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# Zinc compartmentation in root, transport into xylem, and absorption into leaf cells in the hyperaccumulating species of *Sedum alfredii* Hance

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**Abstract** *Sedum alfredii* Hance can accumulate Zn in shoots over 2%. Leaf and stem Zn concentrations of the hyperaccumulating ecotype (HE) were 24- and 28-fold higher, respectively, than those of the nonhyperaccumulating ecotype (NHE), whereas 1.4-fold more Zn was accumulated in the roots of the NHE. Approximately 2.7-fold more Zn was stored in the root vacuoles of the NHE, and thus became unavailable for loading into the xylem and subsequent translocation to shoot. Long-term efflux of absorbed $^{65}$Zn indicated that $^{65}$Zn activity was 6.8-fold higher in shoots but 3.7-fold lower in roots of the HE. At lower Zn levels (10 and 100 μM), there were no significant differences in $^{65}$Zn uptake by leaf sections and intact leaf protoplasts between the two ecotypes except that 1.5-fold more $^{65}$Zn was accumulated in leaf sections of the HE than in those of the NHE after exposure to 100 μM for 48 h. At 1,000 μM Zn, however, approximately 2.1-fold more Zn was taken up by the HE leaf sections and 1.5-fold more $^{65}$Zn taken up by the HE protoplasts as compared to the NHE at exposure times $>16$ h and $>10$ min, respectively. Treatments with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or ruptured protoplasts strongly inhibited $^{65}$Zn uptake into leaf protoplasts for both ecotypes. Citric acid and Val concentrations in leaves and stems significantly increased for the HE, but decreased or had minimal changes for the NHE in response to raised Zn levels. These results indicate that altered Zn transport across tonoplast in the root and stimulated Zn uptake in the leaf cells are the major mechanisms involved in the strong Zn hyperaccumulation observed in *S. alfredii* H.

**Keywords** Compartmentation · Hyperaccumulator Organic and amino acids · Protoplast · Zinc

**Abbreviations** CCCP: Carbonyl cyanide *m*-chloro phenylhydrazone · FAD: Fluorescein diacetate · PBS: Phosphate buffer solution · HE: Hyperaccumulating ecotype · NHE: Nonhyperaccumulating ecotype

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## Introduction

Metal-tolerant plants have the ability to survive and reproduce on soils containing high levels of metals in forms that are toxic or inimical to other plants (Macnair and Baker 1994). Metal-hyperaccumulating plants can store large amounts of metals in their aerial parts (Baker and Walker 1990), which make hyperaccumulators suitable for phytoremediation of metal-polluted soils (Brooks 1998; Baker et al. 2000; McGrath and Zhao 2003). Several metal-hyperaccumulator plant species (Baker and Brooks 1989) have been identified to have genetic potential for successful phytoremediation of contaminated soils. Transferring the genes conferring the hyperaccumulating phenotype to plants that produce more shoot biomass has been suggested as a potential avenue to make phytoremediation a more viable commercial technology (Brown et al. 1995a). However, progress towards this goal has been hindered by a lack of understanding of the basic biochemical, physiological, and molecular mechanisms involved in heavy metal hyperaccumulation (Van der Lelie et al. 2001).

Zinc is one of the most important metal contaminants in industrialized countries (Nriagu and Pacyna 1988), and

numerous studies have been conducted on the species *Thlaspi caerulescens* (Vázquez et al. 1994; Brown et al. 1994, 1995a, b; Lasat et al. 1996, 1998; Küpper et al. 1999; Salt et al. 1999; Frey et al. 2000; Assunçao et al. 2003a, b; Piñeros and Kochian 2003) and, to a lesser extent, on *Arabidopsis halleri* (Küpper et al. 1999, 2000; Bert et al. 2000; Zhao et al. 2000). Radiotracer efflux analysis is useful to investigate ion compartmentation in a semi-quantitative manner. It has been employed to provide valuable information on the efflux and compartmentation of several ions, such as $K^+$ (Kochian and Lucas 1982), $Na^+$ (Pierce and Higinbotham 1970), $Cd^{2+}$ (Rauser 1987), $Cu^{2+}$ (Thornton 1991), and $Zn^{2+}$ (Santa Maria and Cogliatti 1988), and $Co^{2+}$ (Macklon et al. 1990). A significant fraction of symplasmic Zn was stored in the root vacuole of *T. arvense*, and became unavailable for loading into the xylem and subsequent translocation to the shoot. In the superaccumulator *T. caerulescens*, however, a smaller fraction of the absorbed Zn was stored in the root vacuole and readily transported back into the cytoplasm (Lasat et al. 1998).

The buffer system in the plant cell comprises of organic acids, amino acids, phenolic compounds, metal carriers, and metal ligands of low molecular weight proteins, and has an important role in metal complexation and transport in the cell (Verkleij and Schat 1989; Ma et al. 1997a, b, 2001). Organic ligands help facilitate the long-distance transport of Zn in xylem (White et al. 1981) and may be instrumental in helping sequester Zn in the leaf cell vacuole. Histidine production and accumulation in the xylem were reported to be involved in Ni hyperaccumulation in shoots of *Alyssum* (Krämer et al. 1996).

*Sedum alfredii* Hance has been identified as a new Zn-hyperaccumulator plant native to China, and can accumulate more than 20,000 mg $kg^{-1}$ Zn in shoots from a hydroponic medium before symptoms of Zn toxicity occur (Yang et al. 2002). This plant has exceptional abilities to tolerate and accumulate high concentrations of Zn/Cd, and the characteristics of large biomass, rapid growth, asexual propagation, and it is a perennial. Therefore, it is an ideal plant for studying mechanisms responsible for hyperaccumulation and the practice of phytoremediation (Yang et al. 2001, 2002, 2004; Ye et al. 2003).

In this study, hydroponically grown HE and NHE *S. alfredii* H seedlings were used to investigate several mechanisms possibly involved in Zn hyperaccumulation: (1) Zn compartmentation in the root and transport from the root symplasm into the shoot (2) Zn transport into leaf cells and (3) roles of low-MW organic ligands in Zn complexation and transport to shoot or leaf cells.

## Materials and methods

### Plant origins and culture

The hyperaccumulating ecotype (HE) of *S. alfredii* H was obtained from an old Pb/Zn mine area in Zhejiang Province in China, and the nonhyperaccumulating ecotype (NHE) of *S. alfredii* H was obtained from a tea garden of Hangzhou in Zhejiang Province.

Healthy and uniform shoots of two ecotypes of *S. alfredii* H were chosen and grown for 2 weeks in the basal nutrient solution containing 2 mM $Ca^{2+}$, 4 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 $MnSO4{\cdot}H_2O$, 1 μM $ZnSO_4{\cdot}7H_2O$, 0.2 μM $CuSO_4{\cdot}5H_2O$, 0.01 μM $(NH_4)_6$ $Mo_7O_{24}$, 100 μM Fe–EDTA. Nutrient solution pH was adjusted daily to 5.5 with 0.1 M NaOH or 0.1 M HCl. Plants were grown under glasshouse conditions with natural light, day/night temperature of 26/20°C and day/night humidity of 70/85%. The nutrient solution was aerated continuously and renewed every 4 days.

### Zinc accumulation in *S. alfredii* H

Seedlings of *S. alfredii* H were precultured for 14 days (for the initiation of new roots) prior to exposure to different $Zn^{2+}$ levels. The treatments were composed of control (1 μM Zn), 50, 250, 500 μM $Zn^{2+}$, supplied as the sulfate, with three replications for each treatment. Nutrient solutions were aerated continuously and renewed every 4 days. The pH was maintained at 5.5. Plants were harvested after exposure to metal treatments for 20 days. At harvest, roots were soaked in 20 mM $Na_2$–EDTA for 15 min to desorb putatively adsorbed $Zn^{2+}$ on root surfaces. The harvested plants were separated into leaves, stems, and roots, and oven-dried at 65°C for 48 h.

The dried plant materials were then ground using a stainless steel mill and passed through a 0.25-mm sieve for elemental analysis. Dry plant samples (0.1 g) of each treatment were ashed in a muffle furnace at 550°C for 5 h. The ash was dissolved in 5 ml 1:1 HCl, and the digest was transferred to a 50-ml volumetric flask, made up to volume and filtered. Concentrations of $Zn^{2+}$ in the filtrate were analyzed using a flame Atomic Absorption Spectrophotometer (AA 6800, Shimadzu, Tokyo).

### Subcellular compartmentation of $^{65}Zn^{2+}$ in roots

The $^{65}$Zn-efflux studies (short- and long-term) were performed following the methods described by Lasat et al. (1998) with minor modifications. In the short-term efflux studies, seedlings of 20-day-old *S. alfredii* H were incubated with roots in 'Plexiglas' uptake wells filled with 50 ml of aerated solution containing 2 mM Mes (pH 6.0), 0.5 mM $CaCl_2$, and 20 μM $^{65}Zn^{2+}$ (45 kBq $l^{-1}$) (as $ZnCl_2$). After 24 h, the radioactive uptake solution was removed, roots were rinsed with deionized water, and wells were refilled with an efflux solution (2 mM Mes–Tris buffer, pH 6.0, 0.5 mM $CaCl_2$, and 20 μM $Zn^{2+}$). At the efflux time intervals of 0, 1, 5, 10, 15, 20, 30, 60, 120, 180, 240, 300, 360 min, an

aliquot of 5-ml efflux solution was collected for $^{65}Zn^{2+}$ analysis using a gamma counter (GR2519, Canberra Co., Merden, CT, USA). For the long-term $^{65}Zn$ efflux studies, roots of *S. alfredii* H seedlings were incubated for 24 h in a plastic container filled with 1,000 ml of the uptake solution containing 20 μM $^{65}Zn^{2+}$ (45 kBq $l^{-1}$). After uptake for 24 h, the radioactive solution was removed, roots were rinsed with deionized water, and the container was refilled with nonradioactively labeled efflux solution. The efflux time intervals were 0, 3, 6, 12, 12, 24, 48 h, respectively. The efflux of $^{65}Zn^{2+}$ from roots was determined by measuring the activities of $^{65}Zn$ in the efflux solution over time.

## Organic acid and free amino acid analysis

Seedlings of *S. alfredii* H were precultured for 14 days (for initiating new roots) before they were exposed to different $Zn^{2+}$ levels. The treatments were: 1 μM (low Zn) and 500 μM (high Zn) for HE, and 1 μM and 50 μM for NHE. Each treatment was replicated three times. Plants were harvested after being exposed to the Zn treatments for 8 days.

### *Organic acid analysis*

Portions of 1 g fresh sample of leaves or stems frozen in liquid $N_2$ were ground for 2 min and placed into 25-ml polypropylene centrifuge tubes, 10 ml of 5% $H_3PO_4$ was added to each tube and the suspension centrifuged at 8,000 *g* for 20 min. The supernatant solution was collected, and 1 g colophony was added. The solution was then shaken for 30 s, allowed to settle for 15 min, and then centrifuged at 8,000 *g* for 15 min. One milliliter of the supernatant solution was transferred into 3-ml polypropylene tubes and 1 ml of PBS was added. The tubes were then stored at −40°C prior to analysis. Organic acids in the supernatant solution were analyzed using a HPLC (Shimadzu LC-10ATvp, Tokyo) with an ion-exchange analytical column $C_{18}$ (5 μm, 250×4.6 mm) and an eluent of 15 mM $KH_2PO_4$ (pH 2.5). Organic acids were detected by a UV detector (Shimadzu SPD-10ATvp) at 205 nm.

### *Free amino acid analysis*

Fresh samples of leaves or stems were frozen in liquid $N_2$, ice-dried at −60°C under a vacuum. The dried plant materials were ground to $<0.25$ mm using a stainless steel mill and stored at 4°C prior to analysis. Two grams of subsamples of leaves or stems were placed into 50-ml volumetric flasks, and the flasks were made up to volume with 0.02 mol $l^{-1}$ HCl, stood for 2 h, and then shaken intermittently. The contents were filtered through a 0.45 μm membrane, and the filtrate purified with an AccQ–Taq column. Amino acids were analyzed using a HPLC (Waters Alliance 2690, Ann Arbor, MI, USA). Amino acids were identified via a LS-50B (Perkin Elmer, Boston, MA, USA) fluorescence detector at an excitation wavelength of 250 nm and an emission wavelength of 395 nm.

## Uptake of $^{65}Zn^{2+}$ by leaf sections

The leaves of 20-day-old seedlings were cut into 10–20 $mm^2$ sections. The leaf sections were then immersed in an aerated uptake solution containing 10, 100, or 1,000 μM $^{65}ZnCl_2$ (45 kBq $l^{-1}$), 2 mM Mes (pH 6.0), and 0.5 mM $CaCl_2$. At different time intervals of up to 48 h, $^{65}Zn^{2+}$ uptake was terminated by replacing radioactive solution with desorbing solution containing 2 mM $CaCl_2$, 2 mM Mes (pH 6.0), and 100 μM $ZnCl_2$. After 20 min of desorption to remove apoplastic $^{65}Zn^{2+}$, the leaf sections were blotted and weighed, and leaf $^{65}Zn^{2+}$ radioactivity was measured using the gamma counter.

## Uptake of $^{65}Zn$ by leaf protoplasts

### *Protoplast isolation*

Leaf protoplasts were isolated following the method described by Lee et al. (2001) and Lasat et al. (1998) with some modifications. Fully expanded leaves of 20-day-old seedlings were chopped into 1–2 $mm^2$ sections. Approximately, 5 g of fresh leaf fragments were transferred to 30 ml of filter-sterilized enzyme mixture composed of 500 mM mannitol, 5 mM Mes–Tris buffer (pH 5.8), 2 mM $CaCl_2$, 1 mM DTT, 0.7 mM $KH_2PO_4$, 0.1% (w/v) BSA, 2% (w/v) cellulase, and 0.2% (w/v) pectolyaseY-23. Tissues were incubated on a horizontal shaker at 30 rpm and 25°C in the dark for 1–2 h, and shaken intermittently to increase the yield of protoplasts. The resulting suspension was filtered through a 0.05 mm stainless sieve and protoplasts were pelletized by centrifugation at 60 *g* for 5 min at 4°C. From this step onwards, the protoplasts suspension was maintained on ice at all times. The pelletized protoplasts were resuspended in protoplast buffer (600 mM mannitol, 5 mM Mes–Tris buffer (pH 5.5), 2 mM $CaCl_2$, 1 mM DTT, 0.7 mM $KH_2PO_4$, and 0.1%(w/v) BSA), and the suspension was centrifuged. After two additional re-suspending processes, the pellet was then resuspended in 1 ml of preuptake solution consisting of 600 mM mannitol, 50 mM sucrose, 5 mM Mes–Tris (pH 5.5), 0.05 mM $CaCl_2$, 1 mM DTT, and 0.7 mM $KH_2PO_4$. The number of protoplasts was determined using a hemocytometer, and the percentage of viable protoplasts was determined using a fluorescence microscope (model E600, Nikon). Protoplast viability was 80 and 78%, respectively, for HE and NHE *S. alfredii* H.

### *$^{65}$Zn-uptake experiments with protoplasts*

The experiments on $^{65}$Zn-uptake by protoplasts were conducted according to the method described by Lasat et al. (1998). Zinc levels in the preuptake solution were 10, 100, and 1,000 μM $^{65}ZnCl_2$ (25 kBq $^{65}$Zn $l^{-1}$). The final protoplast concentration in the uptake solution was $6.4\times10^7$and $7.8\times10^7$ protoplasts $ml^{-1}$ for HE and NHE *S. alfredii* H, respectively. At different time intervals of up to 16 min, a 100-μl aliquot of the radioactive protoplast suspension was removed and the uptake of $^{65}Zn^{2+}$ into protoplasts was quantified using a gamma detector.

To determine whether Zn accumulation in *S. alfredii* H protoplasts was caused by $^{65}Zn^{2+}$ movement across the plasma membrane into the cytosol or by the binding of Zn to negatively charged sites associated with the external surface of the plasma membrane, the time course of Zn uptake in intact versus ruptured *S. alfredii* H protoplasts was studied. To rupture the plasma membrane, protoplasts were frozen in liquid $N_2$ and then thawed at room temperature. A stock of ruptured and intact protoplasts was used to conduct a time-course study of $^{65}Zn^{2+}$ uptake from a 10 μM Zn solution. Furthermore, the effect of the metabolic inhibitor CCCP on the time-dependent kinetics of $^{65}$Zn uptake in protoplasts was examined. In this experiment, 10 μM CCCP was added to the protoplast stock 30 min prior to beginning of the experiment. The time-course study was conducted in an uptake solution containing 10, 100, or 1,000 μM $^{65}Zn^{2+}$(25 kBq $l^{-1}$), as described above.

### Statistical analysis of data

All data were statistically analyzed 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.

## Results

### Plant growth and Zn accumulation in *S. alfredii* H

At Zn levels from 1 to 500 μM, HE *S. alfredii* grew normally, and there were no visual toxic symptoms. However, plant growth of NHE was significantly inhibited at Zn levels ≥250 μM, with toxic symptoms of smaller, wilting leaves and putrescence on root tip. Shoot biomass of HE increased but that of NHE decreased with increasing Zn levels (Table 1). An increase in root biomass occurred for HE with increasing Zn levels, and high Zn level (500 μM) increased root biomass by 29%. In contrast, root dry weight of NHE decreased by 46% at 500 μM Zn. These results indicate that shoot and root growth of NHE was inhibited by Zn levels ≥250 μM, whereas stimulation of shoot, and especially root growth, occurred in HE at 500 μM Zn.

Zinc concentrations varied greatly in the leaves and stems, but were similar in the roots between the two ecotypes. After 20 days growth in solution with various Zn levels (1–500 μM), NHE accumulated more $Zn^{2+}$ in roots, whereas most of the Zn was translocated to the shoot in HE. At the 50 μM $Zn^{2+}$, leaf and stem Zn concentrations of the HE were 24- and 28-fold higher, respectively, than those of the NHE, whereas 1.4-fold more Zn was accumulated in the roots of the NHE than in the HE (Table 2). At Zn levels ≤ 500 μM, Zn concentrations in the tissue decreased in the order: stem > leaf > root for the HE, but root > stem > leaf for the NHE. These results indicate that HE *S. alfredii* has an extraordinary ability to absorb and transport Zn to the shoot.

### Zn compartmentation in the root

A short-term (6 h) study was conducted on the kinetics of $^{65}Zn^{2+}$ efflux from the roots of two ecotypes *S. alfredii* in order to understand the subcellular compartmentation of $^{65}Zn^{2+}$ in roots. The first-order kinetic plot of $^{65}Zn^{2+}$ efflux from *S. alfredii* roots (log $^{65}$Zn remaining in the roots as a function of time) could be divided into three linear phases representing $^{65}Zn^{2+}$ efflux from three compartments in series: the vacuole, cytoplasm, and cell wall (Fig. 1). The straight line represents the slowest-exchanging phases (120–360 min), i.e., $^{65}Zn^{2+}$ efflux from the vacuole (Fig. 1a). Subtraction of the linear component from total efflux data (Fig. 1a) yielded a second curve (Fig. 1b), which represents $^{65}Zn^{2+}$ efflux from the cytoplasm (30–60 min) and cell wall (0–20 min). Further subtracting the linear phase associated with the cytoplasmic efflux from the data points resulted in a third curve (Fig. 1c), which reflects $^{65}Zn^{2+}$ efflux from the cell wall (0–20 min). The half-times for $^{65}Zn^{2+}$ efflux from the vacuole, cytoplasm, and cell wall were estimated from the slope of the lines in Fig. 1a–c, and $^{65}Zn^{2+}$ distribution in the different organs of root cells at the termination of the radioisotope-loading period could be calculated from the *y*-axis intercepts of these lines in Fig. 1a, b, and c, respectively (Table 3). The results indicate that at the end of a 24-h radioisotope-loading period, comparable amounts of $^{65}Zn^{2+}$ were accumulated in the roots of the two ecotypes of *S. alfredii*. The $^{65}Zn^{2+}$ compartmentation in the root cell wall was slightly higher in the HE (63.5%) than in the NHE (55.8%) of *S. alfredii* H, whereas similar distribution of $^{65}Zn^{2+}$ in cytoplasm (around 31%) of the two ecotypes *S. alfredii* occurred (Table 3). However, approximately 2.7-fold more $^{65}Zn^{2+}$ was accumulated in the root vacuole of the NHE than the HE (Table 3; Fig. 1a). The half-time for $^{65}Zn^{2+}$ efflux from the vacuole was 40% shorter in the HE than the NHE, indicating that Zn efflux from the root vacuole of the HE is approximately 1.8-fold faster than that in the NHE (Table 3). The

**Table 1** Effects of Zn levels on the yields of two ecotypes *Sedum alfredii* H

| Zn levels (μM) | Nonhyperaccumulating ecotype [NHE (g plant$^{-1}$ DW)] | | | Hyperaccumulating ecotype [HE (g plant$^{-1}$ DW)] | | |
|---|---|---|---|---|---|---|
| | Leaves | Stems | Roots | Leaves | Stems | Roots |
| 1 | 0.38 | 0.20 | 0.071 | 0.41 | 0.22 | 0.068 |
| 50 | 0.33 | 0.18 | 0.066 | 0.42 | 0.19 | 0.075 |
| 250 | 0.29 | 0.16 | 0.051 | 0.39 | 0.20 | 0.081 |
| 500 | 0.21 | 0.14 | 0.038 | 0.44 | 0.21 | 0.088 |
| $LSD_{0.05}$ | 0.065 | 0.028 | 0.021 | 0.07 | 0.035 | 0.018 |

The seedlings were precultured for 14 days before they were exposed to different Zn levels, plants were harvested after exposed to the metal treatment for 20 days. The data are means of three replications, and $LSD_{0.05}$ represents the least significant differences ($P < 0.05$) among the corresponding group of data (column)

**Table 2** Zinc accumulation (g kg$^{-1}$ DW) in two ecotypes *S. alfredii* H exposed to different Zn levels in nutrient solution

| Zn levels (μM) | HE | | | NHE | | |
|---|---|---|---|---|---|---|
| | Leaves (g kg$^{-1}$) | Stems (g kg$^{-1}$) | Roots (g kg$^{-1}$) | Leaves (g kg$^{-1}$) | Shoots (g kg$^{-1}$) | Roots (g kg$^{-1}$) |
| 1 | 9.89 ± 0.52 | 11.20 ± 0.61 | 1.31 ± 0.20 | 0.49 ± 0.11 | 0.52 ± 0.10 | 0.71 ± 0.09 |
| 50 | 12.00 ± 1.21 | 16.81 ± 0.98 | 2.11 ± 0.18 | 0.50 ± 0.09 | 0.59 ± 0.14 | 2.94 ± 0.21 |
| 250 | 14.95 ± 0.92 | 20.10 ± 1.60 | 3.24 ± 0.56 | 0.72 ± 0.08 | 0.94 ± 0.26 | 5.21 ± 0.52 |
| 500 | 17.36 ± 1.23 | 21.94 ± 1.94 | 3.69 ± 0.51 | 0.65 ± 0.07 | 0.92 ± 0.19 | 6.81 ± 0.50 |
| $LSD_{0.05}$ | 2.646 | 2.817 | 0.939 | 0.192 | 0.226 | 2.097 |
| $LSD_{0.01}$ | 3.626 | 3.860 | 1.287 | 0.251 | 0.310 | 2.876 |

The results are presented as means ± SE of three replications, and $LSD_{0.05}$ represents the least significant differences ($P < 0.05$) among the corresponding group of data (column)

half-times of $^{65}Zn^{2+}$ efflux from the cell wall and cytoplasm were similar for the HE and NHE (Table 3).

In the long-term (48 h) efflux experiment, $^{65}Zn^{2+}$ efflux from the root of HE *S. alfredii* could be resolved into two components, with the first 12 h initial rapid phase followed by a subsequent 36 h slower phase. Approximately 3.7-fold more $Zn^{2+}$ remained in the NHE roots, as compared with the HE at the end of the 48-h washout period (Fig. 2a). However, during the same period, 6.8-fold more $^{65}Zn^{2+}$ was translocated from the root to the shoot of HE (Fig. 2b). These results indicate that less $^{65}Zn^{2+}$ being compartmented into root vacuole is closely associated with the faster $^{65}Zn^{2+}$ translocation to the shoot of the hyperaccumulator *S. alfredii* H.

## Uptake of $^{65}Zn^{2+}$ into leaf tissues

$^{65}Zn^{2+}$ accumulation in leaf sections was biphasic. The initial rapid phase may involve Zn movement into the apoplast through the residual cuticle and cut edges of the leaf sections (Fig. 3). At 10 μM Zn, there were no detectable differences in leaf Zn accumulation between the two ecotypes *S. alfredii* for all exposure times (Fig. 3a). At 100 μM Zn, $^{65}Zn$ uptake by leaf sections were similar between the two ecotypes except for HE leaves which took up 1.5-fold more Zn than NHE ($P < 0.05$) at the uptake period of 48 h (Fig. 3b). However, at 1,000 μM Zn, approximately 2.1-fold more Zn was accumulated in HE leaf sections for exposure times > 10 h (Fig. 3c).

**Table 3** $^{65}Zn^{2+}$ compartmentation in roots of HE and NHE *S. alfredii* H

| Species | Cell wall | Cytoplasm | Vacuole |
|---|---|---|---|
| NHE | | | |
| $^{65}Zn^{2+}$ (Bq) | 56,312 | 31,300 | 13,400 |
| $^{65}Zn^{2+}$ (%) | 55.8 | 30.9 | 13.3 |
| $t_{1/2\ (min)}$ | 9.0 | 29.1 | 298.5 |
| HE | | | |
| $^{65}Zn^{2+}$ (Bq) | 62,795 | 30,900 | 4,950 |
| $^{65}Zn^{2+}$ (%) | 63.5 | 31.3 | 5.2 |
| $t_{1/2\ (min)}$ | 8.7 | 36.1 | 214.1 |

Bq values were obtained from the intersection of the extrapolated linear components shown in Fig. 1 at the *y* axis

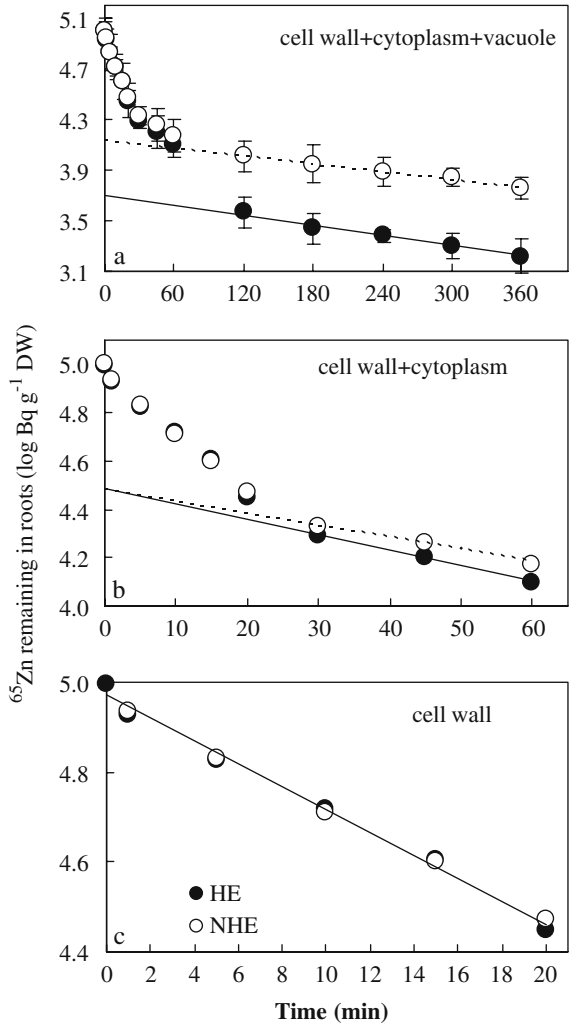


**Fig. 1 a–c** Short-term efflux of $^{65}Zn^{2+}$ from roots of *Sedum alfredii* H. After 24-h incubation in an uptake solution containing 20 μM $^{65}Zn^{2+}$, and the radioactive uptake solution was replaced with an identical, nonlabeled solution containing 20 μM $Zn^{2+}$. Efflux of $^{65}Zn^{2+}$ from roots into the external solution was subsequently measured during a 6-h period. *Lines* represent regressions of the linear portion of each curve extrapolated to the *y* axis. The *curve* shown in **b** was derived by subtracting the linear component in **a** from the data points in **a**. *Curve* in **c** was derived from the *curve* in **b** in a similar manner. *Data points* in **a** represent means ± SE of three replications

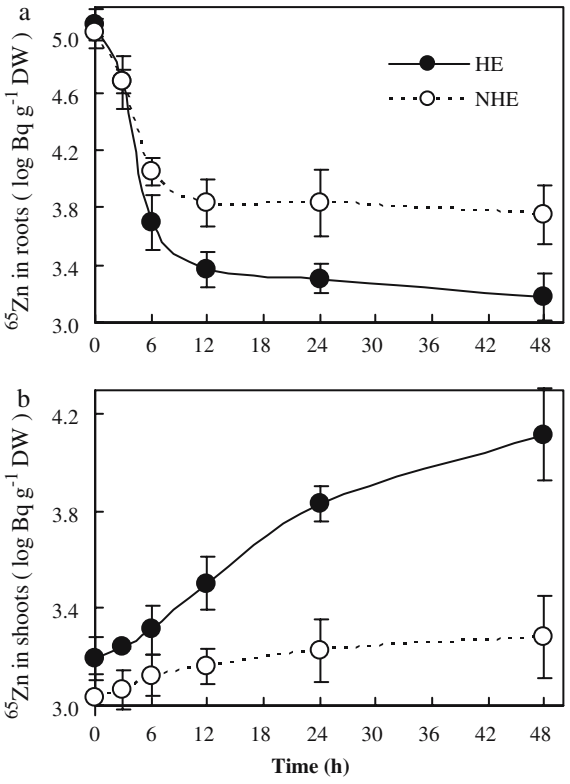


**Fig. 2 a, b** Long-term efflux of $^{65}Zn^{2+}$ from roots (**a**) and $^{65}Zn^{2+}$ translocation to shoots (**b**) of *S. alfredii* H. Seedlings were immersed with roots in a 20 μM $^{65}Zn^{2+}$ uptake solution. After a 24-h loading period, roots were rinsed in deionized water, and the radioactive uptake solution was replaced with an identical, nonlabeled solution containing 20 μM $Zn^{2+}$. At different time intervals up to 48 h, one seedling of each ecotype *S. alfredii* H was removed; roots were excised, blotted, and oven dried at 65°C; root dry weight was recorded, and gamma activity was measured. *Data points* represent means ± SE of three replications

## Uptake of $^{65}Zn^{2+}$ into leaf protoplasts

Leaf Zn transport was also studied at the cellular level using protoplasts isolated from leaves of the two ecotypes *S. alfredii*. The time dependence of $^{65}Zn^{2+}$ uptake was measured in solutions containing 10, 100, and 1,000 μM $^{65}Zn^{2+}$ (Fig. 4). There were no significant differences in $^{65}Zn^{2+}$ uptake by the isolated protoplasts between the two ecotypes of *S. alfredii* at 10 or 100 μM Zn. $^{65}Zn^{2+}$ uptake was 1.5-fold higher by the protoplasts isolated from HE leaves as compared to NHE at 1,000 μM Zn for more than 10 min, which probably resulted from different induction of Zn transport across the tonoplast in HE and NHE *S. alfredii*. Exposure of protoplasts to the protonophore and metabolic inhibitor CCCP (10 μM) elicited an inhibition > 50% of $^{65}Zn^{2+}$ uptake into the protoplasts of both ecotypes *S. alfredii* (Fig. 4). At the lower $^{65}Zn^{2+}$ levels (10 and 100 μM $^{65}Zn^{2+}$), there were no detectable differences in protoplasts $^{65}Zn^{2+}$ between the two ecotypes *S. alfredii* when exposure to CCCP and at higher $^{65}Zn^{2+}$ level (1,000 μM $^{65}Zn^{2+}$). There was a weak tendency of higher $^{65}Zn^{2+}$ uptake in HE protoplasts, but the difference was not statistically significant.

Uptake characteristic of $^{65}Zn^{2+}$ into liquid $N_2$-ruptured protoplasts was similar to those when exposure of protoplasts to CCCP (Fig. 4). $^{65}Zn^{2+}$ uptake was saturated rapidly (in less than 2 min), with minimal subsequent accumulation of $^{65}Zn^{2+}$. At the end of a 16-min uptake period, approximately 2.4- and 2.3-fold more $^{65}Zn^{2+}$ were accumulated in intact than ruptured protoplasts of the HE and NHE, respectively (Fig. 4).

Organic acid and free amino acid in *S. alfredii* H

In the leaves and stems of two ecotypes *S. alfredii*, the predominant organic acids were malic, followed by oxalic and citric acids. Fumarate, pyruvate, acetic acid, and amber acid were not detected (Table 4). When Zn level in solution increased from 1 to 500 μM, oxalic acid concentrations in leaves and stems increased significantly for both ecotypes. Citric acid increased by 2.5-fold in leaves and 1.6-fold in stems of HE, respectively, whereas minimal changes in citric acid concentrations in both leaves and stems occurred for NHE. Malic acid increased only in leaves of HE when Zn level was raised from 1 to 50 μM (Table 4). Both ecotypes contained similar levels of malic acid, but citric and oxalic acid concentrations in leaves and stems were higher for HE than for NHE at both low and high Zn levels.

The predominant free amino acids were asparagine (Asp), serine (Ser), arginine (Arg), alanine (Ala), γ-aminobutyric (GABA), and leucine (Leu) in leaves and stems for both ecotypes *S. alfredii* (Tables 5, 6). Free methionine (Met), glycine (Gly) and cysteine (Cys) were not detected. Differential responses of free amino acid concentrations to Zn levels occurred between the two ecotypes. Arg was the most abundant free amino acid in both ecotypes but its production was not responsive to increasing Zn level. Response of Pro concentration in leaves and stems to Zn levels was strong in both ecotypes. Both leaf and stem concentrations of free Asp, Ser, Ala, and Gaba in the HE slightly increased in response to rising Zn level, but were not statistically significant. Asp in both leaves and stems and GABA in leaves of the NHE significantly decreased at 50 μM Zn (Tables 5, 6). Val concentration was raised by 3.6-fold in leaves and by 1.9-fold in stems of the HE when grown at 500 μM Zn, exhibiting the greatest response to Zn level among all the free amino acids. An increase in free Lys occurred in leaves and stems of the HE. Dramatic decreases in stem Val concentration were measured in the NHE when Zn levels were raised from 1 to 50 μM (Tables 5, 6). These results suggest that Val and Asp may be involved in Zn loading into leaf cells and Zn hyperaccumulation in *S. alfredii* H.

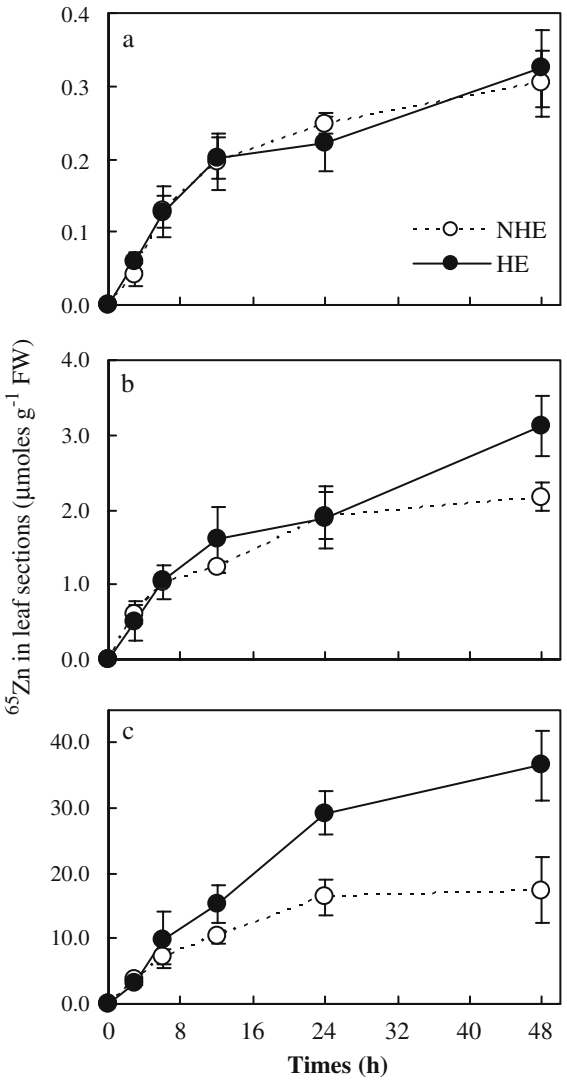


**Fig. 3 a–c** Time course of $^{65}Zn^{2+}$ accumulation in leaf sections of the two ecotypes of *S. alfredii* H. Leaves of HE and NHE were cut into 10–20 mm$^2$ sections and immersed in an aerated uptake solution containing 2 mM Mes–Tris buffer, pH 6.0, 0.5 mM $CaCl_2$, 10 μM (**a**), 100 μM (**b**), or 1,000 μM (**c**) $^{65}ZnCl_2$ (45 kBq l$^{-1}$). After exposures for up to 48 h, leaf sections were treated with desorbing solution (see Material and methods), blotted and weighed, and leaf $^{65}Zn^{2+}$ radioactivity was measured with the gamma counter. *Data points and error bars* represent means ± SE of three replications

**Table 4** Concentration of organic acid (μmol g$^{-1}$ FW) in leaves and stems of two ecotypes *S. alfredii* H

| Organ | Organic acids | HE | | NHE | | LSD $_{0.05}$ |
|---|---|---|---|---|---|---|
| | | 1 μM Zn | 500 μM Zn | 1 μM Zn | 50 μM Zn | |
| Leaves | Malic acid | 48.42 | 51.38 | 49.40 | 46.09 | 10.038 |
| | Oxalic acid | 10.39 | 21.38 | 8.43 | 14.90 | 2.747 |
| | Citric acid | 0.92 | 2.28 | 0.81 | 0.94 | 0.240 |
| Stems | Malic acid | 39.98 | 36.39 | 39.90 | 41.10 | 7.724 |
| | Oxalic acid | 5.96 | 8.70 | 3.24 | 6.36 | 1.526 |
| | Citric acid | 1.89 | 3.06 | 1.80 | 2.01 | 0.360 |

Plants were grown at 1 and 50 or 500 μM $Zn^{2+}$ for 8 days after being precultured in nutrition solution for 22 days. The results are presented as means of three replications, and $LSD_{0.05}$ represents the least significant differences ($P < 0.05$) among the corresponding group of data (line)

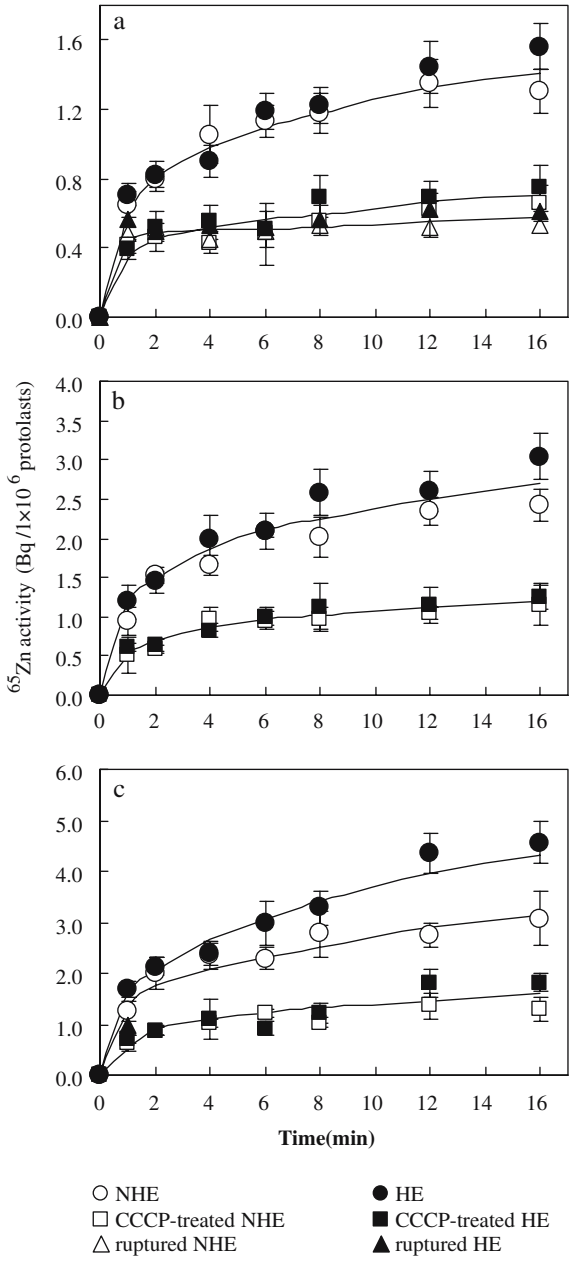


**Fig. 4 a–c** Time course of $^{65}Zn^{2+}$ accumulation in protoplasts isolated from the two ecotypes *S. alfredii* H. Protoplasts isolated from HE and NHE leaves were suspended in an uptake buffer containing 10 μM (**a**), 100 μM (**b**), or 1,000 μM (**c**) $^{65}ZnCl_2$. At different time intervals of up to 16 min, protoplasts were pelleted by centrifugation and the gamma activity was measured. A stock of ruptured protoplasts was used to conduct a time-course study of $^{65}Zn^{2+}$ uptake from a 10 μM Zn solution **a**. For the experiments with CCCP, protoplasts were exposed to 10 μM CCCP for 30 min prior to the uptake experiment

## Discussion

Zinc hyperaccumulation in plants could be controlled by various processes (Yang et al. 2005), including stimulated root absorption and xylem transport as well as leaf cell uptake, which have been reported to be involved in Zn hyperaccumulation in *T. caerulescens* (Vázquez et al. 1994; Lasat et al. 1996, 1998; Küpper et al. 1999; Salt et al. 1999; Frey et al. 2000; Piñeros and Kochian 2003; Cosio et al. 2004) and *Arabidopsis halleri* (Bert et al. 2000; Küpper et al. 2000; Zhao et al. 2000). *S. alfredii* H, belonging to Cruassulaceae family, has been recently identified as Zn/Cd hyperaccumulator native to China (Yang et al. 2002, 2004). Studies on the phytogenetic tree of over 10 *Sedum* species using RAPD methods revealed that the HE and NHE ecotype of *S. alfredii* have the closest genetic linking (data not shown), and belong to the same species. HE *S. alfredii* could accumulate Zn to over 2.0% in shoots at optimal Zn level (500 μM) and over 1.0% even at the low Zn level (1.0 μM) (Table 1). At 50 μM $Zn^{2+}$, leaf and stem Zn concentrations of HE were 24- and 28-fold higher, respectively, than those of NHE, with Zn tolerance and hyperaccumulation ability being close to those of *T. caerulescens* and *A. halleri*. This short-term Zn uptake kinetics study indicated a small difference in the Zn uptake constant ($K_m$) but 3.5 times greater maximum $^{65}Zn$ influx velocity ($V_{max}$) in HE than NHE *S. alfredii* (Li et al. 2005a), which are in agreement with the results obtained from the hyperaccumulator *T. caerulescens* (Lasat et al. 1996). The stimulated Zn influx rate in *T. caerulescens* is considered to be partly regulated by a constitutively high expression of ZNT1, a high affinity Zn transporter, in both roots and shoots (Pence et al. 2000), but whether *S. alfredii* has similar molecular mechanisms is unknown. More Zn (1.4-fold) was accumulated in the roots of NHE compared to the HE (Table 2), which may be attributed to the greater amount of Zn sequestered in the root vacuole of NHE than HE (Fig. 1; Table 3). This portion of Zn may be unavailable for loading into the xylem. The long-term efflux of absorbed $^{65}Zn$ indicated that residual $^{65}Zn$ was 6.8-fold higher in the shoots, but 3.7-fold lower in the roots of the HE, implying that Zn absorbed by roots of this hyperaccumulator is readily available for loading into the xylem and transport to shoots. Zinc xylem translocation was reported to be fivefold greater in *T. caerulescens* as compared to *T. arvense* (Lasat et al. 1998). Certain processes associated with Zn loading into the xylem and xylem translocation may also be stimulated in HE *S. alfredii*.

As one of the major storage sites for Zn is the leaf vacuole (Vázquez et al. 1994), there is a possibility that Zn influx across the leaf cell plasma membrane is stimulated in HE *S. alfredii*. At lower Zn levels (10 and 100 μM), there were no significant differences in $^{65}Zn$ uptake into leaf sections for both ecotypes for all exposure times except that HE leaf sections took up significantly higher $^{65}Zn$ ($P < 0.01$) than NHE only when exposure to100 μM Zn for 48 h (Fig. 3). However, at the higher Zn level (1,000 μM) $^{65}Zn$ accumulation in leaves of HE was significantly stimulated with increasing exposure times, indicating that Zn transport is stimulated at the leaf cell plasma membrane in HE *S. alfredii* as well, consistent with reports for *T. caerulescens* (Lasat

**Table 5** Concentrations of free amino acids (μmol 100 $g^{-1}$ FW) in leaves of two ecotypes *S. alfredii* H

| Amino acids | HE | | NHE | | $LSD_{0.05}$ |
|---|---|---|---|---|---|
| | 1 μM $Zn^{2+}$ | 500 μM $Zn^{2+}$ | 1 μM $Zn^{2+}$ | 50 μM $Zn^{2+}$ | |
| Asp | 52.37 ± 9.14 | 64.60 ± 11.65 | 89.44 ± 16.12 | 59.40 ± 7.86 | 21.886 |
| Ser | 93.22 ± 15.99 | 124.60 ± 22.46 | 79.90 ± 14.40 | 59.00 ± 7.70 | 29.928 |
| Glu | 20.73 ± 3.59 | 26.53 ± 4.78 | 25.10 ± 4.52 | 26.82 ± 3.55 | 7.805 |
| His | 13.62 ± 2.36 | 14.10 ± 2.54 | 14.90 ± 2.69 | 16.50 ± 2.18 | 4.612 |
| Arg | 106.95 ± 18.54 | 109.90 ± 19.81 | 17.30 ± 3.12 | 24.45 ± 3.23 | 25.885 |
| Thr | 23.53 ± 4.08 | 22.70 ± 4.09 | 24.30 ± 4.38 | 25.20 ± 3.33 | 7.506 |
| Ala | 55.07 ± 9.54 | 64.02 ± 11.54 | 61.72 ± 11.12 | 49.40 ± 6.54 | 18.614 |
| Gaba | 60.83 ± 15.42 | 71.00 ± 12.80 | 48.03 ± 8.65 | 32.43 ± 4.28 | 20.848 |
| Pro | – | 10.53 ± 2.01 | 18.20 ± 3.28 | 29.50 ± 3.90 | – |
| Tyr | – | – | 11.50 ± 2.07 | 10.50 ± 1.39 | – |
| Val | 17.94 ± 4.93 | 65.80 ± 11.86 | 18.80 ± 3.39 | 13.40 ± 1.77 | 12.621 |
| Lys | 13.62 ± 4.18 | 28.70 ± 5.17 | 23.94 ± 4.31 | 23.90 ± 3.16 | 8.026 |
| Ile | 11.30 ± 3.27 | 17.54 ± 3.15 | 23.40 ± 4.22 | 3.10 ± 0.41 | 7.004 |
| Leu | 50.58 ± 1.043 | 47.60 ± 8.58 | 51.10 ± 9.23 | 50.10 ± 6.63 | 16.608 |
| Phe | 18.78 ± 5.23 | 15.70 ± 2.83 | 21.80 ± 3.93 | 20.60 ± 2.73 | 7.188 |
| Total | 538.44 | 683.23 | 52.93 | 47.21 | |

Plants were grown with 1 and 50 or 500 μM $Zn^{2+}$ for 8 days after being precultured in nutrition solution for 22 days. The results are presented as means ± SE of three replications, and $LSD_{0.05}$ represents the least significant differences ($P < 0.05$) among the corresponding group of data (line)

et al. 1998). Short-term $^{65}Zn^{2+}$ uptake by protoplasts isolated from leaves of *S. alfredii* increased in response to rising Zn levels, which is similar to those of *T. caerulescens* and *A. halleri* (Lasat et al. 1998; Cosio et al. 2004). Both are characterized of a biphasic pattern with an initial rapid linear component followed by a slower one (Fig. 3). There were no obvious differences in Zn uptake by isolated protoplasts between HE and NHE at low Zn levels (10 and 100 μM), but, higher $^{65}Zn$ uptake by the HE protoplasts than by the NHE ($P < 0.05$) occurred when exposed to 1,000 μM Zn for longer than 10 min (Fig. 3). This may be attributed to the enhanced transport of Zn to the vacuoles by induction of high Zn on a tonoplast transporter, and/or on Zn complexing chelator production in cytoplasm for HE leaf protoplasts. The discrepancy in stimulated $^{65}Zn$ uptake between the isolated protoplasts of HE *S. alfredii* at the high Zn level and that reported in *T. caerulescens* (Lasat et al. 1998) may be due to the fact that the exposure time (12 min) was too short or leaf cells of *T. caerulescens* possess smaller vacuoles than *S. alfredii*. Although leaf growth of NHE *S. alfredii* significantly reduced by long-term (14 days) exposure to Zn at the levels >250 μM (Table 1), the inhibition of high Zn levels on intact shoot growth of NHE occurred in only 2 days after treatment. At 1,000 μM Zn, root dehydrogenase activity markedly decreased in the first 24 h of treatment for both ecotypes of *S. alfredii*, whereas the root dehydrogenase activity of HE *S. alfredii* was fully recovered in 2 days after treatment but it was not for NHE (Li et al. 2005b). Therefore, high Zn stress (1,000 μM) may have similar effects on leaf protoplasts for both ecotypes during the short exposure time (16 min). Further study with longer exposure times and optimal Zn levels are needed to prove the stimulated Zn uptake by isolated protoplasts of the hyperaccumulator. The treatment with CCCP, an anionic protonophore and metabolic inhibitor (DiTomaso et al. 1992), or with liquid $N_2$-rupture, strongly inhibited $^{65}Zn^{2+}$ transport into protoplasts for both ecotypes (Fig. 4). These results indicate that the enhanced Zn transport into leaf cells could have partly resulted from a stimulated transport system operating in

**Table 6** Concentrations of free amino acids (μmol 100 $g^{-1}$ FW) in stems of two ecotypes *S. alfredii* H

| Amino acids | HE | | NHE | | $LSD_{0.05}$ |
|---|---|---|---|---|---|
| | 1 μM $Zn^{2+}$ | 500 μM $Zn^{2+}$ | 1 μM $Zn^{2+}$ | 50 μM $Zn^{2+}$ | |
| Asp | 58.24 ± 3.73 | 63.09 ± 5.69 | 99.06 ± 6.11 | 67.60 ± 13.46 | 15.327 |
| Ser | 83.25 ± 13.15 | 78.07 ± 11.24 | 91.45 ± 12.35 | 70.92 ± 8.81 | 21.577 |
| Glu | 21.49 ± 2.46 | 30.37 ± 4.82 | 29.25 ± 2.54 | 24.82 ± 4.43 | 6.985 |
| His | 14.42 ± 0.76 | 11.41 ± 1.80 | 15.38 ± 2.75 | 13.04 ± 2.26 | 3.856 |
| Arg | 66.24 ± 6.42 | 64.69 ± 10.22 | 18.85 ± 3.37 | 20.28 ± 3.59 | 12.270 |
| Thr | 17.80 ± 2.81 | 17.42 ± 0.84 | 15.26 ± 2.72 | 20.12 ± 3.51 | 5.077 |
| Ala | 30.59 ± 2.07 | 44.57 ± 5.51 | 37.12 ± 10.78 | 39.50 ± 7.07 | 13.344 |
| Gaba | 29.92 ± 4.72 | 35.57 ± 5.62 | 54.27 ± 9.67 | 22.81 ± 4.07 | 12.072 |
| Pro | – | 8.47 ± 1.34 | 9.15 ± 1.60 | 20.63 ± 3.68 | – |
| Tyr | – | – | – | 10.84 ± 1.94 | – |
| Val | 16.04 ± 1.06 | 30.35 ± 4.75 | 21.26 ± 3.80 | 13.84 ± 2.47 | 6.290 |
| Lys | 9.47 ± 1.50 | 15.47 ± 2.44 | 17.48 ± 2.61 | 19.76 ± 3.53 | 4.905 |
| Ile | 7.84 ± 1.24 | 9.76 ± 1.54 | 12.68 ± 2.27 | 15.24 ± 2.72 | 3.802 |
| Leu | 29.67 ± 4.69 | 27.14 ± 4.29 | 25.74 ± 3.32 | 32.06 ± 5.73 | 8.644 |
| Phe | 16.03 ± 2.53 | 13.27 ± 0.47 | 12.85 ± 2.30 | 12.35 ± 2.21 | 3.856 |
| Total | 400.99 | 449.68 | 459.90 | 403.89 | |

Plants were grown with 1 and 50 or 500 μM $Zn^{2+}$ for 8 days after being precultured in nutrition solution for 22 days. The results are presented as means ± SE of three replications, and $LSD_{0.05}$ represents the least significant differences ($P < 0.05$) among the corresponding group of data (line)

the leaf cell tonoplast, which could effectively move cytoplasmic Zn into the vacuole, though it is considered that there exist regulation sites before Zn enters into leaf protoplasts of *T. caerulescens* and *A. halleri* (Lasat et al. 1998; Cosio et al. 2004). Therefore, stimulated Zn uptake into the leaf cells, especially across the leaf tonoplast, may play a critical role in the strong Zn hyperaccumulation expressed in *S. alfredii*.

Carboxylic and amino acids, such as citric, malic, and histidine are potential ligands for heavy metals and could have a role in metal tolerance and detoxification (Rauser 1990). However, strong evidences for a function of organic acids in metal tolerance, such as close correlations between amounts of organic acids produced and exposure to a metal, has not been produced. It is likely that organic ligands facilitate the long-distance transport of Zn (White et al. 1981) and might be instrumental in helping sequester Zn in the leaf cell vacuole. The results from this study demonstrated a significant increase in concentrations of malic, citric, and oxalic acids in leaves of HE in response to raised Zn levels, whereas only oxalic acid in leaves and stems of NHE (Table 4). Our related studies revealed that application of citric or oxalic acid to nutrient solution increased Zn concentrations in leaves, stems, and roots of HE, whereas for NHE, addition of citric acid increased root Zn accumulation by six times, but not for leaf or stem Zn accumulation. The water extractability of Zn in leaves of HE was significantly enhanced by application of citric acid (data not shown). All these results imply that citric acid may be involved in long-distance transport of Zn into leaf cells for HE and amino acids may have a role in Zn transport into leaf cells and His production has been reported to be involved in Ni hyperaccumulation in shoots of *Alyssum* (Krämer et al. 1996). In *T. caerulescens* the increased Zn translocation in xylem was not related to stimulated production of any particular amino acids (Lasat et al. 1998). In the present study, we found that the concentrations of Val and Lys increased in leaves and stems for HE, but decreased or had minimal change for NHE in response to raised Zn levels. Val concentration in leaves of HE increased by over 3.6 times when grown at optimal Zn compared to that at low Zn level, which is the greatest response to Zn level among all the amino acids determined (Table. 5, 6). These results indicate that stimulated production of Val and other responsive amino acids may be involved in Zn loading into leaf cells of the hyperaccumulator *S. alfredii*. Further studies are needed to clarify the roles of particular amino and organic acid production in stimulated Zn transport and re-absorption of xylem-borne Zn into leaf cells of *S. alfredii*.

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