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Research article

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

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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. 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 (Zhao and McGrath, 2009). The hyperaccumulators are capable of accumulating more than 100-fold higher amounts of metals in shoots than are most plants (Baker et al., 2000). The abundance of metals accumulated in the shoots generally results from efficient root uptake and xylem transport (Zhao et al., 2006; Lu et al., 2008), 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 (Sinclair and Krämer, 2012; Clemens et al., 2002). 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 (Sinclair and Krämer, 2012).

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

Abbreviations: HP, hyperaccumulating population; NHP, non-hyperaccumulating population; PBS, phosphate buffer solution; EXAFS, Extended X-ray absorption fine structure; μ-XRF, micro-synchrotron X-ray fluorescence.

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for the formation of metal–organic acid complexes (Haydon and Cobbett, 2007). 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 (Sarret et al., 2002; Salt et al., 1999). Vacuolar oxygen coordination of the metals was suggested in the leaves of *N. caerulescens* (Kupper et al., 2004). 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 (Salt et al., 1999). Ligands may also be secreted from cells to act as chaperones or buffers, thus enhancing the solubility or mobility of metals within plants (Sinclair and Krämer, 2012). 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 (Deinlein et al., 2012). 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 (Kerkeb and Kramer, 2003; Kramer et al., 1996).

Sedum alfredii Hance is a Zn/Cd cohyperaccumulator and lead (Pb) accumulator discovered in a Pb/Zn-rich region of China (Tian et al., 2010; Yang et al., 2004). This plant species is one of a few non-brassica Zn/Cd cohyperaccumulators identified so far (Kramer, 2010); 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 *S. alfredii* was mainly focused on rhizosphere processes, transport, and cellular distribution (Li et al., 2011; Long et al., 2009; Lu et al., 2013a; Tian et al., 2009). 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 (Tian et al., 2011), whereas Pb was largely retained in the cell walls during transportation in plants of *S. alfredii* (Tian et al., 2010). Zinc in stems and leaves of *S. alfredii* was preferentially located in the epidermis with a second storage in mesophyll cells (Tian et al., 2009), 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 (Salt et al., 1999; Kupper et al., 2004; Tian et al., 2011; Sarret et al., 2003). Metal speciation in plants can be tissue- or age-dependent, as reported for the hyperaccumulator *N. caerulescens* (Kupper et al., 2004). 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 *S. 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 $(NH_4)_6$ Mo_7O_{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 μmol m^{-2} 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 preculture 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. (2006).

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 10 K, essentially as described previously (Tian et al., 2010). Zinc K-edge spectra were recorded on Beamline 7-3 with an upstream Rh-coated collimating mirror, a Si(220) double-crystal monochromator, and a downstream focusing mirror. The incident X-ray intensity was monitored using a Kr-filled ionization chamber. The monochromator energy of each spectrum was calibrated using Zn metal foil between the second and third ionization chambers; its absorption edge was calibrated to an edge of 9659 eV. Zinc K_α fluorescences were recorded using a 30 element germanium detector (Canberra Industries, Meriden, CT) equipped with Soller slits and copper filters.

Fresh plant tissues were ground under liquid nitrogen and pressed into 2-mm path length Lucite sample holders with Kapton tape windows cooled in liquid nitrogen. To minimize breakdown and mixing of cellular components within the plant material, care was taken to keep the tissue frozen at all times during measurement. During data collection, samples were maintained approximately at 10 K in a liquid helium flow cryostat to minimize the loss of intensity in the signal. Zinc at room temperature gives a high signal-to-noise ratio because its Debye temperature is very low, samples were kept at the low temperature to avoid this and to minimize the oscillations of the low Zn ligands (i.e., O and C). Model complexes of Zn were measured as standards of the most important potential types of ligands in plants. Spectra of standard Zn species including ZnO (solid), $Zn(NO_3)_2$ (solid), aqueous Zn [$Zn(NO_3)_2$] (solution), Zn–citrate (solution), Zn–malate (solution), Zn–oxalate (solution), Zn–phytate (solution), Zn–histidine (solution), Zn–succinate (solution), Zn–glutathione (solution), 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 $Zn(NO_3)_2$, pH 6. Root cell wall material was exposed to 0.5 mM