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Cu isotopic compositions in *Elsholtzia splendens*: Influence of soil condition and growth period on Cu isotopic fractionation in plant tissue

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ABSTRACT

This study investigates the magnitude and direction of Cu isotopic fractionation in the Cu-tolerant, strategy I plant, *Elsholtzia splendens*, considering the effect of soil condition and plant growth cycle. Uptake of Cu by *E. splendens* from soil was found to favor light Cu isotopic enrichment ($\delta^{65}Cu < 0‰$) due to reduction at the soil–root interface. The magnitude of fractionation between soil and plant was found to be dependent on free Cu ion species in soil solution correlated with pH value of soil other than the phytoavailable component of soil or total soil for the same parent soil.

Cu isotopic fractionation occurs in plant, the fractionation direction and magnitude would vary between different plant organs (i.e., root and stem) as well as different plant tissues (i.e., xylem and phloem). Remobilization of Cu associated with plant senescence was found to have a considerable effect on Cu isotopic fractionation within the plant, and the fractionation factor was found to change with the degree of remobilization. Overall, the results show that the Cu isotopic composition is useful as a tracer for probing the mechanisms of Cu translocation and retranslocation between soil and plants, and within plants.

1. Introduction

Copper is an essential micronutrient for biological organisms and plays a vital role in the normal metabolism and growth of plants over the entire life cycle. Plant growth is an important biogeochemical process that links the lithosphere, hydrosphere, atmosphere, and biosphere. Higher plants take up large amounts of metallic nutrients such as Ca, Mg, and K, as well as metallic micronutrients such as Cu, from soil and transport these nutrients to plant tissues. The variation in atomic mass among isotopes of a given element results in differential uptake and translocation of the different isotopes, resulting in isotopic fractionation in plant tissue. The isotopic composition of plants therefore has great potential as a means of tracing biogeochemical cycling between the geosphere and biosphere, and for understanding the pathway and mechanism of translocation into and within the biological system.

There is now a considerable body of research on heavy metal isotopes in plants, including Fe (Arnold et al., 2015; Guelke and von Blanckenburg, 2007), Cu (Jouvin et al., 2012; Ryan et al., 2013; Weinstein et al., 2011), Zn (Couder et al., 2015; Moynier et al., 2009; Viers et al., 2015, Viers et al., 2007; Weiss et al., 2005), Ca (Page et al., 2008), and Mg (Black et al., 2008; Bolou-Bi et al., 2010).

This study investigates the isotopic fractionation of Cu in plants with respect to enrichment or depletion of the ^{65}Cu isotope. Cu isotopic fractionation associated with plant uptake and translocation of metallic nutrients was first reported by Weinstein et al. (2011), who found light Cu isotope enrichment in plants relative to soil (e.g., Virginia wild rye, $-0.94‰ < \Delta^{65}Cu_{plant(stem,leaves,seeds)-soil} < -0.51‰$; hairy-leaved sedge, $-0.79‰ < \Delta^{65}Cu_{plant(leaf,seeds,stem)-soil} < -0.33‰$). Jouvin et al. (2012) and Ryan et al. (2013) carried out research on Cu isotopic fractionation in plants grown in EDTA, DEDTA + NTA, and high- and low-Fe nutrient solutions during uptake in a hydroponic plant experiment. They found light Cu isotope enrichment of $\Delta^{65}Cu_{whole\ plant-solution} \approx -1‰$ in tomato plants (dicot), and $-0.20‰ < \Delta^{65}Cu_{whole\ plant-soil} < -0.11‰$ in oat plants (monocot). The results show that Cu isotope fractionation occurs during the uptake of Cu by the plant and also within the plant itself. Compared with strategy II plants (e.g., oat, including the monocotyledons and graminaceous plants), strategy I plants (e.g., tomato, including the dicots and non-grass monocots) display relatively large degrees of Cu isotopic fractionation within the plant and between the plant and nutrient solution due to acidification of the rhizosphere through proton excretion via plasmalemma H^+-ATPase, which makes the metals more soluble. Strategy II plants, by contrast, rely on the chelation of metals rather than a reduction process (Jouvin et al., 2012; Ryan et al., 2013). However, further research is needed to understand the variation in Cu isotopic fractionation in soil–plant systems, the influence of parameters such as phytoavailable Cu concentration and the pH

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value of soil, and the effect of growth cycle on Cu isotopic fractionation during Cu uptake and translocation within the plant. Zinc isotopic compositions in bamboo leaves and Cu isotopic compositions in hairy-leaved sedge have been shown to be correlated with plant height (Moynier et al., 2009; Weinstein et al., 2011). However, it remains necessary to determine the correlation between plant height and isotopic fractionation in other plant organs, particularly the branches which provide nutrition to leaves directly, in order to understand the detailed isotope redistribution process and mechanisms within plants.

Recent reports have suggested that Cu isotopic fractionation occurs during plant growth, and that Cu isotopic fractionation is particularly strong in strategy I plants (Jouvin et al., 2012; Ryan et al., 2013; Weinstein et al., 2011). The aim of the present study is to systematically investigate the Cu isotopic fractionation associated with plant growth in the soil–plant system using the Cu-tolerant strategy I plant *Elsholtzia splendens*, and to better understand the possible mechanisms and factors controlling the variations in Cu isotopic compositions in such plants. *E. splendens* is an excellent plant for such a study due to its relatively high Cu content (Jiang et al., 2003; Yang et al., 1998; Yang et al., 2002). Experimentation using this plant species could also help in understanding the mechanisms of uptake, transport, and storage of Cu in copper-tolerant plants.

2. Materials and methods

2.1. Experiment design

Two sets of experiments were conducted. Set I, conducted at the Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China (32°2′24″N; 118°46′48″E), was designed to investigate the influence of soil conditions on Cu isotopic fractionation. For this set of experiments, specimens of *E. splendens* were cultivated in four different soils: natural soil (denoted CK soil), natural soil with elemental sulfur (S^0, denoted S soil), natural soil with ethylenediamine disuccinic acid ($C_{10}H_{16}N_2O_8$; EDDS) (denoted EDDS soil), and natural soil with sulfur and EDDS (denoted S + EDDS soil). Sulfur and EDDS were added to the soil to investigate the influence of changes to soil acidity (by S addition) and Cu complexation (by EDDS addition) on Cu isotopic fractionation. No Cu was added to the parent soil.

Experiment Set II was conducted in a natural field environment (suburbs of Hangzhou City, Zhejiang Province, China) (29°57′17″N; 119°56′4″E) to examine the influences of growth cycle on Cu isotopic fractionation.

2.2. Laboratory cultivation experiments

The soil used for cultivating plants was a mixture of two surface (0–20 cm depth) alluvial soil samples (fluvio-marine yellow soil) collected in the suburbs of Hangzhou City, Zhejiang Province, China. Freshly collected soil was air dried and passed through a 2 mm-mesh nylon sieve before use. To the parent natural soil was added a standard nutrient mix of 120 mg N as NH_4NO_3, 80 mg P as KH_2PO_4, and 120 mg K as KCl and KH_2PO_4 per kg to the CK soil. S soil was prepared by the addition of 200 mmol sulfur as a powder per kg to CK soil. EDDS soil was prepared by the addition of 3.0 mmol EDDS as Na_3EDDS per kg to CK soil. S + EDDS soil was prepared by the addition of 200 mmol sulfur and 3.0 mmol EDDS as above per kg to CK soil.

Seeds of *E. splendens* were collected from a copper mine site in Zhuji County, Zhejiang Province China (29°57′17″N; 119°56′4″E). Seeds were selected for uniformity and washed several times with deionized water. In a pot containing 1.3 kg of soil at room temperature (20–25 °C), the washed seeds were germinated to a stage where plants displayed up to three true leaves. The seedlings were then thinned to six plants per pot after growth for one month.

One pot of plants was prepared for each soil type, and plants were harvested after growth for four months. Two additional pots of plants, one grown in CK soil and the other in S soil, were also prepared. To these pots was added 3.0 mmol EDDS per kg at four months, and the plants were grown for one more month and then harvested after growth for a total of five months.

The plants were separated into roots, stems and branches, and leaves using ceramic scissors, washed sequentially in deionized water, ultrapure water, then deactivated enzymes at 105 °C for 30 min, dried at 80 °C for 48 h, and finally ground.

2.3. Field cultivation experiments

The field-grown plants and corresponding soil samples were collected on 20 November 2015 after growth for approximately nine months, when the plants were in the later stage of anthesis and seeds had begun to form. Plant samples were separated into roots, stems and branches, leaves, and flowers using ceramic scissors, with samples segregated into height intervals of 0–20 and 20–40 cm. Plant samples were prepared in the same manner as for laboratory-cultivated specimens.

2.4. Chemical purification and Cu isotope measurement

2.4.1. Chemical purification

A1 g sample of fresh field soil was dissolved in 40 mL of 0.43 mol/L acetic acid (HOAc) following reported procedures (Quevauviller, 1998; Ure et al., 1993). The soil samples were dispersed by immersion of the sample vial in an ultrasonic bath for 16 h at room temperature, and then centrifuged. The supernatant, regarded as soil solution (the phytoavailable solution component), was decanted into a polytetrafluoroethylene (PTFE) vial, dried, and redissolved in 2 mL of HCl + HNO_3 (v:v = 3:1) to digest organic matter. A 0.05 g directed treatment total soil sample and the dried residual soil after extracted using HOAc were digested in 2 mL of HCl + HNO_3 (v:v = 3:1) in PTFE vials on a hotplate at 120 °C for 12 h, then dried and digested in 2 mL HF + HNO_3 (v:v = 4:1) on a hotplate at 120 °C for 12 h, and finally dried.

Pulverized plant samples (50–900 mg of roots, stems, leaves) were digested in 2–6 mL HNO_3 for 24 h at ambient temperature, and then in 2–6 mL HCl + HNO_3 (v:v = 3:1) and 0.1 mL HF for 12 h at 120 °C, then in 0.2 mL $HClO_4$ for 12 h at 150 °C. Samples were then evaporated to dryness and redissolved in 1 mL HNO_3 to drive off $HClO_4$, then evaporated to dryness on a hotplate at 150 °C for 12 h. This evaporation process was repeated three times. All samples were subsequently dried and redissolved in 6 mol/L HCl, and evaporated to dryness on a hotplate at 120 °C for 12 h, repeated three times, following Li et al. (2008, 2011).

The digest extracts were redissolved in 6 mol/L HCl for Cu purification following the method of Tang et al. (2006). AG-MP-1 anion exchange resin (BioRad, USA) was used to separate Cu from matrix elements. The Cu fraction from the columns was collected in PTFE vials and evaporated to dryness on a hotplate. Samples were redissolved in 0.1 mL/L HNO_3. Matrix elements (e.g. Na, Al) were measured by inductively coupled plasma mass spectrometry (ICP-MS; Nu Plasma HR MC-ICP-MS, Nu Instruments, UK) to determine the suitability of Cu extracts for analysis of Cu isotopic ratios.

2.4.2. Cu isotope measurement

The Cu isotopic ratio ($^{65}Cu/^{63}Cu$) of purified samples was determined by ICP-MS (Nu Plasma HR MC-ICP-MS, Nu Instruments, UK), and mass bias was corrected through standard-sample-standard bracketing (Cai et al., 2006; Zhu et al., 2000). The Cu isotopic compositions were expressed as $\delta^{65}Cu$ values relative to NIST 976 Cu isotopic reference material according to

$$\delta^{65}Cu = \left[\frac{\left(^{65}Cu/^{63}Cu\right)_{sample}}{\left(^{65}Cu/^{63}Cu\right)_{NIST979}} - 1\right] \times 1000 \quad (1)$$

The long-term repeatability of Cu isotopic compositions measurements using this instrument is better than 0.005‰ amu^{-1} at the 95% confidence level. Details of the mass spectrometry method have been described elsewhere (Cai et al., 2006).

Cu isotopic compositions in plant tissue samples were calculated using the following mass balance equation:

$$\delta^{65}Cu_{whole\ plant} = \sum_i \delta^{65}Cu_i \times F_i \quad (2)$$

where F_i is the fraction of Cu in a given tissue sample i, and $\delta^{65}Cu_i$ (‰) is the Cu isotopic composition in the tissue samples.

The isotopic fractionation between two components i and j is defined in the same manner as in Moynier et al. (2009) and Weinstein et al. (2011), as follows:

$$\Delta^{65}Cu_{i-j} = \left(\delta^{65}Cu\right)_i - \left(\delta^{65}Cu\right)_j \quad (3)$$

Two sigma errors (2σ) for all the analyses were determined by multiple analyses of the samples and the uncertainty for a given sample was calculated by error propagation using the Cu isotopic composition of individual fraction (Kusonwiriyawong et al., 2016):

$$\text{Uncertainty} = \sqrt{\sum_i^n (F_i \times 2\sigma_i)^2} \quad (4)$$

where F_i is the fraction of Cu in a given tissue sample i, and experimental analytical deviation (2σ) is given as two standard deviations for sample i.

3. Results

3.1. Biomass and Cu concentrations in plants

Plant biomass and Cu concentrations are reported in Tables 1 and 2, and Cu concentrations are presented in Fig. 1. The results show that sulfur treatment led to an increase in the dry biomass of roots and stems + branches of plants, but had no significant influence on the biomass of leaves.

In all *E. splendens* specimens, the Cu concentrations are higher in root tissue than in all other plant tissues. The main binding sites for Cu in root cell walls make this an important tissue for Cu storage in *E. splendens* (Peng et al., 2005). The Cu concentrations in the roots of laboratory-cultivated plants are much higher than those in field-grown plants. With the exception of EDDS and S + EDDS leaves, however, Cu concentrations in aboveground tissues are higher in field-grown plants than in laboratory-cultivated plants. The application of EDDS to soil at four months led to a significant increase in plant Cu concentration, consistent with previous research (Bucheli-Witschel and Egli, 2001; Cooper et al., 1999; Jiang et al., 2003; Qiu et al., 2006; Sun et al., 2006; Wu et al., 2007), indicating that the growth period and growth cycle of the

Table 1
Cu isotopic compositions and concentrations in soil.

Sample	$\delta^{65}Cu$	2σ	n	Cu (μg/g)
Laboratory soils				
Parent soil	0.23	0.11	2	325
CK soil	0.28	0.11	4	329
S soil	0.19	0.20	4	314
EDDS soil	0.24	0.04	4	319
S + EDDS soil	0.22	0.19	4	317
Field soil				
Total soil	−0.12	0.01	2	137
Phytoavailable component	0.18	0.04	2	/
Residual	−0.28	0.07	2	/

External reproducibility is 0.10 (2sd) for $\delta^{65}Cu$ and ~20% for biomass.

Table 2
Cu isotopic compositions, Cu concentrations and biomass of *E. splendens* specimens cultivated in different soils.

Sample name	Average $\delta^{65}Cu_{plant}$	$\Delta^{65}Cu_{plant-soil}$	2σ	n	Cu (μg/g) dry matter	Biomass (g) dry matter
Laboratory-cultivated plants						
CK root	0.05	−0.23	0.11	4	126	5.34
CK stem + branches	−0.54	−0.82	0.09	4	3	15.80
CK leaves	−0.18	−0.46	0.05	4	4	21.40
CK whole plant	−0.01	−0.28	/	/	19	42.54
S root	−0.12	−0.31	0.06	4	102	9.07
S stem + branches	−0.60	−0.79	0.08	4	3	18.10
S leaves	−0.07	−0.26	0.04	4	5	20.60
S whole plant	−0.14	−0.33	/	/	22	47.77
EDDS root	−0.39	−0.62	0.08	4	138	4.94
EDDS stem + branches	−0.60	−0.83	0.26	4	4	16.10
EDDS leaves	−0.50	−0.74	0.12	4	36	21.40
EDDS whole plant	−0.45	−0.69	/	/	36	42.44
S + EDDS root	−0.41	−0.63	0.08	4	136	7.73
S + EDDS stem + branches	−0.60	−0.90	0.07	4	10	20.00
S + EDDS leaves	−0.68	−0.78	0.03	4	28	19.40
S + EDDS whole plant	−0.48	−0.70	/	/	38	47.13
Field-cultivated plants						
Root	−0.64	−0.52	0.06	2	60	2.81
0–20 cm *plant height interval*						
Stem	−0.72	−0.60	0.01	2	7	15.30
Branches	−0.83	−0.76	0.13	2	36	7.90
Leaves	−1.54	−1.42	0.20	2	17	1.45
Flowers	−0.74	−0.62	0.00	2	14	6.02
20–40 cm *plant height interval*						
Stem	−0.65	−0.53	0.06	2	20	6.95
Branches	−0.65	−0.44	0.23	2	16	3.33
Leaves	−0.59	−0.47	0.10	2	10	0.92
Flowers	−1.03	−0.91	0.07	2	23	4.06

Sample name	$\delta^{65}Cu_{plant}$	Uncertainty
Cu isotopic fractionation calculated based on Eqs. (2) and (4) for laboratory-cultivated plants		
Ck stem + branches + leaves	−0.30	0.04
Ck whole plant	−0.01	0.09
S stem + branches + leaves	−0.24	0.04
S whole plant	−0.14	0.05
EDDS stem + branches + leaves	−0.51	0.11
EDDS whole plant	−0.45	0.07
S + EDDS stem + branches + leaves	−0.59	0.03
S + EDDS whole plant	−0.48	0.05
Cu isotopic fractionation calculated based on Eq. (2) and (4) for field-grown plants		
0–20 cm *plant height interval*		
Leaves + flowers	−0.92	0.06
Branches + leaves + flowers	−0.85	0.09
Stem + branches + leaves + flowers	−0.82	0.07
20–40 cm *plant height interval*		
Leaves + flowers	−0.98	0.06
Branches + leaves + flowers	−0.87	0.09
Stem + branches + leaves + flowers	−0.77	0.05
Whole plant		
Root	−0.64	0.06
Stem + branches	−0.75	0.07
Stem + branches + leaves + flowers	−0.80	0.05
Leaves	−1.28	0.27
Flowers	−0.89	0.07
Aboveground tissue	−0.80	0.05
Whole plant	−0.77	0.04

External reproducibility is 0.10 (2sd) for $\delta^{65}Cu$ and ~20% biomass.

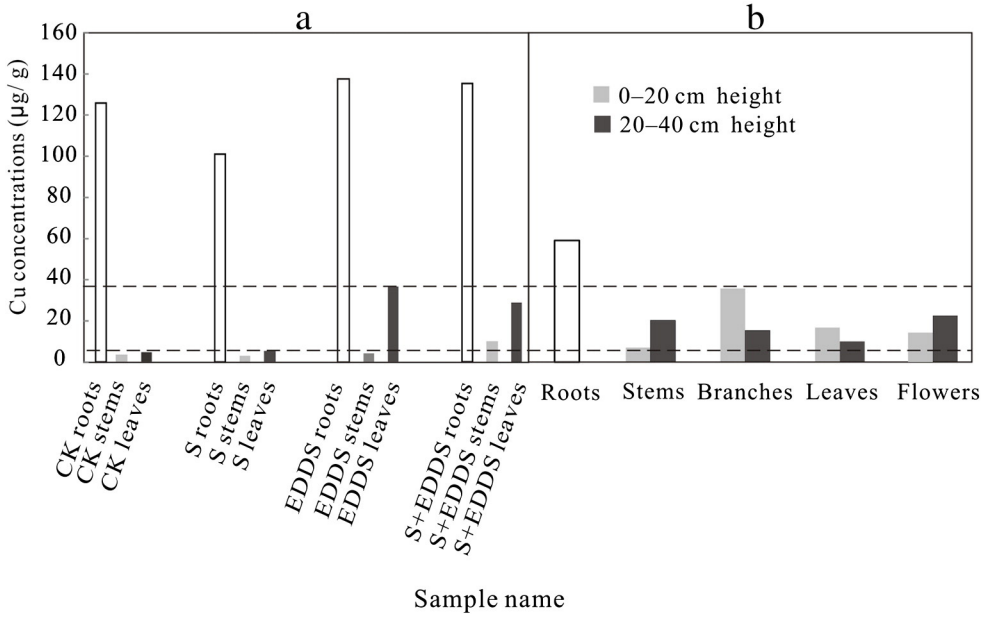

Fig. 1. Cu concentrations in tissue from (a) laboratory-cultivated and (b) field-grown specimens of *E. splendens*.

plant have significant influences on plant Cu concentration. For the field-grown plants, Cu concentrations in the same tissues vary with plant height interval, and the range of variation between lower and upper intervals differs according to tissue type.

3.2. Cu isotopic compositions in soil and plants

Cu isotopic compositions in soil and Cu isotopic fractionation between plants and soil are listed in Tables 1 and 2, and illustrated in Figs. 2 and 3. The key observations from these results are as follows.

(1) The Cu isotopic compositions of the parent soil and the soils used to cultivate *E. splendens* in the laboratory are the same within analytical error. The Cu isotopic compositions of soils used for laboratory plant growth are therefore uniform and it is not necessary to consider the potential influences of variations in Cu isotopic compositions on the isotopic fractionation in these soil-plant systems.

(2) The Cu isotopic composition in the phytoavailable component of field soil is 0.30‰ heavier than in the total soil, indicating that the phytoavailable component is not a source of light Cu isotopic enrichment in the plant.

(3) The Cu isotopic composition in laboratory soil is 0.35‰ heavier than in the field soil.

(4) Cu isotopic fractionation between plant tissues and soil range from −1.42‰ to −0.23‰, and the results show systematic light isotopic enrichment compared with corresponding soils. The highest light Cu isotopic enrichment occurred in stems and branches in laboratory-cultivated specimens, and in the lower leaves in field-grown specimens.

(5) The Cu isotopic compositions in tissues of *E. splendens* cultivated in all laboratory soils show a common trend of $\Delta^{65}Cu_{root-soil} > \Delta^{65}Cu_{leaves-soil} > \Delta^{65}Cu_{stem+branches-soil}$, although the magnitude of variation differs with soil type (Fig. 2).

(6) All specimens, cultivated in both the laboratory and the field, show the same general trend of heavy Cu isotopic enrichment in roots and light isotopic enrichment in stems and branches.

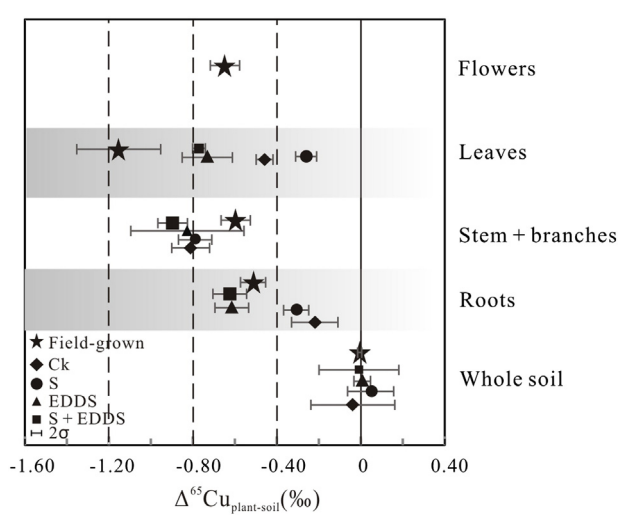

Fig. 2. Cu isotopic fractionation between soil and plants in laboratory- and field-cultivated *E. splendens* specimens. Error bars represent the 2σ of our analytical procedure (Tables 1, 2).

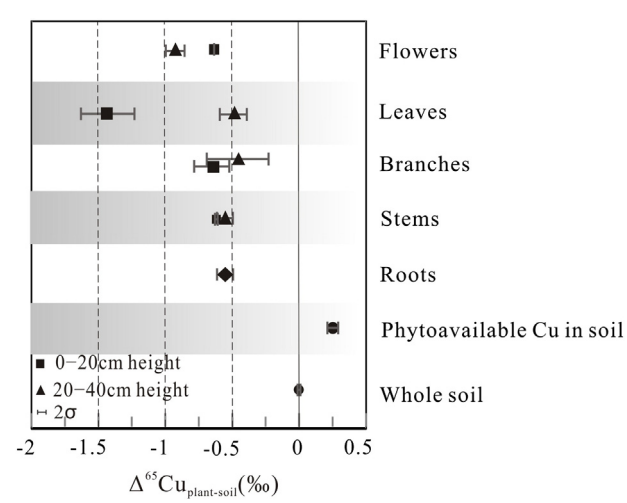

Fig. 3. Cu isotopic compositions in field-cultivated *E. splendens* specimens and in field soil. Error bars represent the 2σ of our analytical procedure (Tables 1, 2).

(7) There is no systematic trend in Cu isotopic fractionation in the leaves and flowers of field-grown plants.
(8) In field-grown specimens, Cu isotopic compositions in leaves and flowers vary with plant height interval, and the variation between lower and upper plant parts differs among tissue types.

4. Discussion

4.1. Cu isotopic compositions in soil solution

Heavy Cu isotope was riched in the 0.43 mol/L HOAc supernatant (Quevauviller, 1998; Ure et al., 1993). The yellow soil used in this experiment was from oxidizing condition and riched in iron oxyhydroxide (goethite), Cu was mainly associated with strongly bound Fe oxide−, silicate– and natural organic matter– (NOM) forms in the soil (Bigalke et al., 2010, 2011). Water is the most important component in soil solution that providing mineral metals for the plant, Cu mainly existed as $Cu(H_2O)_n^{2+}$ ($n = 5, 6$) and Cu(II)-NOM forms in soil solution. The slightly positive Cu isotopic compositions of the soil solution are similar to the pore water, acid mine drainage waters, river and ocean waters, the extractable fractions in soil (Kimball et al., 2009; Kusonwiriyawong et al., 2016; Mathur et al., 2012; Vance et al., 2008). The differences in bond strength or distance of Cu–complexes and elemental vibrational frequencies lead to the Cu isotopic fractionation (Bigalke et al., 2010; Kusonwiriyawong et al., 2016). The most reasonable explanation for positive $\Delta^{65}Cu_{\text{soil solution–total soil}}$ value was that the preferential incorporation of the heavy Cu isotope into the solution (Mathur et al., 2005; Mathur et al., 2012).

4.2. Cu in plant

4.2.1. Cu species in plant

Most of plants are rich in water, and the ratios of water to the whole weight are ca. 75%. Water is the most important component in cellular protoplasm and plays an essential role in the process of metabolism of plants. Cu(I) would present as Cu(I)-S protein-bound complexes in roots and leaves of plants (Ryan et al., 2013), however, Cu(II) mostly exist as Cu(II)-complex, and <0.05% of free Cu(II) ($Cu–H_2O$) in plant sap (Liao et al., 2000). Due to very small proportion of free Cu(II), Cu isotopic fractionation would be induced mostly by the transport of Cu(II)-complex and translocation of Cu between different Cu(II)-complex species or between Cu(II)-complex and Cu(I)-complex species, as detailed below.

4.2.2. Cu isotopic fractionation between neighboring tissues in E. splendens

As a mineral nutrient element, higher plants take up Cu via the roots from the growth environment and transport the metal throughout the plant, from soil to roots, to stem, to branches, to leaves and flowers, in this general sequence. Any given organ is therefore a recipient of nutrients from upstream organs in the transport sequence, and also a reservoir of nutrients for distribution to downstream organs. To understand Cu isotopic fractionation, it is therefore necessary to look past the apparent difference in Cu isotopic compositions between neighboring organs and consider the sequential accumulation and distribution of Cu isotopes and the fractionation that occurs in each step. For example, the difference in Cu isotopic composition between the whole plant and soil represents the Cu isotopic fractionation at the soil–root interface, while the difference in Cu isotopic composition between the aboveground tissues and the whole plant represents the Cu isotopic fractionation at the root–stem interface. The Cu isotopic fractionation in *E. splendens* is assessed in this way by considering sequential groupings of tissues. The Cu isotopic composition of a given tissue is calculated based on mass balance theory using Eq. (2), and the uncertainty is calculated following Eq. (4) using two times the analytical deviation. The calculated results are listed in Table 2 and illustrated in Fig. 4. (See Fig. 5.)

Considering the adsorption of Cu on root tissue as identified in previous studies (see details in Section 4.2.3.1), heavy Cu isotope is expected to be preferentially translocated into aboveground tissues at the root–stem interface. A trend towards light Cu isotope enrichment occurs from stem to branches, flowers + leaves, although Cu isotopic fractionation is weak compared with the analytical uncertainties. Systematic heavy isotopic enrichment was found in the leaves of laboratory-cultivated specimens. However, obvious differences exist in the distribution of Cu isotopes in leaves with height in the field-grown specimens, with heavy Cu isotope enrichment in the upper leaves and light Cu isotope enrichment in the lower leaves.

4.2.3. Uptake

4.2.3.1. Reduction at the soil solution–root interface during uptake. The isotopic composition of the whole plant reflects the isotopic composition taken up by the roots from the phytoavailable component of the soil. The Cu isotopic compositions of the phytoavailable component of the CK, S, EDDS, and S + EDDS soils were not measured in this study, and as such it is not possible to accurately determine the magnitude of Cu isotopic fractionation during uptake in the present *E. splendens* specimens.

Cu in the whole field-grown plant includes two components: Cu taken up into the plant, and Cu adsorbed on the roots. Biomolecular responses to oxidative stress are therefore likely in yellow soil. Also present in bacterial surfaces, humic acid and natural organic matter, trace Cu(0) is expected to exist in the rhizosphere of *E. splendens* at the soil–root interface (Fulda et al., 2013; González et al., 2016; Manceau et al., 2008; Navarret et al., 2011). Cu(II) mainly complexes to carboxylic groups (Cu(II)-O/N), and adsorbed on root cell walls in *E. splendens* (Liu et al., 2014; Shi et al., 2004). Cu is therefore considered to be adsorbed on the root tissue of *E. splendens* as Cu(II) and Cu(0).

Assuming that (i) ~70% of total Cu in the present *E. splendens* specimens is bound to the root cell wall (Peng et al., 2005), (ii) adsorption occurs at a ratio of 10% Cu(0) and 90% Cu(II)-complex as found in devitalized bacteria samples (González et al., 2016), (iii) similar Cu isotopic compositions occur in Cu(0) as in Cu(II)-complex as found in Cu(0) and precursor Cu(II) mineral in previous research (Markl et al., 2006), and (iv) the isotopic fractionation between Cu adsorbed on root tissue and soil solution is 0.49‰, as found for Cu adsorbed on heat-killed bacteria at pH 5.1 ($pH_{\text{field soil}} = 6.3$; Navarret et al., 2011), then $\delta^{65}Cu_{\text{taken up by root}}$ should be −4.13‰ based on Eq. (5) (i.e., $F_{Cu(0)\text{ adsorbed on root}}(10\% \times 70\%) \times \delta^{65}Cu_{Cu(0)\text{ adsorbed on root}}(0.18 + 0.49)‰ + F_{Cu(II)\text{ adsorbed on root}}(90\% \times 70\%) \times \delta^{65}Cu_{Cu(II)\text{ adsorbed on root}}(0.18 + 0.49)‰ + F_{Cu\text{ taken up by root}}(30\%) \times \delta^{65}Cu_{\text{taken up by root}} = \delta^{65}Cu_{\text{whole plant}}(-0.77‰)$, where F_i is the fraction of Cu in a given tissue) and Eq. (3), and $\delta^{65}Cu_{\text{taken up by whole plant − phytoavailable Cu in soil solution}}$ in the field-grown plant is expected to be −4.33‰.

Such a large negative fractionation indicates the presence of a dominant reductive uptake mechanism; i.e., the reduction of Cu(II) to Cu(I) during uptake into the symplasm (xylem), as Cu reduction is known to induce such significant light Cu isotopic enrichment (−3.06‰ to −4.03‰; Criss, 1999; Ehrlich et al., 2004; Zhu et al., 2002), in addition to diffusion processes, due to weak Cu isotopic enrichment (about −0.3‰; Rodushkin et al., 2004). Although both Cu(II) and Cu(0) would exist in the rhizosphere of *E. splendens*, Cu(I) is taken up by the plant as a product of Cu(II) reduction to Cu(I), and Cu(0) found in the rhizosphere of *E. splendens* is expected to be stored on the root surface.

Cu(II) is thought to be reduced by FRO-type reductase at the soil–root interface (Zheng et al., 2005) and transported as Cu^+ by transporter protein COPT1 (Sancenón et al., 2004). Our results provide strong evidence for the importance of a reduction process.

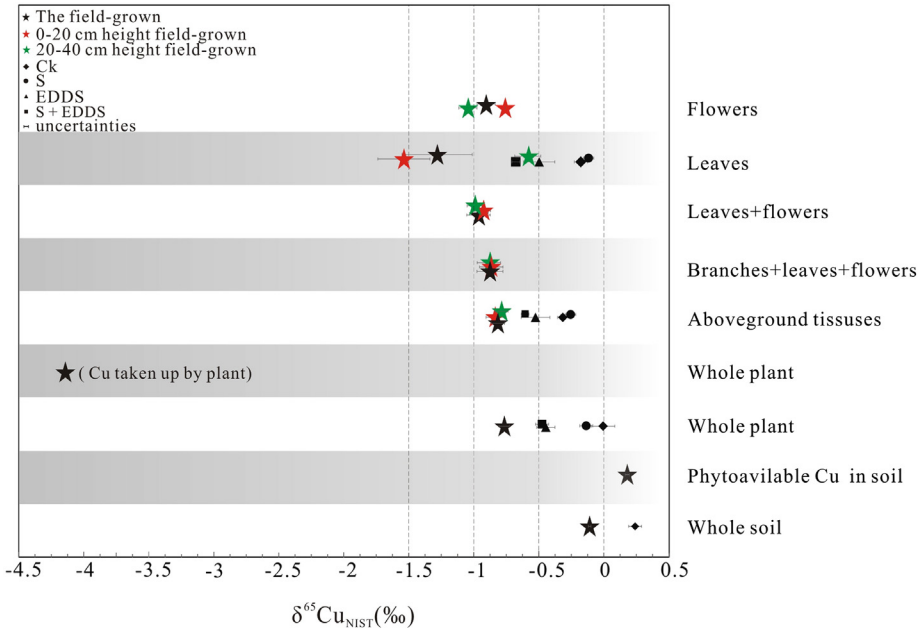

Fig. 4. Variations in Cu isotopic compositions of cultivated *E. splendens* specimens and in soil. Error bars refer to the uncertainties of Cu isotopic compositions in given samples and the 2σ of our analytical procedure, respectively (Tables 1, 2).

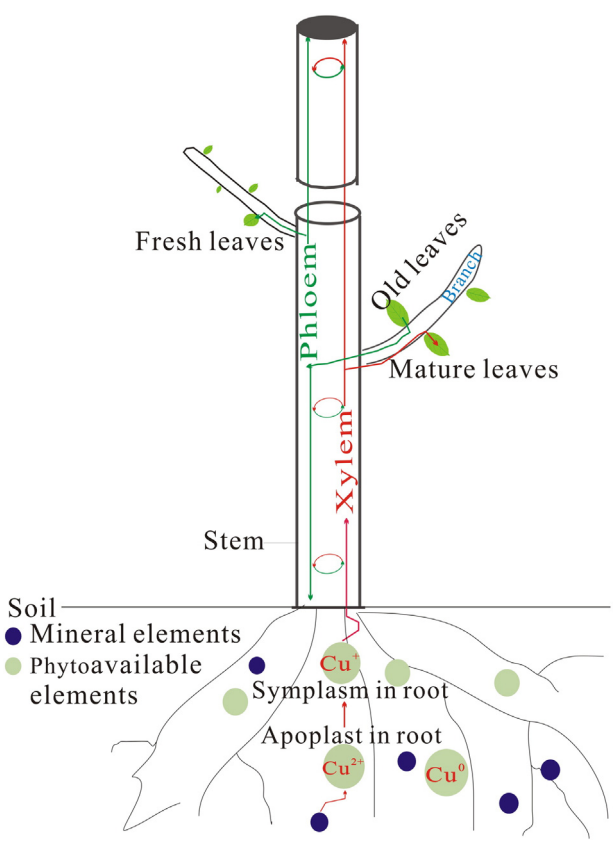

Fig. 5. Cu uptake and translocation pathways in *E. splendens*. Arrows represent the long-distance circulation of Cu within the plant. Cu(II) is reduced by FRO-type reductase at the interface between the soil solution and root, and taken up into the plant as Cu(I). Cu(I) is mainly stored in small detoxifying Cu clusters. A small proportion of Cu(I) is oxidized to Cu(II) at the interface between root (apoplast) and xylem (symplasm), translocated to the xylem through HMA family transporter proteins, and then transported in stem and branch tissue (xylem and phloem) as a Cu(II)-nicotianamine complex within the transpiration stream. Cu is remobilized from old tissues to the phloem through the *ysl* family of proteins.

4.2.3.2. Factors that influence Cu isotopic fractionation during uptake. Even though the reduction process has a strong influence on Cu isotopic fractionation during uptake, other factors also influence Cu isotopic fractionation. Although the CK, S, EDDS, and S + EDDS soils were prepared from the same parent soil, the Cu concentrations contents in the corresponding plants differ. High Cu concentrations were found in plants cultivated in EDDS and S + EDDS soils, particularly in leaves (Table 2), showing that EDDS promotes the uptake of Cu into these plants. In this study, Cu concentrations in whole plant follow the trend $[Cu]_{CK\ plant} < [Cu]_{S\ plant} < [Cu]_{EDDS\ plant} < [Cu]_{S\ +\ EDDS\ plant}$ (Table 2). As Cu uptake is a passive adsorption process (Song et al., 2004), the phytoavailable Cu (Cu_{phyto}) concentration in soil is therefore expected to follow the trend $[Cu_{phyto}]_{CK} < [Cu_{phyto}]_S < [Cu_{phyto}]_{EDDS} < [Cu_{phyto}]_{S\ +\ EDDS}$ in laboratory-cultivated soil solution, consistent with the actual experiment condition. These results show that elemental S and EDDS both enhance the Cu_{phyto} concentration in soil solution. Due to the oxidation of S, the acidity of the S and S + EDDS soils is higher than that of the parent soil: $acidity_{CK\ soil} < acidity_{S\ soil} < acidity_{EDDS\ soil} < acidity_{S\ +\ EDDS\ soil}$. This suggests that the higher the soil acidity, the higher the Cu_{phyto} concentration in soil (for the same parent soil). Song et al. (2004) found that acid enhances the Cu free ion (Cu_{free}) concentration in soil solution relative to the same parent soil, and is therefore expected to follow the trend $[Cu_{free}]_{CK} < [Cu_{free}]_S < [Cu_{free}]_{EDDS} < [Cu_{free}]_{S\ +\ EDDS}$ in laboratory-cultivated soil solution, indicating that the Cu concentration in *E. splendens* should depend on the Cu free ion concentration in the soil solution.

EDDS has high selectivity and high affinity for metal cations, forming strong bonds with Cu (log*k* for EDDS-Cu is 17). In the EDDS soils, Cu is therefore expected to be present mainly as a complex with EDDS in the soil solution. As equatorial Cu–O/N bonds are shorter than the bonds in aquo complexes (Harding, 1999; Korshin et al., 1998; Sheals et al., 2001; Xia et al., 1997) and ^{65}Cu is enriched in the species in which Cu is more strongly bound, heavy Cu isotope are expected to be partitioned into Cu — EDDS species in EDDS and S + EDDS soil solutions relative to the soil solution (Balistrieri et al., 2008; Pokrovsky et al., 2008), resulting in light Cu isotope enrichment in the phytoavailable component of EDDS and S + EDDS soil solutions based on mass balance. In soil solutions with added S, heavy Cu isotope is expected to be partitioned into free Cu ion species due to the oxidation of elemental S (Nor and Tabatabai, 1977), which causes preferential heavy isotope

enrichment in free Cu ion species. However, as the S oxidation process is slow compared with the complexation of Cu with EDDS, the effect of S addition on $\delta^{65}Cu_{free\ ions\ in\ soil}$ may be minor (Nor and Tabatabai, 1977).

Although it is not possible to determine the actual value, $\delta^{65}Cu$ for free Cu ions in soil solution is likely to follow the trend $\delta^{65}Cu_{S\ soil} > \delta^{65}Cu_{CK\ soil} > \delta^{65}Cu_{S+EDDS\ soil} > \delta^{65}Cu_{EDDS\ soil}$. This is consistent with the trend in $\Delta^{65}Cu_{plant-soil}$ ($\Delta^{65}Cu_{S\ plant-soil} > \Delta^{65}Cu_{CK\ plant-soil} > \Delta^{65}Cu_{S+EDDS\ plant-soil} > \Delta^{65}Cu_{EDDS\ plant-soil}$) and the pH of the soil solution($pH_{CK\ soil} < pH_{S\ soil} < pH_{EDDS\ soil} < pH_{S+EDDS\ soil}$), which indicates that free Cu ions may be the dominant species contributing to Cu flux at the root cell membrane (Gioia et al., 2008; Johnson et al., 2002).

The variation in Cu isotopic compositions of the laboratory-cultivated specimens reflects the variation in the Cu isotopic compositions of isotope reservoirs. The Cu isotopic composition in plants is expected to be related to the isotopic composition of ionic Cu species in the soil solution, modulated by the reduction process, and to be correlated with the pH of the soil solution for the same parent soil, independent of the Cu isotopic composition of the phytoavailable species and total soil.

Although Wu et al. (2007) found EDDS in the xylem of *E. splendens*, the light Cu isotopic enrichment found in EDDS and S + EDDS plants in the present study shows that *E. splendens* does not take up Cu as a complex, since the Cu isotopic composition of the Cu-EDDS complex is heavier than that in ionic Cu species. This is consistent with previous research that found that lighter Zn and Cu isotopes are partitioned into plants cultivated in solution with a higher metal affinity (Jouvin et al., 2012; Weiss et al., 2005).

In summary, the present results show that strategy I plants such as *E. splendens* favor the light Cu isotope at the soil solution–root interface during the uptake process, modulated by a dominant reductive uptake mechanism, and that the Cu isotopic composition in plants is related to free Cu ion species and correlated with the pH of the soil solution for the same parent soil, largely independent of the Cu isotopic composition of the phytoavailable species and total soil.

4.2.4. Translocation of Cu in plants

4.2.4.1. Translocation of Cu from root to aboveground tissues. The $\Delta^{65}Cu_{aboveground\ tissue-root}$ value for the field-grown plants is +0.17 ± 0.08‰, compared with −0.28 ± 0.12‰, −0.12 ± 0.07‰, −0.12 0.14‰, and −0.18 ± 0.09‰ for plants cultivated in CK, S, EDDS, and S + EDDS soils, respectively. However, as discussed in Section 4.2.3.1, considering Cu adsorbed on root tissue, $\delta^{65}Cu_{taken\ up\ in\ root}$ is −4.13‰ and $\Delta^{65}Cu_{aboveground\ tissue-whole\ plant}$ in field-grown plant is expected to be +3.33‰. Even without accurate Cu isotopic fractionation data, it seems likely that heavy enrichment occurs in aboveground tissue through the xylem relative to root tissue. Both complexation and oxidation products tend to be heavy isotope enriched; e.g., 0.27‰ Cu isotopic fractionation for complexation (Bigalke et al., 2010), and +1.9‰ to +5.3‰ for oxidation (Asael et al., 2007; Balistrieri et al., 2008; Mathur et al., 2005). As the magnitude of fractionation induced by Cu complexation in plants is expected to be small, it appears likely that Cu(I) is oxidized to Cu(II) at the interface between root (apoplast) and xylem (symplasm). This would be consistent with previous findings that Cu may exist mainly as nicotianamine-Cu(II) in the xylem and phloem (branch) (Curie et al., 2009; Jouvin et al., 2012; Liao et al., 2000; Pich and Scholz, 1996). Although Cu was taken up as Cu(I), and then the most of Cu through uptake was stored probably as Cu(I)–complexes as in tomato (Ryan et al., 2013), such a large isotopic enrichment shows that oxidation could play an important role in Cu isotopic fractionation relative to Cu complexation through a heavy metal ATPase family transporter (Kobayashi et al., 2008) in Cu translocation at apoplast-symplasm interface from the root to aboveground tissues.

4.2.4.2. Transport of Cu in stems and branches. As mineral elements are taken up by the plant root and translocated throughout the plant, Cu in the upper stems and branches is translocated upward from lower stems and branches. It is generally assumed that two main mechanisms are involved in the transport of Cu into stems and branches: diffusion of Cu over relatively short distances according to a concentration gradient (Moynier et al., 2009; Weinstein et al., 2011), and convective mass flow in the plant, controlled by transpiration (Barberon and Geldner, 2014; Couder et al., 2015; Lorenz et al., 1994).

As Cu concentrations in plant stems in the 0–20 cm height interval are distinctly lower than those in the 20–40 cm height interval (Table 2), the present specimens show no positive Cu concentration gradient from lower to upper stems, indicating that the condition for diffusion is not met. In addition, there is no clear variation (within uncertainty) in the Cu isotopic compositions of stem and branch tissues with respect to plant height interval ($\Delta^{65}Cu_{(stem\ 0-20\ cm)-(stem\ 20-40\ cm)} = -0.07 \pm 0.06‰$, $\Delta^{65}Cu_{(branches\ 0-20\ cm)-(branches\ 20-40\ cm)} = -0.18 \pm 0.26‰$), as also found for Zn isotopic fractionation in bamboo stem tissue (Moynier et al., 2009). Theoretically, light isotope enrichment should occur at the end point as a result of diffusion (Rodushkin et al., 2004), which is also inconsistent with the present results. It therefore seems unlikely that diffusion is a dominant transport process for Cu in the stem and branch tissue of *E. splendens*.

The increasing Cu concentration in stems from the bottom of the plant upward shows that convection (i.e., transpiration flow) could play an important role in the translocation of Cu in stems and branches. The main convection process for Cu would be transpiration pull in the stem tissue. Cu exists as Cu(II) complexes (mainly Cu(II)-nitrogenous compounds, particularly Cu (II)-nicotianamine) in xylem and phloem (Curie et al., 2009; Liao et al., 2000; Pich and Scholz, 1996). No Cu isotopic fractionation with height interval was found in stem tissue, indicating that convective translocation does not involve Cu isotopic fractionation in this state.

Cu isotopic compositions in branches + leaves + flowers ($\Delta^{65}Cu_{(0-20\ cm)-(20-40\ cm)} = -0.04 \pm 0.13‰$) and in leaves + flowers ($\Delta^{65}Cu_{(0-20\ cm)-(20-40\ cm)} = 0.06 \pm 0.09‰$) with height interval are the same within uncertainty. This shows that the exchange of Cu between the stem and upper tissues (branches), and between the branches and upper tissues (leaves + flowers), is similar in different height intervals despite the difference in growth period of tissues associated with plant height.

In summary, the results show that convective transpiration flow may control Cu transport in stems and branches, but this process does not lead to Cu isotopic fractionation, and the mechanism of Cu exchange between stem and branches is similar in the lower and upper parts of the plant, unaffected by growth period.

4.2.4.3. Translocation of Cu from stems and branches to leaves. The Cu isotopic fractionations ($\Delta^{65}Cu_{leaves-(stems+branches+leaves)}$) for specimens grown in CK, S, EDDS, S + EDDS soils are 0.12 ± 0.06‰, 0.12 ± 0.06‰, 0.01 ± 0.16‰, and −0.09 ± 0.04‰ respectively, showing a slight heavy Cu isotope enrichment during translocation from branches to young leaves at the branches–leaves interface. As discussed in Section 4.2.3.1, slight heavy Cu isotopic enrichment in leaves is representative of the complex reaction with heavy metal transporting Atpase5 (HMA5-Cu) at the branches–leaves interface, as found in previous research (Kobayashi et al., 2008), without an oxidation reaction. This is also consistent with the finding that Cu is present in the xylem tissue as Cu(II) (Liao et al., 2000). Although most Cu is present in leaves as Cu(I), as reported for tomato (Ryan et al., 2013), the reduction reaction is expected to occur inside the leaves.

However, the Cu isotopic composition of lower leaves is considerably lighter than in upper leaves in field-grown plant ($\Delta^{65}Cu_{(leaves\ 0-20\ cm)-(leaves\ 20-40\ cm)} = -0.95 \pm 0.22‰$) (Table 2; Fig. 2), in disagreement with previous investigations using hairy-leaved sedge, which showed that the isotopic composition of leaves is correlated with height, with light Cu isotope enrichment in upper leaves (Moynier et al., 2009; Weinstein et al., 2011). Due to the similar (within uncertainty)

Cu isotopic compositions of branches and branches + leaves + flowers with height (see Section 4.2.4.2), the strong light Cu isotopic enrichment in the lower leaves indicates the action of a Cu isotope redistribution process between leaves and flowers. This view is consistent with previous research showing that Cu is remobilized from old leaves to new tissues during plant senescence (Curie et al., 2009; Garnett and Graham, 2005; Waters et al., 2006). In the present study, laboratory-cultivated *E. splendens* plants were harvested after 4–5 months of growth, and the field-grown plants were harvested after 9–10 months of growth at the later stage of anthesis. The present results show that the growth cycle appears to have an effect on Cu isotopic fractionation in plants.

A previous study using a plant species with a double mutation in the yellow stripe-like (*ysl*) oligopeptide transporter proteins *ysl1* and *ysl3* showed that both of these transporters play a role in Fe, Cu, and Zn remobilization from leaf tissue (Curie et al., 2009; Waters et al., 2006). Burkhead et al. (2009) suggested that *ysl2* and *ysl3* have an as yet incompletely characterized biochemical role in the mobilization of Cu from vegetative tissue for seed loading. This previous research and the results of the present study indicate that remobilization of Cu likely occurs by *ysl* from old leaves to new tissue, which would lead to Cu isotopic fractionation. Combined with the previous researches, the variation with height in Cu isotopic composition in field-grown *E. splendens* leaves may be induced by the retranslocation of Cu from older leaves at anthesis, independent of plant species.

The present results indicate that active transport plays an important role in the translocation of Cu at the interface between stems + branches (xylem) and leaves, which is expected to lead to slight heavy Cu isotopic enrichment in leaves. Remobilization of Cu from old leaves during plant senescence is considered to have a strong influence on the Cu isotopic composition of leaves and flowers, as discussed in Section 4.2.4.4.

4.2.4.4. Translocation of Cu from leaves to flowers through branches. The Cu isotopic composition of leaves + flowers is invariant with height within uncertainty ($\Delta^{65}Cu_{(leaves\ +\ flowers\ 0–20\ cm)\ -\ (leaves\ +\ flowers\ 20–40\ cm)} = 0.06 \pm 0.06‰$). However, separately, the Cu isotopic compositions of flowers and leaves do vary with height. Flowers display heavy isotope enrichment in lower flowers with respect to upper flowers ($\Delta^{65}Cu_{(flowers\ 0–20\ cm)\ -\ (flowers\ 20–40\ cm)} = 0.29 \pm 0.07‰$), while leaves display heavy isotope enrichment in lower leaves with respect to upper leaves ($\Delta^{65}Cu_{(leaves\ 0–20\ cm)\ -\ (leaves\ 20–40\ cm)} = -0.95 \pm 0.22‰$), as discussed in Section 4.2.4.3. The calculated differences in Cu isotopic fractionation between leaves and flowers with height, based on mass balance theory, are the same within uncertainty: $\delta^{65}Cu_{(leaves\ +\ flowers\ 0–20\ cm)} = -0.92 \pm 0.06‰$ and $\delta^{65}Cu_{(leaves\ +\ flowers\ 20–40\ cm)} = -0.98 \pm 0.06‰$. The Cu isotopic compositions of leaves and flowers therefore vary in a complementary manner, with lighter Cu isotope enrichment in leaves and heavier Cu isotope enrichment in flowers. The phloem has been recognized to supply Cu for flowers (Curie et al., 2009). The variation in the Cu isotopic composition of flowers with plant height could indicate that the Cu isotopic composition of the phloem of branches varies with height. The present results indicate that Cu redistribution between leaves and flowers appears likely to occur, and that most of the Cu in flowers originates from a reservoir of Cu remobilized from old leaves through the transport channels and phloem of branches, leading to variations in the Cu isotopic composition of leaves, as discussed in Section 4.2.4.3. These isotope results provide strong evidence for the redistribution of Cu between old tissues and flowers.

The fraction of Cu content in flowers compared with that in leaves + flowers is 77.4% and 91.0%, respectively, based on Eq.(6) ($F_{flowers} = Content_{Cu\ in\ flowers} / (Content_{Cu\ in\ flowers\ +\ leaves})$), it indicates the degree of remobilization of Cu from the lower leaves is smaller than that in the upper leaves. The Cu isotopic fractionation between leaves and flowers ($\Delta^{65}Cu_{flowers\ -\ leaves\ +\ flowers}$) is $0.18 \pm 0.06‰$ in the lower part and $-0.05 \pm 0.09‰$ in the upper part, corresponding to fractionation factors of 1.0008 and 0.9996, respectively, based on Eq. (7) ($\alpha_{flowers\ -\ leaves} = (\delta^{65}Cu_{flowers} + 1000) / (\delta^{65}Cu_{leaves} + 1000)$). Heavy Cu isotope may be retranslocated by *ysl* family transporter proteins from old leaves to the phloem of branches first, as discussed in Section 4.2.4.3, then retranslocated to flowers. However, with increasing degree of remobilization the fractionation factor would change, and the inverse Cu isotopic fractionation may occur at the leaves–branches–flowers interface because Cu is likely to be stored in more than one form in the different organs (e.g., epidermis, mesophyll, or bio macromolecule-chloroplast) in leaves. The remobilization of Cu from different Cu species would therefore lead to a difference in the magnitude and direction of Cu isotopic fractionation at the exchange interface.

The remobilization of Cu is a complex process, and in the absence of detailed Cu isotopic composition data for plants at different stages of the growth cycle and information on the Cu species in leaves and flowers, it is not possible to determine the influence of Cu remobilization on Cu isotopic composition and the mechanism of Cu retranslocation at the leaves–branches–flowers interface. Further research is needed.

5. Postulate of Cu isotopic composition of xylem and phloem

As discussed in Section 4.2.4.3, leaf tissue was found to display slight heavy Cu isotope enrichment. As the xylem provides Cu to young leaves (Curie et al., 2009), the Cu isotopic composition of mature leaves should reflect that in the xylem. The Cu isotopic composition in xylem may therefore be heavier than that in stems + branches. In terms of mass balance, the Cu isotope composition of other parts of the stem and branches (e.g., phloem) should be lighter than that in the xylem, consistent with previous research showing that seeds display light Cu isotope enrichment in Virginia wild rye and hairy-leaved sedge plants (Weinstein et al., 2011), and shoots (phloem) display light Cu isotopic enrichment compared with mature leaves (xylem) (Ryan et al., 2013).

Although the Cu isotopic compositions of stem and branch tissue within the uncertainties are uniform (within uncertainty) in the longitudinal direction, differences in Cu isotopic composition among plant tissues are expected to exist in the transverse direction (i.e., xylem and phloem). However, further research is needed to determine in detail the difference in Cu isotopic composition between xylem and phloem tissue. Such studies would advance our understanding of the transport process of Cu in plants using Cu isotopic composition.

6. Conclusions

Based on the results of the present and previous studies, Cu uptake and translocation in *E. splendens* are expected to follow the process illustrated in Fig. 5. Mineral elements are taken up by the plant via the root surface (root apoplast) from rhizosphere soil, then loaded into the root tissue and translocated throughout the plant.

Cu isotopic fractionation occurs during uptake and translocation as follows. 1) The plant takes up Cu(I). Reduction at the interface between the soil solution and root is an important factor in the fractionation of light Cu isotope the plant during the uptake process, the magnitude of which may be related to the Cu isotopic composition of the ionic Cu species in the soil solution. 2) Oxidation at the interface between root (apoplast) and xylem (symplasm) leads to heavy Cu isotope enrichment in aboveground tissues relative to Cu taken up by root tissue. 3) Cu is transported in stem and branch tissue (xylem and phloem) as a Cu(II)-nicotianamine complex via transpiration, with no associated Cu isotopic fractionation. 4) Cu remobilization during plant senescence may affect the magnitude and direction of Cu isotopic fractionation within the plant. Further research is needed to elucidate the fractionation mechanisms, particularly in the xylem and phloem, and clarify the influence of Cu speciation in soil solutions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:http://dx.doi.org/10.1016/j.chemgeo.2016.09.036. These data include the Google map of the most important areas described in this article.

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