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Title:

Efficient phloem transport significantly remobilizes cadmium from old to young organs in a hyperaccumulator Sedum alfredii

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Abstract

Our knowledge of cadmium (Cd) in hyperaccumulators mainly concerns root uptake, xylem translocation and foliar detoxification, while little attention has been paid to the role of phloem remobilization. We investigated Cd distribution in different organs of the hyperaccumulating ecotype (HE) of Sedum alfredii and compared its Cd phloem transport with that of the non-hyperaccumulating ecotype (NHE). In HE, results of micro X-ray fluorescence revealed that Cd preferentially accumulated in younger organs compared to the older, and its primary distribution sites changed from parenchyma to vascular/epidermal cells with increased organ age. Strong Cd signals in phloem cells were observed in HE old stems. Pre-stored Cd was readily exported from older to growing leaves, which could be accelerated by leaf senescence. Short-term feeding experiments showed that phloem-mediated Cd transport is rapid and efficient in HE. HE relocated 44% of the total leaf-labelled Cd to other organs, while over 90% Cd was retained in labelled leaves of NHE. High Cd was detected in HE phloem exudates but not in those from NHE leaves. In conclusion, Cd phloem transport is efficient and important for dominating the age-dependent Cd allocation in plants of HE S. alfredii.

Keywords

Cadmium; μ-XRF; Phloem; Remobilization; Age

Abbreviations

HE: Hyperaccumulating Ecotype;

NHE: Non Hyperaccumulating Ecotype

1. Introduction

Cadmium (Cd) is a toxic heavy metal that has a negative impact on human health and threatens food safety, particularly in industrialized zones [1, 2]. About 7% of the soils in China were found to be contaminated with Cd (according to the soil quality standard in China) [3]. Cd in soils is easily accumulated to the edible parts of crops, and consequently become concentrated in the human body through the food chain [4, 5]. A few plant species identified as Cd hyperaccumulators can accumulate more than 100 mg kg^{-1} DW Cd in shoots, and thereby have great potential as metal extractors in phytoremediation of Cd contaminated soils [6]. A better understanding of the mechanisms controlling Cd accumulation in those plants is therefore important for developing strategies that promote the practical phytoextraction of Cd-polluted soils.

Metal hyperaccumulation in plants has classically been attributed to rapid root uptake, enhanced root-to-shoot translocation, as well as efficient metal sequestration and detoxification in the shoots [7, 8, 9]. However, little is known regarding the subsequent activities of the excess heavy metals stored in the shoots of hyperaccumulators. Nutrients are mobile and often reallocated from old to developing tissues to drive growth, while there is little evidence in the literature that heavy metals follow the same path as nutrients. Recently, significant leaf-age effects on metal accumulation in hyperaccumulators were observed in terms of manganese (Mn) [10], zinc (Zn) [11, 12] and nickel (Ni) [13]. Although the results were apparently metal- and species-dependent, older tissues usually showed higher metal concentrations than developing tissues. The age-dependent variation of Cd accumulation was also observed in the hyperaccumulator Noccaea caerulescens, but the results were contradictory. Cadmium contents were found to be higher in young than mature leaves in one study [14] but the reverse was reported in another study [12]. Moreover, a third study found that Cd accumulation was the same in all leaves regardless of age [15]. Hence, the information concerning the distribution of Cd between organs in hyperaccumulators in the literature is inconclusive and much uncertainty remains concerning how Cd is governed at the whole plant level corresponding to development.

Very recent studies suggest that a considerable amount of metals may be remobilized by phloem transport in hyperaccumulators. For instance, relatively high level of Ni and Zn were detected in phloem exudates from leaves of the hyperaccumulator N. caerulescens [16]. Isaure et al. [17] found significant Cd signals in leaf phloem tissues of the hyperaccumulator Arabidopsis halleri by using micro X-ray fluorescence (μ-XRF) analysis. In Sedum alfredii Hance, a Chinese Zn/Cd co-hyperaccumulator [18], our previous study reported an enhanced ability for Zn remobilization via phloem transport [19], and a high level of Cd in young tissues and phloem sites triggered by calcium (Ca) deficiency [20]. Taken together, these findings strongly suggest that remobilization of Cd from mature to developing organs via phloem transport occurs in the hyperaccumulator plants. In the present study, a range of different experiments were performed to test this hypothesis by using the hyperaccumulator S. alfredii. The Cd levels in the different leaves and stems of S. alfredii plants were determined following short and long term exposures to the heavy metal. Moreover, XRF imaging of Cd was used to determine the relative contents of this metal in different tissues particularly the phloem of S. alfredii (hyperaccumulating ecotype, HE) and the non-hyperaccumulating ecotype (NHE). Re-allocation of Cd by phloem transport from mature leaves to young tissues was also compared for the two contrasting ecotypes of S. alfredii.

2. Experimental

2.1 Plant pre-culture

Sedum alfredii (HE) seeds were collected from an old Pb/Zn mine in Zhejiang province, China, while those of the NHE were obtained from a tea garden near Hangzhou, Zhejiang province [18]. The plants were grown in non-contaminated soil for several generations, and healthy and uniform shoots were selected and pre-cultured as previously reported [21]. The nutrient solution contains 2 mM $Ca(NO_3)_2$, 0.1 mM KH_2PO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 0.7 mM K_2SO_4, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.01 μM $(NH_4)_6 \cdot Mo_7O_{24}$, and 100 μM Fe-EDTA, with the pH between 5.5-5.8. Plants were grown under natural light in a greenhouse equipped with a cooling system, with day/night temperatures of 26 °C /20 °C and a day/night humidity of 70%/85%.

2.2 Long-term Cd treatment on the HE S. alfredii plants

After pre-culture, the HE seedlings were treated with 50 μM $CdCl_2$ (ionic activity of Cd^{2+} was 1.2 μM) for 84 d. Leaves and stems were harvested according to their age, and numbered from the young to the old (from 1-4), and tissues numbered 4 existed before Cd treatment but kept growing during treatment. For elemental analysis, the samples were washed, dried, weighed, and analyzed for Cd using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500a and Agilent ICPS-7510, USA) after digestion with HNO_3 and H_2O_2. For μ-XRF mapping, targeted stems and leaves were cross-sectioned with a cryotome (CM 1850, Germany) at -20°C as previously reported [22]. Sections in good condition were selected and freeze-dried for 48 h. The μ-XRF analysis was carried out on Stanford Synchrotron Radiation Lightsource (SSRL) beamline 14-3 (Menlo Park, CA, USA). The μ-XRF maps were obtained by rastering the beam in 10 μm steps (with a count time of 100 ms per step). The maps were produced using the software package SMAK version 0.34, S-4 (http://www.sams-xrays.com/smak).

2.3 Redistribution of pre-stored Cd in the HE S. alfredii plants

After being pre-cultured for 2 wk, the HE S. alfredii seedlings were treated with or without 100 μM $CdCl_2$ (ionic activity of Cd^{2+} was 9.5 μM) for 5 d. Part of the leaves and whole roots were then removed, with only the seedling apexes (with nine to ten young leaves) and the fully expanded mature leaves (nearly eight leaves) retained, in order to clearly distinguish the "sink" and "source." The seedlings were then washed and stored in the dark for one night for wound healing. The plants were then re-cultured in a nutrient solution for 6 wk. Before and after re-culture, the seedlings were separated into newly emerged tissues, original young tissues, original mature leaves, stems and new roots, and subsequently harvested. Cd concentrations in the plant samples were determined by ICP-MS as mentioned above.

In a separate experiment, 2-wk-old HE S. alfredii seedlings were treated with or without 100 μM $CdCl_2$ in a nutrient solution for 5 d and then transferred to deionized water for re-culture. To investigate the effects of senescence on Cd remobilization, all the mature leaves on the HE S. alfredii pre-treated plants were randomly divided into two equal parts, and one part was covered with aluminium foil. The plants were harvested after 4 wk, and the elements in the different tissues were analyzed.

2.4 Redistribution of foliar-applied Cd in the HE and NHE plants

The HE and NHE S. alfredii seedlings were pre-cultured in a full nutrition solution for 6 wk. One fully expanded mature leaf was chosen as the labelled leaf in each seedling. Labelled leaves were cut about 2 mm from their tips to facilitate Cd uptake. Subsequently, the tips (about 1/5 of the leaf area) were immersed in a labelling solution with 0, 10, or 100 μM $CdCl_2$ for 7 d (Fig. 5a). Each treatment had four replicates. At harvest, the seedlings were divided into the youngest tissues, mature leaves, labelled leaves, old leaves, stem, and roots. The samples were dried and digested with HNO_3 to determine the Cd content by ICP-MS.

In another experiment, selected mature leaves of 6-wk-old HE seedlings were treated with 100 μM $CdCl_2$. Petioles of the labelled leaves and adjacent stems were collected

at 6 h, 24 h, and 5 d, and then used for section making and μ-XRF analysis.

2.5 Fluorescence imaging of phloem tissue

As a phloem-mobile compound, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS-acetate, Sigma) has been previously used to trace phloem transport in Arabidopsis thaliana and barley [23, 24, 25]. Mature leaves of 6-wk-old seedlings of HE S. alfredii were selected and treated with 10 mM HPTS-acetate as the same way of Cd foliar application. After HPTS-acetate loading for 1, 3, or 6 h, sections of the labelled leaves and adjacent stems from 3-6 individual plants were cut and observed under a Nikon Eclipse E600 epi-fluorescence microscope (Nikon, Tokyo, Japan).

2.6 Collection and analysis of phloem exudates

After 6 wk of pre-culture, the HE and NHE S. alfredii seedlings were hydroponically treated with or without 10 μM $CdCl_2$ (ionic activity of Cd^{2+} was about 0.10 μM) for 5 d. Phloem exudates were collected from mature leaves according to the protocol of Tetyuk et al. [26], with some modifications [27]. Phloem exudates from six individual seedlings were considered as one replicate, and each treatment had five replicates. Glucose was measured by the dinitrosalicylic acid method [28], and sucrose was measured by the alkaline hydrolysis-resorcinol method [29]. A subsample of 5.0 mL of phloem exudates was mixed with 2 mL of 2% (w/v) HNO_3 for metal content analysis by ICP-MS. In a separate experiment, HE S. alfredii seedlings were treated with 10 μM $CdCl_2$ for 4 wk, and the phloem exudates were collected and the metal concentrations were determined.

2.7 Statistical Analysis

All data were statistically analyzed using the SPSS package (version 11.0; SPSS Inc., Chicago, IL, USA), one-way or two-way ANOVA was performed on the data sets depending on the overall number of factors, and the mean and SD of each treatment as well as least significant difference (LSD; $P < 0.05$ and $P < 0.01$) for each set of corresponding data were calculated.

3. Results

3.1 Variation of Cd accumulation in shoots of HE S. alfredii

Variations in Cd concentrations were observed in the different stems and leaves of HE S. alfredii after long-term Cd exposure (Fig. 1). The youngest stems and leaves (location of No. 1) showed the highest Cd concentrations, followed by the mature stems and leaves (location of No. 2). The old leaves (location of No. 3 and 4), those exhibiting the typical symptoms of senescence (e.g. partially tuning yellow), contained the lowest levels of Cd (Fig. 1).

3.2 Distribution patterns of Cd in the different organs

Analysis of Cd in the stem and leaves cross-sections by μ-XRF imaging confirmed the age-specific variations in the Cd levels of HE S. alfredii (Fig. 2, Fig S1). In the youngest stem at top position (No. 1), Cd was mainly distributed to parenchyma cells in both pith and cortex, whereas, Cd in the vascular tissues was in very low levels (Fig. 2b). In the stem, at a lower position (No. 2), high Cd signals were noticed in xylem, epidermis and cortex, but the metal contents in pith became lower than those in the youngest stem (Fig. 2b). In the old stems (No. 3 and No. 4), very high Cd levels were observed in vascular bundles and epidermis, but not in the pith and cortex parenchyma cells. Interestingly, the tissues near the junctions of stems and leaves always showed a higher Cd concentration than the same kind of tissues far away from the junctions. Moreover, considerable Cd signals were observed at the curvilinear outer layers of the vascular bundles in old stems (No. 3 and No. 4), where the phloem tissues were localized (Fig. 2b, c). Age-specific variations in the cellular distribution patterns of Cd were also observed in the leaves, showing decreased Cd contents in mesophyll cells of old leaves as compared with the young and mature leaf cells (Fig. S1).

3.3 Re-allocation of pre-stored Cd to the new growing tissues

After pre-treated with 0 or 100 μM $CdCl_2$ for 5 d, the HE seedlings were moved to nutrient solutions without Cd to evaluate the metal remobilization from storage sites to the new growing tissues (Fig. 3). All the roots were removed to avoid direct xylem

transport of Cd from the root cells and apoplastic space to the new growing tissues. After 6-wk re-culture, the Cd concentration in the newly emerged tissues was 433.98 ± 28.76 μg g^{-1} DW, while Cd levels in the other tissues had significantly decreased (Fig. 3b). Taking biomass into account, approximately 12% of the total Cd stored in mature leaves was exported out for reallocation (Fig. 3c). Furthermore, dark-induced senescence of mature leaves significantly increased Cd remobilization out of the storage sites in these leaves (Fig. 4).

3.4 Re-allocation efficiency of foliar-applied Cd in HE and NHE S. alfredii

After Cd application to selected mature leaves for 7 d (Fig. 5a), considerable amounts of Cd were re-allocated from the labelled sites to other plant tissues in HE S. alfredii, particularly to the stems and young leaves (Fig. 5b, c). In contrast, the Cd concentrations in the non-labelled tissues of NHE plants were too low to be determined, although a high Cd level was found in both HE and NHE Cd-labelled leaves. It was shown that only 56% of the leaf-absorbed Cd remained in labelled leaves of HE, while over 90% Cd was retained in labelled leaves of NHE (Fig. 5c). Very little Cd was found in the tissues of HE and NHE seedlings that had a foliar application of Cd-free solution.

The significant difference in Cd remobilization of two ecotypes was also confirmed by the μ-XRF analysis. As shown in Fig. 6, significant localization of Cd in the vascular bundles was observed in the cross-sections of petiole and stem collected from Cd-labelled HE seedlings, with no Cd signal observed in the control samples (data not shown). The Cd fluorescence intensities in the cross-sections of NHE petiole and stem were too low to be determined (Fig. S2). In short-term experiment, abundant Cd was observed in stem vascular bundles just after foliar application for 6 h (Fig. S3), suggesting that remobilization from the labelled leaves to the other plant tissues was very rapid. Moreover, a peak Cd signal was observed in the vascular tissues at the junctions of the stem and labelled leaves (Fig. 7a), where a phloem mobile tracer (HPTS) also exhibited high fluorescence intensity (Fig. 7b).

3.5 Determination of Cd in phloem exudates

Phloem exudates were collected from mature leaves of HE and NHE S. alfredii plants treated with 10 μM $CdCl_2$ for 5 d, the purity of which was evaluated by their glucose and sucrose contents. We found no glucose, but high levels of sucrose in the phloem exudates (Fig. 8a), suggesting that there was no significant contamination by cell sap [30]. Cd treatment significantly decreased the sucrose content of phloem exudates from NHE plants as compared to that in the controls, but did not significantly change the sucrose levels in the HE samples. The Cd content of HE phloem exudate was 359.0 ± 12.6 μg mg^{-1}, while that of NHE phloem exudate was less than 10 μg mg^{-1} (Fig. 8b). After exposure of 10 μM $CdCl_2$ for 4 wk, Cd concentration was much higher than that of magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), and copper (Cu) in phloem exudates of HE seedlings (Table S1).

4. Discussion

4.1 Cd storage and remobilization in S. alfredii shoots

Metal accumulation in different organs and tissues of hyperaccumulators were generally metal-, species-, and even condition-dependent [10-15]. The data presented here demonstrated that in both short-term and long-term studies, Cd was preferentially localized in the young organs and was less abundant in the older ones in shoots of HE S. alfredii (Fig. 1, 2). As previously reported, Cd was mainly stored in the stem and leaf parenchymal cells consisting of large vacuoles for the metal detoxification in this plant species [22, 32]. The data presented here not only confirms these findings but also shows significantly decreased Cd contents in the parenchyma of old leaves and stems (Fig. 2; Fig. S1). It is likely that Cd stored in the old stems and leaves has been exported out of these storage sites (parenchymal cells) due to cells' senescence, and this is confirmed by an enhanced depletion of Cd in the leaves suffering from artificially dark-induced senescence (Fig. 4). Küpper et al. [12] also suggested that Cd redistribution might occur during leaf senescence in the Cd hyperaccumulator N. caerulescens (Ganges ecotype) but the reasons for this remain obscure. It may be that Cd is recycled alongside other nutrients in the hyperaccumulators, rather than remaining in the storage sites. Moving the Cd pre-treated seedlings of HE S. alfredii to a non-Cd solution confirmed that considerable amounts of Cd pre-stored in leaves was exported out of its storage sites and subsequently translocated into the young tissues after several days (Fig. 3). The above results point out that age-dependent variation of Cd accumulation occurs in HE S. alfredii, which is partially resulted from the metal reallocation within the plants.

4.2 Phloem remobilization of Cd

It is generally accepted that remobilization of elements out of the old/mature tissues to the young tissues occurs via the phloem instead of xylem transport, since the latter is unidirectional [33]. Moreover, low transpiration rates largely restrict xylem transport in the newly emerging leaves [34], although phloem-to-xylem transfer sometimes occurs. The clear localization of Cd in the phloem sites of old stems (Fig. 2b, c) strongly demonstrates that phloem-mediated transport of Cd involves in Cd remobilization from

the older to younger leaves in HE S.alfredii, may resulting in Cd preferential accumulation to developing tissues. It is therefore that the efficiency of phloem-mediated transport of Cd was important for Cd remobilization from old to young tissues in hyperaccumulators, which finally affects the metal accumulation in different parts of the plants.

The efficiency of phloem-mediated transport of Cd in the HE S. alfredii was further compared with its NHE plants by leaf application, which is a widely used method to test metal mobility in plants [31, 35, 36, 37]. A higher percentage of the total Cd absorbed by the labelled leaves was transferred to the other tissues in the HE (40%) than in the NHEs (<10%) (Fig. 5c), suggesting a much higher efficiency of the phloem-mediated Cd translocation process in the former. This is confirmed by strong Cd signals noted in the vascular bundles of petiole and stem nearby the labelled leaves (Fig. 6), as well as in the phloem zone at junctions between petioles and stems in HE plants (Fig. 7), but not in NHE plants (Fig. S2). Since foliar-applied Cd could be detected in stem vascular bundles as early as 6 h in the HE seedlings (Fig. S3), the phloem-mediated transport of Cd is a very rapid process in HE.

Additional direct evidence for efficient phloem-mediated Cd transport in HE S. alfredii was the extremely high levels of Cd in phloem exudates from its mature leaves as compared with the NHEs (Fig. 8). Limited to the collecting method, we were not able to determine the exact Cd concentration in phloem flow, but the relative abundance between different elements is also informative and makes the comparison between different studies feasible [16]. The Cd level was 2- to 50- fold higher than those of Mg, Zn, Fe, Cu, and Mn in the phloem exudate of HE mature leaves (Table S1). This is quite different from those reported in other plants, such as rice [38], Brassica napus [39], and castor bean [40], where the Cd concentration in phloem exudates was equal to or less than the concentration of essential metals.

Taken together, these results provide convincing evidence that the phloem transport of

Cd is efficient in HE. This finding agrees with other observations concerning enhanced Zn and Ni remobilization in hyperaccumulators [16, 19]. Hence, efficient phloem-mediated Cd transport may be as important as root uptake processes, high shoot-to-root ratios, and detoxification pathways in Cd hyperaccumulating plants [9].

4.3 Physiological and practical implications of Cd allocation and relocation

Understanding why hyperaccumulators allocate and remobilize Cd from the mature organs to the youngest developing leaves via the phloem is important in developing new concepts of processes that enable heavy metal accumulation [8]. While we cannot discount the possibility that a lack of ion transporter selectivity drives Cd remobilization because Cd and Ca transport pathways are interrelated in S. alfredii [20, 21, 22, 41], preferential Cd accumulation in the younger leaves clearly does not impair the viability of the plants, suggesting it may have some physiological advantages. One possible advantage of Cd accumulation in the developing organs of HE plants is that it affords a degree of protection from herbivores. Preliminary experiments have shown that the Cd-rich HE plants were much more resistant to infestation by Aphis fabae Scopoli, a typical black bean aphid that feeds on the phloem of young tissues (unpublished data). It is tempting to suggest therefore that Cd serves as a defence compound in the phloem and that Cd accumulation forms an integrated element in the overall defence strategy of the HE.

This reallocation of Cd mediated by phloem transport could also be a metal tolerance mechanism to avoid overaccumulation of Cd in certain leaves and stems. Even though hyperaccumulators are of high Cd tolerance, overaccumulation of the metal can still cause serious disorders and inhibit carbon gain [9, 12, 18]. Manipulating Cd allocation at the whole plant level may contribute to the survival of a perennial plant and thereby enhance its metal accumulation. Moreover, metal loss with fallen leaves in hyperaccumulator/accumulator plants was noticed in both lab research and field survey [10, 18, 19]. For instance, more than half of Cd retained in old tissues and lost with senesced leaves in the Cd accumulator Solanum nigrum [31]. This may reduce the

efficiency of long-term phytoremediation for the Cd contaminated soils. Improved phloem transport and remobilization of the metal from older to younger tissues in plants therefore helps to reduce metal loss and increase metal accumulation, promoting the phytoremediation efficiency.

5. Conclusion

This study clearly points out that rapid phloem-mediated Cd transport underpins redistribution of the metal from older to developing leaves in the hyperaccumulator S. alfredii. Mechanisms involved in the efficient phloem remobilization of Cd in this plant species are therefore a fundamental importance not only in terms of understanding metal tolerance and its potential benefits but also for developing strategies for soil remediation.

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Figure legends

Fig. 1 Cadmium concentration variation in different stems and leaves collected from plants of the hyperaccumulating ecotype (HE) of Sedum alfredii treated with 50 μM $CdCl_2$ for 84 d. Number 1, 2, 3, and 4, in (a) - (c), represent different stems and leaves from younger to older ones, and those of No. 4 existed before Cd treatment. (b) Cd concentrations of stems. (c) Cd concentrations of leaves. Scale bar in (a) represents 1 cm. Different letters in (b) and (c) indicate significant differences between the organs with different ages ($P<0.05$).

Fig. 2 In vivo imaging of Cd by μ-XRF in different stems collected from plants of the hyperaccumulating ecotype (HE) of Sedum alfredii treated with 50 μM $CdCl_2$ for 84 d. Number 1, 2, 3, 4 represent stems from younger to older ones as marked in Fig. 1. The red colour depicts the elemental concentration in each map, which was scaled to the maximum value for each map. Scale bar: 1000 μm.

Fig. 3 Cadmium concentrations (b) and distributions (c) in different tissues of the hyperaccumulating ecotype (HE) of Sedum alfredii pre-treated with Cd and re-cultured in nutrient solution. The experiment was conducted as shown in (a). Intact seedlings were pre-treated with or without 100 μM $CdCl_2$ for 5 d, then total roots and part leaves were excised, leaving only the top youngest tissues and eight mature leaves. The plants were re-cultured in the nutrient solution for 6 weeks. Samples were harvested and analyzed before and after re-culture. Bars represent means ± SD (n=4), and asterisks denote significant differences ($P<0.01$) within the same tissues before and after re-culture.

Fig. 4 Effect of dark-induced senescence on Cd redistribution in the hyperaccumulating ecotype (HE) of Sedum alfredii. The experiment was conducted as shown in Fig. 3a. Seedlings were pre-cultured with 100 μM $CdCl_2$ and then re-cultured with water for 4 weeks. During the re-culture, mature leaves were randomly divided into two equal parts, one of which was covered with aluminum foil and the other was uncovered. After re-culture, the plants were harvested for elemental determination of newly emerged tissues,

original young tissues, original mature leaves (covered), original mature leaves, stems and new roots. Bars represent means ± SD (n=4), and asterisks represent significant ($P<0.05$) differences in Cd accumulations between the two parts of the original mature leaves.

Fig. 5 Cadmium redistribution from labelled leaves to other tissues in the hyperaccumulating ecotype (HE) and non-hyperaccumulating ecotype (NHE) of Sedum alfredii. (a) Diagrammatic sketch of the leaf-application experiment. A fully expanded mature leaf was labelled with different Cd levels (0, 10, and 100 μM $CdCl_2$) for 7 d, and then different plant tissues were harvested. The red circles numbered 1 and 2 represent petiole and stem cross-sections, respectively. (b) Cadmium concentrations in the youngest tissues, mature leaves, labelled leaves, old leaves, stems and roots of plants under different treatments. (c) Relative Cd distribution (percentage of total Cd) in seedlings of the two ecotypes treated with 10 μM. Every treatment included four replicates, and the experiments were independently performed twice. Bars represent means ± SD (n=4), and (b) and (c) share the same labels.

Fig. 6 In vivo imaging of Cd by μ-XRF in the petiole (a) and stem (b) of the hyperaccumulating ecotype (HE) of Sedum alfredii after leaf labelling with 100 μM $CdCl_2$ for 5 d. The red colour depicts the elemental concentration in each map, which was scaled to the maximum value for each map. UE, upper epidermis; M, mesophyll; VB, vascular bundles; LE, lower epidermis. Scale bar: 500 μm.

Fig. 7 Cadmium and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) transport from petiole to stem via the phloem in the hyperaccumulating ecotype (HE) of Sedum alfredii. (a) μ-XRF imaging of Cd, chlorine (Cl), and phosphorous (P) distributions at the junctions between the stem and leaves labelled with 100 μM $CdCl_2$ for 24 h. (b) Section of the stem of HE S. alfredii after application with HPTS-acetate to the cut edge of the leaf tip for 6 h. The fluorescence emitted by HPTS indicates phloem regions. VB, vascular bundles; Ep, epidermis. Scale bar: 500 μm.

Fig. 8 Concentrations of sucrose (a) and Cd (b) in phloem exudates collected from mature leaves of non-hyperaccumulating (NHE) and hyperaccumulating ecotypes (HE) of Sedum alfredii treated with 0 (control, CK) or 10 μM $CdCl_2$ for 5 d. Bars represent means ± SD (n=5), and different letters denote significant ($P < 0.001$) differences within treatments.

Supplementary material

Fig. S1 In vivo imaging of Cd by μ-XRF in different petioles and leaves of the hyperaccumulating ecotype (HE) of Sedum alfredii exposure to Cd for long term.

Fig. S2 In vivo imaging of Cd, P and Cl by μ-XRF in the petiole and stem of the non-hyperaccumulating ecotype (NHE) of Sedum alfredii after Cd foliar application.

Fig. S3 In vivo imaging of Cd by μ-XRF in the leaf and stem of the hyperaccumulating ecotype (HE) of Sedum alfredii after leaf labelling with Cd for 6 h. **Table S1** Relative content of elements in the phloem exudates collected from the mature leaves of the hyperaccumulating Sedum alfredii treated with or without Cd.

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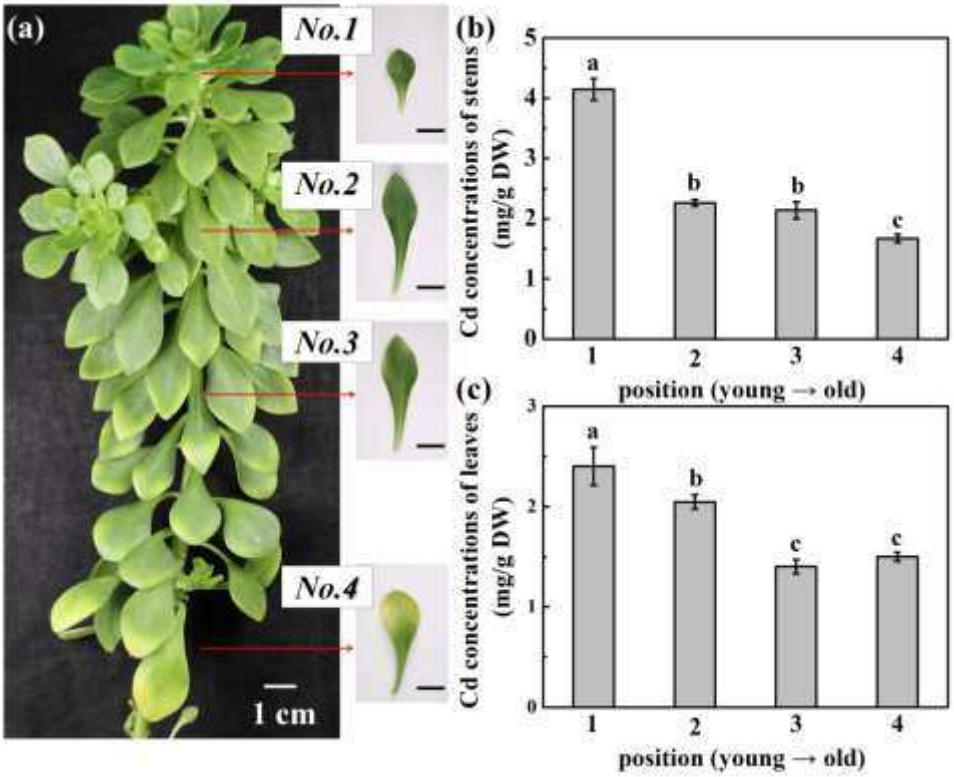

Fig. 1 Cadmium concentration variation in different stems and leaves collected from plants of the hyperaccumulating ecotype (HE) of Sedum alfredii treated with 50 μM $CdCl_2$ for 84 d. Number 1, 2, 3, and 4, in (a) - (c), represent different stems and leaves from younger to older ones, and those of No. 4 existed before Cd treatment. (b) Cd concentrations of stems. (c) Cd concentrations of leaves. Scale bar in (a) represents 1 cm. Different letters in (b) and (c) indicate significant differences between the organs with different ages ($P<0.05$).

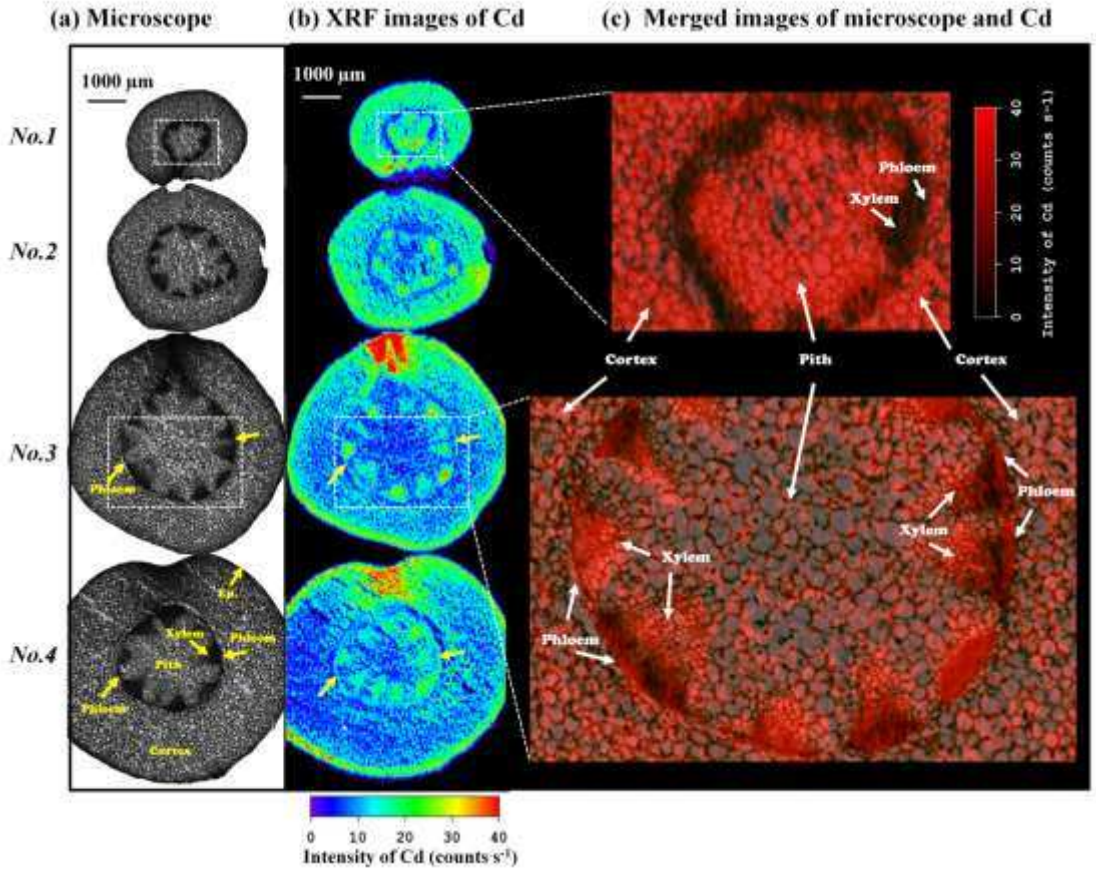

Fig. 2 In vivo imaging of Cd by μ-XRF in different stems collected from plants of the hyperaccumulating ecotype (HE) of Sedum alfredii treated with 50 μM $CdCl_2$ for 84 d. Number 1, 2, 3, 4 represent stems from younger to older ones as marked in Fig. 1. The red colour depicts the elemental concentration in each map, which was scaled to the maximum value for each map. Scale bar: 1000 μm.

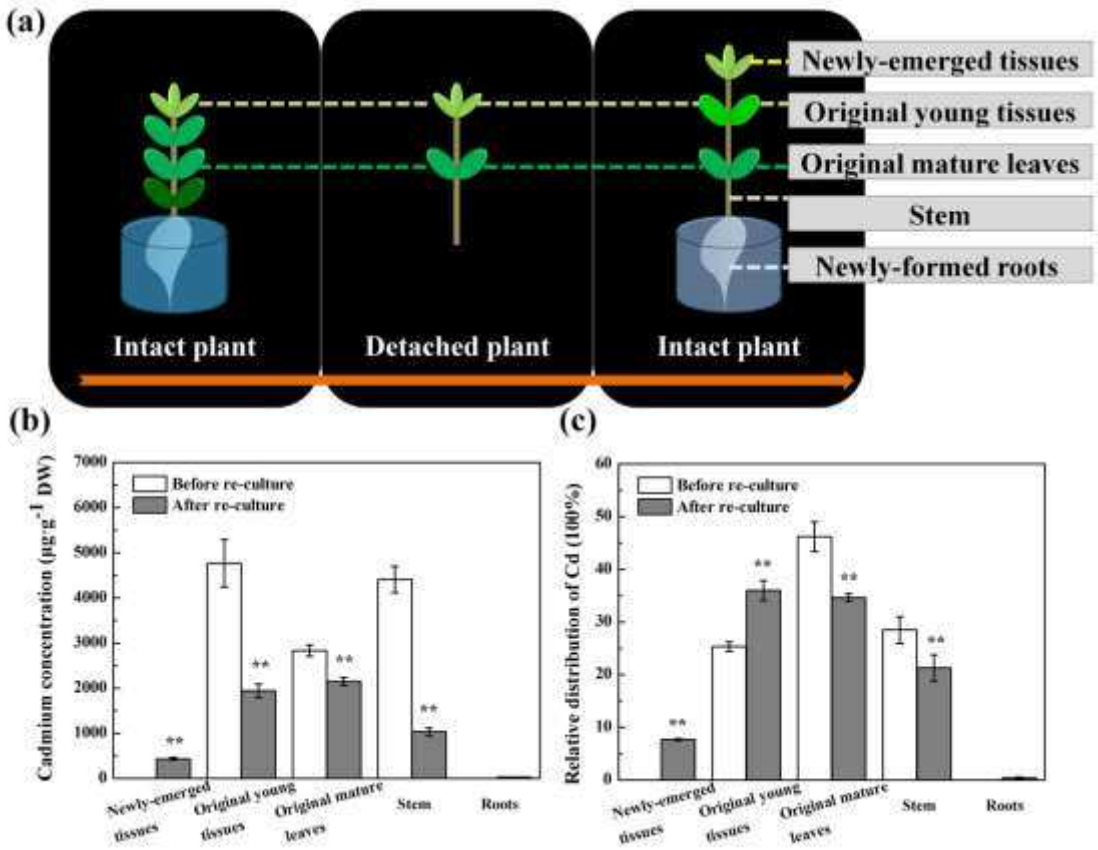

Fig. 3 Cadmium concentrations (b) and distributions (c) in different tissues of the hyperaccumulating ecotype (HE) of Sedum alfredii pre-treated with Cd and re-cultured in nutrient solution. The experiment was conducted as shown in (a). Intact seedlings were pre-treated with or without 100 μM $CdCl_2$ for 5 d, then total roots and part leaves were excised, leaving only the top youngest tissues and eight mature leaves. The plants were re-cultured in the nutrient solution for 6 weeks. Samples were harvested and analyzed before and after re-culture. Bars represent means ± SD (n=4), and asterisks denote significant differences ($P<0.01$) within the same tissues before and after re-culture.

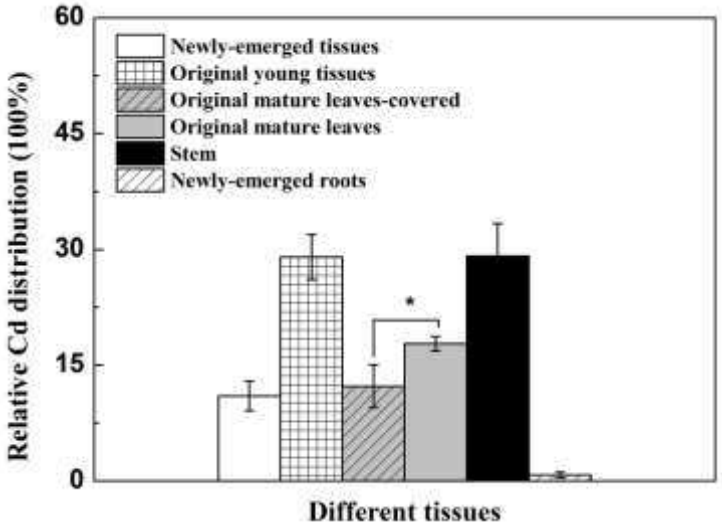

Fig. 4 Effect of dark-induced senescence on Cd redistribution in the hyperaccumulating ecotype (HE) of Sedum alfredii. The experiment was conducted as shown in Fig. 3a. Seedlings were pre-cultured with 100 μM $CdCl_2$ and then re-cultured with water for 4 weeks. During the re-culture, mature leaves were randomly divided into two equal parts, one of which was covered with aluminum foil and the other was uncovered. After re-culture, the plants were harvested for elemental determination of newly emerged tissues, original young tissues, original mature leaves (covered), original mature leaves, stems and new roots. Bars represent means ± SD (n=4), and asterisks represent significant ($P<0.05$) differences in Cd accumulations between the two parts of the original mature leaves.

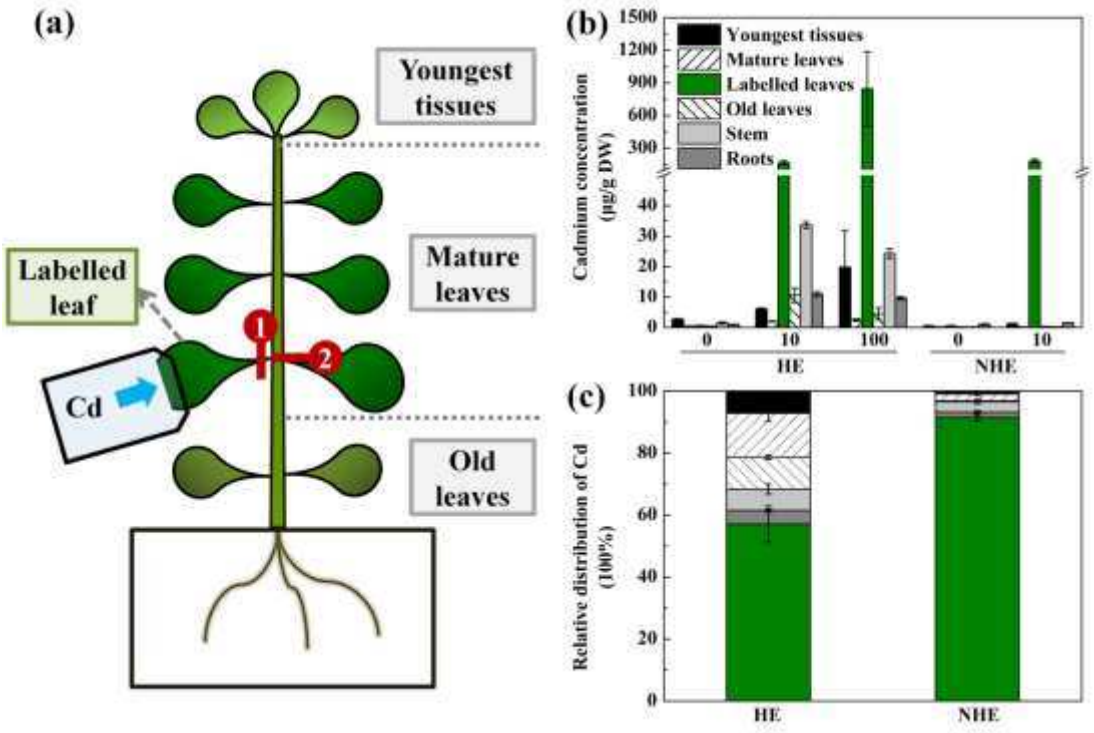

Fig. 5 Cadmium redistribution from labelled leaves to other tissues in the hyperaccumulating ecotype (HE) and non-hyperaccumulating ecotype (NHE) of Sedum alfredii. (a) Diagrammatic sketch of the leaf-application experiment. A fully expanded mature leaf was labelled with different Cd levels (0, 10, 100 μM $CdCl_2$) for 7 d, and then different plant tissues were harvested. The red circles numbered 1 and 2 represent petiole and stem cross-sections, respectively. (b) Cadmium concentrations in the youngest tissues, mature leaves, labelled leaves, old leaves, stems and roots of plants under different treatments. (c) Relative Cd distribution (percentage of total Cd) in seedlings of the two ecotypes treated with 10 μM. Every treatment included four replicates, and the experiments were independently performed twice. Bars represent means ± SD (n=4), and (b) and (c) share the same labels.

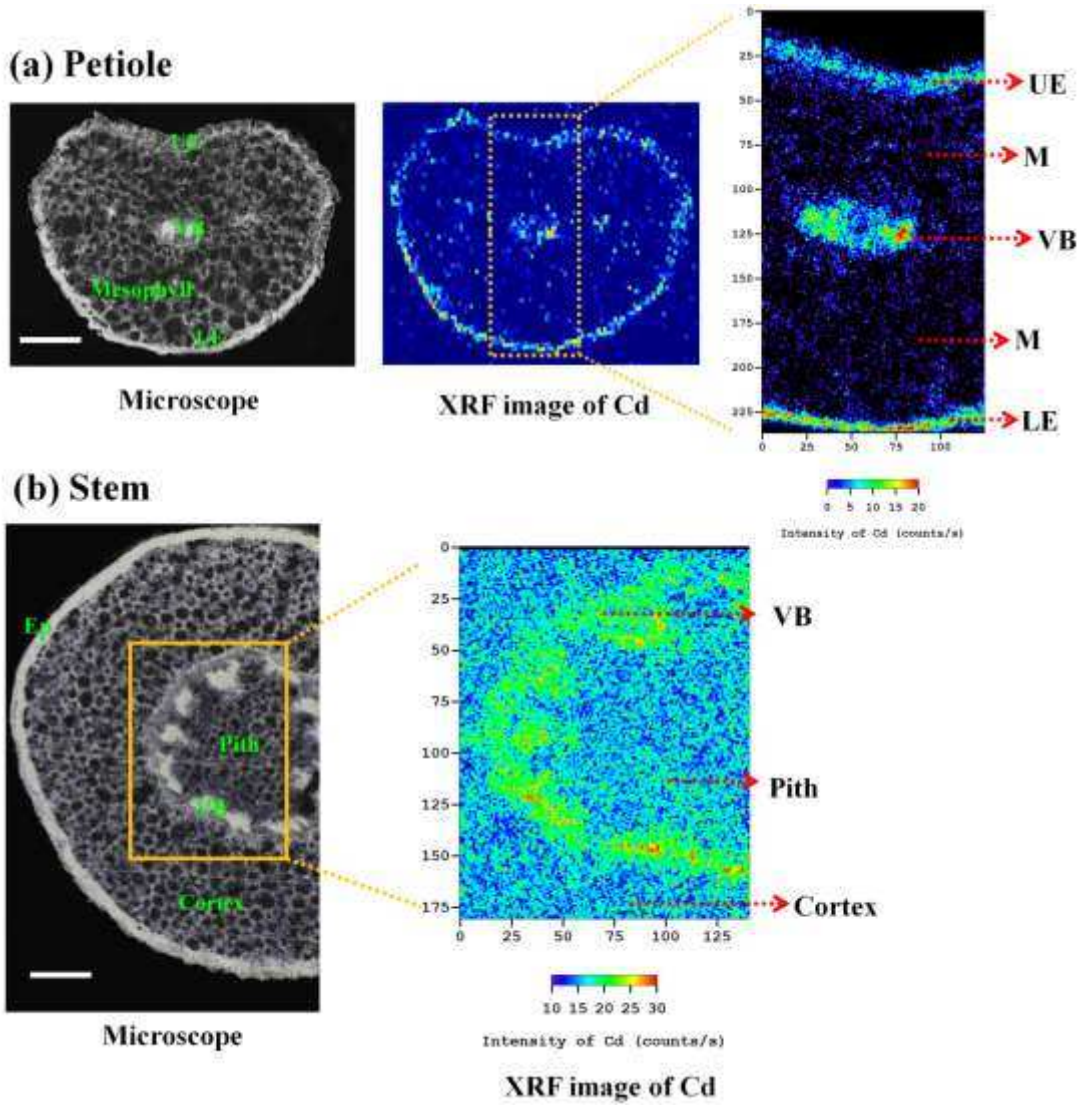

Fig. 6 In vivo imaging of Cd by μ-XRF in the petiole (a) and stem (b) of the hyperaccumulating ecotype (HE) of Sedum alfredii after leaf labelling with 100 μM $CdCl_2$ for 5 d. The red colour depicts the elemental concentration in each map, which was scaled to the maximum value for each map. UE, upper epidermis; M, mesophyll; VB, vascular bundles; LE, lower epidermis. Scale bar: 500 μm.

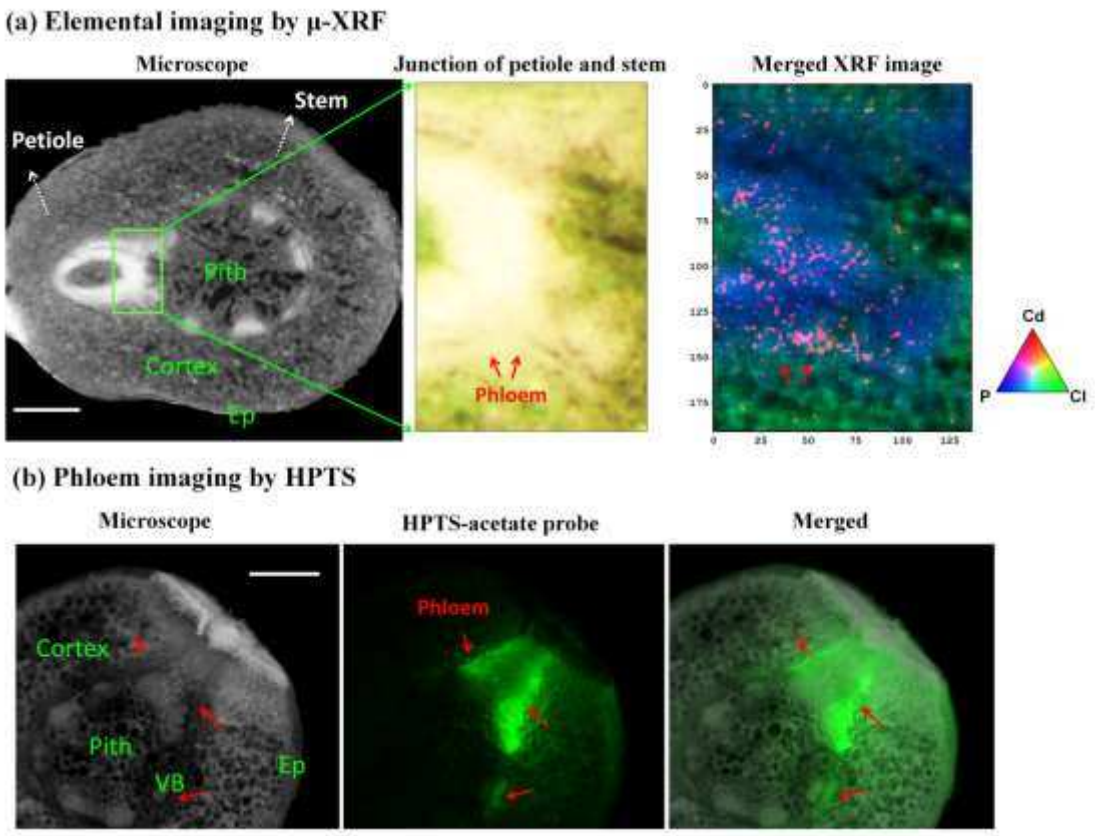

Fig. 7 Cadmium and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) transport from petiole to stem via the phloem in the hyperaccumulating ecotype (HE) of Sedum alfredii. (a) μ-XRF imaging of Cd, chlorine (Cl), and phosphorous (P) distributions at the junctions between the stem and leaves labelled with 100 μM $CdCl_2$ for 24 h. (b) Section of the stem of HE S. alfredii after application with HPTS-acetate to the cut edge of the leaf tip for 6 h. The fluorescence emitted by HPTS indicates phloem regions. VB, vascular bundles; Ep, epidermis. Scale bar: 500 μm.

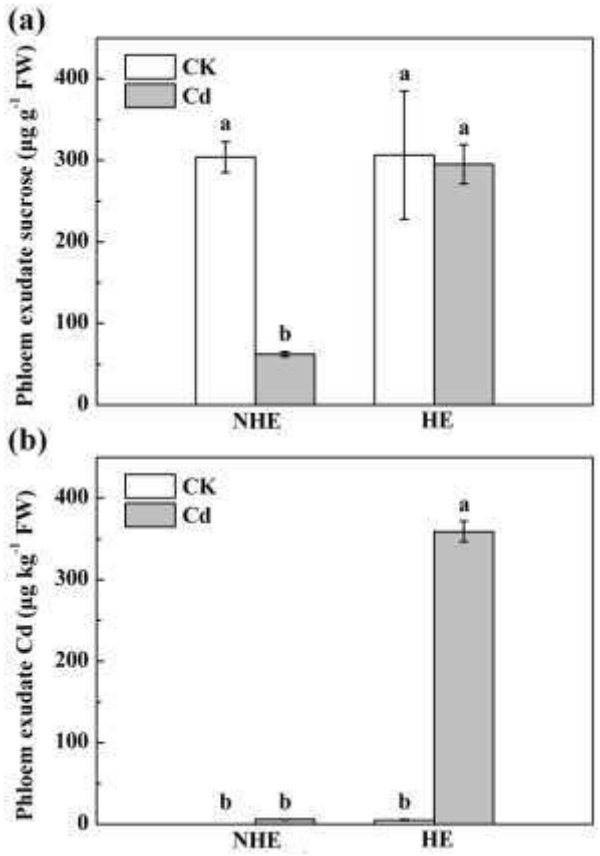

Fig. 8 Concentrations of sucrose (a) and Cd (b) in phloem exudates collected from mature leaves of non-hyperaccumulating (NHE) and hyperaccumulating ecotypes (HE) of Sedum alfredii treated with 0 (control, CK) or 10 μM $CdCl_2$ for 5 d. Bars represent means ± SD (n=5), and different letters denote significant ($P<0.01$) differences within treatments.

Supplementary material

Title :

Efficient phloem transport significantly remobilizes cadmium from old to young organs in a hyperaccumulator Sedum alfredii

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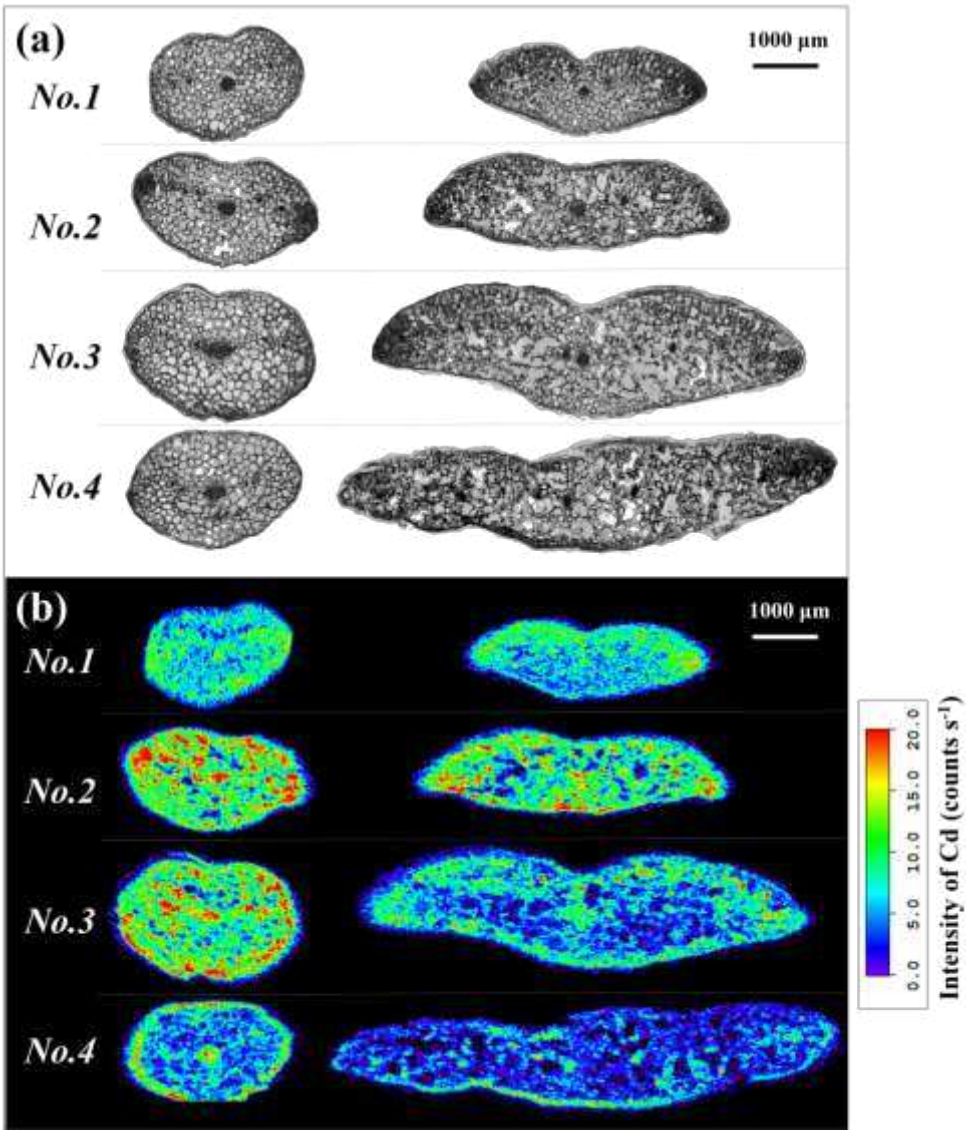

Fig. S1 In vivo imaging of Cd by μ-XRF in different petioles (a) and leaves (b) of different ages collected from plants of the hyperaccumulating ecotype (HE) of Sedum alfredii treated with 50 μM $CdCl_2$ for 84 d. Number 1, 2, 3, 4 represent the petioles and leaves from younger to older ones as marked in Fig. 1. The red colour depicts the elemental concentration in each map, which was scaled to the maximum value for each map. Scale bar: 1000 μm.

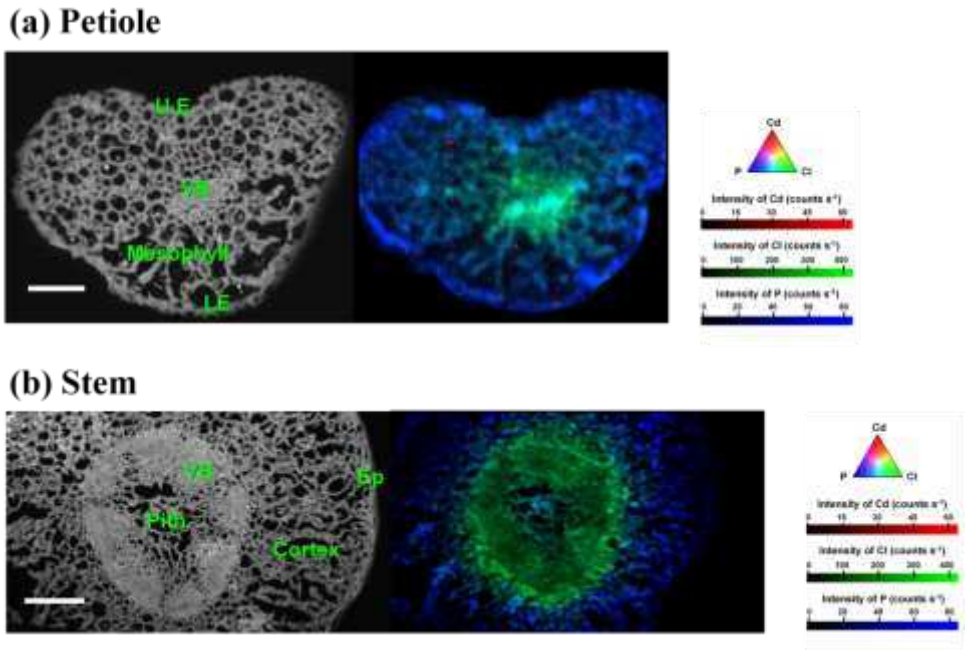

Fig. S2 In vivo imaging of Cd (red), P (green) and Cl (blue) by µ-XRF in the petiole (a) and stem (b) of the non-hyperaccumulating ecotype (NHE) of Sedum alfredii after leaf labelling with 100 µM $CdCl_2$ for 5 d. Pixel brightness is displayed in RGB mode, with the brightest spots corresponding to the highest elemental fluorescence. UE, upper epidermis; M, mesophyll; VB, vascular bundles; LE, lower epidermis. Ep, epidermis. Scale bar: 500 µm.

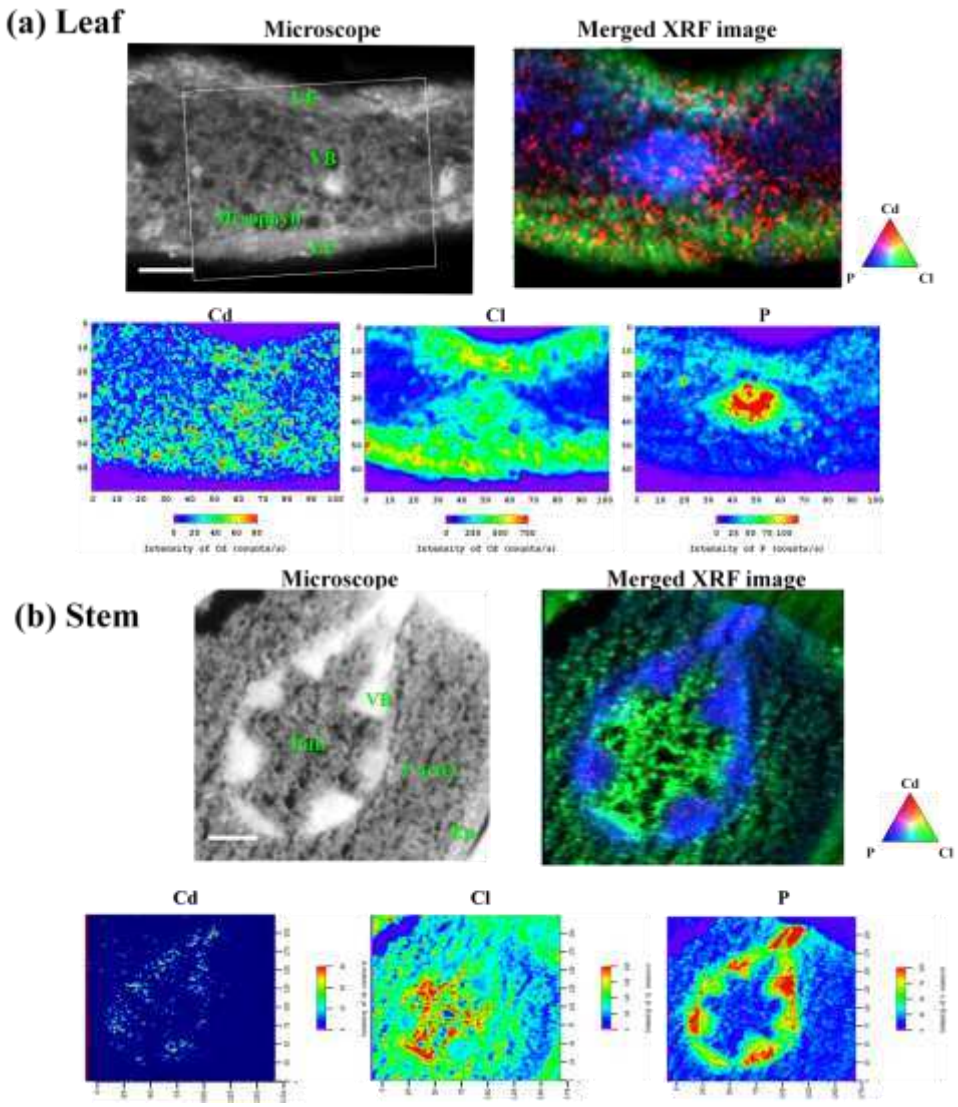

Fig. S3 In vivo imaging of Cd by μ-XRF in the leaf (a) and stem (b) of the hyperaccumulating ecotype (HE) of Sedum alfredii after leaf labelling with 100 μM $CdCl_2$ for 6 h. Distributions of Cd (red), phosphorous (P) (green) and sulphur (S) (blue) in the merged XRF images are shown, with the brightest spots corresponding to the highest elemental fluorescence. The red colour depicts the elemental concentration, which was scaled to the maximum value for the individual map for Cd, P, and Cl. U.E., upper epidermis; M., mesophyll; V.B., vascular bundles; L.E., lower epidermis; Ep., epidermis. Scale bar: 500 μm.

Table S1 Relative content of elements (K, Ca, Mg, Fe, Mn, Cu, Zn, Cd) in the phloem exudates collected from the mature leaves of the hyperaccumulating Sedum alfredii treated with 0 (control, CK) or 10 μM $CdCl_2$ for four weeks.

Treatment	Relative elemental concentration (% of each K content)							
	K	Ca	Mg	Fe	Mn	Cu	Zn	Cd
CK	100	31.5±4.4	2.87±0.84	0.357±0.091	0.0903±0.025	0.0127±0.0046	1.63±0.38	/
Cd	100	29.8±6.0	2.63±0.23	0.224±0.056	0.0496±0.010 **	0.0118±0.0073	1.39±0.20	4.40±1.2**

Relative concentration= (element content)/ (K content in the same exudate)*100%. Data represents means ± SD (n=6), and asterisks denote significant ($P<0.01$) differences within treatments.