Burkhead *et al.*, 2009) and the XANES data, which suggest that *c.* 80% of all Cu present in the leaves of both treatments is Cu(I) (Fig. 3b). In addition, dissociation of the Cu(II)-NA complex for Cu transport into leaf tissues would also favour the lighter isotope.

Translocation fractionation between green and chlorotic leaves appears to be different, with no apparent isotopic discrimination observed between chlorotic leaves and stems ($\Delta^{65}Cu_{chlorotic\ leaves} - \Delta^{65}Cu_{chlorotic\ leaves\text{-}stem} = -0.04‰$; Table 2). This indicates that there may be two different mechanisms of Cu transport at play in green and chlorotic leaves. It is not possible to determine whether this difference between chlorotic (young) and green (old) leaves is a result of the imposed Fe deficiency or simply a consequence of harvesting young and old leaves separately, as old and new leaves were not separated from the Fe-sufficient plants for comparison. Differences in isotopic signature may be related to the relative fraction of Cu contained in the given tissues. Green leaves are older and contain Cu that was translocated early in growth, when relatively more Cu was translocated from roots to

shoots. As growth progresses, the roots begin to take up an excess of Cu which is stored in the roots, whereas Cu in the shoots continues to be regulated. This results in F_{shoot} becoming smaller, and hence $\delta^{65}Cu_{shoot}$ becoming more positive. This could potentially cause a lighter isotope enrichment in older leaves, relative to young leaves, as predicted by a Rayleigh curve (Notes S1).

Conceptual model of Cu uptake and translocation The uptake and translocation of Cu in strategy I tomato plants appears to be driven by redox-selective processes, as evidenced by the strong Cu isotope fractionations between the nutrient solution and different plant tissues. XAS data indicated a dominance of Cu(I) species throughout roots and leaves, and suggested that excess root Cu is being stored in detoxifying small Cu clusters, bridged by S ligands. A conceptual model of the uptake and translocation processes for tomato plants based on the findings of this study, combined with the known importance of Cu-NA for Cu transport through vascular tissue from the literature (Pich & Scholz, 1996; Curie *et al.*, 2009), is presented in Fig. 4(a). We propose that the uptake of Cu occurs predominantly as Cu(I), with oxidation to Cu(II) required for xylem loading, followed by complexation, most probably with NA, for translocation.

Oat plants (strategy II)

Cu uptake Oat plants showed an overall small enrichment in the light Cu isotope, relative to the nutrient solution, with no significant difference between Fe treatments (Fig. 1). The observed Cu isotope fractionations for oat plants are similar to the $\Delta^{65}Cu_{root\text{-}solution}$ values of wheat and rice reported by Jouvin *et al.* (2012) (−0.48‰ to −0.11‰). The small fractionation of Cu isotopes on uptake into oat plants is too small to be attributed to a reductive uptake mechanism, which induces larger fractionation (Navarrete *et al.*, 2011; Jouvin *et al.*, 2012). ZIP divalent cation transporters have been suggested as potential Cu(II) transporters (Wintz *et al.*, 2003; Schaaf *et al.*, 2004). They are mostly nonspecific for Cu, and hence do not involve specific Cu binding. Uptake into oat roots may also be affected by the exclusive strategy II phytosiderophore complexation mechanism. Heavy Cu isotopes would be expected to preferentially form the Cu(II)-phytosiderophore complex (Guelke & Von Blanckenburg, 2007; Bigalke *et al.*, 2010a,b); however, absorption, possibly via the Fe-phytosiderophore membrane yellow-stripe-like (YSL) transporter, should not induce any significant Cu isotope fractionation, given that the complex is too large for mass-dependent fractionation (Schaaf *et al.*, 2004; Palmer & Guerinot, 2009). The production of phytosiderophore is enhanced under Fe deficiency, given that it is predominantly an Fe-acquiring mechanism; hence, the increased Cu uptake under Fe deficiency may have resulted from an increase in Cu-phytosiderophore absorption. A combination of these mechanisms may be contributing to Cu uptake in oats.

XANES Cu speciation analysis found that the majority of Cu in oat roots for both Fe treatments was present as Cu(I) species, as was the case for tomato roots (Fig. 3a). However, unlike tomatoes, no evidence for reduced Cu uptake was found. EXAFS data indicate that the root Cu is bound with S-containing ligands in

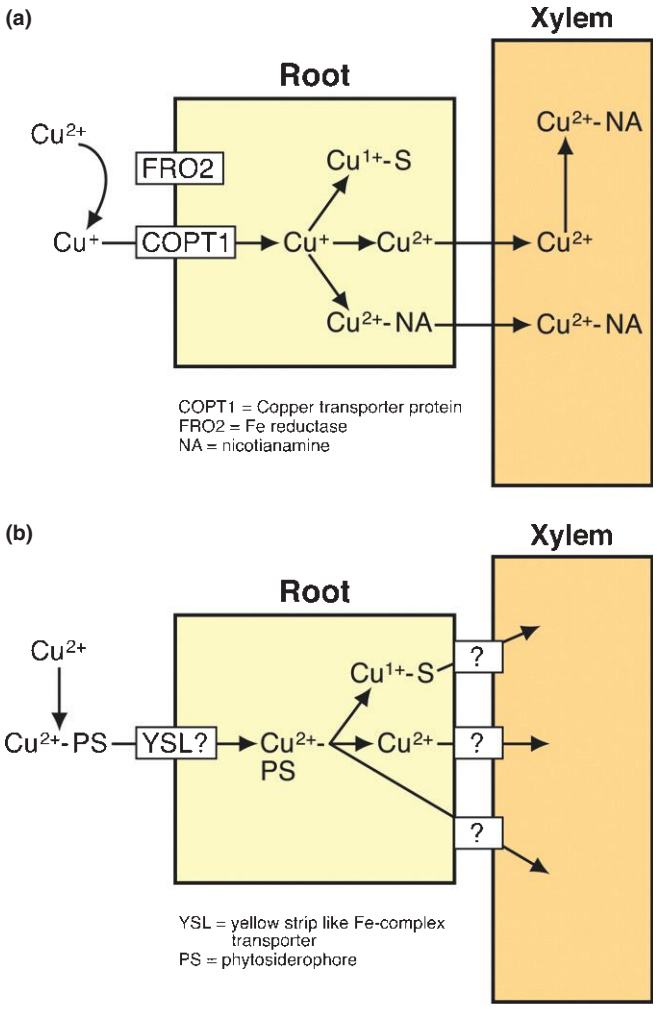

Fig. 4 (a) A conceptual model of the uptake and translocation mechanisms for Cu in strategy I tomato (*Solanum lycopersicum*) plants based on the findings in this study. (b) A conceptual model of the uptake and translocation mechanisms for Cu in strategy II oat (*Avena sativa*) plants based on the findings in this study.

small Cu clusters, as found for the Fe-deficient tomato plants (Figs S5, S6). This suggests that Cu(II) was taken up into roots and, once absorbed, was reduced and stored as Cu(I) metallothionein/phytochelatin-type complexes as a method of detoxification, given the excess amount of Cu taken up into the roots. This suggests that, although the uptake mechanisms for strategy I and II plants differ, the method of storing and detoxifying excess Cu is similar in these nonaccumulator plants.

Cu translocation The oat plants studied showed a constant Cu isotope ratio, within error, throughout the plant, indicating that no significant fractionation of Cu isotopes occurred during translocation ($\Delta^{65}Cu_{shoot} - \Delta^{65}Cu_{shoot\text{-}root} = -0.04‰$ and +0.04‰ for Fe-sufficient and Fe-deficient plants, respectively; Table 2). Similar results have been found for Fe in other strategy II plants (wheat) with a constant isotopic signature throughout the plant, whereas strategy I plants showed significant fractionation (Guelke & Von Blanckenburg, 2007; Guelke-Stelling & von Blanckenburg, 2012). These authors suggested that complexation-driven

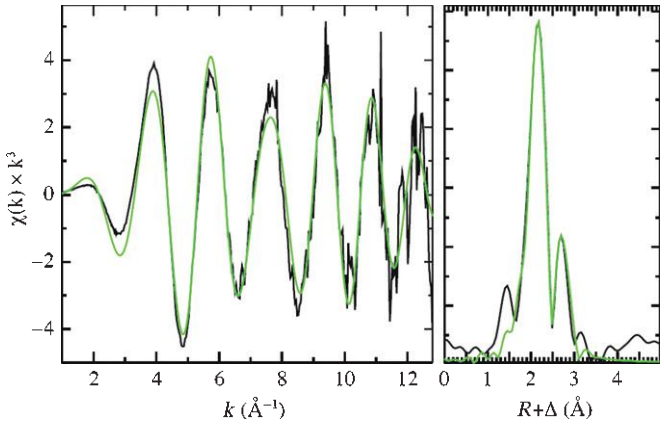

Fig. 5 Example extended X-ray absorption fine structure (EXAFS) spectra (left) and Fourier transform (right) of tomato (*Solanum lycopersicum*) roots grown in an iron (Fe)-deficient nutrient solution. Black line, experimental data; green line, fitted data. Fitted parameters can be found in Table 3.

processes that lacked redox cycling, or that involved only quantitative redox changes, were responsible for the relatively conserved Fe isotope signature. Given the similar lack of redox cycling observed for Cu, it is suggested that, in order to conserve the Cu isotope signature between roots and stems, translocation of Cu(I) present in roots occurs, or Cu(I) is quantitatively oxidized to Cu (II) after moving into the xylem; that is, no selection for oxidized species occurs at the root–stem boundary, and hence no fractionation is observed. Further investigations are required to confirm this proposed mechanism for Cu transport.

The translocation of Cu from stem to leaves (green), as determined by $\Delta^{65}Cu_{leaves} - \Delta^{65}Cu_{leaves\text{-}stem}$, showed no Cu isotope fractionation ($\Delta^{65}Cu_{leaves} - \Delta^{65}Cu_{leaves\text{-}stem} = -0.02‰$ and 0‰ for Fe-sufficient and Fe-deficient plants, respectively; Table 2). The XANES data for oat leaves indicate that 22% of the Cu in Fe-sufficient leaves and 14% of the Cu in Fe-deficient leaves is Cu(II), with the majority of Cu present as Cu(I) (Fig. 3b). It is unclear what mechanisms are responsible for the translocation of Cu from stems to leaves in the oat plants, but the small Cu isotope fractionation observed suggests that it is not a redox-selective process and, possibly, more of a complexation-driven mechanism.

As observed with strategy I tomato plants, Cu isotope fractionation following the translocation of Cu from the stems was different when moving Cu to green leaves vs chlorotic leaves (0‰ and −0.27‰ for green and chlorotic leaves, respectively; Table 2). Young, chlorotic leaves contained isotopically light Cu, as opposed to the lack of isotopic discrimination in chlorotic tomato leaves. The reason behind this difference between the two species is unclear at this stage. Evidently, translocation between roots and shoots is significantly different between the two plant species studied; hence, it is reasonable to conclude that the translocation mechanisms between stems and leaves would also differ.

Conceptual model of Cu uptake and translocation The uptake and translocation of Cu in strategy II oat plants does not appear to be driven by redox-selective processes, but by complexation-driven processes. As is the case with tomatoes, XAS found Cu(I) to be the dominant Cu species present in the roots and leaves of oat plants. A conceptual model of the uptake and translocation processes for Cu in oats, based on the findings in this study, is presented in Fig. 4(b). More research is required to elucidate the mechanisms driving Cu translocation in strategy II plants.

Concluding remarks

The results obtained here align well with findings reported previously for Fe uptake and translocation in strategy I and II plants (Guelke & Von Blanckenburg, 2007). The Cu concentrations used in this study were high to enable all Cu to remain as free Cu^{2+} for clear interpretation of the isotope data, and to generate plants with sufficiently high Cu concentrations to allow XAS analysis. Although further research is needed to elucidate the role of Cu concentration on the uptake and translocation of Cu in soil–plant systems, it is likely that the uptake mechanisms suggested in this study hold for monocots and dicots at various Cu concentrations. This has been shown with Fe, where dicots continually have significantly lighter Fe isotopic signatures than monocots, although the monocot $\delta^{56}Fe$ appears to become slightly more negative during high Fe supply, because of the use of the strategy I mechanism under Fe-sufficient conditions (Guelke & Von Blanckenburg, 2007; Kiczka *et al.*, 2010; Guelke-Stelling & von Blanckenburg, 2012). Although uptake mechanisms are not expected to change drastically with Cu supply, Cu speciation at lower Cu concentrations may. Cu-S species would probably be less dominant, given that less immobilization of Cu would be required.

The combination of isotopic and intact tissue speciation techniques has provided useful, new information on the behaviour of Cu in plant systems. Both species studied store and immobilize Cu using similar Cu-bridged S complexes that resemble metallothioneins. However, the uptake and translocation mechanisms employed by the strategy I and II species studied are evidently different, with tomatoes relying heavily on redox-selective transport, and with oats showing no sign of dominant redox selection in either uptake or translocation. To the authors' knowledge, this is the first identification that different Cu uptake and translocation mechanisms may exist between monocot and dicot plant species. Further studies using molecular techniques will be required to confirm the suggested mechanisms.

Acknowledgements

We acknowledge travel funding provided by the International Synchrotron Access Program (ISAP) managed by the Australian Synchrotron and funded by the Australian Government. Technical support from Claire Wright and Claire Weekley has been indispensible and is greatly appreciated.

References

Alaoui-Sossé B, Genet P, Vinit-Dunand F, Toussaint M-L, Epron D, Badot P-M. 2004. Effect of copper on growth in cucumber plants (*Cucumis sativus*) and its relationships with carbohydrate accumulation and changes in ion contents. *Plant Science* **166**: 1213–1218.

Asael D, Matthews A, Bar-Matthews M, Halicz L. 2007. Copper isotope fractionation in sedimentary copper mineralization (Tinma Valley, Israel). *Chemical Geology* **243**: 238–254.

Balistrieri LS, Borrok DM, Wanty RB, Ridley WI. 2008. Fractionation of Cu and Zn isotopes during adsorption onto amorphous Fe(III) oxyhydroxide: experimental mixing of acid rock drainage and ambient river water. *Geochimica et Cosmochimica Acta* **72**: 311–328.

Bernal M, Casero D, Singh V, Wilson GT, Grande A, Yang H, Dodani SC, Pellegrini M, Huijser P, Connolly EL *et al.* 2012. Transcriptome sequencing identifies SPL7-regulated copper acquisition genes FRO4/FRO5 and the copper dependence of iron homeostasis in Arabidopsis. *Plant Cell* **24**: 738–761.

Bigalke M, Weyer S, Wilcke W. 2010a. Stable copper isotopes: a novel tool to trace copper behavior in hydromorphic soils. *Soil Science Society of America Journal* **74**: 60–73.

Bigalke M, Weyer S, Wilcke W. 2010b. Copper isotope fractionation during complexation with insolubilized humic acid. *Environmental Science & Technology* **44**: 5496–5502.

Broadley M, Brown P, Cakmak I, Rengel Z, Zhao F. 2012. Function of nutrients: micronutrients. In: Marschner P, ed. *Mineral nutrition of higher plants*. Waltham, MA, USA: Academic Press, 210–211.

Brown KR, Keller GL, Pickering IJ, Harris HH, George GN, Winge DR. 2002. Structures of the cuprous-thiolate clusters of the Mac1 and Ace1 transcriptional activators. *Biochemistry* **41**: 6469–6476.

Burkhead JL, Reynolds KAG, Abdel-Ghany SE, Cohu CM, Pilon M. 2009. Copper homeostasis. *New Phytologist* **182**: 799–816.

Chen C-C, Chen Y-Y, Tang IC, Liang H-M, Lai C-C, Chiou J-M, Yeh K-C. 2011. Arabidopsis SUMO E3 ligase SIZ1 is involved in excess copper tolerance. *Plant Physiology* **156**: 2225–2234.

Cobbett C, Goldsbrough P. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* **53**: 159–182.

Collins RN, Merrington G, McLaughlin MJ, Knudsen C. 2002. Uptake of intact zinc-ethylenediaminetetraacetic acid from soil is dependent on plant species and complex concentration. *Environmental Toxicology and Chemistry* **21**: 1940–1945.

Criss RE. 1999. *Principles of stable isotope distribution*. New York, NY, USA: Oxford University Press.

Curie C, Cassin G, Couch D, Divol F, Higuchi K, Le Jean M, Misson J, Schikora A, Czernic P, Mari S. 2009. Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Annals of Botany* **103**: 1–11.

Ehrlich S, Butler I, Halicz L, Rickard D, Oldroyd A, Matthews A. 2004. Experimental study of the copper isotope fractionation between aqueous Cu(II) and covellite, CuS. *Chemical Geology* **209**: 259–269.

Fernandes J, Henriques F. 1991. Biochemical, physiological, and structural effects of excess copper in plants. *The Botanical Review* **57**: 246–273.

Flemming CA, Trevors JT. 1989. Copper toxicity and chemistry in the environment – a review. *Water Air and Soil Pollution* **44**: 143–158.

Georgatsou E, Mavrogiannis LA, Fragiadakis GS, Alexandraki D. 1997. The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *Journal of Biological Chemistry* **272**: 13786–13792.

Guelke M, Von Blanckenburg F. 2007. Fractionation of stable iron isotopes in higher plants. *Environmental Science & Technology* **41**: 1896–1901.

Guelke-Stelling M, von Blanckenburg F. 2012. Fe isotope fractionation caused by translocation of iron during growth of bean and oat as models of strategy I and II plants. *Plant and Soil* **352**: 217–231.

Guerinot ML, Yi Y. 1994. Iron – nutritious, noxious, and not readily available. *Plant Physiology* **104**: 815–820.

Huett DO, Maier NA, Sparrow LA, Piggott TJ. 1997. Vegetable crops. In: Reuter DJ, Robinson JB, eds. *Plant analysis: an interpretation manual*. Collingwood, Vic., Australia: CSIRO Publishing, 449–456.

Iwasaki K, Takahashi E. 1989. Effects of charge characteristics of Cu-chelates on the Cu uptake from the solution by Italian ryegrass and red clover. *Soil Science and Plant Nutrition* **35**: 145–150.

Jouvin D, Weiss DJ, Mason TFM, Bravin MN, Louvat P, Zhao F, Ferec F, Hinsinger P, Benedetti MF. 2012. Stable isotopes of Cu and Zn in higher plants: evidence for Cu reduction at the root surface and two conceptual models for isotopic fractionation processes. *Environmental Science & Technology* **46**: 2652–2660.

Kau LS, Spirasolomon DJ, Pennerhahn JE, Hodgson KO, Solomon EI. 1987. X-Ray absorption edge determination of the oxidation state and coordination number of copper – application to the type-3 site in *Rhus vernicifera* laccase and its reaction with oxygen. *Journal of the American Chemical Society* **109**: 6433–6442.

Kavner A, John SG, Sass S, Boyle EA. 2008. Redox-driven stable isotope fractionation in transition metals: application to Zn electroplating. *Geochimica et Cosmochimica Acta* **72**: 1731–1741.

Kiczka M, Wiederhold JG, Kraemer SM, Bourdon B, Kretzschmar R. 2010. Iron isotope fractionation during Fe uptake and translocation in alpine plants. *Environmental Science & Technology* **44**: 6144–6150.

Kopittke PM, Menzies NW, de Jonge MD, McKenna BA, Donner E, Webb RI, Paterson DJ, Howard DL, Ryan CG, Glover CJ *et al.* 2011. *In-situ* distribution and speciation of toxic copper, nickel, and zinc in hydrated roots of cowpea. *Plant Physiology* **156**: 663–673.

Kuepper H, Goetz B, Mijovilovich A, Kuepper FC, Meyer-Klaucke W. 2009. Complexation and toxicity of copper in higher plants. I. Characterization of copper accumulation, speciation, and toxicity in *Crassula helmsii* as a new copper accumulator. *Plant Physiology* **151**: 702–714.

Laurie SH, Tancock NP, McGrath SP, Sanders JR. 1991. Influence of complexation on the uptake by plants of iron, manganese, copper and zinc. II. Effect of DTPA in a multi-metal and computer simulation study. *Journal of Experimental Botany* **41**: 515–519.

Lee M, del Rosario MC, Harris HH, Blankenship RE, Guss JM, Freeman HC. 2009. The crystal structure of auracyanin A at 1.85 Å resolution: the structures and functions of auracyanins A and B, two almost identical "blue" copper proteins, in the photosynthetic bacterium *Chloroflexus aurantiacus*. *Journal of Biological Inorganic Chemistry* **14**: 329–345.

Liao MT, Hedley MJ, Woolley DJ, Brooks RR, Nichols MA. 2000. Copper uptake and translocation in chicory (*Cichorium intybus* L. cv Grasslands Puna) and tomato (*Lycopersicon esculentum* Mill. cv Rondy) plants grown in NFT system. II. The role of nicotianamine and histidine in xylem sap copper transport. *Plant and Soil* **223**: 243–252.

Marechal CN, Telouk P, Albarede F. 1999. Precise analysis of copper and zinc isotopic compositions by plasma-source mass spectrometry. *Chemical Geology* **156**: 251–273.

Marschner H, Romheld V. 1994. Strategies of plants for acquisition of iron. *Plant and Soil* **165**: 261–274.

Martins LL, Mourato MP. 2006. Effect of excess copper on tomato plants: growth parameters, enzyme activities, chlorophyll, and mineral content. *Journal of Plant Nutrition* **29**: 2179–2198.

Mathur R, Ruiz J, Titley S, Liermann L, Buss H, Brantley S. 2005. Cu isotopic fractionation in the supergene environment with and without bacteria. *Geochimica et Cosmochimica Acta* **69**: 5233–5246.

Michaud AM, Chappellaz C, Hinsinger P. 2008. Copper phytotoxicity affects root elongation and iron nutrition in durum wheat (*Triticum turgidum durum L.*). *Plant and Soil* **310**: 151–165.

Mijovilovich A, Leitenmaier B, Meyer-Klaucke W, Kroneck PMH, Gotz B, Kupper H. 2009. Complexation and toxicity of copper in higher plants. II. Different mechanisms for copper versus cadmium detoxification in the copper-sensitive cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges Ecotype). *Plant Physiology* **151**: 715–731.

Mills HA, Jones JB. 1996. *Plant analysis handbook II*. Athens, GA, USA: Micromacro Publishing.

Navarrete JU, Borrok DM, Viveros M, Ellzey JT. 2011. Copper isotope fractionation during surface adsorption and intracellular incorporation by bacteria. *Geochimica et Cosmochimica Acta* **75**: 784–799.

Palmer CM, Guerinot ML. 2009. Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nature Chemical Biology* **5**: 333–340.

Parker D, Norvell WA. 1999. Advances in solution culture methods for plant mineral nutrition research. *Advances in agronomy* **65**: 151–213.

Pich A, Scholz G. 1996. Translocation of copper and other micronutrients in tomato plants (*Lycopersicon esculentum* Mill): nicotianamine-stimulated copper transport in the xylem. *Journal of Experimental Botany* **47**: 41–47.

Polette LA, Gardea-Torresdey JL, Chianelli RR, George GN, Pickering IJ, Arenas J. 2000. XAS and microscopy studies of the uptake and biotransformation of copper in *Larrea tridentata* (creosote bush). *Microchemical Journal* **65**: 227–236.

Reichman SM, Menzies NW, Asher CJ, Mulligan DR. 2006. Responses of four Australian tree species to toxic concentrations of copper in solution culture. *Journal of Plant Nutrition* **29**: 1127–1141.

Reuter DJ, Edwards DG, Wilhelm NS. 1997. Temperate and tropical crops. In: Reuter DJ, Robinson JB, eds. *Plant analysis: an interpretation manual.* Collingwood, Vic., Australia: CSIRO Publishing, 170–174.

Robinson NJ, Procter CM, Connolly EL, Guerinot ML. 1999. A ferric-chelate reductase for iron uptake from soils. *Nature* **397**: 694–697.

Sancenon V, Puig S, Mira H, Thiele DJ, Penarrubia L. 2003. Identification of a copper transporter family in *Arabidopsis thaliana*. *Plant Molecular Biology* **51**: 577–587.

Schaaf G, Ludewig U, Erenoglu BE, Mori S, Kitahara T, von Wiren N. 2004. ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. *Journal of Biological Chemistry* **279**: 9091–9096.

Schmidt W, Bartels M, Tittel J, Fuhner C. 1997. Physiological effects of copper on iron acquisition processes in *Plantago*. *New Phytologist* **135**: 659–666.

Weinstein C, Moynier F, Wang K, Paniello R, Foriel J, Catalano J, Pichat S. 2011. Isotopic fractionation of Cu in plants. *Chemical Geology* **286**: 266–271.

Weiss DJ, Mason TFD, Zhao FJ, Kirk GJD, Coles BJ, Horstwood MSA. 2005. Isotopic discrimination of zinc in higher plants. *New Phytologist* **165**: 703–710.

Welch RM, Norvell WA, Schaefer SC, Shaff JE, Kochian LV. 1993. Induction of iron(III) and copper(II) reduction in pea (*Pisum-sativum* L) roots by Fe and Cu status – does the root-cell plasmalemma Fe(III)-chelate reductase perform a general role in regulating cation uptake. *Planta* **190**: 555–561.

Wintz H, Fox T, Wu YY, Feng V, Chen WQ, Chang HS, Zhu T, Vulpe C. 2003. Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. *Journal of Biological Chemistry* **278**: 47644–47653.

Yruela I. 2009. Copper in plants: acquisition, transport and interactions. *Functional Plant Biology* **36**: 409–430.

Zhu XK, Guo Y, Williams RJP, O'Nions RK, Matthews A, Belshaw NS, Canters GW, de Waal EC, Weser U, Burgess BK *et al.* 2002. Mass fractionation processes of transition metal isotopes. *Earth and Planetary Science Letters* **200**: 47–62.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Element elution profiles of the standard reference material National Institute of Standards and Technology (NIST) 1573a (tomato leaf) from the anion exchange resin column used to separate and purify Cu for isotope analysis.

Fig. S2 Standard compound Cu K-edge X-ray absorption near-edge structure (XANES) spectra used for principal component analysis.

Fig. S3 Measured and fitted X-ray absorption near-edge structure (XANES) spectra of root samples of tomato and oat plants.

Fig. S4 Measured and fitted X-ray absorption near-edge structure (XANES) spectra of leaf samples of tomato and oat plants.

Fig. S5 Extended X-ray absorption fine structure (EXAFS) spectra and Fourier transform of oat roots grown in an Fe-sufficient nutrient solution.

Fig. S6 Extended X-ray absorption fine structure (EXAFS) spectra and Fourier transform of oat roots grown in an Fe-deficient nutrient solution.

Fig. S7 The δ^{65}Cu value in the root and shoot of tomatoes as a function of the fraction of Cu translocated to the shoot.

Table S1 The GEOCHEM predicted species distribution in the three different nutrient solutions used for plant growth: Fe-*N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-dipropionic acid (HBED) solution used for tomato plants, Fe-ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) (EDDHA) solution used for oat plants, and a no Fe solution, used on both tomato and oat plants to induce Fe deficiency

Table S2 Copper recoveries following digestion and column purification of National Institute of Standards and Technology (NIST) 1573a standard reference material

Table S3 Summary of the anion exchange column purification procedure for Cu using AG-MP-1 resin

Table S4 Macro- and micronutrient concentrations ($mg\,kg^{-1}$) for tomato and oat plant tissues grown in Fe-sufficient (+Fe) or Fe-deficient (−Fe) conditions

Table S5 Linear combination fitting of X-ray absorption near-edge structure (XANES) Cu K-edge spectra for tomato and oat plants grown in Fe-sufficient (+Fe) and Fe-deficient (−Fe) nutrient solutions

Table S6 Results for extended X-ray absorption fine structure (EXAFS) curve fitting of tomato and oat plant roots

Methods S1 A more detailed overview of the methods used to grow and digest plants, as additional information on the Cu purification procedure and isotope measurement conditions.

Methods S2 A description of how X-ray absorption spectroscopy (XAS) model compounds were prepared, analysed and interpreted.

Notes S1 A Rayleigh fractionation model is presented and used to explain in more detail the root to shoot copper fractionation observed and discussed in the main text.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.