Fractionation of Stable Cadmium Isotopes in the Cadmium Tolerant *Ricinus communis* and Hyperaccumulator *Solanum nigrum*

Rongfei Wei¹, Qingjun Guo¹, Hanjie Wen², Congqiang Liu², Junxing Yang¹, Marc Peters¹, Jian Hu², Guangxu Zhu², Hanzhi Zhang³, Liyan Tian¹, Xiaokun Han¹, Jie Ma¹, Chuanwei Zhu², & Yingxin Wan⁴

Cadmium (Cd) isotopes provide new insights into Cd uptake, transport and storage mechanisms in plants. Therefore, the present study adopted the Cd-tolerant *Ricinus communis* and Cd-hyperaccumulator *Solanum nigrum*, which were cultured under controlled conditions in a nutrient solution with variable Cd supply, to test the isotopic fractionation of Cd during plant uptake. The Cd isotope compositions of nutrient solutions and organs of the plants were measured by multiple collector inductively coupled plasma mass spectrometry (MC-ICPMS). The mass balance of Cd isotope yields isotope fractionations between plant and Cd source (δ¹¹⁴/¹¹⁰Cdorgans-solution) of −0.70‰ to −0.22‰ in *Ricinus communis* and −0.51‰ to −0.33‰ in *Solanum nigrum*. Moreover, Cd isotope fractionation during Cd transport from stem to leaf differs between the Cd-tolerant and hyperaccumulator species. Based on these results, the processes (diffusion, adsorption, uptake or complexation), which may induce Cd isotope fractionation in plants, have been discussed. Overall, the present study indicates potential applications of Cd isotopes for investigating plant physiology.

Cadmium (Cd) is a highly toxic heavy metal that can be accumulated in the human body through the food chain¹–³. The health risks of environmental Cd pollution have caused global concern, since the ‘itai-itai’ disease caused by chronic Cd poisoning appeared in Japan in the 1950s⁴. As a cost-effective and environmentally sustainable strategy⁵, phytoremediation could be used in the remediation and sustainable management of Cd polluted soils⁶. The mechanisms of Cd uptake, transport, and storage in plants are of high interest with respect to phytoremediation of Cd polluted soils.

Metal isotope signatures can be applied to identify the chemical process controlling metal transformation in plants and organisms⁷. Previous researchers have studied the metal toxicity in plants using different concentrations and forms of heavy metals⁸–¹⁰. At present, some studies have comprehensively investigated the distribution of metal isotopes in plants, including isotopes of Fe⁹,¹⁰, Zn¹¹–¹³, Cu¹⁴,¹⁵, Ca¹⁶–²⁰, Mg¹¹,¹², and Ni²³. Overall, these studies suggested that the identification of different isotopes within higher plants had specific mode of transport. Hence, metal isotopes could be used as valuable tracers when researching metal uptake, storage and translocation processes within plants.

High precision multiple collector inductively coupled plasma mass spectrometer (MC-ICPMS) has extended the application range of Cd isotopes. Cd stable isotopes were initially used to study mass-dependent fractionation in ordinary chondrites and lunar samples, generated by partial evaporation and condensation²⁴–²⁶. In addition, some studies reported that anthropogenic processes might lead to Cd isotopic fractions, suggesting that Cd stable isotopes could be used as tracers for anthropogenic Cd pollution of the environment²⁷–³⁰. Moreover, many studies have focused on the marine environment, suggesting that biological uptake and utilisation of dissolved seawater Cd generated significant Cd isotope fractionation in the oceans³¹–³⁸.

¹ Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing 100101, China. ² Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550002, China. ³ Shenyang Academy of Environmental Science, Shenyang 110016, China. ⁴ College of Applied Arts and Science of Beijing Union University, Beijing 100191, China. Correspondence and requests for materials should be addressed to Q.G. (email: Guoqj@igsnrr.ac.cn)
However, to date there has been limited research on Cd isotopic composition in plants. In the present study, three Cd tolerant *Ricinus communis* cultivars (Zibo-5, Zibo-6, Zibo-8) and one Cd hyperaccumulator *Solanum nigrum* cultivar were used to study Cd uptake and translocation. These three *R. communis* cultivars were all Cd-hyperaccumulators, and *S. nigrum* was a relatively fast-growing and high-biomass Cd-hyperaccumulator used to develop new techniques for phytoextraction. We conducted hydroponic culture experiments with these plant species and two nutrient solutions with differing Cd concentrations to 1) characterise the Cd isotope fractionation associated with Cd transfer in the Cd-tolerant and -hyperaccumulator species; and 2) explore possible mechanisms of Cd mobilisation from the solution to various physiological compartments.

**Materials and Methods**

**Plant Growth.** Seeds of three *R. communis* cultivars (Zibo.5, Zibo.6 and Zibo.8) and *S. nigrum* were obtained from the Zibo Academy of Agricultural Sciences (Shandong, China) and the Institute of Applied Ecology, Chinese Academy of Sciences (Shenyang, China), respectively. All seeds were washed in running deionized water before germination in the substrate for 14 d. The seedlings were then transferred into polycarbonate pots containing half strength Hoagland’s solution. The macronutrient solution consisted of 2 mmol·L⁻¹ Ca(NO₃)₂, 2.5 mmol·L⁻¹ KNO₃, 0.3 mmol·L⁻¹ KH₂PO₄, 1 mmol·L⁻¹ MgSO₄ and 0.5 mmol·L⁻¹ NH₄NO₃ as well as the micronutrient solution consisted of 0.25 μmol·L⁻¹ H₂BO₃, 0.25 μmol·L⁻¹ MnSO₄, 0.25 mmol·L⁻¹ CoCl₂, 12.5 mmol·L⁻¹ KI, 7.5 mmol·L⁻¹ ZnSO₄, 0.25 mmol·L⁻¹ CuSO₄, 2.5 mmol·L⁻¹ Na₂MoO₄ and 25 μmol·L⁻¹ Fe-EDTA. After 7 d, CdCl₂·2.5H₂O was added to the Cd concentration of 2 mg·L⁻¹ (Low Cd) and 5 mg·L⁻¹ (High Cd). No Cd was added to the control check (CK).

Plants were cultivated under controlled conditions (16 h photoperiod with a white light intensity of 350 μmol photons m⁻² s⁻¹; day: night temperatures 25 °C: 18 °C; relative humidity 60% ~ 70%). The isotopic composition (δ₁¹⁴/₁¹⁰Cd_spex) of the initial nutrient solution relative to Spex Cd standard solution was +0.14 ± 0.08‰ (2SD, n = 3).

**Sample Preparation.** Three plant samples as replicates were harvested 30 d after their transplantation, washed with tap water, and then rinsed thrice with deionized water. Each plant was divided into root, stem and leaf. Plant materials were freeze-dried and weighed prior analysis.

0.2 g of plant samples were digested in concentrated aristar grade HNO₃ (5 mL) and HF (1 mL) for 48 h in acid-cleaned Teflon beakers. The closed beakers were placed on a hot plate for 8 h at 80 °C and then at 160 °C because it constitutes less than 0.0132‰ of the indigenous Cd present in plant samples. Standard-sample bracketing was applied in this study to correct the mass bias. The instrumental reproducibility based on repetitive 114/110Cd measurements of Spex Cd standard solution was 0.09‰ (2SD, N = 214). The accuracy of the measurements was verified by measuring the Münster Cd standard solution and the results (+0.53 ± 0.08‰ of δ₁¹⁴/₁¹⁰Cd) were in good agreement with previously published values.

To express the isotope fractionation between two components A and B, we used δ₁¹⁴/₁¹⁰Cd_A-B that equated the difference δ₁¹⁴/₁¹⁰Cd_A – δ₁¹⁴/₁¹⁰Cd_B.

The isotopic composition of the whole plant Cd δ₁¹⁴/₁¹⁰Cd_WP has been established according to the following:

\[ \delta^{114/110}_{Cd, WP}(M_{Cd, root} + M_{Cd, shoot}) = \delta^{114/110}_{Cd, root}M_{Cd, root} + \delta^{114/110}_{Cd, shoot}M_{Cd, shoot} \] (2)

The Cd isotope composition of shoot and the whole plant (WP) relative to Spex Cd standard solution, as well as the isotopic variation between the different organs are shown in Table 2.

**Data Analysis.** Cadmium bioconcentration factor (BCF) was defined as the ratio of Cd in shoot or root of the plant to that in the nutrient solution. Cadmium translocation factor (TF) was described as the ratio of Cd in the...
The two tested plant species exhibit a consistent gradient that progressively increases from upper to lower organs. The Cd mass in the root of *S. nigrum* is much lower than that in the shoot under low Cd conditions. The Cd mass in *R. communis* is also lower in the root than that in the shoot independently of low or high Cd conditions. In contrast, *S. nigrum* exhibits a significant gradient with a progressive increase from upper to lower organs, by the order of leaf > stem > root, independently of the Cd concentration in the nutrient solution. In contrast, the Cd concentration in the leaf of *S. nigrum* is higher than that in the stem under low Cd conditions.

It is essential to precisely determine mass-balances for Cd in the different organs when Cd transfer in the plants is investigated. The Cd mass is calculated using the dry weight and Cd concentrations of the plant organs as shown in Fig. 1c–f. The total Cd mass in *S. nigrum* and *R. communis* under soil condition are lower than those under hydroponic conditions, considering that Cd in soil occurs in complicated forms because of its association with many physicochemical environments that impact Cd availability. The root BCFs of different cultivars increase by the order of *Zibo*-5 > *Zibo*-8 > *Zibo*-6 > *S. nigrum* under low Cd conditions, whereas they increase by the order of *Zibo*-5 > *Zibo*-8 > *S. nigrum* > *Zibo*-6 under high Cd conditions. The shoot BCFs of different cultivars increase by the order of *S. nigrum* > *Zibo*-8 > *Zibo*-5 > *Zibo*-6 under low Cd conditions, whereas they increase by the order of *S. nigrum* > *Zibo*-5 > *Zibo*-8 > *Zibo*-6 under high Cd conditions. Consequently, the root BCFs are highest in *Zibo*-5, followed by *Zibo*-8, whereas the shoot BCFs are highest in *S. nigrum*.

### Cd bioconcentration factor, translocation factor and tolerance index of *R. communis* and *S. nigrum*.

All bioconcentration factors (BCFs) of the four plant cultivars are higher than 1 (Table 1). The BCFs of the four cultivars under soil condition are lower than those under hydroponic conditions, considering that Cd in soil occurs in complicated forms because of its association with many physicochemical environments that impact Cd availability. The root BCFs of different cultivars increase by the order of *Zibo*-5 > *Zibo*-8 > *Zibo*-6 > *S. nigrum* under low Cd conditions, whereas they increase by the order of *Zibo*-5 > *Zibo*-8 > *S. nigrum* > *Zibo*-6 under high Cd conditions. The shoot BCFs of different cultivars increase by the order of *S. nigrum* > *Zibo*-5 > *Zibo*-6 > *Zibo*-8 under low Cd conditions, whereas they increase by the order of *S. nigrum* > *Zibo*-5 > *Zibo*-8 > *Zibo*-6 under high Cd conditions. Consequently, the root BCFs are highest in *Zibo*-5, followed by *Zibo*-8, whereas the shoot BCFs are highest in *S. nigrum*.

The translocation factors (TFs) of four plant cultivars are low, which indicates that the Cd concentration is higher in root than that in shoot. The TfDs of different cultivars increase by the order of *S. nigrum* > *Zibo*-8 > *Zibo*-6 > *Zibo*-5 under low Cd conditions, whereas they increase by the order of *S. nigrum* > *Zibo*-6 > *Zibo*-5 > *Zibo*-8 under high Cd conditions. Thus, *S. nigrum* accumulates the highest Cd concentrations during Cd translocation from root to shoot, whereas *Zibo*-5 accumulates the least, regardless of Cd concentration in solution.

A tolerance index (TI) based on biomass exposed to heavy metals is used to evaluate the heavy metal toxicity in the plants. The TIs of different cultivars increase by the order of *Zibo*-6 > *Zibo*-5 > *Zibo*-8 > *S. nigrum* under...
Low Cd conditions, whereas they increase by the order of Zibo-5 > Zibo-6 > Zibo-8 > S. nigrum under high Cd conditions. *R. communis* reveals higher TI than *S. nigrum* under hydroponic conditions, showing higher Cd tolerance of *R. communis* than *S. nigrum*.

According to an independent samples T-test (p < 0.05), the Cd treatments exert significant effects on transport and accumulation in Zibo-6, Zibo-8, and *S. nigrum* but have no significant effects on shoot accumulation and transport in Zibo-5. Overall, *R. communis* is characterised by a higher Cd tolerance, whereas *S. nigrum* has a higher potential to translocate Cd from root to shoot. In the organs of these four plant cultivars, more Cd is

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**Figure 1.** Cd concentration (a,b), dry weight (c,d), Cd mass (e,f) and Cd distribution (g,h) of root, stem, and leaf of three *R. communis* cultivars and *S. nigrum* during the 2ppm and 5ppm Cd solution conditions. Error bars show standard deviation (SD) of the three replicates.
accumulated in the root of *R. communis*, whereas more Cd is translocated from root to shoot in *S. nigrum* than *R. communis*.

**Cd isotopic composition in *R. communis* and *S. nigrum***. The four plant cultivars reveal small differences in $\delta^{114/110}$Cd$_{\text{stem-root}}$ (Table 2). The stem of Zibo-5 is enriched in lighter isotopes relative to the root, whereas Zibo-6 and *S. nigrum* are enriched in heavy isotopes relative to the root. In contrast, the $\delta^{114/110}$Cd$_{\text{stem-root}}$ values of Zibo-8 behave differently under low and high Cd conditions. In low Cd conditions, the stem of Zibo-8 is depleted of heavy isotopes relative to the root, which is consistent with Zibo-5, whereas, in high Cd conditions, the stem of Zibo-8 is enriched in heavy isotopes relative to the root, which is consistent with Zibo-6 and *S. nigrum*. The three *R. communis* cultivars show similar distributions of heavy and light Cd isotopes in stem and leaf, which are different to *S. nigrum*. The leaf of the three *R. communis* cultivars is all enriched in lighter isotopes relative to the stem, whereas those of *S. nigrum* are depleted of light isotopes relative to the stem (Fig. 2).

The Cd isotope compositions in the organs of Zibo-5, Zibo-6, and *S. nigrum* under low and high Cd conditions behave similarly, but differently to Zibo-8. The observed isotopic fractionations between the solution and organs increase by the order of $\delta^{114/110}$Cd$_{\text{root-solution}} > \delta^{114/110}$Cd$_{\text{stem-solution}} > \delta^{114/110}$Cd$_{\text{leaf-solution}}$ for Zibo-5, whereas they increase in the reverse order by $\delta^{114/110}$Cd$_{\text{leaf-solution}} > \delta^{114/110}$Cd$_{\text{stem-solution}} > \delta^{114/110}$Cd$_{\text{root-solution}}$ for *S. nigrum*. In contrast, the isotope value of $\delta^{114/110}$Cd$_{\text{stem-solution}}$ in Zibo-6 is larger than values of $\delta^{114/110}$Cd$_{\text{root-solution}}$ and $\delta^{114/110}$Cd$_{\text{leaf-solution}}$. The Cd isotopic fractionation between the solution and organs of Zibo-8 under low conditions behave similar to Zibo-5, but those under high conditions behave similarly to Zibo-6.

**Discussion**

The average $\delta^{114/110}$Cd$_{\text{solution}}$ values observed from solution to plants for Zibo-5, Zibo-6, Zibo-8, and *S. nigrum* are $-0.36\%$, $-0.40\%$, $-0.30\%$, and $-0.46\%$, respectively (Table 2). The observed enrichment of light Cd isotopes is consistent with previous studies on other metal isotopes (e.g. Cu, Fe, Zn, Ca) in plants, except for Mg exhibiting isotopically heavy plant biomass$^{8,13,15,23,46}$. The physiological and molecular mechanisms of Cd hyperaccumulation and tolerance include root proliferation in Cd-rich substrate, influx into cytosol or vacuole by specific and non-specific transporters, and complexation of Cd by certain ligands in cells$^{47}$. Based on the physiological and molecular mechanisms of Cd in higher plants, the speciation and diffusion in solution, adsorption on the root cell walls, uptake by ZIP proteins (Zinc-regulated transporter, iron-regulated transporter protein), complexation by phytosiderophores in solution and uptake of the entire complex through the membrane may

### Table 2. $\delta^{114/110}$Cd values in root, shoot, and whole plant (WP) of the three *R. communis* cultivars and *S. nigrum* relative to Spex Cd standard solution, as well as the isotopic variations between different organs.

| Organ                  | Zibo-5 | Zibo-6 | Zibo-8 | *S. nigrum* |
|------------------------|--------|--------|--------|-------------|
| Root                   | -0.12  | -0.23  | -0.08  | -0.25       |
| Shoot                  | -0.22  | -0.05  | -0.13  | -0.19       |
| Root-Solution          | -0.35  | -0.49  | -0.31  | -0.44       |
| Stem-Root              | -0.08  | 0.19   | -0.03  | 0.02        |
| Leaf-Stem              | -0.18  | -0.04  | -0.14  | 0.09        |
| WP-Solution            | -0.37  | -0.46  | -0.32  | -0.41       |
| Shoot-WP               | -0.08  | 0.15   | -0.04  | 0.03        |

Figure 2. Cd isotope compositions (reported as $\delta^{114/110}$Cd$_{\text{spex}}$) in final solution, root, stem, and leaf of three *R. communis* cultivars and *S. nigrum*. Error bars show standard deviation (SD) of the three replicates.
solution-root interface of the lighter Cd isotope. Therefore, diffusion may be a dominant process, leading to Cd isotope fractionation at the xylem of It may be attributed to the complexation with organic acids, phytochelatins (PCs), and metallothionein in the Cd isotope. In comparison, Pallavicini the Spex Cd standard solution. This further suggests that these two plant species preferentially take up lighter for

tation, previous work14 also showed that complexation with organic ligands led to an enrichment of heavy isotope  with Cd, which catalyse the translocation of Cd from root

Two possible abiotic processes could lead to isotope fractionation at the solution-root interface: diffusion and adsorption. Rodushkin et al.48 found that lighter isotopes diffused faster than heavier isotopes and free ions diffused faster than complex ions. Diffusion from solution to root could lead to an enrichment of the lighter isotopes at the root surface. In addition, adsorption could also result in Cd isotope fractionation. A previous study49 showed a small Cd isotope fractionation occurred during sorption of Cd to synthetic birnessite from low ionic strength solution, with lighter isotopes sorbed and heavier isotopes remaining in solution. In the present study, the $\delta^{114/110}$Cd Root-Solution in $R$. communis and S. nigrum is $-0.51\%$ to $-0.28\%$ (Table 2). The root is enriched in the lighter Cd isotope. Therefore, diffusion may be a dominant process, leading to Cd isotope fractionation at the solution-root interface of $R$. communis and S. nigrum.

Cd transport across the root cell and other cell membranes are possibly metabolically controlled14. In addition, within plants Cd can be transported along the electrochemical gradient via carrier proteins and ion channels or against the electrochemical gradient via electrogenic pumps13,14. Carrier-mediated transport favours heavy isotopes because it involves covalent binding to a carrier protein on the outer side of the membrane, with subsequent release on the inner side as a result of conformational changes in the carrier13. Conversely, transport through ion channels or via electrogenic pumps favours light isotopes because of its greater diffusion coefficient14. The observed net enrichment of the lighter isotopes in root and the differences between the plant cultivars, therefore, suggest that membrane transport is dominated by ion channels and electrogenic pumps rather than by carrier-mediated transport.

The differences in Cd isotope fractionation from root to stem of four plant cultivars might be due to the different Cd supply limitation, which is associated with the tolerance of plants. Although the Cd mass in nutrient solutions is sufficiently supplied, the Cd mass translocated in the extracellular and cellular plant organs might be limited in different plant cultivars. The magnitudes of the isotopic shifts during the solution-to-organ transfer slightly increase with decreasing Cd concentrations in the organs (Fig. 3). Moreover, the plant biomass is higher under low Cd conditions than that under high Cd conditions (Fig. 1c,d). Therefore, the Cd stress affects the magnitude of the isotopic shift during the solution-to-organ transfer. Gault-Ringold et al.34 proposed that Cd uptake of phytoplankton did not result in no net Cd isotopic fractionation under ‘supply-limited’ condition, but it could be kinetically driven resulting in Cd isotope fractionation under sufficiently high Cd levels. This could explain the different Cd isotope fractionation from root to stem between the cultivars.

The variation in the Cd isotopic composition between stem and leaf in $R$. communis and S. nigrum is distinct. It may be attributed to the complexation along with organic acids, phytochelatins (PCs), and metallothionein in the xylem of S. nigrum. Sun et al.50 identified that complexation with organic acids, phytochelatins (PCs), and metallothionein was an important mechanism for Cd detoxification, transportation and storage in S. nigrum. In addition, previous work14 also showed that complexation with organic ligands led to an enrichment of heavy isotope in the organs. Figure 1g,h show that a higher amount of Cd is stored in the root of $R$. communis, whereas more Cd is translocated to the stem and leaf of S. nigrum. This can be explained by the complexation of organic acids, phytochelatins (PCs), and metallothionein in S. nigrum with Cd, which catalyse the translocation of Cd from root to shoot. The observed difference between root and shoot in the Cd tolerant and -hyperaccumulator species may reflect the different Cd transportation mechanisms of the species.

Until recently, limited studies have reported the Cd isotopic composition in plants, including Cyperus alternifolius ($-0.37\%$ of $\delta^{114/110}$Cd$_{Spex}$), Pteris vittata ($-0.34\%$ of $\delta^{114/110}$Cd$_{Spex}$) and some birch leaves (ranged from $+0.30\%$ to $+1.3\%$ of $\delta^{114/110}$Cd$_{Spex}$). In the present study, all Cd isotopic compositions of plants determined for $R$. communis ($-0.40\%$ to $-0.01\%$) and S. nigrum ($-0.25\%$ to $-0.10\%$) show negative values relative to the Spex Cd standard solution. This further suggests that these two plant species preferentially take up lighter Cd isotope. In comparison, Pallavicini et al.51 reported that the $\delta^{114/110}$Cd$_{Spex}$ values of birch leaves favoured the

![Figure 3. Relationships between the Cd concentration and $\delta^{114/110}$Cd in the organs of $R$. communis and S. nigrum under different Cd conditions.](image-url)
enrichment of heavier Cd isotopes. Wei et al.\textsuperscript{42} suggested that different Cd isotopic compositions in different plant samples could result from distinct mechanisms of Cd accumulation in plants or different sources of Cd (from soil or nutrient solution).

The Cd isotopic composition of \textit{R. communis} and \textit{S. nigrum} are enriched with Cd isotope reservoirs in nature. Fig. 4 shows Cd isotope investigations on natural materials, such as meteorites and lunar rocks\textsuperscript{24–26,52}, seawater\textsuperscript{34–38,53–56}, samples from Pb-Zn smelting and refining plants\textsuperscript{28,29}, and soil polluted by the emissions from plants\textsuperscript{57,58}. Compared with the Cd isotope values in those materials, the variation of Cd isotopic compositions in plants is small. However, plants represent a reservoir of Cd isotopes in nature. In previous studies\textsuperscript{28,29,57,58}, the $\delta^{114/110}\text{Cd}_{\text{spex}}$ values of source featured with ‘slag (+0.4‰) > GSS-1 (+0.1‰) > GSD-12 (-0.4‰) > dust (-0.6‰) > Zinc oxide ore (-1.2‰) > residue (-1.4‰) > Primary Zinc ore (-1.6‰)’ (Fig. 4). In the present study, the $\delta^{114/110}\text{Cd}_{\text{spex}}$ ranges for \textit{R. communis} (-0.40‰ to -0.01‰) and \textit{S. nigrum} (-0.25‰ to -0.10‰) were between the $\delta^{114/110}\text{Cd}_{\text{spex}}$ values of GSS-1 (soil) and GSD-12 (sediment).

**Conclusions**

In the present study, the Cd isotope measurements show an isotopic shift to lighter isotopes during Cd transport from the nutrient solution to the plant organs of the Cd-tolerant \textit{R. communis} and the Cd-hyperaccumulator \textit{S. nigrum}. The observed isotope fractionation is enriched with the Cd isotope reservoirs in nature. In addition, the variation of the Cd isotopic compositions in leaf and stem differs between \textit{R. communis} and \textit{S. nigrum} implying different mechanisms of Cd translocation to the xylem in the Cd-tolerant and -hyperaccumulator species. Cd isotope fractionations of different organs provide new information to identify the chemical processes controlling Cd uptake and translocation in plants and organisms. Plant uptake is an important factor of isotopic variation in the Cd biogeochemical cycle. Thus, Cd isotope fractionation by plants needs to be taken into account in future investigations on environmental pollution using Cd isotopes. Overall, studies on Cd isotopes in plants lay the groundwork for understanding the biogeochemical Cd cycle and mechanisms of plant Cd acquisition and allocation.

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Author Contributions
Q.J.G. and H.J.W. proposed and organized the project. R.F.W., Q.J.G. and H.J.W. discussed and designed the experiment. R.F.W., Q.J.G., J.X.Y., M.P., G.X.Z., H.Z.Z., L.Y.T. and X.K.H. carried out the experiments. R.F.W., Q.J.G., H.J.W., C.Q.L., J.H., J.M. and C.W.Z analyzed and interpreted the data together. Q.J.G. and R.F.W. wrote the main manuscript text. M.P. and Y.X.W. revised the manuscript. All the authors participated in discussions of the research.

Additional Information
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