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REGULAR ARTICLE

Nickel translocation via the phloem in the hyperaccumulator *Noccaea caerulescens* (Brassicaceae)

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Abstract
Aims This study evaluated the effect of phloem translocation on Ni accumulation in the hyperaccumulator *Noccaea caerulescens*.
Methods The first experiment assessed the metal and organic compound concentrations in phloem sap. Leaves were cut from plants grown in nutrient solutions added with 100 μM Ni or Zn, and then used for phloem sap extraction by means of the EDTA-stimulated exudation method. In the second experiment, $^{61}Ni^{2+}$ was applied to old leaves as a foliar spray to assess bidirectional movement of Ni in phloem.
Results In the first experiment, enrichment of Ni or Zn was found in phloem exudates, indicating high Ni and Zn phloem loading and translocation capacity in *N. caerulescens*. Amino acids, *e.g.* nicotianamine and histidine, were present at low concentrations in exudates, which are insufficient for Ni and Zn chelation. On the contrary, concentrations of organic acids, especially malate, were high in all treatments, which may be involved in Ni and Zn chelation in phloem. The second experiment showed that 89 % of ^{61}Ni exported from old leaves was translocated upward to young leaves, whereas only 11 % moved downward to roots.
Conclusions Phloem sap of *N. caerulescens* is enriched in Ni and malate, the majority of which moves upward to young tissues. Phloem translocation may play an important role for Ni accumulation in young leaves of *N. caerulescens*.

Keywords Nickel · Hyperaccumulator · *Noccaea caerulescens* · Phloem

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Introduction

Hyperaccumulating plants are capable of accumulating extraordinary concentrations of heavy metals, *e.g.* Ni, Zn and Cd, in their shoots (Baker and Brooks 1989; Krämer 2010). Metal hyperaccumulation is a rare phenomenon in higher plants. To date, only 500

hyperaccumulating species have been identified globally, of which the majority are Ni hyperaccumulators (van der Ent et al. 2013).

The unique metal absorption and transport ability of hyperaccumulators has stimulated much academic interest for elucidating metal homeostasis mechanisms *in planta* and much advance has been achieved in recent decades. Generally, the physiological processes associated with hyperaccumulation include: i) stimulated metal influx across root cell plasma membranes (Pence et al. 2000); ii) reduced metal sequestration in root vacuoles (Lasat et al. 1998); iii) increased loading into xylem for transport to shoots (Papoyan and Kochian 2004; Hanikenne et al. 2008) and iv) stimulated metal influx across leaf cell plasma membranes and sequestration in leaf vacuoles (Küpper et al. 2001; Küpper et al. 2004). However, although phloem translocation is also an important physiological process in plants, little is known about the role of this process for metal accumulation in hyperaccumulators.

Field investigations have discovered that Ni hyperaccumulators growing on ultramafic soils can accumulate high concentrations of Ni in flowers and seeds (Robinson et al. 1997; Zhang et al. 2014; Groeber et al. 2015). As reproductive organs are usually considered to have weak xylem transport but strong phloem translocation activities (Taiz and Zeiger 2010), these results suggest that Ni may be efficiently translocated via phloem in these hyperaccumulators. Furthermore, Ni enrichment in phloem has been documented in a number of woody hyperaccumulators growing on tropical ultramafic soils. For instance, 16.9 % of Ni was found in the freeze-dried phloem sap of the Ni hyperaccumulator *Phyllanthus balgooyi* from Sabah, Malaysia (van der Ent and Mulligan 2015); and up to 25.7 % Ni was recorded in the latex of *Pycnandra acuminata* from New Caledonia (Jaffre et al. 1976), amounting to the highest Ni concentration ever found in living material.

Phloem sap is slightly alkaline (pH = 7–8) and enriched with organic compounds, *e.g.* sugars, amino and organic acids (Taiz and Zeiger 2010). Metal ions, *e.g.* Ni^{2+} and Zn^{2+}, are usually chelated with organic complexes, and amino acids are favorable for metal complexation in such alkaline condition (Harris et al. 2012). Zinc-nicotianamine (NA) complex has been found in the phloem sap of castor bean and rice (Nishiyama et al. 2012; Hazama et al. 2014). In addition to NA, Harris et al. (2012) predicted that cysteine (Cys) also plays a role for Zn chelation in phloem sap, according to a speciation model. The only study regarding Ni speciation in phloem sap was carried out by Wiersma and van Goor (1979), who found that Ni was bound to organic compounds with a molecular weight in the range of 1000–5000 in phloem sap of *Ricinus communis*. However, no information is available for Ni complexation in phloem sap of hyperaccumulators. Thus, if high concentrations of Ni are present in the phloem of hyperaccumulators, it is of interest to investigate the Ni chemical speciation in this compartment.

Nickel is rather mobile in phloem and can be readily transferred from sources to sinks (Neumann and Chamel 1986; Page and Feller 2005; Page et al. 2006). Both downward and upward movements exist during phloem translocation. Riesen and Feller (2005) found that radioactive ^{63}Ni fed into the leaf lamina in wheat seedlings was rapidly transferred up to younger leaves and down to roots. Fismes et al. (2005) also found that in three vegetables (lettuce, radish and bean), ^{63}Ni migrated throughout the whole plants following foliar application, and mainly toward young leaves, seeds and roots. Therefore, Ni accumulation in young growing tissues could be affected by phloem translocation, in particular when phloem flow carries large quantities of Ni.

Noccaea caerulescens (J. & C. Presl) F. K. Mey (formerly *Thlaspi caerulescens*) is a herbaceous Ni and Zn hyperaccumulator extensively used as a model species in the study of hyperaccumulation mechanisms (Milner and Kochian 2008; Krämer 2010). To date, little attention has been paid to its Ni phloem translocation ability. Therefore, the objectives of this study were : i) to assess the Ni enrichment in phloem; ii) to investigate the potential chelating ligands for Ni in phloem sap; and iii) to clarify the impact of phloem translocation on Ni accumulation in young tissues of *N. caerulescens*.

Materials and methods

Seed germination and plant cultivation

Seeds of *N. caerulescens* (originating from Puy de Wolf in France, a population that grows on ultramafic soils) were sown on peat matrix (Fafard Custom Growing Mix, Canada) and germinated in the dark at 22 °C for 7 days. Seedlings were then transferred to a growth chamber. Growth conditions were 22/18 °C day/night temperatures, 60 % relative humidity, 16 h photoperiod and 120 μmol s^{-1} m^{-2} light intensity. After 1 month, 40

seedlings were transferred to 4 L nutrient solution for hydroponic pre-culture. The standard solution contained the following nutrients (in μM): 1000 $Ca(NO_3)_2$, 1000 KNO_3, 500 $MgSO_4$, 100 KH_2PO_4, 50 KCl, 10 H_3BO_3,1 $MnCl_2$, 0.2 $CuSO_4$, 5 $ZnSO_4$, 0.2 Na_2MoO_4, 20 Fe(III)-EDDHA. Two mM 2-morpholinoethanesulphonic acid (MES) was used to buffer the pH, which was adjusted to 5.8 by the addition of 5 % KOH.

Phloem exudate extraction from expanding and old leaves of *Noccaea caerulescens*

After 1 month of pre-cultivation in standard solution, plants of uniform size were selected for treatment. Sets of two plants were transplanted to a container filled with 2 L nutrient solution. Three treatments were set up, i.e. CK (control; Ni 0, Zn 5 μM), Zn100 (Ni 0, Zn 100 μM) and Ni100 (Ni 100, Zn 5 μM). All treatments were replicated three times. Solutions were renewed every 6 days.

After 5 months of treatment, leaves were cut from the rosette and used for phloem exudate extraction, the method of which was adapted from Tetyuk et al. (2013). Briefly, 30 old leaves (>2 months of age) from each pot were cut at the base of the petioles and then placed in Petri dishes containing 20 mM K_2-EDTA solution. Leaves were recut at the base of the petioles and then transferred to a centrifuge tube (5 ml, Eppendorf) containing 4 mL of 20 mM K_2-EDTA, with the cut end of leaves submerged in the solution. Potassium-EDTA was used to prevent sealing of phloem sieve elements. After 1 h, leaves were washed by Milli-Q water and then transferred to a new tube, which contained another extraction solution (4 mL Milli-Q water). The final extraction period was 5 h with the tubes placed in a moist and illuminated environment to minimize leaf transpiration. Phloem exudate solution was frozen in liquid N_2, followed by lyophilization, and finally re-dissolved in 1 mL of Milli-Q water. In addition, 30 expanding leaves (ca. 1 month of age) from each pot were also cut from the rosette and used for phloem exudate collection. Phloem exudates were then divided into several aliquots for analysis of mineral nutrient elements, amino acids and organic acids.

Mineral nutrient elements (Ni, Zn and Fe) were determined by inductively coupled plasma - mass spectrometry (ICP-MS) (iCAP Q, Thermo Scientific, USA). Iron, a micronutrient element in plants, was analyzed in this experiment because its concentrations in plant tissues (ca. 1.8 μmol g^{-1}; (Epstein and Bloom 2005)) and phloem saps (40–80 μM; (Hocking 1983; Schmidke and Stephan 1995; Yoneyama et al. 2010; Ando et al. 2012)) generally remain stable. Therefore, Fe can be regarded as a reference element, with which the concentrations of other elements in phloem sap can be compared.

For amino acid compounds, special attention was paid to nicotianamine (NA) and histidine (His), which have high chelating affinities with Ni and Zn and were previously reported to play a role in metal chelation and hyperaccumulation (Kramer et al. 1996; Mari et al. 2006; Richau et al. 2009; Kozhevnikova et al. 2014). Nicotianamine was determined by liquid chromatography - tandem mass spectrometry (LC-MS/MS) (TSQ Quantum Ultra, Thermo Scientific, USA) with C18 ion exchange analytical column (1.9 μm , 100 mm × 2.1 mm; Hypeisil GOLD, Thermo Scientific, USA) applying the following gradient system: 0 min, 10 % eluent A (acetonitrile) and 90 % eluent B (0.1 % HCOOH / water); 3 min, 90 % eluent A; 4.1 min, 10 % eluent A (flow rate 0.3 mL/min; sample injection volume 5 μL). The MS profiles were carried out in the following conditions: source heater temperature, 300 °C; sheath gas (N_2), 45 arbitrary units; auxiliary gas (N_2), 5 arbitrary units; spray voltage, +3.0 kV; capillary temperature, 300 °C. The quantitative analysis of NA was detected in SRM mode. Three product ions $(M+H)^+$ were acquired, i.e. *m/z* 286.2, 185.2 and 114.1, with source collision induced dissociation (CID) energy of 10 , 18 and 27 V, respectively, while *m/z* 304.2 → *m/z* 185.2 was used for quantitative ion pairs. Nicotianamine standard was bought from Toronto Research Chemicals Inc., Canada. Twenty common amino acids were determined by amino acid analyzer (Syknm-S7130, Germany), according to National Standard Guideline of China for Analysis of Amino Acids (JY/T019-1996).

Malate and citrate, which are the most abundant organic acids and play important roles for metal chelation in shoots of *N. caerulescens* (Tolrà et al. 1996; Montargès-Pelletier et al. 2008), were determined by ion spectrometry (IC) (DX 600, Dionex, USA) according to AS11 and AS11-HC Anion-Exchange Column Instruction Manual (Dionex, USA). In brief, the analysis was conducted using a 4-mm diameter AS11 model pre-column and column. A gradient program, ranging from 3 to 20 mM of eluent (NaOH), was used to separate the acids in 30 min, with a flow rate of 1.0 mL min^{-1}.

After phloem extraction, the expanding and old leaves, along with roots and young leaves (10–15 days of age), were put in paper bags and dried in 60 °C, and then digested by concentrated HNO_3. Heavy metal concentrations in digested solutions were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (iCAP 6500 Duo, Thermo Scientific, USA).

The EDTA-stimulated phloem exudation method has long been used in many studies and is efficient for phloem sap collection (King and Zeevaart 1974; Chen et al. 2001; Deeken et al. 2008; Guelette et al. 2012). The phloem exudate collected by this method could be used to analyze proteins, small molecules, lipids, and RNAs (Tetyuk et al. 2013). However, the main focus of this study is on mineral element (Ni, Zn and Fe) in phloem sap. Thus, to test the relevance of this method for mineral element extraction, we conducted a preliminary experiment. After 4 months of pretreatment, 4 plants with uniform size were selected and each was placed in a 1 L pot. Plants were growing in 0.75 L nutrient solution, with the addition of 100 μM $NiSO_4$, RbCl (rubidium chloride) and $SrCl_2$ (strontium chloride), respectively, and solutions were renewed every 3 days. After 20 day of treatment, old leaves were cut from rosette and used for phloem exudate extraction, according to the method described earlier. Three treatments were set up: 1) 1 h of Milli-Q water (MQW) + 5 h of MQW extraction; 2) 1 h of EDTA-K_2 + 5 h of MQW extraction; 3) 1 h of EDTA-K_2 + 1 h of MQW extraction. Phloem exudates were then determined for their Ni, Rb and Sr concentrations by ICP-MS. In addition, metal concentrations in leaves were also analyzed. Strontium is a phloem-immobile element, while Rb is easily transported in phloem (Kuppelwieser and Feller 1991). Therefore, Sr concentration is expected to be low, while Rb should be enriched in phloem. The results of this experiment were quite corroborated with the expectation. The leaf exudation extracted by EDTA contained extremely low amount of Sr and had high Rb/Sr ratio (Table S1 and Figure S1, Supplementary Material), indicating its phloem origin. On the contrary, the water-extracted exudation had a high Sr concentration and low Rb/Sr ratio. In particular, the Rb/Sr ratio in the exudation was quite similar to that in leaves, indicating that this exudation could originate from leaf mesophyll cells. The Ni behavior was quite similar to that of Rb. Therefore, it can be concluded that the EDTA-facilitated extraction method is efficient for phloem exudate collection and subsequent metal determination. Furthermore, to test Ni accumulation during short-term Ni exposure, a young leaf (ca. 15 days of age) and an old leaf (>2 months of age) were cut from each plant in the 6th day of treatment, and then dried and digested. Nickel and Sr concentrations in leaves were then determined.

Foliar application of ^{61}Ni in *Noccaea caerulescens*

To avoid contamination from other sources, we chose a rare Ni stable isotope - ^{61}Ni (natural abundance: 0.0114) as the Ni^{2+} source. A certain volume of 10 μg mL^{-1} $^{61}Ni(NO_3)_2$ (^{61}Ni abundance: 0.9944; Inorganic Ventures, USA) solution was dried at 90 °C and concentrated to 1 mM. The pH was adjusted to 5.8 by 5 % KOH and 0.01 (v/v) Silwet L-77 spray adjuvant was added in the solution to increase leaf tissue permeability.

The method of foliar application was adapted from Lu et al. (2013). Plant seedlings were cultivated in nutrient solution for 3 months and then transferred to 1 L containers. All the fully-expanded leaves, except one, were cut to ensure the remaining leaf was the oldest and located in the lowest position of the rosette. This remaining leaf was then soaked in 5.0 mL of the prepared ^{61}Ni solution for 10 s. Each plant was treated as one replicate, and there were 3 replicates. The foliar application was conducted once every day for 3 days, and plants were cultivated for another 3 days before harvesting. All plants were constantly exposed to nutrient solution without added Ni during the whole cultivation period.

At harvest, the ^{61}Ni-spiked leaf in each plant was cut and discarded. Then the plants were divided into roots and shoots. Samples were dried and then digested by concentrated HNO_3. ^{61}Nickel concentration was determined by ICP-MS (iCAP Q, Thermo Scientific), whilst ^{60}Ni (natural abundance: 0.2622) was also analyzed to monitor Ni contamination from other sources.

Results

Ni and Zn concentrations in leaves and phloem exudates of expanding and old leaves

The Ni and Zn concentrations of different plant parts in Ni100 and Zn100 treatments are presented in Table 1. After the long-term treatment (5 months), all leaves accumulated high levels of Ni or Zn, and concentrations

Table 1 Ni and Zn concentrations in different plant parts of *Noccaea caerulescens* in the phloem exudate extraction experiment

Treatment	Element		Roots	Young leaves (10–15 days)	Expanding leaves (30 day)	Old leaves (>60 day)
Ni100	Ni	μM	33.6 ± 4.5	72.5 ± 10.4	107 ± 33	130 ± 29
Zn100	Zn		46.4 ± 11.7	82.9 ± 3.1	129 ± 12	161 ± 13

Data are means ± standard deviation of three replicates

increased with aging. Nickel and Zn concentrations could reach 130 and 161 μmol g^{-1} dry weight, respectively, in fully-expanded old leaves (>2 months of age), indicating the exceptional Ni and Zn accumulation ability in *N. caerulescens*. It is noticeable that young leaves (10 – 15 days of age) could also accumulate up to 72.5 μmol g^{-1} of Ni or 82.9 μmol g^{-1} of Zn. However, young leaves contained only 8.5 μmol g^{-1} Ni, after the short-term treatment (6 days) (Fig S2, Supplementary Material). The great difference for Ni accumulation between the long- and short-term treatments suggests that young leaves should have additional Ni sources after the long-term treatment.

For phloem exudates, significant variation could be observed between expanding and old leaves. The Ni and Zn concentrations in exudates were 56 and 101 μM, respectively in old leaves, while a 27 and 26 % reduction was recorded in expanding leaves (Fig. 1b, d). These results suggest that old leaves are more efficient in loading metals into phloem tissues, and may act as the main metal source for phloem translocation.

Iron concentrations in leaves and phloem exudates are shown in Fig. 1e, f. Stable concentrations of Fe were found in leaves (~ 2.5 μmol g^{-1}) of *N. caerulescens* growing in all treatments, which is similar to non-hyperaccumulating plants (ca. 1.8 μmol g^{-1}; Epstein and Bloom 2005). Iron concentrations in phloem exudates also remained quite stable (1.0–2.9 μM). Therefore, it could be speculated that Fe concentrations in phloem sap of *N. caerulescens* could be comparable to that of non-hyperaccumulating plants. It is noticeable that concentrations of Ni and Zn in phloem exudates were far greater than that of Fe (ca. 20 and 70 times, respectively), which indirectly indicates that exudates are enriched with Ni and Zn.

Organic compounds in phloem exudate of old leaves

To investigate the possible chelating ligands for Ni and Zn during phloem translocation, relevant amino acids and organic acids in exudates were determined (Fig. 2). Concentrations of amino acids were relatively low compared to those of Ni and Zn in phloem exudates. In particular, only trace concentrations (< 1 μM) of NA and His were detected in all treatments, which are negligible to chelate Ni and Zn in exudates. Thus, amino acids seem to play a minor role in Ni and Zn chelation during phloem translocation in *N. caerulescens*.

In contrast, concentrations of malate, the prevailing organic acid in the phloem exudates, were constitutionally high (149–169 μM) in all treatments, which were themselves sufficient to chelate all Ni or Zn (1:1) in the exudates, implying that organic acids should play an important role for Ni and Zn speciation in phloem sap of *N. caerulescens*.

Upward and downward movement of Ni in phloem

Table 2 shows the results from ^{61}Ni foliar application experiment. According to ^{60}Ni concentration in *N. caerulescens*, and the natural abundances of ^{60}Ni and ^{61}Ni, we calculated that less than 1 % of the total ^{61}Ni content was brought by contamination, which could be neglected. Thus, it can be considered all of the ^{61}Ni in plants was exported from the spiked leaf via phloem translocation. And the results show that 89 % of the ^{61}Ni moved upward to shoots, and 11 % was transported downward to the roots, suggesting that young growing leaves are the main sink for phloem flow in *N. caerulescens*.

Discussion

Because the EDTA-stimulated exudation method can only produce dilute phloem exudates, we cannot determine the true Ni and Zn concentrations in the original phloem saps. However, according to the ratio between Fe concentration in diluted exudates from this study (1.0–2.9 μM), and that in phloem saps from publications (40–80 μM; (Hocking 1983; Schmidke and Stephan 1995; Yoneyama et al. 2010; Ando et al. 2012), we

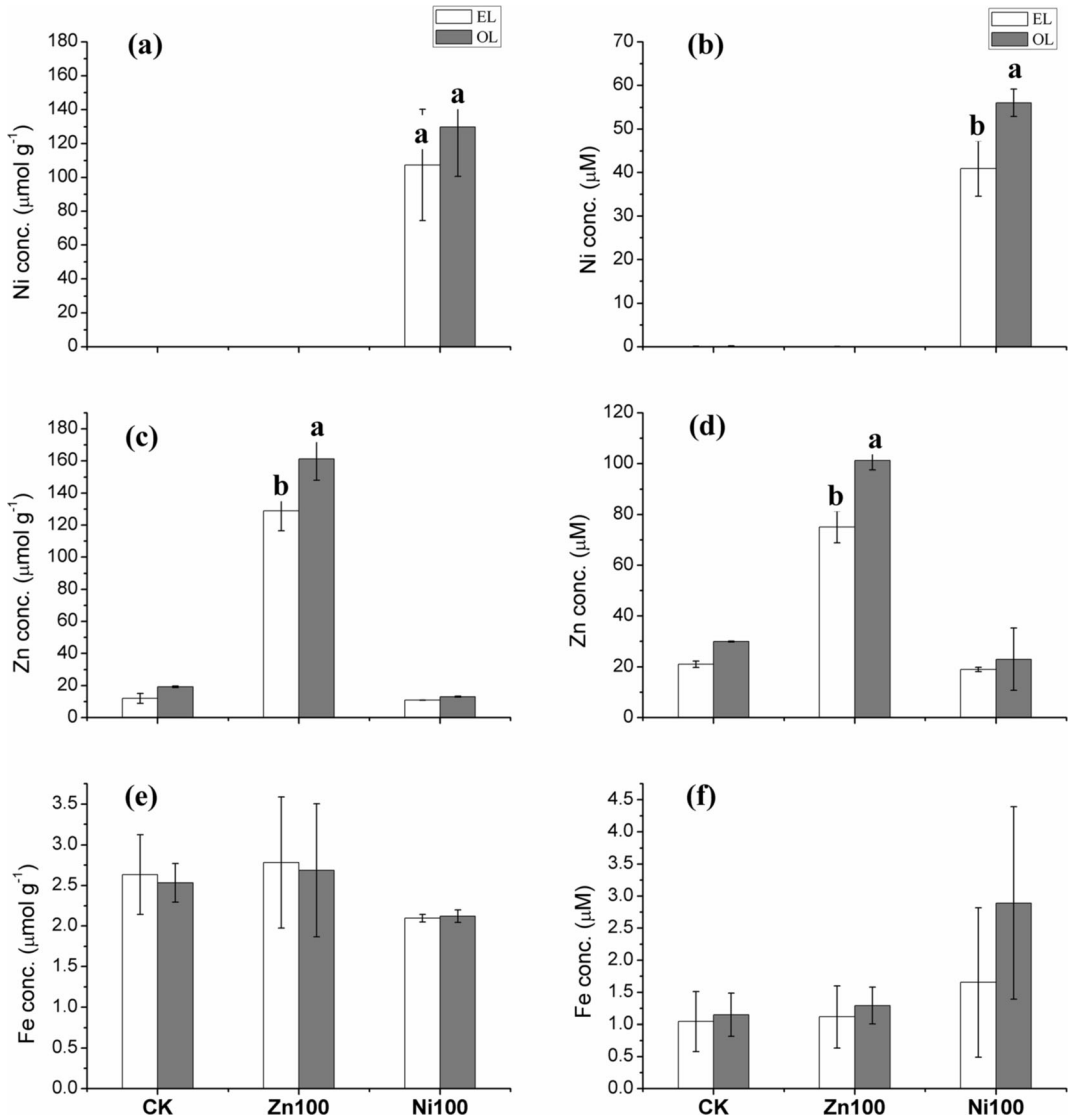

Fig. 1 Concentrations of Ni, Zn and Fe in leaves (**a**, **c**, **e**) and phloem exudates (**b**, **d**, **f**) of expanding and old leaves of *Noccaea caerulescens*. *EL* expanding leaves, *OL* old leaves. Data are means ± standard deviation of three replicates. Different letters in the column indicate values significantly different at $P < 0.05$, Duncan's Test

estimated that Ni and Zn concentrations in original phloem sap of *N. caerulescens* could reach 800 to 1600 μM and 2800 to 5600 μM, respectively. These concentrations are far greater than those in phloem sap of non-hyperaccumulating plants as well as in xylem fluids of hyperaccumulators (Álvarez-Fernández et al. 2014 and the references therein). Nickel enrichment in phloem has been documented in a number of woody hyperaccumulators growing on tropical ultramafic soils, such as the earlier noted *P. balgooyi*, *P. acuminata*, and also in *Euphorbia helenae* subsp. *grandifolia* which grows in Cuba (Reeves et al. 1996). Moreover, enhanced ability for Zn remobilization via phloem was found in the Zn hyperaccumulator *Sedum alfredii* (Lu et al. 2013). These results, along with our study, suggest that Ni or Zn enrichment in phloem may be a common phenomenon in hyperaccumulating species.

In addition, high Ni and Zn concentration in phloem saps also suggests that *N. caerulescens* has extraordinary phloem loading capability for heavy metals. Little is known about how mineral nutrients are loaded into phloem tissue. According to our knowledge on the

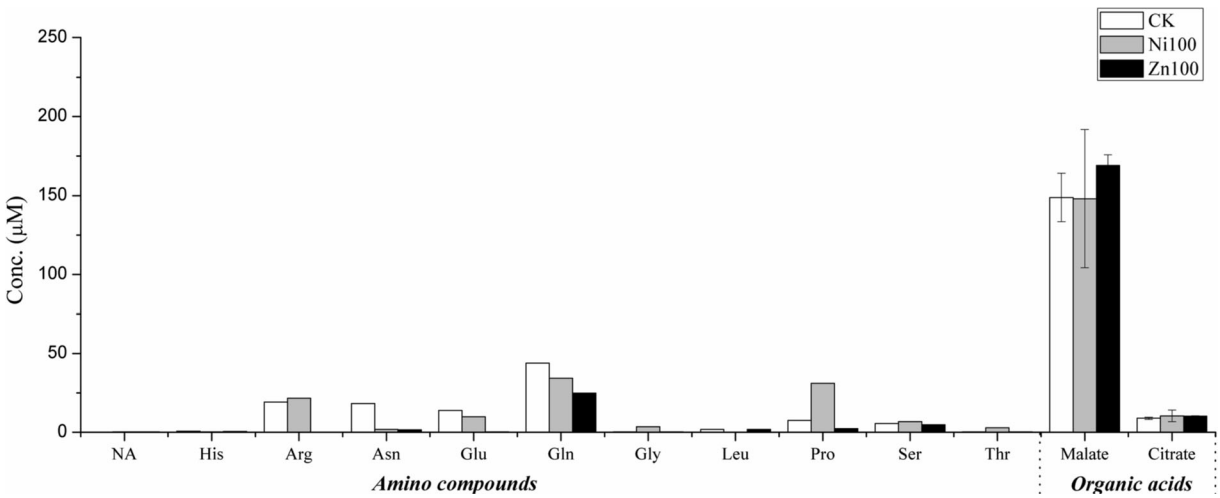

Fig. 2 Concentrations of amino acids and organic acids in phloem exudate of *Noccaea caerulescens*. Data of amino acids have one replicate; data of organic acids are means of two replicates

sucrose phloem loading process, both apoplastic and symplastic pathways exist in higher plants. In *Arabidopsis thaliana*, apoplastic loading is the most likely sucrose transport pathway due to the lack of plasmodesmata connections between the phloem parenchyma and the sieve element-companion cell complex (Haritatos et al. 2000). *Noccaea caerulescens*, which shares approximately 88 % nucleotide sequence identity within coding regions with *A. thaliana* (Peer et al. 2006), may have similar phloem structure. If this is indeed the case, then phloem tissues in *N. caerulescens* should have great Ni and Zn absorption ability by taking up large quantities of Ni and Zn from ambient apoplastic fluid.

For non-hyperaccumulating plants, Ni and Zn in phloem sap are present at low concentrations, which can be easily chelated by the redundant amino acids, and even high molecular weight organic molecules (Wiersma and van Goor 1979; Nishiyama et al. 2012; Hazama et al. 2014). However, it should be noted that Ni and Zn are not micronutrients in the hyperaccumulator *N. caerulescens*. Their concentrations in phloem sap are several tens of times higher than those in non-hyperaccumulating plants. Therefore, Ni and Zn chelation with high molecular weight organic compound (i.e. S- and N-donors) are unlikely to happen, due to the substantial amounts of ligands that would be required. The relatively low concentrations of amino acids in phloem sap are also insufficient for chelating all Ni or Zn. Therefore, organic acids, which present in high levels in phloem sap of *N. caerulescens*, seem to be the best candidates for Ni and Zn chelation, as complexation with organic acids can also increase the metal mobilities in phloem (Miranda et al. 2001; Epstein and Bloom 2005). In fact, citrate has already been discovered to be the main Ni chelator in the latex of the Ni hyperaccumulator *P. acuminata* (Schaumloffel et al. 2003).

From foliar ^{61}Ni application experiment, we observed that most of the Ni (89 %) exported from the spiked old leaf was transferred upward to young growing leaves, while only 11 % was translocated down to roots. This Ni partitioning pattern in phloem is quite similar to that of sucrose (Fondy and Geiger 1980).

Table 2 Biomass and ^{61}Ni concentration in different parts of *Noccaea caerulescens* from foliar ^{61}Ni application experiment

Plant parts	Biomass (mg)	Concentration (μg g^{-1})		^{61}Ni content (μg)	Ni content ratio
		^{60}Ni	^{61}Ni		
Root	43.8 ± 11.2	1.01 ± 0.26	5.34 ± 1.79	0.226 ± 0.071	11 %
Shoot	56.7 ± 8.3	0.484 ± 0.048	33.5 ± 13.8	1.86 ± 0.68	89 %

Data are means ± standard deviation of three replicates

Young leaves are stronger sinks than roots, which can deplete the sucrose content in the sieve elements more readily and thus increase the pressure gradient and the rate of phloem translocation towards themselves (Taiz and Zeiger 2010). Therefore, it could be speculated that Ni moves passively in the sieve elements, following the sucrose flow, and Ni partitioning in *N. caerulescens* might be a reflection of sucrose partitioning in plants.

The observation of high Ni concentrations in phloem saps and large Ni influx into young leaves suggests that phloem translocation may play an important role for Ni accumulation in these tissues. A rough calculation can be made to estimate the contribution of phloem translocation for Ni accumulation in young leaves, when diurnal change and competition between sinks are left out. In Ni100 treatment, 30 old leaves exported 0.057 μmol of Ni in 5 h, thus the Ni exporting rate was 0.0114 μmol h^{-1}. If 89 % of the Ni had moved up to a young leaf (0.01 g dry weight), then the leaf would gain 24.7 μmol g^{-1} Ni in 24 h. Similarly, phloem-based Zn was estimated to be 48.3 μmol g^{-1}. This is a non-negligible sum of Ni or Zn compared to the Ni or Zn concentrations in young leaves (72.5 and 82.9 μmol g^{-1} for Ni and Zn, respectively; Table 1), which may partly explain the fast accumulation of Ni or Zn in young leaves in comparison to expanding and old leaves, in particular when young leaves are considered to be strong sinks for phloem sap. As a matter of fact, we had tested the Ni accumulation in young leaves without any phloem supply. In the preliminary experiment, low concentrations of Ni were accumulated in both young and old leaves (8.5 and 3.7 μmol g^{-1} of Ni, respectively; Fig S2, Supplementary Material) after short-term Ni exposure (6 days). Due to the strong sequestration ability in leaves of *N. caerulescens* (Milner

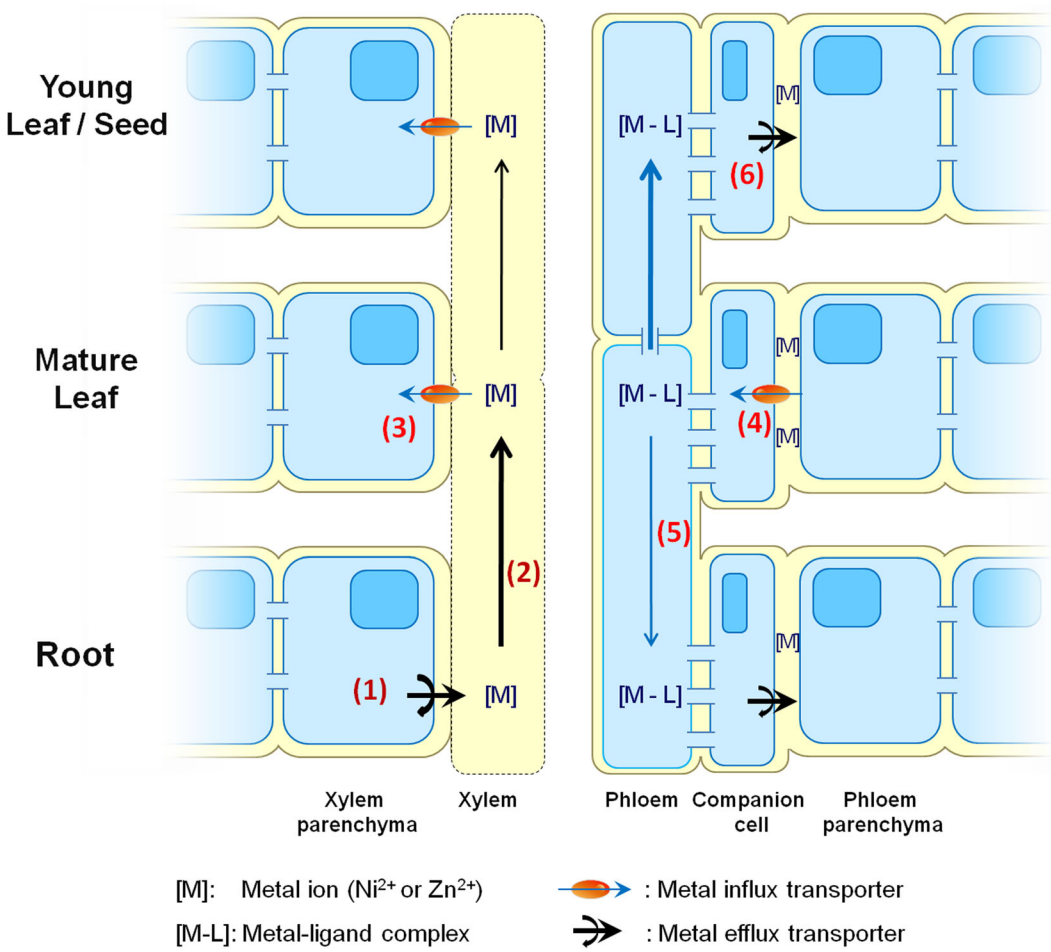

Fig. 3 Conceptual model for long distance transport of heavy metals in *Noccaea caerulescens*. (1) xylem loading; (2) xylem transport; (3) xylem unloading; (4) phloem loading; (5) phloem translocation; (6) phloem unloading

and Kochian 2008), little Ni was expected to be exported out via phloem in such low Ni concentrations. In addition, similar Ni/Sr ratio between young and old leaves (Fig S2) also suggested that Ni in these leaves came from the same source, i.e. xylem transport, as Sr is an indicator for xylem transport (Kuppelwieser and Feller 1991). Thus, it could be concluded that Ni accumulated in young leaves after 6-days Ni exposure was mainly imported by xylem, and its Ni accumulation was much lower than that with both xylem and phloem supplies.

On the basis of this study, along with knowledge from the literature, we put forward a conceptual model for long distance transport of heavy metals (Ni or Zn) in *N. caerulescens* (Fig. 3). Six physiological processes are included:

(1) Heavy metals are efficiently exported from the root symplast and loaded into xylem vessels, which may be due to the high expression of specific efflux transporter(s), *e.g.* HMA4 (Papoyan and Kochian 2004).
(2) Metals move up to shoots following the xylem flow, most of which find their final destination in mature leaves due to the strong transpiration in these tissues, while young leaves receive relatively small amount of metals. Metals are presented mainly as free hydrated cations during xylem transport (Salt et al. 1999; Centofanti et al. 2013).
(3) As xylem flow reaches minor veins and fills in the apoplastic space in leaves, metal ions are unloaded into the leaf symplast, which is the main storage site for heavy metals (Milner and Kochian 2008).
(4) Phloem companion cells, which are soaked in metal-rich apoplastic fluid, may contain large amount of metal influx transporters in cell membranes, which are able to take up large quantities of metal ions into the cytosols. The imported metal ions are chelated by a variety of organic molecules, e.g. organic and amino acids.
(5) Metal-ligand complexes are then transferred from companion cells to phloem vessels via plasmodesmata. Metals move passively in sieve elements, following the phloem flow, which is mainly driven by osmotic pressure generated by photosynthate (mainly sucrose) concentration gradients between sources and sinks (Taiz and Zeiger 2010). Upward movement appears to be the main direction for phloem translocation.
(6) When reaching sink organs, metals may be exported out from phloem tissues to the apoplast again, which are then taken up by the surrounding cells (Lu et al. 2013).

To conclude, phloem flow in *N. caerulescens* transports significant amount of Ni, probably chelated by organic acids (e.g. malate), from mature leaves to young leaves and thus makes a great contribution to Ni accumulation in these tissues.

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