Title:

Speciation and localization of Zn in the hyperaccumulator *Sedum alfredii* by extended X-ray absorption fine structure and micro-X-ray fluorescence

Authors:

Lingli Lu¹, Xingcheng Liao¹, John Labavitch², Xiaoe Yang¹, Erik Nelson³, Yonghua Du⁴, Patrick H. Brown², and Shengke Tian^{1,2*}

Abstract

Differences in metal homeostasis among related plant species can give important information of metal hyperaccumulation mechanisms. Speciation and distribution of Zn were investigated in a hyperaccumulating population of *Sedum alfredii* by using extended X-ray absorption fine structure and micro-synchrotron X-ray fluorescence (μ -XRF), respectively. The hyperaccumulator uses complexation with oxygen donor ligands for Zn storage in leaves and stems, and variations in the Zn speciation was noted in different tissues. The dominant chemical form of Zn in leaves was most probably a complex with malate, the most prevalent organic acid in *S. alfredii* leaves. In stems, Zn was mainly associated with malate and cell walls, while Zn-citrate and Zn-cell wall complexes dominated in the roots. Two-dimensional μ -XRF images revealed age-dependent differences in Zn localization in *S. alfredii* stems and leaves. In old leaves of *S. alfredii*, Zn was high in the midrib, margin regions and the petiole, whereas distribution of Zn was essentially uniform in young leaves. Zinc was preferentially sequestered by cells near vascular bundles in young stems, but was highly localized to vascular bundles and the outer cortex layer of old stems. The results suggest that tissue- and age-dependent variations of Zn speciation and distribution occurred in the hyperaccumulator *S. alfredii*, with most of the Zn complexed with malate in the leaves, but a shift to cell wall- and citric acid-Zn complexes during transportation and storage in stems and roots. This implies that biotransformation in Zn complexation occurred during transportation and storage processes in the plants of *S. alfredii*.

- 1 **Keywords:** cellular distribution; hyperaccumulation; *Sedum alfredii*; speciation; zinc
- 2 **Abbreviations** HP: hyperaccumulating population; NHP: non-hyperaccumulating population;
- 3 PBS: phosphate buffer solution; EXAFS: Extended X-ray absorption fine structure; μ -XRF:
- 4 micro-synchrotron X-ray fluorescence

1) Introduction

Metal hyperaccumulators are of great interest for their potential use in development of phytoremediation strategies for metal-contaminated soils and for bio-fortification of trace nutritional elements, as well as research with model plants on plant adaptation to harsh environmental conditions [1]. The hyperaccumulators are capable of accumulating more than 100-fold higher amounts of metals in shoots than are most plants [2]. The abundance of metals accumulated in the shoots generally results from efficient root uptake and xylem transport [3, 4], and inevitably involves an enhanced metal homeostatic system to maintain the normal cell functions in shoots. Metal homeostasis in plants has been suggested to be maintained by a tightly regulated network of low molecular-weight ligands, membrane transport and metal-binding proteins, as well as regulators [5, 6]. By forming complexes with metal ions, ligands function to sequester, detoxify and store high levels of metals. The important metal-binding ligands most often reported are nicotianamine, histidine, phytochelatins and organic acids [5].

In hyperaccumulators, organic acids generally serve as chelators acting in metal tolerance and accumulation. Organic acid concentrations can reach very high levels in the vacuoles of shoot cells, with the acidic environment of vacuoles providing favorable sites for the formation of metal-organic acid complexes [7]. In the Zn/Cd hyperaccumulators *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) and *Arabidopsis halleri*, Zn-citrate and Zn-malate are the most abundant Zn-ligand complexes, respectively [8, 9]. Vacuolar oxygen

coordination of the metals was suggested in the leaves of *N. caerulescens* [10]. Other ligands have also been reported to play important roles in metal accumulation in hyperaccumulators. For instance, X-ray absorption fine structure studies in the Zn hyperaccumulator *N. caerulescens* suggested that Zn-histidine was the second most abundant Zn-ligand species, playing an important role in root Zn homeostasis [9]. Ligands may also be secreted from cells to act as chaperones or buffers, thus enhancing the solubility or mobility of metals within plants [5]. Recent studies on the Zn hyperaccumulator *A. halleri* proposed that nicotianamine formed complexes with Zn in root cells and facilitated symplastic passage of Zn toward the xylem [11]. In the Ni hyperaccumulator *Alyssum lesbiacum* and the accumulator *Brassica juncea*, free histidine has a high affinity for the binding of transition metal cations, and plays an important role in Ni release into xylem [12, 13].

Sedum alfredii Hance is a Zn/Cd cohyperaccumulator and lead (Pb) accumulator discovered in a Pb/Zn-rich region of China [14, 15]. This plant species is one of a few non-brassica Zn/Cd cohyperaccumulators identified so far [16]; the mechanisms involved in metal accumulation by *S. alfredii* are much less studied, thus largely unknown when compared with the strategies used by *N. caerulescens* and *A. halleri*. Previous research on the Zn hyperaccumulation process of *Sedum alfredii* was mainly focused on rhizosphere processes, transport, and cellular distribution [17-20]. Our previous studies on *S. alfredii* indicated that a majority of the Cd was accumulated in the parenchyma cells, likely bound with malate, which was indicative of a critical role in vacuolar Cd sequestration [21], whereas

Pb was largely retained in the cell walls during transportation in plants of *S. alfredii* [14]. Zinc in stems and leaves of *S. alfredii* was preferentially located in the epidermis with a second storage in mesophyll cells [20], which is significantly different from *S. alfredii*'s localization of Cd or Pb. However, information for Zn-associated ligands in *S. alfredii* remains unknown. The aim of this study is to investigate Zn ligands environments in the *S. alfredii* hyper-accumulating population (HP) and compare this with that of the nonhyperaccumulating population (NHP) by using extended X-ray absorption fine structure (EXAFS) measurements performed on intact frozen plant tissues. The EXAFS technique is an element-specific method that is particularly suited for analyzing the *in vivo* ligand environments in plants [9, 10, 21, 22]. Metal speciation in plants can be tissue- or age-dependent, as reported for the hyperaccumulator *N. caerulescens* [10]. Therefore, age-dependent differences in Zn speciation and distribution patterns are described in the present study.

2) Methods

2.1 Plant culture

Seedlings of two contrasting populations of *Sedum alfredii* were cultivated hydroponically. Seeds of a Zn/Cd hyperaccumulating population of *S. alfredii* (HP) were collected from an old Pb/Zn mine area in Zhejiang Province, and seeds for the non-hyperaccumulating population (NHP) of *S. alfredii* were obtained from a tea plantation of Hangzhou in Zhejiang Province, China. The seeds of the two populations were germinated on a mixture of perlite

and vermiculite moistened with deionized water. Four weeks after germination, groups of plants were subjected to 4 d exposure to 1/4, 1/2 or full strength nutrient solution containing 2.0 mM Ca^{2+} , 4.0 mM NO_3^- , 1.6 mM K^+ , 0.1 mM H_2PO_4^- , 0.5 mM Mg^{2+} , 1.2 mM SO_4^{2-} , 0.1 mM Cl^- , 10 μM H_3BO_3 , 0.5 μM MnSO_4 , 5.0 μM ZnSO_4 , 0.2 μM CuSO_4 , 0.01 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 100 μM Fe-EDTA. Nutrient solution pH was adjusted daily to 5.8 with 0.1 N NaOH or HCl. Plants were grown in a growth chamber with a 16/8 h photoperiod at 400 $\mu\text{mol m}^{-2}\text{s}^{-1}$, day/night temperature of 26/20°C, and day/night humidity of 70/85%. The nutrient solution was continuously aerated and renewed every 3 d. After the 4-week pre-culture period, triplicate sets of the HP and NHP plants were treated with different Zn levels (5 μM , and 100 μM ZnSO_4) in nutrient solution for 30 d under the indicated growth chamber conditions.

2.2 Organic acid assay

Organic acids in roots, stems, young and mature leaves of HP and NHP plants treated with different Zn levels (5 μM , and 100 μM ZnSO_4) for 30 d were analyzed according to the methods described by Yang et al. [23].

2.3 Bulk EXAFS analysis

EXAFS data of powdered tissues (roots, stems, young and mature leaves) from the *S. alfredii* plants were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) with the storage ring SPEAR-3 operating at 3 GeV and with ring currents of 80-100 mA. X-ray absorption spectroscopy of bulk tissues was carried out on SSRL Beamline 7-3 at 10K, essentially as described previously [14]. Zinc K-edge spectra were recorded on Beamline 7-3

1 with an upstream Rh-coated collimating mirror, a Si(220) double-crystal monochromator, and
2 a downstream focusing mirror. The incident X-ray intensity was monitored using a Kr-filled
3 ionization chamber. The monochromator energy of each spectrum was calibrated using Zn
4 metal foil between the second and third ionization chambers; its absorption edge was
5 calibrated to an edge of 9659 eV. Zinc K_{α} fluorescences were recorded using a 30 element
6 germanium detector (Canberra Industries, Meriden, CT) equipped with Soller slits and copper
7 filters.

8 Fresh plant tissues were ground under liquid nitrogen and pressed into 2-mm path length
9 Lucite sample holders with Kapton tape windows cooled in liquid nitrogen. To minimize
10 breakdown and mixing of cellular components within the plant material, care was taken to
11 keep the tissue frozen at all times during measurement. During data collection, samples were
12 maintained approximately at 10°K in a liquid helium flow cryostat to minimize the loss of
13 intensity in the signal. Zinc at room temperature gives a high signal-to-noise ratio because its
14 Debye temperature is very low, samples were kept at the low temperature to avoid this and to
15 minimize the oscillations of the low Zn ligands (i.e., O and C). Model complexes of Zn were
16 measured as standards of the most important potential types of ligands in plants. Spectra of
17 standard Zn species including ZnO (solid), $Zn(NO_3)_2$ (solid), aqueous Zn [$Zn(NO_3)_2$]
18 (solution), Zn-citrate (solution), Zn-malate (solution), Zn-oxalate (solution), Zn-phytate
19 (solution), Zn-histidine (solution), Zn-succinate (solution), Zn-glutathione (solution),
20 Zn-cysteine (solution), and Zn-cell wall (solution) were collected. All reference solutions

were prepared in 30% glycerol to prevent ice crystal formation. The complexes of Zn-citrate, Zn-malate, Zn-oxalate, Zn-phytate, Zn-histidine, Zn-succinate, Zn-glutathione, and Zn-cysteine were made by adding 5.0 mM citrate, malate, oxalate, phytate, histidine, succinate, glutathione, and cysteine to an aqueous solution of 0.5 mM $\text{Zn}(\text{NO}_3)_2$, pH 6. Root cell wall material was exposed to 0.5 mM $\text{Zn}(\text{NO}_3)_2$ in hydroponic solution (final pH=6) for 72 h, rinsed in deionized water, and frozen in liquid nitrogen for storage. Spectra of all plant samples and standard solution samples were recorded in fluorescence mode, while spectra of solid standard samples were recorded in transmission mode. Detuning of the primary beam by 50% was performed to reject higher harmonic reflections.

Multiple scans (8-16, depending on Zn concentration) were collected and averaged for each sample to improve the signal-to-noise ratio. The spectrum data of each sample were calibrated to the Zn K-edge (9659 eV) in the program SixPack [24]. Normalization of the EXAFS spectra was carried out according to standard methods using the SixPack program suite, the spectra were normalized to unit step height using a linear pre- and post-edge background subtraction. The spectra were transformed to k -space based on E_0 equal to 9659 eV. The k -function was extracted from the raw data by subtracting the atomic background using a cubic-spline consisting of 7 knots set at equal distances fitted to k^3 -weighted data and k^3 -weighted $\chi(k)$ functions were Fourier transformed over the 1.5 to 12 $\text{\AA}^{-1}$ range using a Kaiser-Bessel window with a smoothing parameter of 4. The k^3 -weighted EXAFS spectra recorded on the plants were least-squares fitted over a wave vector (k) range of 1.5-12 $\text{\AA}^{-1}$

using a combination of the Zn compounds standards. Best fits were derived by incrementally increasing the number of fit components and minimizing the fit residuals. Fits were optimized by minimizing the residual of the fit, defined as the normalized root square difference between the data and the fit. The range for the fit was varied as a function of data quality and in order to test contributions from minor components. In parallel, the structural parameters for the second Zn coordination shells were determined with simulation using SixPack [24]. The EXAFS spectra were Fourier transformed over a k range of 3-12 $\text{\AA}^{-1}$, and the contributions of the first and second shells were simulated in k and R space. Phase and amplitude functions were calculated by FEFF8 for Zn-O and Zn-C pairs, Zn histidine dehydrate for Zn-N and Zn-C pairs, and hopeite for Zn-O, Zn-P and Zn-Zn pairs.

2.3 μ -XRF mapping

Leaves and stems at different developmental stages were collected from the plants and surface rinsed with deionized water. Sections (100 μm thick) of stem samples were prepared with a cryotome (Leica CM1950) according to Tian et al. [14]. Sections in good condition were selected and freeze dried at -20°C for 3 d. Leaf samples were immediately fixed with liquid nitrogen and freeze-dried under -20°C for 3 d.

The μ -XRF analysis of Zn and other elements of the plant samples were carried out on Stanford Synchrotron Radiation Laboratory (SSRL) Beamline 2-3 with the same equipment conditions described in our previous study [14]. The elemental distribution maps were collected using a 5 μm pixel size. Dwell time per point was 200 milliseconds. The beam

energy was set to 15 Kev during mapping. The fluorescence energies windowed for this investigation were P, S, K, Ca, Mn, Fe, and Zn. Leaf samples were performed at the SSRL using Beamline 10-2. Experiments on SSRL Beamline 10-2 were recorded at 13 500 eV, using a 20 μm (H) $\times$ 20 μm (V) beam spot size, a 100 μm $\times$ 100 μm pixel size, and 100 ms dwell time per pixel. The spectra were calculated using the software package SMAK, version 0.34, S-4 (<http://www-ssrl.slac.stanford.edu/~swebb/smak.htm>). Normalized X-ray fluorescence intensities were scaled to red (maximum) and to blue (minimum) for individual elements.

2.4 Statistical analysis of data

All data were statistically analysed using the SPSS package (Version #11.0), analysis of variance (ANOVA) was performed on the data sets and the mean and SE of each treatment as well as LSD ($P < 0.05$ and $P < 0.01$) for each set of corresponding data were calculated.

3) Results

3.1 Zn concentrations and organic acids

Plants of HP and NHP *S. alfredii* treated with 5 μM or 100 μM Zn for 30 d were subjected to assays of Zn concentration and organic acid components. As shown in Fig. 1, Zn concentration in the HP plant samples was many times higher than that of the NHP plants, regardless of the Zn treatment levels. In the HP plants, Zn concentration was highest in the

1 stems, followed by leaves, while least in the roots. This agrees with our previous study [20].

2 For both populations, Zn in mature leaves was higher than that in the young leaves.

3 In the stems and leaves of both HP and NHP *S. alfredii*, the predominant organic acids
4 were malic acid followed by succinic, citric and oxalic acids (Table 1). Other organic acids
5 such as acetic, fumaric, and amber acid were not detected. This is consistent with results
6 previously reported by Yang et al. [23] and Tian et al. [21]. Regardless of the treatments, in
7 the plant tissues of both HP and NHP *S. alfredii*, concentrations of malic acid was highest in
8 the mature and young leaves, followed by stems, and was lowest in the roots. No significant
9 differences in malic acid concentrations were observed in the two different populations of *S.*
10 *alfredii*. Treatment with 100 μM Zn did not significantly alter (increase or decrease) the
11 malic acid concentrations in the stems and leaves of the HP or NHP plants. However, citric
12 and oxalic acid concentrations increased significantly in the stems and leaves of the HP *S.*
13 *alfredii* treated with 100 μM Zn as compared to the 5 μM controls.

14 3.2 Speciation of Zn measured by EXAFS

15 Speciation of Zn in different tissues of the two *S. alfredii* populations was determined by
16 EXAFS under very low temperature using hydrated plant samples. EXAFS spectra of the
17 plant samples are shown in Fig. 2. The structures of the first and second coordination shells
18 of the plant samples were analyzed in detail by FEFF simulations (Table 2). The results show
19 that oxygen is the exclusive first shell Zn neighbor for all the plant samples. Zinc-O distances
20 range between 1.95 and 2.05 Å, which suggests a mixture of tetrahedral and octahedral

environments. In the second coordination shell, 0.9-2.2 C atoms are encountered at an average distance of 2.78-3.07 Å. The shorter Zn-C distance of 2.78 Å is in agreement with distance in carboxyl (Table 2).

Zinc model complexes were generated and measured simultaneously as they represent the important potential types of ligands in plants (Fig. 3) and this facilitated the evaluation of the *in vivo* EXAFS data. Derived structural parameters of model Zn compounds are listed in Table 3. The spectra for aqueous Zn, Zn malate, Zn citrate and Zn succinate in solution have a similar frequency and slightly different shape, while Zn-oxalate and Zn-histidine have distinctive features on the second oscillation (Fig. 3). The Zn-S interactions can be unambiguously distinguished from Zn-O and Zn-N interactions because of the larger Zn-S bond distance (Fig. 3). The spectra for Zn-cell wall has lower frequency oscillations than those for Zn-oxalate and aqueous Zn²⁺, which reflects short Zn-O distances.[25]

The Zn K-edge EXAFS spectra of the plant samples were quantitatively analyzed by linear combination fitting (LCF) to a library of Zn standard spectra. Principal component analysis was not used here because of the relatively small number of spectra and their similarity to each other. For each spectrum, a single component was first tested, and an additional component was allowed if NSS (normalized sum-square) was decreased by at least 10%. Using this procedure, all spectra were correctly simulated by three components. Satisfactory fits were defined by NSS increase within 5% of that for the best fit [25]. The curves representing the best-fit linear combinations are plotted as dashed lines in Fig.4. The

corresponding fitting parameters are reported in Table 4. Four types of components were identified, including Zn malate, Zn citrate, aqueous Zn and Zn-cell wall. Zinc-glutathione and Zn-cysteine were not noted in the fits. The results showed that the ligand environment in the plant samples differed depending on the plant tissues sampled (Table 4). Based on these spectra, the dominant chemical form of Zn in leaves of both HP and NHP plants appears to be similar to Zn-malate complex, although a higher percentage of Zn coordinated with malate was observed in the HP leaves as compared with that in NHP. By comparison with young leaves, the mature leaves contained higher proportions of Zn-malate, 67.8% and 52.5% for HP and NHP, respectively, whereas aqueous Zn was higher in the young leaves. More Zn was associated with cell wall components in the NHP plants than in the HP plants, and the proportion of Zn-cell wall complex was highest in the roots, followed by stems, with the least in the leaves of the two *S. alfredii* populations (Table 4).

3.3 Localization of Zn by μ -XRF mapping

To visualize the spatial Zn distribution in age-dependent stems and leaves of HP *S. alfredii*, μ -XRF mapping was performed on stems and leaves at different developmental stages from plants treated with 100 μ M Zn for 30 d. Two-dimensional μ -XRF images showed an age-dependent difference of Zn localization in HP stems and leaves (Figs. 5 and 6). In young stem cross-sections (Fig. 5A), Zn was highly concentrated in the pith tissues around the vascular bundles, which consists of abundant parenchyma cells with large vacuoles. However, in the mid- and old-stem cross-sections, preferential localization of Zn was observed in the

vascular bundles and the outer cortex layer (Fig. 5B and 5C). In the mature leaves of HP *S. alfredii*, Zn fluorescence intensity was high in the midrib, margin regions and the petiole (Fig. 6B). Judging from the intensity of the elemental signal, the content of Zn in these areas was more than 10-fold higher than in the remainder of the leaf (data not shown). In young leaves of HP plants, Zn accumulation was essentially uniform, although the fluorescence intensity was slightly richer in the area near the petiole/margins (Fig. 6D). Zinc concentrations in these Zn-enriched regions were 5 times greater than in the rest of the leaf, as quantified from signal intensities (data not shown).

4) Discussion

Metal elements accumulated in plant tissues must become associated with ligands for phytodetoxification. Strong ligands, like the PCs and metallothioneins that detoxify heavy metals in many plants, however, do not play a major role in the metal complexation that occurs in hyperaccumulator plants [10, 26]. Results based on the X-ray absorption spectroscopy analysis reported herein for Zn speciation in the HP *S. alfredii* suggest that the majority of the Zn in these plants is in association with oxygen ligands (Table 2), rather than S ligands such as PCs, metallothioneins or other Cys-rich peptides. Hyperaccumulating plants accumulate extremely large amounts of metals, especially in their aerial parts, as observed for HP *S. alfredii* (Fig. 1). It is therefore an economical strategy for hyperaccumulator plants to store the metals in less active cell parts and/or bind them with organic acids which normally accumulate to relatively high tissue concentrations in the plants, rather than investing energy

1 in the synthesis of the large amounts of strong ligands such as PCs for complexation and
2 detoxification [16]. Investigations of Zn speciation in other hyperaccumulators also have
3 indicated that organic acids such as malate, citrate, were the predominant ligands for Zn in
4 the aerial parts [8-10].

5 *Sedum alfredii* belongs to the family Crassulaceae, which is known to possess a capacity
6 for Crassulacean acid metabolism (CAM). In CAM plants, the diurnal separation of
7 carboxylation processes mediated by phosphoenolpyruvate carboxylase and Rubisco
8 generally resulted in fluctuating levels of malate stored in a large central vacuole [27].
9 Determination of the organic acid component in the HP *S. alfredii* confirmed that malate was
10 the most abundant organic acid in the plants (Table 1), as reported in our previous studies [21,
11 28]. Vacuole provides a favorable acidic environment for the formation of metal-organic acid
12 complexes [7], and has been suggested as the primary site of accumulation for a number of
13 heavy metals [29]. Linear combination fitting analysis of the EXAFS spectra revealed that the
14 dominant (about 70% in leaves and 47% in stems) form of Zn species in leaves and stems of
15 HP *S. alfredii* had a spectrum similar to that of a Zn-malate complex (Table 4). Our previous
16 study also showed that leaf mesophyll and stem cortex cells, which normally contain large
17 vacuoles, played an important role in coping with the accumulated Zn, although epidermal
18 layers serve as important storage sites for Zn in the *S. alfredii* [20]. The above evidences
19 suggest that vacuolar sequestration of Zn *via* association with malic acid may be an important
20 strategy to detoxify the extremely high metal levels in shoots, especially in the leaves.

1 The malate concentration in the leaves and stems of HP *S. alfredii* was not increased
2 significantly by the elevated Zn levels (100 μ M) in the nutrient solution provided (Table 1),
3 suggesting that malate synthesis was not required to accommodate the Zn. This result is
4 similar to that previously reported for HP *S. alfredii* under Cd stress [21] and for another
5 hyperaccumulator, *N. caerulescens* [30]. Thus, metal association with organic acids in the
6 hyperaccumulator does not appear to be a specific response to metal hyperaccumulation or
7 hypertolerance, but rather it seems to be based on the constitutively high organic acid
8 concentrations stored in the vacuole, as suggested by Kramer [16]. This point-of-view is also
9 supported by the association of Zn mainly with malate in the leaves of NHP *S. alfredii*.
10 Efficient metal transport across the tonoplast reduces its availability and potentially negative
11 impact in the cell cytoplasm and thus supports a higher influx of the metal into the plant cells.
12 Thus, transporters involved in Zn efflux into the vacuoles are of great importance for
13 understanding the molecular mechanisms of Zn hyperaccumulation in *S. alfredii*. MTP1 is a
14 Zn transporter in the vacuolar membrane that mediates Zn detoxification and drives leaf Zn
15 accumulation in Zn hyperaccumulator plants *A. thaliana* and *T. goesingense* [31-33]. Our
16 previous studies also suggest that a transporter localized to the tonoplast, SaMTP1, plays an
17 important role in Zn accumulation in shoots of the HP *S. alfredii* [34].

18 The results of this study also indicated tissue- and age-dependent variations in Zn
19 speciation and distribution patterns in the HP *S. alfredii*, suggesting that biotransformation of
20 the metal occurred during transportation and storage processes. Evidence for

1 biotransformation of metals within plants exists from other hyperaccumulators. For instance,
2 in the nickel hyperaccumulator *Alyssum murale*, Ni is transported from roots to shoots
3 complexed with histidine [13], although when it is stored within leaf dermal tissues it is
4 complexed with malate or other organic acids [35]. In Zn-hyperaccumulator *N. caerulescens*,
5 histidine also plays an important role in Zn homeostasis in the roots, whereas organic acids
6 are involved in xylem transport and Zn storage in shoots [9]. In our study, while most of the
7 Zn complexation in leaves was as Zn-malate compounds, in stems and roots a considerable
8 amount of Zn was associated with cell walls and citric acid, with higher proportions of the
9 two compounds in the roots (Table 4).

10 Our work with HP *S. alfredii* suggested that a considerable proportion (36.7–42.3%) of Zn
11 was associated with citric acid during xylem transportation [19]. The results of the present
12 study (Table 4) further implied an important role for citric acid-Zn associations during Zn
13 transportation or storage in stems and roots, and the increases in citric acid content following
14 the exposure of HP plants to 100 μ M Zn (Table 1) may be an additional indication of the
15 organic acid's involvement in Zn transport and detoxification. The involvement of citric acid
16 in Cd uptake and speciation processes in roots of HP *S. alfredii* has been reported [21, 36]. It
17 is therefore worthwhile to identify the exact role(s) of citric acid involved in the plant's
18 management of xylem transport and root storage of Zn in *S. alfredii*. While our data are
19 suggestive, LCF analysis of EXAFS spectra is not free of uncertainties and limitations, and
20 the accuracy of the interpretations of spectra is largely affected by the choice of the

1 references used in the fitting procedure [14, 37]. The references selected in this study did not
2 include some other ligands, such as nicotianamine, which has been proposed to form
3 complexes with Zn in root cells, thus facilitating symplastic passage of Zn toward the xylem
4 in hyperaccumulators [11, 38]. The possibility of Zn associations with untested ligands
5 cannot be completely ruled out.

6 In contrast with the accumulation of Zn in leaf tissues, a higher proportion of Zn was
7 associated with cell walls in stems and roots of HP *S. alfredii* (Table 4), suggesting different
8 strategies for Zn storage in different tissues. Metal compartmentalization in less bioactive
9 sites such as cell walls has been hypothesized as one of the possible mechanisms for
10 detoxification of heavy metals in plants [39]. In HP *S. alfredii*, a higher proportion of Zn was
11 localized to parenchyma cells in young tissues, but Zn was preferentially distributed in the
12 vascular tissues, outer cortex layer, or epidermis, in mature and old plant tissues (Figs. 5, 6),
13 especially for the stems. The vascular bundles and collenchyma tissues generally have a
14 greater content of cell wall material than do the mesophyll and stem parenchyma cells.
15 Metals in plant roots are also frequently bound with cell walls, as reported in tobacco for Zn
16 [40]. The Zn in cell wall complexes reported here may be an indication of Zn that was in
17 transit to another, perhaps more permanent, location, as reported for Pb in the HP *S. alfredii*
18 [14]. Further studies may help to explain the higher number of Zn-cell wall in roots and stems,
19 when compared with the leaves.

In conclusion, the present study (1) demonstrates the utility of EXAFS analysis of frozen hydrated plant samples under very low temperature for the localization of metal elements that have been accumulated by plants and the speciation of Zn in different tissues of the HP *S. alfredii* and (2) shows that Zn is largely associated with malate, the constitutively highest synthesized organic acid, in leaves, while citric acid and cell walls played important roles in Zn transportation or storage in its stems and roots. The results of this study also suggested an age-dependent variation of Zn speciation and distribution for different tissues of HP *S. alfredii*. Further investigation of spatial metal speciation by using XRF- μ -XAS is worthwhile to give more specific information of the metal ligand environments at the cellular level.

5) Acknowledgements

This work was supported by projects from the National Natural Science Foundation of China (31300040, 31471939), Zhejiang Provincial Natural Science Foundation of China (LR14C150001) and the Fundamental Research Funds for the Central Universities (2014QNA6011). Portions of this research were carried out at the Stanford Synchrotron Radiation Lightsource, a Directorate of SLAC National Accelerator Laboratory and an Office of Science User Facility operated for the U.S. Department of Energy Office of Science by Stanford University. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by the National Institutes of Health, National Institute of General Medical Sciences (including P41GM103393) and the

National Center for Research Resources (P41RR001209). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of NIGMS, NCRR or NIH. Authors sincerely thank all the staff of BL 7-3, and BL 2-3 at the Stanford Synchrotron Radiation Lightsource, particularly Serena DeBeer, Sam Webb, Ben Kocar and Matthew Latimer, for their support.

6) Contributions

Lingli Lu, Xingcheng Liao, Xiaoe Yang and Shengke Tian designed and performed the research, Shengke Tian, Erik Nelson, and Yonghua Du analyzed the EXAFS data, Lingli Lu, John Labavitch and Patrick H. Brown wrote the paper.

7) References

- [1] F.J. Zhao, S.P. McGrath, Biofortification and phytoremediation, *Current Opinion on Plant Biology*, 12 (2009) 373-380.
- [2] A.J.M. Baker, S.P. McGrath, R.D. Reeves, J.A.C. Smith, Metal hyperaccumulator plants: A review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils, **In: *Phytoremediation of Contaminated Soil and Water*, Terry N. and G.S. Banuelos (Eds.) CRC Press, Boca Raton (2000) 85-107.**
- [3] F.J. Zhao, R.F. Jiang, S.J. Dunham, S.P. McGrath, Cadmium uptake, translocation and tolerance in the hyperaccumulator *Arabidopsis halleri*, *New Phytologist*, 172 (2006) 646-654.
- [4] L.L. Lu, S.K. Tian, X.E. Yang, X.C. Wang, P. Brown, T.Q. Li, Z.L. He, Enhanced root-to-shoot translocation of cadmium in the hyperaccumulating ecotype of *Sedum alfredii*, *Journal of Experimental Botany*, 59 (2008) 3203-3213.

- [5] S.A. Sinclair, U. Krämer, The zinc homeostasis network of land plants, *Biochimica et Biophysica Acta*, **1823** (2012) 1553-1567.
- [6] S. Clemens, M.G. Palmgren, U. Kramer, A long way ahead: understanding and engineering plant metal accumulation, *Trends in Plant Science*, **7** (2002) 309-315.
- [7] M.J. Haydon, C.S. Cobbett, Transporters of ligands for essential metal ions in plants, *New Phytologist*, **174** (2007) 499-506.
- [8] G. Sarret, P. Saumitou-Laprade, V. Bert, O. Proux, J.L. Hazemann, A.S. Traverse, M.A. Marcus, A. Manceau, Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*, *Plant Physiology*, **130** (2002) 1815-1826.
- [9] D.E. Salt, R.C. Prince, A.J.M. Baker, I. Raskin, I.J. Pickering, Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy, *Environmental Science & Technology*, **33** (1999) 713-717.
- [10] H. Kupper, A. Mijovilovich, W. Meyer-Klaucke, P.M.H. Kroneck, Tissue- and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by X-ray absorption spectroscopy, *Plant Physiology*, **134** (2004) 748-757.
- [11] U. Deinlein, M. Weber, H. Schmidt, S. Rensch, A. Trampczynska, T.H. Hansen, S. Husted, J.K. Schjoerring, I.N. Talke, U. Kramer, S. Clemens, Elevated nicotianamine levels in *Arabidopsis halleri* roots play a key role in zinc hyperaccumulation, *Plant Cell*, **24** (2012) 708-723.
- [12] L. Kerkeb, U. Kramer, The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*, *Plant Physiology*, **131** (2003) 716-724.
- [13] U. Kramer, J.D. CotterHowells, J.M. Charnock, A.J.M. Baker, J.A.C. Smith, Free histidine as a metal chelator in plants that accumulate nickel, *Nature*, **379** (1996) 635-638.
- [14] S.K. Tian, L.L. Lu, X.E. Yang, S.M. Webb, Y.H. Du, P.H. Brown, Spatial imaging and speciation of lead in the accumulator plant *Sedum alfredii* by microscopically focused synchrotron X-ray investigation, *Environmental Science & Technology*, **44** (2010) 5920-5926.
- [15] X.E. Yang, X.X. Long, H.B. Ye, Z.L. He, D.V. Calvert, P.J. Stoffella, Cadmium tolerance and hyperaccumulation in a new Zn-hyperaccumulating plant species (*Sedum alfredii* Hance), *Plant and Soil*, **259** (2004) 181-189.
- [16] U. Kramer, Metal hyperaccumulation in plants, *Annual Review of Plant Biology*, **61** (2010) 517-534.

- 1 [17] T.Q. Li, Z.Z. Di, E. Islam, H. Jiang, X.E. Yang, Rhizosphere characteristics of zinc
2 hyperaccumulator *Sedum alfredii* involved in zinc accumulation, Journal of Hazardous
3 Materials, 185 (2011) 818-823.
- 4 [18] X.X. Long, Y.G. Zhang, D. Jun, Q.X. Zhou, Zinc, cadmium and lead accumulation and
5 characteristics of rhizosphere microbial population associated with hyperaccumulator *Sedum*
6 *alfredii* hance under natural conditions, Bulletin of Environmental Contamination and
7 Toxicology, 82 (2009) 460-467.
- 8 [19] L. Lu, S. Tian, J. Zhang, X. Yang, J.M. Labavitch, S.M. Webb, M. Latimer, P.H. Brown,
9 Efficient xylem transport and phloem remobilization of Zn in the hyperaccumulator plant
10 species *Sedum alfredii*, New Phytologist, 198 (2013) 721-731.
- 11 [20] S.K. Tian, L.L. Lu, X.E. Yang, J.M. Labavitch, Y.Y. Huang, P. Brown, Stem and leaf
12 sequestration of zinc at the cellular level in the hyperaccumulator *Sedum alfredii*, New
13 Phytologist, 182 (2009) 116-126.
- 14 [21] S.K. Tian, L.L. Lu, J. Labavitch, X.E. Yang, Z.L. He, H.N. Hu, R. Sarangi, M. Newville,
15 J. Commisso, P. Brown, Cellular sequestration of cadmium in the hyperaccumulator plant
16 species *Sedum alfredii*, Plant Physiology, 157 (2011) 1914-1925.
- 17 [22] G. Sarret, W.H. Schroeder, M.A. Marcus, N. Geoffroy, A. Manceau, Localization and
18 speciation of Zn in mycorrhized roots by mu SXRF and mu EXAFS, Journal De Physique Iv,
19 107 (2003) 1193-1196.
- 20 [23] X.E. Yang, T.Q. Li, J.C. Yang, Z.L. He, L.L. Lu, F.H. Meng, Zinc compartmentation in
21 root, transport into xylem, and absorption into leaf cells in the hyperaccumulating species of
22 *Sedum alfredii* Hance, Planta, 224 (2006) 185-195.
- 23 [24] S.M. Webb, SIXpack: a graphical user interface for XAS analysis using IFEFFIT,
24 Physica Scripta, T115 (2005) 1011-1014.
- 25 [25] G. Sarret, G. Willems, M.P. Isaure, M.A. Marcus, S.C. Fakra, H. Frerot, S. Pairis, N.
26 Geoffroy, A. Manceau, P. Saumitou-Laprade, Zinc distribution and speciation in *Arabidopsis*
27 *halleri* x *Arabidopsis lyrata* progenies presenting various zinc accumulation capacities, New
28 Phytologist, 184 (2009) 581-595.
- 29 [26] H. Kupper, B. Gotz, A. Mijovilovich, F.C. Kupper, W. Meyer-Klaucke, Complexation
30 and Toxicity of Copper in Higher Plants. I. Characterization of copper accumulation,
31 speciation, and toxicity in *Crassula helmsii* as a new copper accumulator, Plant Physiology,
32 151 (2009) 702-714.
- 33 [27] A.M. Borland, T. Taybi, Synchronization of metabolic processes in plants with
34 Crassulacean acid metabolism, Journal of Experimental Botany, 55 (2004) 1255-1265.

- [28] X. Yang, T.Q. Li, J.C. Yang, Z.L. He, L.L. Lu, F.H. Meng, Zinc compartmentation in root, transport into xylem, and absorption into leaf cells in the hyperaccumulating species of *Sedum alfredii* Hance, *Planta*, 224 (2006) 185-195.
- [29] S. Conn, M. Gilliam, Comparative physiology of elemental distributions in plants, *Annals of Botany*, 105 (2010) 1081-1102.
- [30] D. Ueno, J.F. Ma, T. Iwashita, F.J. Zhao, S.P. McGrath, Identification of the form of Cd in the leaves of a superior Cd-accumulating ecotype of *Thlaspi caerulescens* using Cd-113-NMR, *Planta*, 221 (2005) 928-936.
- [31] A.G. Desbrosses-Fonrouge, K. Voigt, A. Schroder, S. Arrivault, S. Thomine, U. Kramer, Arabidopsis thaliana MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation, *Febs Letters*, 579 (2005) 4165-4174.
- [32] J.L. Gustin, M.E. Loureiro, D. Kim, G. Na, M. Tikhonova, D.E. Salt, MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants, *Plant Journal*, 57 (2009) 1116-1127.
- [33] Y. Kobae, T. Uemura, M.H. Sato, M. Ohnishi, T. Mimura, T. Nakagawa, M. Maeshima, Zinc transporter of Arabidopsis thaliana AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis, *Plant and Cell Physiology*, 45 (2004) 1749-1758.
- [34] M. Zhang, T. Senoura, X.E. Yang, N.K. Nishizawa, Functional analysis of metal tolerance proteins isolated from Zn/Cd hyperaccumulating ecotype and non-hyperaccumulating ecotype of *Sedum alfredii* Hance, *Febs Letters*, 585 (2011) 2604-2609.
- [35] D.H. McNear, R.L. Chaney, D.L. Sparks, The hyperaccumulator Alyssum murale uses complexation with nitrogen and oxygen donor ligands for Ni transport and storage, *Phytochemistry*, 71 (2010) 188-200.
- [36] L.L. Lu, S.K. Tian, X.E. Yang, H.Y. Peng, T.Q. Li, Improved cadmium uptake and accumulation in the hyperaccumulator *Sedum alfredii*: the impact of citric acid and tartaric acid, *Journal of Zhejiang University-Science B*, 14 (2013) 106-114.
- [37] E. Lombi, J. Susini, Synchrotron-based techniques for plant and soil science: opportunities, challenges and future perspectives, *Plant and Soil*, 320 (2009) 1-35.
- [38] A. Trampczynska, H. Kupper, W. Meyer-Klaucke, H. Schmidt, S. Clemens, Nicotianamine forms complexes with Zn(II) in vivo, *Metallomics*, 2 (2010) 57-66.
- [39] J.L. Hall, Cellular mechanisms for heavy metal detoxification and tolerance, *Journal of Experimental Botany*, 53 (2002) 1-11.

1 [40] A. Straczek, G. Sarret, A. Manceau, P. Hinsinger, N. Geoffroy, B. Jaillard, Zinc
2 distribution and speciation in roots of various genotypes of tobacco exposed to Zn,
3 Environmental and Experimental Botany, 63 (2008) 80-90.

4

5

Table 1 Concentrations of organic acids in roots, stems, and leaves of the two *S. alfredii* populations under Zn exposure

Population	Sample	Zn levels	Malate	Citrate	Oxalate	Succinate	Total
		μM	$\mu mol g^{-1} FW$				
HP	Young leaves	5	46.6 $\pm$ 2.3	2.12 $\pm$ 0.41	1.25 $\pm$ 0.11	4.32 $\pm$ 0.18	54.32
		100	49.8 $\pm$ 4.2	3.74 $\pm$ 0.51	1.39 $\pm$ 0.22	3.98 $\pm$ 0.43	58.88
	Mature leaves	5	53.4 $\pm$ 4.4	2.74 $\pm$ 0.32	1.43 $\pm$ 0.13	2.54 $\pm$ 0.21	60.13
		100	58.8 $\pm$ 3.8	4.13 $\pm$ 0.45	2.32 $\pm$ 0.18	4.77 $\pm$ 0.65	70.01
	Stems	5	37.2 $\pm$ 4.6	3.62 $\pm$ 0.41	1.51 $\pm$ 0.21	5.22 $\pm$ 1.14	47.59
		100	39.9 $\pm$ 3.2	4.98 $\pm$ 0.33	2.19 $\pm$ 0.38	3.92 $\pm$ 0.32	50.97
	Roots	5	7.8 $\pm$ 0.5	1.31 $\pm$ 0.41	0.82 $\pm$ 0.05	4.12 $\pm$ 0.92	14.08
		100	8.8 $\pm$ 0.7	1.78 $\pm$ 0.31	1.32 $\pm$ 0.19	2.89 $\pm$ 0.19	14.76
NHP	Young leaves	5	43.76 $\pm$ 3.66	0.84 $\pm$ 0.12	2.09 $\pm$ 0.13	5.38 $\pm$ 0.73	52.07
		100	45.81 $\pm$ 4.12	1.32 $\pm$ 0.21	1.74 $\pm$ 0.32	3.19 $\pm$ 2.01	52.06
	Mature leaves	5	48.32 $\pm$ 5.34	0.54 $\pm$ 0.16	1.82 $\pm$ 0.21	4.33 $\pm$ 0.58	55.01
		100	42.81 $\pm$ 3.91	0.64 $\pm$ 0.13	2.91 $\pm$ 0.32	2.89 $\pm$ 0.61	49.25
	Stems	5	32.12 $\pm$ 4.98	0.34 $\pm$ 0.12	2.38 $\pm$ 0.34	3.23 $\pm$ 0.34	38.07
		100	35.19 $\pm$ 3.81	0.81 $\pm$ 0.21	3.87 $\pm$ 0.52	3.77 $\pm$ 0.25	43.64
	Roots	5	8.92 $\pm$ 0.45	0.73 $\pm$ 0.21	0.89 $\pm$ 0.25	2.13 $\pm$ 0.20	12.67
		100	7.48 $\pm$ 0.31	0.89 $\pm$ 0.16	0.79 $\pm$ 0.18	3.44 $\pm$ 0.19	12.6

Plants were treated with 5 or 100 μM Zn for 30 d. The results are presented as means $\pm$ SEs for three replications.

Table 2 First and second shell structural parameters derived from the spectra shown in Fig. 2

Plant Samples		Type	N	R(Å)	σ^2 (10 ⁻³ Å)	R factor	
HP	Young leaves	Zn-O	5.8±0.2	2.02±0.02	0.0101	20	
		Zn-C	1.9±0.3	2.94±0.03			
	Mature leaves	Zn-O	5.6±0.2	1.98±0.03	0.0101	10	
		Zn-C	1.8±0.3	2.91±0.02			
	Stems	Zn-O	5.3±0.1	2.05±0.02	0.0055	35	
		Zn-C	1.2±0.2	2.87±0.05	0.0101		
	Roots	Zn-O	5.8±0.4	2.01±0.03	0.013	21	
		Zn-C	2.2±0.2	2.78±0.05	0.009		
	NHP	Young leaves	Zn-O	4.8±0.2	2.03±0.03	0.010	23
			Zn-C	1.1±0.1	2.87±0.02	0.0048	
Mature leaves		Zn-O	5.4±0.3	1.97±0.04	0.0102	35	
		Zn-C	0.9±0.1	3.07±0.02	0.0048		
Stems		Zn-O	4.8±0.3	1.95±0.01	0.0066	24	
		Zn-C	1.1±0.2	3.03±0.02	0.0101		
Roots		Zn-O	4.2±0.3	1.95±0.01	0.0090	29	

N, number of atoms; R, interatomic distance, σ^2 : Debye waller factor, R factor: reliability factor for goodness of fit. All spectra were recorded on samples in the frozen-hydrate condition.

1 **Table 3** First and second shell structural parameters derived from the spectra shown in Fig. 3

Sample	Type	N	R(Å)	σ^2 (10^{-3} Å)	R factor
ZnO	O	4.0±0.1	1.95±0.01	0.004	
	Zn	12.0±0.1	3.28±0.03	0.009	12
	Zn	6.0±0.1	4.51±0.07	0.007	
Zn(NO ₃) ₂	O	5.8±0.4	2.09±0.05	0.007	17
Aqueous Zn	O	6.2±0.3	2.03±0.03	0.001	18
Zn-Citrate	O	6.3±0.2	2.02±0.04	0.008	
	C	3.9±0.3	2.85±0.02	0.011	16
Zn-Malate	O	5.9±0.1	2.05±0.03	0.010	
	C	2.5±0.3	2.84±0.02	0.009	19
Zn-Phytate	O	4.1±0.2	1.98±0.01	0.009	
	P	1.0±0.1	3.12±0.02	0.007	18
Zn-Histidine	N	4.0±0.1	2.06±0.01	0.006	
	C	3.5±0.1	2.96±0.05	0.011	14
Zn-Succinate	O	6.3±0.7	2.03±0.04	0.009	19
Zn-glutathione	S	4.0±0.1	2.32±0.01	0.006	9
Zn-cysteine	S	3.9±0.2	2.34±0.02	0.005	10
Zn-cell wall	O	2.4±0.1	1.91±0.01		
	O	1.4±0.2	2.05±0.02	0.009	12
	C	0.9±0.1	2.88±0.03		

2 N, number of atoms; R, interatomic distance, σ^2 : Debye waller factor, R factor: reliability
3 factor for goodness of fit. All spectra were recorded on samples in the frozen-hydrate
4 condition.

1 **Table 4** Zinc species identified by linear combination fit (LCF) analyses of powdered plant
2 sample spectra.

Plant samples		Zn species (%)				Sum	Residual (%)
		Zn ²⁺	Zn-Malate	Zn-Citrate	Zn-Cell wall		
HP	Young leaves	29.5±3.8	57.4±5.1	-	18.4±4.7	105.3	8.2
	Mature leaves	17.4±2.7	67.8±3.9	-	21.7±4.2	106.9	11.4
	stems	16.4±1.8	41.4±1.2	13.5±3.6	35.8±3.6	107.1	9.1
	roots	27.7±2.4	-	33.9±6.2	42.5±3.2	104.1	19.7
NHP	Young leaves	24.7±3.1	42.5±6.2	-	38.7±2.7	105.9	18.2
	Mature leaves	18.4±2.4	52.5±7.7	-	31.4±5.3	102.3	15.3
	stems	21.5±3.8	27.8±3.1	17.1±3.4	41.5±4.4	107.9	8.2
	roots	26.3±2.3	-	16.8±2.9	57.6±3.1	100.7	5.7

3

Figure legends

Figure 1 Total Zn concentration in different tissues of two *S. alfredii* populations. Leaves with different development stages, stems and roots were collected for each population. Values shown represent the mean $\pm$ SE of 15 plants. DW, dry weight.

Figure 2 Zinc K-edge extended X-ray absorption fine structure (EXAFS) spectra recorded on roots, stems, and young and mature leaves of HP and NHP *S. alfredii*. All samples were analyzed in the frozen hydrated state.

Figure 3 Zinc K-edge K^3 -weighted extended X-ray absorption fine structure (EXAFS) spectra for model compounds (solid lines) with best linear combination fits (dashed black lines) (a). Stacked radial structure functions (not corrected for phase shift) from Zn K-edge K^3 -weighted EXAFS data (b). Solid and dashed lines are the Fourier transform magnitude and imaginary part, respectively.

Figure 4 Zinc K-edge K^3 -weighted extended X-ray absorption fine structure (EXAFS) spectra for powdered plant samples of HP *S. alfredii* treated with 100 μ M Zn (solid lines) with best linear combination fits (dashed black lines) (a). Stacked radial structure functions (not corrected for phase shift) from Zn K-edge K^3 -weighted EXAFS data (b). Solid and dashed lines are the Fourier transform magnitude and imaginary part, respectively.

Figure 5 μ -XRF imaging of Zn distribution in young stem (A), mid-aged stem (B) and old stem (C) cross-sections from HP *S. alfredii* treated with 100 μ M Zn for 30 d. The microscope

1 images show the stem region selected for μ -XRF imaging. The red color, depicting the
2 highest Zn concentrations, was scaled to the maximum value for each map.

3 **Figure 6** μ -XRF imaging of Zn distribution in young and mature leaf from HP *S. alfredii*
4 treated with 100 μ M Zn for 30 d. The camera images show the leaf region (A, young leaf; C,
5 mature leaf) selected for μ -XRF imaging (B, young leaf; D, mature leaf). The red color,
6 depicting elemental concentrations in each map, was scaled to the maximum value for each
7 map.

Figure 1 Total Zn concentration in different tissues of two *S. alfredii* populations. Leaves with different development stages, stems and roots were collected for each population. Values shown represent the mean $\pm$ SE of 15 plants. DW, dry weight.

12

13 **Figure 2** Zinc K-edge extended X-ray absorption fine structure (EXAFS) spectra recorded on
 14 roots, stems, and young and mature leaves of HP and NHP *S. alfredii*. All samples were
 15 analyzed in the frozen hydrated state.

Figure 3 Zinc K-edge K^3 -weighted extended X-ray absorption fine structure (EXAFS) spectra for model compounds (solid lines) with best linear combination fits (dashed black lines) (a). Stacked radial structure functions (not corrected for phase shift) from Zn K-edge K^3 -weighted EXAFS data (b). Solid and dashed lines are the Fourier transform magnitude and imaginary part, respectively.

Figure 4 Zinc K-edge K^3 -weighted extended X-ray absorption fine structure (EXAFS) spectra for powdered plant samples of HP *S. alfredii* treated with 100 μM Zn (solid lines) with best linear combination fits (dashed black lines) (a). Stacked radial structure functions (not corrected for phase shift) from Zn K-edge K^3 -weighted EXAFS data (b). Solid and dashed lines are the Fourier transform magnitude and imaginary part, respectively.

Figure 5 μ -XRF imaging of Zn distribution in young stem (A), mid-aged stem (B) and old stem (C) cross-sections from HP *S. alfredii* treated with 100 μ M Zn for 30 d. The microscope images show the stem region selected for μ -XRF imaging. The red color, depicting the highest Zn concentrations, was scaled to the maximum value for each map.

34

35 **Figure 6** μ-XRF imaging of Zn distribution in young and mature leaf from HP *S. alfredii*

36 treated with 100 μM Zn for 30 d. The camera images show the leaf region (A, young leaf; C,

37 mature leaf) selected for μ-XRF imaging (B, young leaf; D, mature leaf). The red color,

38 depicting the highest Zn concentrations, was scaled to the maximum value for each map.

Contributions

Lingli Lu, Xingcheng Liao, Xiaoe Yang and Shengke Tian designed and performed the research, Shengke Tian, Erik Nelson, and Yonghua Du analyzed the EXAFS data, Lingli Lu, John Labavitch and Patrick H. Brown wrote the paper.