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Root and shoot transcriptome analysis of two ecotypes of *Noccaea caerulescens* uncovers the role of *NcNramp1* in Cd hyperaccumulation

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SUMMARY

The Zn/Cd hyperaccumulator, *Noccaea caerulescens*, has been studied extensively for its ability to accumulate high levels of Zn and Cd in its leaves. Previous studies have indicated that the Zn and Cd hyperaccumulation trait exhibited by this species involves different transport and tolerance mechanisms. It has also been well documented that certain ecotypes of *N. caerulescens* are much better Cd hyperaccumulators than others. However, there does not seem to be much ecotypic variation for Zn hyperaccumulation in *N. caerulescens*. In this study we employed a comparative transcriptomics approach to look at root and shoot gene expression in Ganges and Prayon plants in response to Cd stress to identify transporter genes that were more highly expressed in either the roots or shoots of the superior Cd accumulator, Ganges. Comparison of the transcriptomes from the two ecotypes of *Noccaea caerulescens* identified a number of genes that encoded metal transporters that were more highly expressed in the Ganges ecotype in response to Cd stress. Characterization of one of these transporters, NcNramp1, showed that it is involved in the influx of Cd across the endodermal plasma membrane and thus may play a key role in Cd flux into the stele and root-to-shoot Cd transport. NcNramp1 may be one of the main transporters involved in Cd hyperaccumulation in *N. caerulescens* and copy number variation appears to be the main reason for high *NcNramp1* gene expression underlying the increased Cd accumulation in the Ganges ecotype.

Keywords: ***Noccaea caerulescens*, Cd hyperaccumulation, hyperaccumulation, cadmium, Nramp, Cd transport.**

INTRODUCTION

The zinc (Zn)/cadmium (Cd) hyperaccumulating species, *Noccaea caerulescens* J. & C. Presl (formally known as *Thlaspi caerulescens*), has served as a useful model for the study of the physiology and molecular biology of the hyperaccumulation of heavy metals in the aerial portions of the plant. While Zn influx and translocation in *N. caerulescens* have been studied extensively (Lasat *et al.*, 2000; Pence *et al.*, 2000; de Guimarães *et al.*, 2009; Milner *et al.*, 2012), somewhat less information is known about Cd hyperaccumulation in this plant species. Leaf Cd concentrations of up to 10 000 mg kg DW^{-1} have been measured in certain ecotypes of *N. caerulescens* without any toxicity symptoms. In comparison, typical shoot Cd concentrations in non-accumulator plant species are between 0.1–10 ppm Cd (Kabata-Pendias and Pendias, 2000). The metal hyperaccumulation phenotype involves increased uptake into the root and translocation to the aerial portions of the plant coupled with highly efficient mechanisms that provide Cd tolerance in the leaf, which involves Cd sequestration in the leaf cell vacuole (Ma *et al.*, 2005). Of considerable current interest is the identification of the genetic and molecular bases of Cd hyperaccumulation.

Several studies have examined the path by which Cd enters into and is transported within *N. caerulescens* (Küpper *et al.*, 1999, 2004, 2007; Bert *et al.*, 2003; Cosio

et al., 2004; Ma *et al.*, 2005; Ueno *et al.*, 2005, 2011; Courbot *et al.*, 2007; Ebbs *et al.*, 2009). There is considerable intraspecific variation between populations of *N. caerulescens* for the capacity to absorb Cd from the soil and accumulate Cd in the shoot. Comparison of the two most widely studied ecotypes of *N. caerulescens* has shown that the Prayon ecotype is considerably less tolerant to Cd than Ganges and other *N. caerulescens* ecotypes collected from southern France (Lombi *et al.*, 2001, 2002; Zhao *et al.*, 2002; Zha *et al.*, 2004; Cosio *et al.*, 2005). While both Prayon and Ganges are both Cd hyperaccumulators, the Ganges ecotype exhibits as much as a five-fold higher V_{max} for root Cd^{2+} influx with no difference in the K_M for root Cd^{2+} uptake (Lombi *et al.*, 2001). When plants were grown under Fe limiting conditions, a three-fold increase in root Cd influx was observed in Ganges, but no change was seen in Prayon (Lombi *et al.*, 2002). With regards to leaf Cd transport, Cosio *et al.* (2004) obtained the rather surprising result using protoplasts isolated from Ganges and Prayon leaf mesophyll tissue that there was little difference in the kinetic parameters for Cd^{2+} influx in Ganges versus Prayon leaf cells, this result suggests that the differences in Cd accumulation in the leaf are driven more by root transport processes. While Ganges plants are better Cd hyperaccumulators than plants of the Prayon ecotype, there is not much difference in Zn hyperaccumulation between the two ecotypes. A comparison of root Zn^{2+} influx showed negligible differences in the kinetic parameters for root Zn uptake between the two ecotypes, a result that suggests that Cd is not moving into the plant on the same transporters as Zn (Lombi *et al.*, 2001; Zhao *et al.*, 2002; Zha *et al.*, 2004). However the kinetics for root Cd^{2+} influx described above suggests that there is increased abundance of a transporter(s) that mediate Cd uptake in the roots of the Ganges ecotype in the roots, but not in the shoots.

With regards to Cd tolerance in leaves of *N. caerulescens*, findings from several laboratories have indicated that vacuolar Cd sequestration may be the primary mechanism (Küpper *et al.*, 1999, 2004, 2007; Cosio *et al.*, 2005; Ma *et al.*, 2005; Ueno *et al.*, 2005, 2011; Ebbs *et al.*, 2009). The P_{1B}-type ATPase, NcHMA3, has been shown to be localized in the leaf cell vacuole and mediates Cd transport into the vacuole both in *Arabidopsis thaliana* (Gravot *et al.*, 2004; Morel *et al.*, 2008) and *N. caerulescens* (Ueno *et al.*, 2011). Over-expression of *NcHMA3* in transgenic Arabidopsis plants resulted in significant increases in Cd tolerance and accumulation (Ueno *et al.*, 2011). A second metal transporter, *NcMTP1* (*ZTP1*), has been proposed to be a major contributor to leaf Zn tolerance and accumulation via the mediation of Zn uptake into the vacuole of cells in the leaf (Assunção *et al.*, 2001). *NcMTP1* is expressed mainly in the shoots of *N. caerulescens* and shows a low level of expression in root tissue. It also appears that there may be more than one copy of *NcMTP1* in the *Noccaea* genome (Assunção *et al.*, 2001). However, it remains unknown if NcMTP1 is involved primarily in vacuolar Zn and not in Cd transport.

In an attempt to identify transporters that are involved in the greater Cd accumulation and tolerance observed in the Ganges ecotype, we conducted a comparative transcriptome analysis in response to Cd for roots and shoots of the Prayon and Ganges ecotypes of *N. caerulescens*. In an earlier publication we used a similar comparative transcriptome analysis and focused solely on shoots to identify NcHMA3, which we showed is a tonoplast-localized transporter highly specific for Cd that is responsible for sequestration of Cd into the leaf vacuoles. We also showed that a higher expression of this gene in Ganges shoots was due to an increased copy number; it may play a major role in Cd hyperaccumulation in leaf cells. In the current study, we conducted comparative transcriptome analysis in both roots and shoots. In the root, we identified and focused this study on NcNramp1, which we characterized for its role in the root transport component of Cd hyperaccumulation in *Noccaea caerulescens*.

RESULTS

Comparative transcriptome analysis

Similar to the approach used by Schat and Kalff (1992) in their study of the role of phytochelatins in metal tolerance in metallophytes, the strategy employed here to identify genes involved in Cd hyperaccumulation sought to compare gene expression in Prayon and Ganges plants exposed to solution Cd concentrations that produced a comparable physiological effect in those plants. To determine this ecotype-specific treatment concentration, it was necessary first to identify the concentration that caused Cd phytotoxicity in each ecotype for relatively brief Cd exposure periods. For the Prayon ecotype of *N. caerulescens*, there were no adverse effects observed following growth for 7 days in nutrient solution that contained 0.1 or 0.5 mM $CdSO_4$ (after hydroponic growth on –Cd nutrient solution). However, when the $CdSO_4$ concentration was increased to 1 mM or higher, Prayon plants became obviously chlorotic, with the severity of the chlorosis increasing with concentration. Ganges plants showed a similar degree of chlorosis at 5 and 10 mM $CdSO_4$, and exhibited no toxicity symptoms at concentrations of 1 mM or lower. Leaf tissues from plants exposed to the highest Cd concentration that did not result in Cd phytotoxicity symptoms on a given ecotype (0.5 mM for Prayon, 1.0 mM for Ganges, grown for 7 days on Cd) were subjected to elemental analysis. Leaf Cd concentrations exceeded 16 100 mg kg dry weight (DW^{-1}) in the Ganges ecotype compared with approximately 790 mg kg DW^{-1} in Prayon, concentrations indicative of Cd hyperaccumulation by each ecotype (Figure S1).

Consequently, solution Cd concentrations of 0.5 mM for Prayon and 1 mM for Ganges were employed for the 7-day exposure period to yield the root and shoot tissues that were used for transcriptome analysis.

To compare the root and shoot transcriptomes from the Ganges and Prayon ecotypes, total RNA from both ecotypes was hybridized to the ATH1 Affymetrix chip. At the sequence level it has been estimated that *N. caerulescens* and *Arabidopsis thaliana* share between 85 and 90% homology in the coding regions (Peer *et al.*, 2003). To account for the differences in sequence between Arabidopsis and *Noccaea*, the probes were selected based on the Hammond *et al.* (2006) study that hybridized the same ATH1 microarrays to *N. caerulescens* genomic DNA to call a better match of the oligo and the rest of the hybridizations as mismatches for background subtraction. It should be noted that Hammond *et al.* (2006) used a different ecotype of *N. caerulescens* from the two ecotypes studied here, so small differences in hybridization efficiencies may exist that may skew the relative expression ratios. Also as the microarray is designed for Arabidopsis and not *N. caerulescens*, the array may not cover the full *N. caerulescens* genome. However, it was estimated by Hammond *et al.* (2006) that the Affymetrix array provides significant coverage of the *N. caerulescens* genome and over 21 000 probe sets were able to be called as unique *N. caerulescens* genes.

Based on the comparison of the two transcriptomes, 289 genes were expressed higher in the roots of Ganges, including 39 transporters (Table 1). By comparison, 85

Table 1 Transporter genes that show higher expression in the roots of Ganges versus Prayon in response to Cd. Significance was determined using a FDR cut off of 0.1

Probe ID	Ratio	FDR	Annotation	AGI code
266963_at	7.272727	$7.24E^{-05}$	Mtp11	At2 g39450
262751_at	7.173601	0.0215448	Cation efflux protein	At1 g16310
259116_at	6.6357	0.00299255	Peptide transporter PTR2-B (histidine transporting protein)	At3 g01350
251902_at	6.289308	$7.24E^{-05}$	Uncoupling protein (ucp/PUMP)	At3 g54110
261639_at	4.535147	0.00755036	Tubulin alpha-2/alpha-4 chain	At1 g50010
260414_at	4.301075	0.00071124	Nitrate transporter (NTL1)	At1 g69850
245513_at	4.253509	0.0338263	SYBL1 like protein	At4 g15780
260298_at	3.815338	0.00677722	Putative oxidoreductase	At1 g80320
260075_at	3.690037	0.00107634	MATE efflux transporter	At1 g73700
247207_at	3.415301	0.00942671	Nucleotide sugar transporter	At5 g65000
252328_at	3.348962	0.00453715	Sece	At3 g48570
262456_at	3.269042	0.0343398	Glucose transporter	At1 g11260
245846_at	3.173596	0.00236008	P-type transporting ATPase	At1 g26130
250712_at	3.103662	0.00271844	Human RAN binding protein 16-like	At5 g06120
266939_at	2.9994	0.00503465	Plasma membrane proton atpase (PMA)	At2 g18960
258861_at	2.993116	0.037972	Putative helicase similar to C-terminal half of transcription-repair coupling factor (TRCF)	At3 g02060
246862_at	2.879355	0.00302709	E2, ubiquitin-conjugating enzyme, putative ubiquitin-conjugating enzyme	At5 g25760
266672_at	2.771619	0.00445816	Putative Na+-dependent inorganic phosphate cotransporter	At2 g29650
250088_at	2.717391	0.0189541	APG5 (autophagy 5)-like protein	At5 g17290
254068_at	2.682403	0.00623274	Abcb28	At4 g25450
264587_at	2.580645	0.0261305	Glr3.4	At1 g05200
259133_at	2.540005	0.0223333	Sugar transporter, putative similar to integral membrane protein	At3 g05400
258293_at	2.522704	0.0119026	Pho1	At3 g23430
253658_at	2.474023	0.0109338	Hma3	At4 g30120
262649_at	2.455193	0.0102055	EXS family protein	At1 g14040
267483_at	2.367424	0.00503465	Utr1	At2 g02810
267092_at	2.318034	0.0287502	Aux1	At2 g38120
245146_at	2.268088	0.0341046	Putative cis-Golgi SNARE protein	At2 g45200
256224_at	2.2041	0.005475	GTP-binding protein (SAR1B) identical to GTP-binding protein (SAR1B)	At1 g56330
266533_s_at	2.157032	0.0376319	Pip2.8	At2 g16850
264748_at	2.144082	0.0325936	Hypothetical protein predicted by genemark.hmm	At1 g70070
266927_at	2.134927	0.0204166	Aquaporin (plasma membrane intrinsic protein 1B)	At2 g45960
261895_at	2.132196	0.016113	Metal ion transporter (NRAMP1)	At1 g80830
254857_at	2.064836	0.0338511	Putative protein acetylcholine regulator unc-18	At4 g12120
245868_at	2.030869	0.00808528	Hypothetical protein	At1 g58032
255505_at	1.990842	0.035359	SAR1/GTP-binding secretory factor	At4 g02080
261881_at	1.987281	0.0407026	Nodulin-like protein	At1 g80760
251257_at	1.860811	0.0292286	ADP-ribosylation factor-like protein ADP-ribosylation factor 1	At3 g62290
264906_at	1.802776	0.0446582	Putative mitochondrial phosphate translocator protein	At2 g17270

genes showed higher expression in the roots of Prayon, including nine transporters (Table S1). In shoots, 78 genes showed higher expression in Ganges, with 12 of the 78 genes encoding transporters (Table S2). Finally, we found 95 genes that were expressed more highly in shoots of Prayon with 18 classified as a transporter (Table S3). Three metal-related transporters were expressed more highly in both roots and shoots of the Ganges ecotype, Nramp1, HMA3 and MTP11. Of these three transporter genes, HMA3 and MTP11 have been shown to be localized to the vacuole (Ueno *et al.*, 2011) or other endomembranes (Delhaize *et al.*, 2007), while Nramp1 is believed to encode a plasma membrane transporter and has been shown to mediate Fe and Mn transport in *Arabidopsis thaliana*, and also suggested to possibly mediate Cd uptake (Curie *et al.*, 2000; Cailliatte *et al.*, 2010). There was twice as much expression of *NcNramp1* in Ganges versus Prayon roots and approximately four-fold higher expression in the shoots of the Ganges ecotype compared with Prayon under the high Cd stress [false discovery rate (FDR) 0.016113; 0.000632] (Figure 1). Of the 39 transporters that were more highly expressed under Cd stress in the roots of Ganges, *Nramp1* stood out as a candidate for cellular Cd influx in the root.

Expression of NcNramp1 under various Zn and Cd conditions

The Ganges and Prayon *NcNramp1* orthologs were isolated using 3′ and 5′ RACE-PCR (polymerase chain reaction). The comparison between the Prayon and Ganges *Nramp1* open reading frames (ORF) showed there is 99% identity between the two genes with 16 base pairs that differ within the 1.6 kb ORF, which results in eight amino acid changes. The comparison of AtNramp1 to the two NcNramp1 orthologs showed 90.1% and 89.9% identity at the amino acid level to the Ganges and Prayon proteins, respectively, and all three ORFs are 533 amino acids in length. Comparison amongst all six members of the AtNramp family suggests that NcNramp1 is most similar to AtNramp1 (Figure S2). Alignments of the DNA and amino acid sequences of each NcNramp1 ortholog along with its AtNramp1 homolog can be seen in Figures S3 and S4.

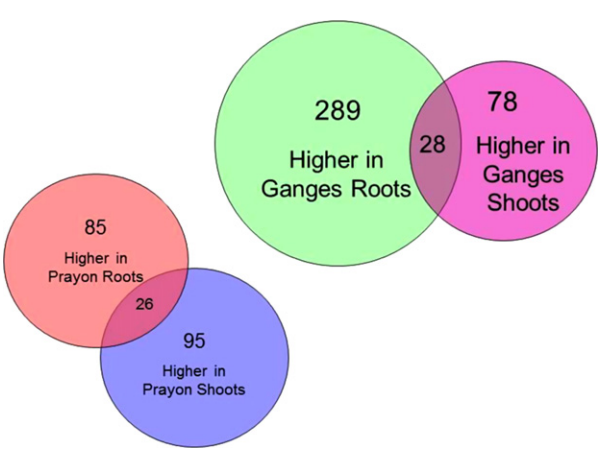

Figure 1. Venn diagram of total number of genes expressed more highly in Ganges or Prayon roots and shoots for plants treated with Cd (1.0 for Ganges and or 0.5 mM for Prayon) for 1 week.
The numbers in the overlapping circles represent common genes expressed more highly in both the roots and shoot of either Ganges or Prayon.

Primers were designed to homologous regions of the *NcNramp1* orthologs and quantitative RT-PCR analysis was used to quantify the relative expression of *NcNramp1* in Ganges and Prayon in response to two Zn and two Cd concentrations to gain greater insight into the possible role of Nramp1 in Cd transport and accumulation. A general theme was observed that in roots and shoots of the Ganges ecotype, *NcNramp1* was always expressed at least five-fold higher under all conditions tested compared with the expression of the Prayon ortholog in the respective tissues (Figure 2). The expression of *NcNramp1* was tested on 3-week-old plants and *NcNramp1* transcript concentrations did not change significantly over the range of Zn or Cd concentrations tested. These concentrations included Zn deficient/cadmium absent (0 μM Zn and Cd), sufficient (10 μM) and high (100 μM) concentrations of Zn, and two concentrations of Cd (1 and 10 μM).

Genome copy number

Recent evidence has suggested that increased expression of metal transporter genes in *N. caerulescens* compared with non-accumulator plant species can be due to increased genome copy number and not increased transcription at a single gene locus (Ó Lochlainn *et al.*, 2011; Ueno *et al.*, 2011). To test whether increased copy number was involved in the higher *NcNRAMP1* expression in Ganges, the genomic copy number of *NcNramp1* was estimated with quantitative real-time PCR. The data were normalized to *NcZNT1*, which is a single-copy gene in *N. caerulescens* (Deniau *et al.*, 2006). The data were also normalized to the C_T value of two indel markers designed from expressed sequence tags (ESTs) (RR11nr025 and RR4nr003) of *N. caerulescens* (Deniau *et al.*, 2006). The genes in Arabidopsis for the ESTs RR11nr025 and RR4nr003 correspond to At3 g26520 and At3 g19820, respectively, which encode tonoplast intrinsic protein and calmodulin protein, respectively. Using this approach which we had used previously to estimate the copy number for *NcHMA3* (Ueno *et al.*, 2011), we found that the copy number for *NcNramp1* in the Ganges ecotype was approximately four times higher than in the Prayon ecotype (Figure 3).

Cd transport characteristics of NcNramp1

We expressed both orthologs of NcNramp1 in yeast and conducted short term (3–10 min) ^{109}Cd uptake to quantify

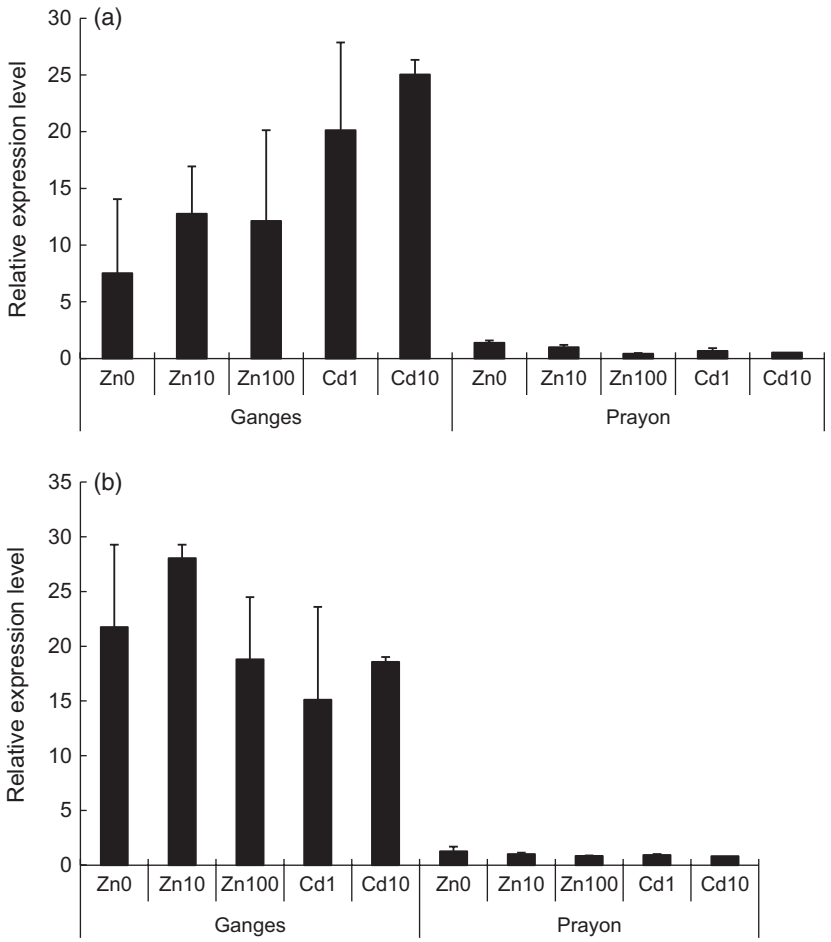

Figure 2. Relative expression levels of *NcNramp1* in the roots and shoots of Ganges and Prayon.
Three week old plants were grown on varying concentrations of Zn (0, 10 or 100 μM) or Cd (1 and 10 μM) for 7 days and *NcNramp1* transcript concentrations were assayed and normalized to *actin* transcript concentrations for comparison in either the root (a) or shoots (b).
Shown is the mean of three biological replicates with the standard error (SE) of the mean.

yeast Cd^{2+} influx mediated by NcNramp1. As seen in Figure 4, both orthologs of NcNramp1 mediated Cd^{2+} influx equally effectively, suggesting that both may be plasma membrane-localized transporters for Cd uptake in *N. caerulescens*.

Localization of NcNramp1 protein in *N. caerulescens* roots

To determine the cell and tissue specific localization of NcNramp1 in *N. caerulescens*, immunostaining was conducted with an antibody raised to the cytosolic C-terminal tail of NcNramp1. Roots of both Ganges and Prayon were probed with the purified antibody and the signal could be seen almost exclusively in the endodermis and stele (Figure 5). The signal was stronger in the Ganges ecotype, and correlated well with the gene expression data both from the transcriptomic analysis and subsequent qRT-PCR analysis. An effort was made to probe shoot tissue of *N. caerulescens* with this antibody but non-specific binding could not be eliminated from shoot tissue and thus we could not identify where NcNramp1 was localized in the shoot. To identify the membrane localization for NcNramp1, subcellular fractions were isolated from roots and probed with the same antibody. NcNramp1 was found in the plasma membrane fraction, co-localized with H^+-ATPase and not V-type ATPase, which is localized in the tonoplast membrane (Figure 6).

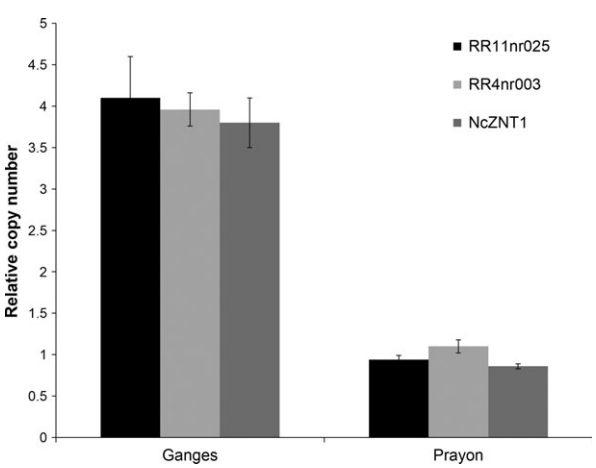

Figure 3. Determination of genome copy number for *NcNramp1*.
Copy number was determined with qPCR and normalized with C_T values for two expressed sequence tags (ESTs), RR11nr025 and RR4nr003 and NcZNT1. Error bars represent ± standard errors (SEs) (n = 3).

Over-expression of *NcNramp1* in transgenic *Arabidopsis thaliana*

To test the function of NcNramp1 *in planta*, both orthologs of *NcNramp1* were expressed under the control of the 35S promoter in stably transformed Arabidopsis plants. Testing of five independent lines overexpressing each *Nramp1* ortholog for Cd tolerance (root growth in transgenic lines/root growth of Col-0) on a toxic concentrations (30 μM) of Cd in nutrient solution showed that after 10 days of growth, all of the lines that expressed either ortholog of *Nramp1* exhibited a 25–45% increase in Cd sensitivity relative to Col-0 (Figure 7a). Additionally, eight of the 10 transgenic lines exhibited significant increases in plant Cd accumulation compared with wild type Arabidopsis, with the increased Cd accumulation ranging from 1.75-fold to nearly three-fold higher in the transgenic lines (Figure 7b).

To test if the over-expression of *NcNramp1* leads only to Cd sensitivity or if it also results in increased sensitivity to other heavy metals, we tested two of the transgenic lines for each ortholog that had been used for the Cd sensitivity/accumulation studies in Figure 7 (P3, P5, G6, and G7), for growth on high concentrations of Fe, Mn and Zn. Lines expressing either *NcNramp1* ortholog exhibited similar root growth (primary root length) compared with Col-0 when grown on high concentrations of Zn and Fe, indicating they were not sensitive to high levels of Fe or Zn (Figure 8). Interestingly, when plants were grown on a high concentration of Mn (1.5 mM), plants that overexpressed either version of *NcNramp1* were more tolerant to the high Mn in the media compared with Col-0 plants

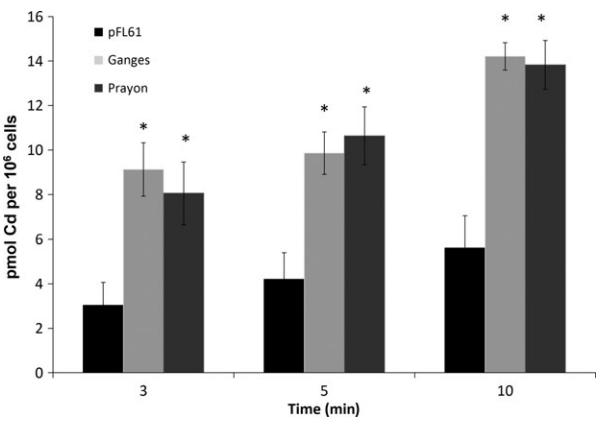

Figure 4. Cd uptake mediated by NcNramp1 expressed in ZHY3 yeast cells.
Yeast cells expressed either the Ganges or Prayon *NcNramp1* or the empty vector pFL61, and radiotracer ($^{109}Cd^{2+}$) flux assays were conducted with 25 μM Cd in SC-URA liquid medium for uptake periods of 3, 5, or 10 min. Shown are the mean flux values with the standard error (SE) of the mean for one of three separate experiments that yielded similar results. Asterisks indicates a *P*-value < 0.05.

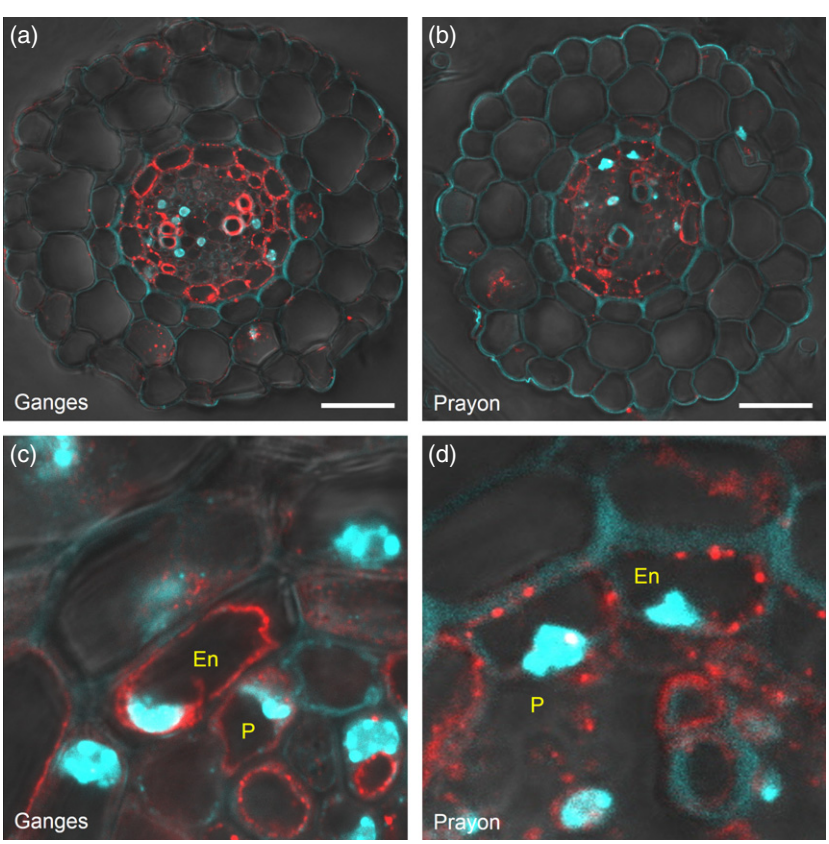

Figure 5. Localization of NcNramp1.
The roots of both Ganges and Prayon plants were used for immunostaining of NcNramp1 protein with a 1:300 antibody dilution. The antibody against NcNramp1 from Ganges and Prayon specific to the sequence C-DIV-DMQLHGRVSTTDVN (positions 516–532 of NcNramp1s).
(a) Ganges root; (b) Prayon root; (c) higher magnification image of Ganges root; and (d) higher magnification image of Prayon root. The red color represents the signal from the NcNramp1 antibody, while the cyan color represents the signal from nucleus and autofluorescence of the cell wall.
Scale bars represent 20 μm. P, pericycle; En, endodermis.

(approximately 40% increase in root growth; Figure 8). It should also be noted the when the over-expression lines and Col-0 were grown on Murashige and Skoog (MS) medium alone, there was no difference in root growth (Figure 8). The mineral content of the four over-expression lines were also measured under the same conditions for which the root length was measured and no significant differences in Fe or Mn content were observed (Figure S3). Zn content was statistically less for one line of each ortholog tested and the general trend was that the Zn content of seedlings was lower than that of Col-0 (Figure S5).

Iron-induced expression of *NcNramp1*

To understand if *NcNramp1* plays a role in the Fe-induced transport of Cd in the Ganges ecotype reported by Lombi *et al.* (2002), western blot analysis was conducted on NcNramp1 protein abundance in plasma membrane fractions isolated from roots of *N. caerulescens* grown on +Fe and – Fe conditions. As seen in Figure 9, there was little to no induction of NcNramp1 protein expression in response to Fe deficiency. To confirm these results and to ensure that Fe deficiency had been imposed, *Nramp1* transcript abundance was assayed in roots of the same plants and again found to not be increased by Fe deficiency. However the transcript abundance for the full length *IRT1* transcript was increased significantly (greater than 30×) in the Ganges ecotype but not in the Prayon ecotype in response to Fe deficiency, as reported previously by Lombi *et al.* (2002) and Plaza *et al.* (2007) (Figure 10).

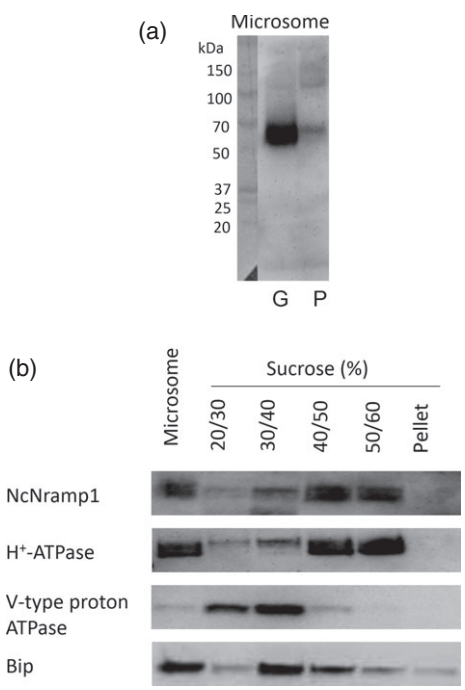

Figure 6. Western blot analysis of NcNramp1 protein in Ganges and Prayon roots. An antibody against NcNramp1 specific to the sequence, C-DIV-DMQLHGRVSTTDVN (positions 516–532 of NcNramp1) was used.
(a) NcNramp1 protein concentrations from the microsomal fraction isolated from Ganges (G) and Prayon (P) roots.
(b) Membrane localization of NcNramp1. For the determination of the subcellular localization of NcNramp1, the microsomal fraction from the ecotype, Ganges, was fractionated through a 20, 30, 40, 50, and 60% (w/v) discontinuous sucrose gradient. The PVDF membrane was treated with the purified primary rabbit anti-NcNramp1 (1:500), V-type ATPase (marker for TP; 1:5000), H^+-ATPase (marker for PM; 1:2000), and ER marker Bip (1:2000). Anti-rabbit IgG (H+L), horseradish peroxidase (HRP) conjugate (1:10 000) was used as a secondary antibody.

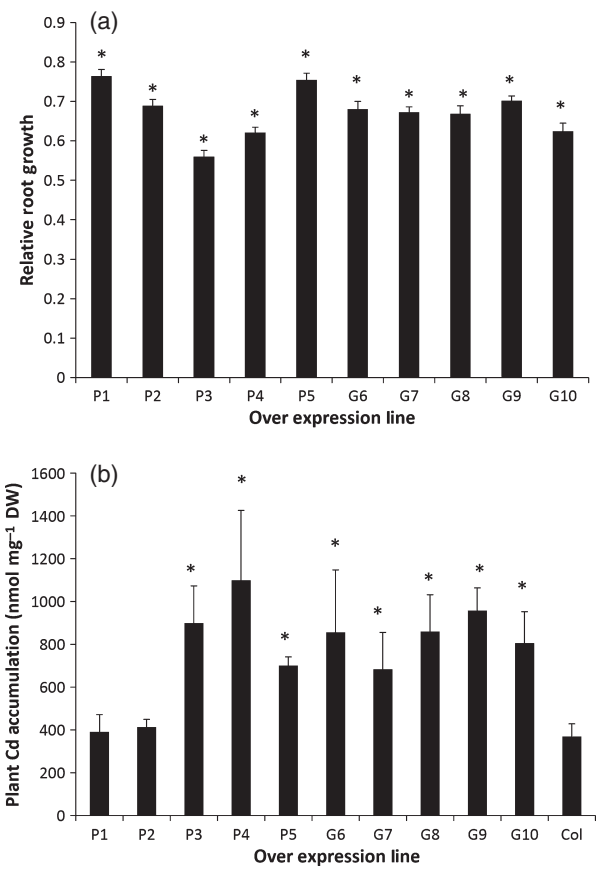

Figure 7. Metal tolerance evaluation in transgenic lines expressing *NcNramp1*.
Relative root growth and Cd accumulation of plants over expressing either *NcNramp1-P* or *NcNramp1-G* grown on liquid medium + Cd was determined.
(a) Primary root growth was compared between each over-expression line grown on MS plus 25 μM $CdSO_4$ relative to root growth for Col-0 grown on the same medium. Root length was determined as described in Experimental Procedures using the RootReader 2D platform (Clark *et al.*, 2013). All transgenic lines were significantly more Cd sensitive than wild type Col-0 plants for relative root growth (*P*-value < 0.05).
(b) Cd concentration of *NcNramp1* overexpressing lines. Cd concentration was determined from the over-expression lines that expressed either the Prayon or Ganges ortholog using ICP-AES analysis on 10-day-old Arabidopsis plants that expressed either *NcNramp1-G*, *NcNramp1-P* or Col-0 alone.
The mean of three biological replicates with at least 15 plants sampled for each biological replicate is shown with the standard error (SE) of the mean. Asterisk symbolizes a significant difference in Cd accumulation relative to Col (*P*-value < 0.05).

DISCUSSION

The ability of certain species to accumulate and tolerate normally toxic concentrations of micronutrients/heavy metals is a rare trait in the plant and animal kingdoms. *Noccaea caerulescens* is one species that has this unique ability and even within this species there is a large variation in its ability to accumulate and tolerate Cd. In an attempt to better understand the molecular mechanism(s) that underlie the differences seen between two populations of *N. caerulescens* for their ability to absorb and accumulate Cd in the shoot, a comparative transcriptomics approach was taken to identify candidate transporter genes involved in Cd hyperaccumulation. From this analysis, 51 genes that encode putative metal transporters were found to be expressed to significantly higher levels in the superior Cd-accumulating Ganges ecotype compared with the Prayon ecotype in response to Cd stress. Of these 51 differentially expressed transporters, only 12 of the transporter genes were more highly expressed in the shoots of Ganges, while 39 transporter genes were more highly expressed in Ganges roots (Figure 1, Tables 1 and S2).

As we had previously used a similar analysis to identify and characterize NcHMA3 as an important Cd transporter in Cd accumulation in the leaf, here we focused on root genes that were more highly expressed in Ganges in response to Cd. We chose here to focus on *NcNramp1* for this study even though it was not the transporter gene most differentially expressed in Ganges versus Prayon roots. The reason for choosing *Nramp1* was based on previous findings that its closest sequence match in *Arabidopsis thaliana* was highly expressed in the epidermis and endodermis of roots, localized to the plasma membrane localized, and has been suggested previously to possibly mediate Cd uptake in Arabidopsis while functioning primarily as an Fe and Mn uptake transporter (Curie *et al.*, 2000; Cailliatte *et al.*, 2010). Characterization of *NcNramp1* has led us to conclude that this transporter is most likely involved in 'basal' Cd hyperaccumulation in the plant species *N. caerulescens* but that it is not involved in the Fe deficiency-induced increase in root Cd uptake reported previously for the Ganges ecotype.

Characterization of *NcNramp1* suggests strongly that it is specifically a Cd uptake transporter in *N. caerulescens*, based on its transport properties when expressed in yeast and on the response of the transgenic over-expression Arabidopsis lines to high Cd, Zn, Mn and Fe concentrations. The transgenic plants that expressed either ortholog of *NcNramp1* did show some tolerance to high Mn in the medium, however the lack of a difference in the accumulation of Mn between the over-expression lines and wild type Arabidopsis plants would preclude the notion that NcNramp1 is involved in root Mn uptake. We currently do not have an explanation for the increased Mn tolerance without altered Mn accumulation in the over-expression lines, and this aspect should be the subject of future study. Arabidopsis plants that overexpress *NcNramp1* accumulate significantly higher concentrations of Cd but not Fe, Zn, or Mn compared with Col-0 plants and exhibit increased sensitivity specifically to Cd toxicity. Furthermore, its unique cell-specific protein localization to the endodermis and stelar cells just internal to the endodermis (Figure 5) also suggest an important role for NcNramp1 in translocation from the root to the shoot that has been

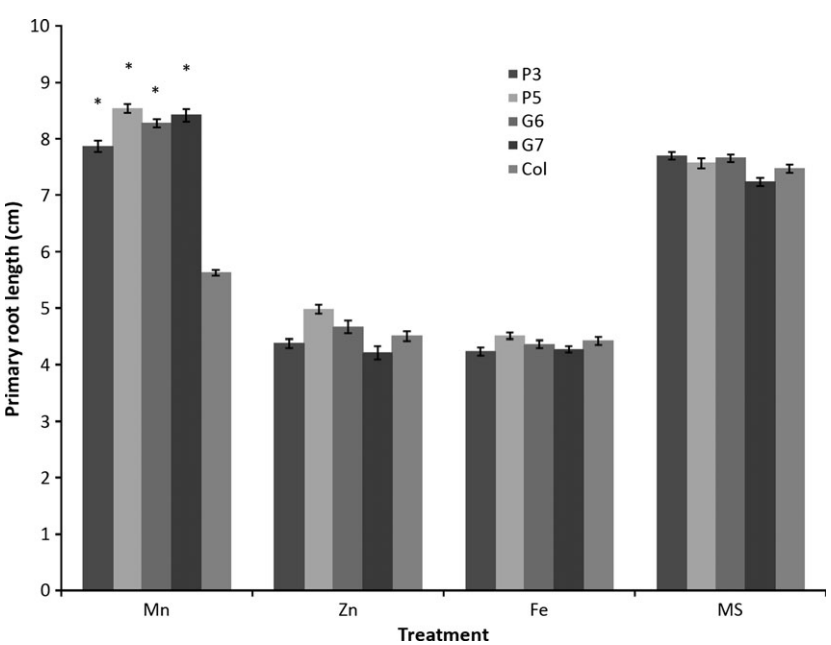

Figure 8. Effect of other heavy metals on relative root growth of *NcNramp1* overexpressing plants.
Two of the five over-expression lines expressing either the Prayon or Ganges *Nramp1* ortholog were selected from those presented in Figure 7 and grown on high concentrations of Zn (300 μM), Fe (250 μM Fe::EDDHA), and Mn (1.5 mM), or on MS alone as a reference. Root length of the primary root was determined as described in Experimental Procedures using the RootReader 2D platform (Clark *et al.*, 2013). The mean relative root growth of three biological replicates with the standard error (SE of the mean is shown.
Asterisks indicates a *P*-value of less than 0.05.

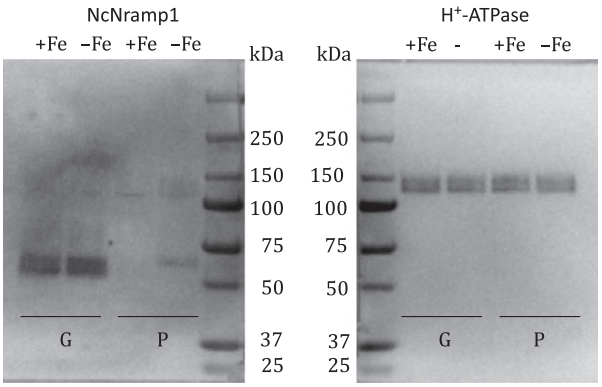

Figure 9. Nramp1 protein concentrations using western blot analysis in response to plant growth under +Fe and –Fe conditions.
The plasma membrane fraction was purified from root microsomal membranes for western blot analysis as described in Experimental Procedures.
Western analysis for the PM H^+-ATPase was conducted to verify that the membrane fraction was plasma membrane in origin.

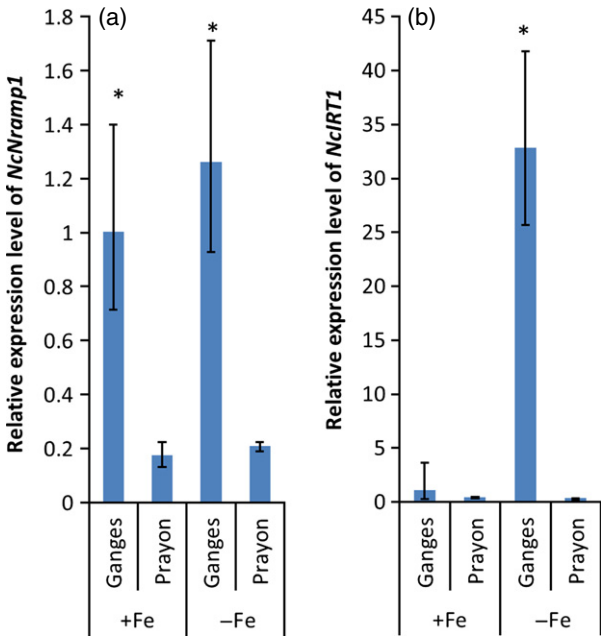

Figure 10. Quantitative real-time PCR analysis of *NcNramp1* (a) and *NcIRT1* (b) transcript abundance in roots of Ganges and Prayon plants grown on +Fe and –Fe conditions.
The mean of three biological replicates with the standard error (SE) of the mean is shown.
Asterisks indicates a *P*-value of less than 0.05.

shown to be a key transport step in Zn/Cd hyperaccumulation in *N. caerulescens* and in another Zn/Cd hyperaccumulator, *Arabidopsis halleri* (Lasat *et al.*, 1996, 1998; Kramer, 2010). The idea here is that increased loading of Cd from the root cortical apoplasm into endodermal cells with subsequent symplastic (and possibly apoplastic) transport to the xylem parenchyma, would provide more Cd for transport from the xylem parenchyma into xylem vessels for translocation to the shoot.

It is also interesting to note that the greater expression of *NcNramp1* in Ganges is due to increased genome copy number. As both orthologs responded in a similar fashion to the various metal treatments tested, there do not appear to be differences in the regulation of expression. Furthermore, both NcNramp1 proteins appear to possess similar Cd uptake properties. Thus the mechanistic basis for the contribution of NcNramp1 to the increased Cd hyperaccumulation in the Ganges ecotype appears to be due to the fact that the Ganges ecotype has at least four genomic copies of *Nramp1* whereas Prayon only has a single copy in its genome. The fact that Ganges has four copies correlates very well with the 4.5-fold higher Cd transport into the roots of Ganges compared with Prayon from Zhao *et al.* (2002). This finding leads us to suggest speculatively that NcNramp1 is a major player in the Cd hyperaccumulation trait in *N. caerulescens*, along with NcHMA3 (Ueno *et al.*, 2011).

It also should be noted that none of the candidate transporters previously hypothesized to play a role in *N. caerulescens* Cd hyperaccumulation was found to be differentially expressed between these two ecotypes of *N. caerulescens*. This list includes the previously characterized *NcIRT1* and *NcZNT1* and other ZIP family members (Pence *et al.*, 2000; Plaza *et al.*, 2007; Milner *et al.*, 2012).

We also found that the larger Cd flux into the root under Fe-deficient conditions in the Ganges population may be more attributable to the up-regulation of the full length *IRT1* rather than Cd transport mediated by Nramp1. A combination of the baseline Cd transport via Nramp1 and the Fe deficiency-induced Cd transport via IRT1, which was strongly induced in the Ganges ecotype and not Prayon (Figure 10b), would fit with the previous publications on this subject in the literature (Lombi *et al.*, 2002; Zhao *et al.*, 2002; Plaza *et al.*, 2007).

The current comparative study has helped to further define the underlying molecular basis for the dramatic differences in Cd hyperaccumulation seen between ecotypes in the *Noccaea* genus, and also helped to identify another possible key player in Cd hyperaccumulation. This study has also presented yet additional evidence that genome duplication of metal transporter genes are involved in heavy metal hyperaccumulation that lead to the increased capacity to tolerate and accumulate the large amounts of Zn, Cd and Ni found in *Noccaea* species. It also fits that the lack of differences in Zn uptake and accumulation presented previously for the Ganges and Prayon ecotypes for Zn uptake are consistent with the findings here that the expression of the Zn transporter gene, *NcZNT1*, or expression of any other *ZIP* family members suggested to be involved in Zn transport were not different between the

two ecotypes. Our understanding of Cd tolerance is still not complete and further study still needs to be done to understand the role that the other transporters found to be higher in the Ganges ecotype play in regards to the increased Cd tolerance. However the findings presented here suggest a significant role for Nramp1 in Cd hyperaccumulation in both *Noccaea caerulescens* populations and also suggests that there are likely to be multiple routes and pathways in which Cd hyperaccumulation occur.

EXPERIMENTAL PROCEDURES

Plant growth conditions

Seeds of the Prayon and Ganges ecotypes of *Noccaea caerulescens* were surface sterilized first in dilute bleach (0.5%) and subsequently with 70% ethanol before being germinated on Murashige and Skoog (MS) plates (MS medium + vitamins at a concentration of 4.43 g L^{-1}, 0.05% 2-(*N*-morpholino)-ethanesulfonic acid (MES) at pH 5.7, 1% sucrose, 1.5% Bacto-agar). The plates were placed in a Percival Scientific growth chamber (Model E-36 L, Boone, IA, USA) at a 30° angle. Germinating seeds were illuminated with a combination of fluorescent and incandescent lights at an intensity of approximately 150 μM m^{-2} sec^{-1} with a 16 h photoperiod and held at ambient humidity with a 24°C/20°C day/night temperature cycle. After 17 days of growth, seedlings from plates that showed no contamination were transferred to a hydroponic solution with the following composition: 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM $NiSO_4$, and 0.1 μM H_2MoO_4. The solution was aerated and buffered with 1 mM MES, titrated to pH 6.0 with KOH. Iron was provided as 10 μM Fe-EDDHA from Sequestrene 138 (Becker-Underwood, Ames, IA, USA). Plants were grown under the same growth chamber conditions as above until the plants reached the 8–10 leaf stage (approximately 32 days). Plants of each ecotype were then transferred to the same nutrient solution supplemented with $CdSO_4$ to final concentrations of 0, 0.1, 0.5, 1, 5, or 10 mM. Each treatment was replicated three times. After a 7-day exposure, plant shoots were inspected visually for evidence of heavy metal toxicity (e.g. chlorosis).

Plant Cd treatment for microarray analysis

Seeds of the Prayon and Ganges ecotypes of *N. caerulescens* were germinated and grown as above to the 8–10 leaf stage. *Noccaea caerulescens* plants were transferred to fresh nutrient solution supplemented with either 0.5 or 1 mM $CdSO_4$, respectively, and subjected to a 7-day treatment, as described above with four replications of each treatment. These concentrations represent the highest concentration from the dose–response experiment that did not produce visual symptoms of toxicity in that ecotype. Following the treatment period, shoots were quickly rinsed, patted dry, and split into two subsamples. One subsample was immediately snap frozen in liquid nitrogen, and stored at −80°C. These samples were shipped by courier on dry ice to the Robert W. Holley Center, Ithaca, NY, USA. The second subsample was dried at 60°C to constant mass and ground to <2 mm. These samples were digested following EPA method 3050b (http://www.epa.gov/epaoswer/hazwaste/test/pdfs/3050b.pdf) and filtered through a 0.45-μm Millipore filter to remove particulates. The tissue extracts were analyzed for Cd using a SpectrAA 220FS Atomic Absorption Spectrometer (Varian Inc., Walnut Creek, CA, USA). Tissue Cd concentrations in shoots of the Prayon and Ganges ecotypes were compared using Student's *t*-test in the SPSS statistical package (SPSS for Windows, Ver. 13.0, http://www-03.ibm.com/software/products/en/spss-stats-standard/).

Microarray analysis

Plants were grown as described above, total RNA was isolated from root and shoot tissue using the Plant RNeasy RNA Mini Kit (Qiagen, Valencia, CA, USA) and double-stranded cDNA was synthesized following standard protocols (Affymetrix, Santa Clara, CA, USA). The synthesized cDNAs were transcribed *in vitro* by T7 RNA polymerase using biotinylated nucleotides to generate biotinylated complementary RNAs (cRNAs); cRNAs were purified using the GeneChip® Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA). cRNAs were then fragmented at 94°C for 35 min to generate cRNA molecules of approximately 35–200 bp in length. *Arabidopsis thaliana* ATH1 GeneChip® arrays (Affymetrix, Santa Clara, CA) were hybridized with 15 μg of fragmented labeled cRNA for 16 h at 45°C. The genomic DNA (gDNA)-based probe-selection strategy described in Hammond *et al.* (2006) was used to process our transcriptome dataset. The CEL files generated by hybridizing *N. caerulescens* genomic DNA samples to Arabidopsis ATH1 GeneChip® arrays were obtained from http://affymetrix.arabidopsis.info/xspecies. These gDNA CEL files were used to mask the probes in the Arabidopsis ATH1 array Chip Description File (CDF) with a gDNA hybridization intensity threshold of 300, using the Xspecies Perl script (Hammond *et al.*, 2006). The probe-masked CDF file was used to process and normalize *N. caerulescens* RNA CEL files at probe level with the RMA algorithm (Irizarry *et al.*, 2003). The detection calls (present, marginal, or absent) for each probe set were obtained using the mas5calls function in the Affy package (Gautier *et al.*, 2004). Only genes with at least one present call across all the compared samples were used to identify differentially expressed genes. Significance of gene expression was determined using the LIMMA test (Smyth, 2004) and raw *P*-values of multiple tests were corrected using the false discovery rate (FDR; Benjamini and Hochberg, 1995). Genes with FDRs < 0.1 were identified as differentially expressed genes.

Cloning of Nramp1 from *N. caerulescens*

Primers were designed based on the Affymetrix Nramp1 probe 261895_AT and other primers were designed for regions of high sequence conservation between the rice and Arabidopsis Nramp families of genes. To acquire the full approximatey 1.6 kb open reading frame (ORF) region, RACE–PCR (rapid amplification of cDNA ends–polymerase chain reaction) was performed using the original fragment to clone the full 5′ and 3′ ends of the gene in Prayon (Clontech RACE kit, Mountain View, CA, USA). Primers with sequences ATGGCGGCTACAGGATCTGGACGACCTCAATTC ATTTC and TCAATTAACATCGGTTGTAGACACTCTA were then used to isolate the full ORF from both ecotypes. Alignments of the *Noccaea* and Arabidopsis *Nramp1* DNA and amino acid sequences were performed using CLC Sequence Viewer 6 (Cambridge, MA, USA).

Quantitative real-time PCR expression analysis

To investigate the expression of *NcNramp1*, seedlings of Ganges (14-day-old) and Prayon (11-day-old) hydroponically cultivated according to Ueno *et al.* (2011), were exposed to various concentrations of Zn (0, 10 or 100 μM) or Cd (1 or 10 μM) for 7 days, or to –Fe or –Mn growth conditions. The plants were divided into shoots and roots, frozen with liquid nitrogen and subjected to RNA extraction using an RNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com/). The total RNA from various tissues were

treated with DNase I (Invitrogen, http://www.invitrogen.com/), and then converted to cDNA using the SuperScript II (Invitrogen) protocol using random oligomers. Expression was determined with THUNDERBIRD™ SYBR qPCR Mix (TOYOBO) by Mastercycler® ep realplex (Eppendorf). The primer sequences for RT-PCR of *NcNramp1* were 5′-CGTTGAGCTCCACTACTCAAAGC-3′ and 5′-GCCAGTAGCTGAACGAGGGATC-3′. *Actin* (forward primer, 5′-GAGACTTTCAATGCCCCTGC-3′; reverse primer, 5′-CCATCTCCAGAGTCGAGCACA-3′) was used as an internal control. Expression data were normalized with the expression level of *Actin* by the $\Delta\Delta C_t$ method. A thermal dissociation curve was performed after each of the two biological replicates to ensure only one product was being amplified. A subsample of the product of the qRT-PCR reaction for each ortholog of *Nramp1* and *actin* was further amplified by PCR, cloned into the pGEM-T® Easy vector (Promega, Madison, WI, USA), and sequenced for target verification.

Estimation of *Nramp1* copy number in genomic DNA

To investigate *Nramp1* copy number in the genomic DNA of Ganges and Prayon, a DNA fragment of this gene was amplified from genomic DNA. Primers and PCR conditions were the same as described in the section 'Quantitative real-time PCR expression analysis' above, except 40 ng of genomic DNA was used as the template, instead of cDNA for each reaction. The data obtained were normalized based on the C_T value for the InDel markers designed from two ESTs, RR11nr025 (GenBank DN925409) and RR4nr003 (GenBank DN923929), of *N. caerulescens* (Deniau *et al.*, 2006) as well as *NcZNT1*. The fragments of two expressed sequences tags (ESTs), RR11nr025 and RR4nr003, were amplified using forward and reverse primers with the sequences: 5′-GTGGTAACATCACTCTCCTCCGTGG-3′ and 5′-AAGCATTTAGCACTCCTACTCCGGC-3′; as well as 5′-GTGGTAACATCACTCTCCTCCGTGG-3′ and 5′-TAGAGAAACAGAGAATCGAAAATAC-3′; respectively (Deniau *et al.*, 2006). The NcZNT1 locus was amplified using primers with sequences 5′-ATCCTCTGTGATGCTGGCGAATC-3′ and 5′-AAGGCTTTAGCAGCTACAAAGAGATTTCC-3′. The sequences of *NcNramp1* primers used were 5′-GAGTTACTTTGGATCATATTGGTT-3′ and 5′-GCATTACAAACAGCG CCACTTACT-3′. The $\Delta\Delta C_t$ values between Nramp1 and the three genomic markers were used to determine relative genome copy number.

Yeast culture and transformation

Saccharomyces cerevisiae strain DY1457 (MATα *ade6 can1 his3 leu2 trp1 ura3*) was obtained from Dr David Eide (University of Wisconsin, USA) and cultured on yeast extract peptone dextrose (YPD) plates (per liter: 10 g yeast extract, 20 g bacto-peptone, 20 g glucose, 15 g Bacto-agar). Transformation of this strain with the target construct (pFL61 vector and the pFL61 vector with the gene of interest) was performed using the lithium acetate/single-stranded carrier DNA/polyethylene glycol (PEG) method. To obtain cells for transformation, a single colony was streaked on a fresh YPD plate and incubated for approximately 2 days at 30°C. Cells scraped from this plate were suspended in 1.0 ml of sterile deionized water and then pelleted by centrifugation (13 000 ***g*** for 30 sec). The supernatant was discarded and the following, in sequence, were layered over the pellet: 240 μl PEG 3350 (50% w/v), 36 μl 1.0 M lithium acetate, 10 μl single-stranded carrier DNA (10 mg ml^{-1}, herring sperm DNA boiled for 5 min to cause denaturation), and plasmid DNA (0.5–1 μg), and sufficient sterile deionized water to provide a final volume of 360 μl. The mixture was vigorously vortexed for up to 1 min and subjected to heat shock at 42°C for 20 min. For some constructs, the transformation mix was held overnight at room temperature to enhance the transformation efficiency. The transformation mix was then centrifuged at 13 000 ***g*** for 30 sec to pellet the cells. After the supernatant was decanted, the cells were resuspended in 1.0 ml of sterile deionized water. Aliquots of the resuspended cells were plated onto a synthetic complete uracil dropout selection medium (referred to hereafter as SC-URA media). The SC-URA media contained per liter: 6.7 g yeast nitrogen base with ammonium sulfate, without amino acids, 2 g synthetic complete amino acid supplement minus uracil, 20 g glucose, 0.1 g adenine sulfate, 15 g bacto-agar. Plates were incubated for 3–5 days at 30°C until transformants were observed. Single colonies were picked from each transformant plate and established on fresh SC-URA plates.

^{109}Cd uptake

ZHY3 yeast strains (MATα *ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3*) that express either the empty pFL61 vector or pFL61 with either *NcNramp1* ortholog were grown to an optical density (OD) of approximately 1 in SC-URA liquid medium. One hundred μl aliquots of the cell suspension were mixed with equal volumes of a radiolabeled $^{109}Cd^{2+}$ solution in SC-URA at a Cd concentration of 25 μM. After an uptake period of 3, 5 or 10 min, the cells were centrifuged through a silicone oil/dinonyl phthalate pad into 10 μl of 40% perchloric acid. ^{109}Cd content of the pellet then was determined by γ detection and converted to Cd^{2+} influx values. Values shown have background and 30 sec cell wall $^{109}Cd^{2+}$ binding measurement subtracted to yield $^{109}Cd^{2+}$ influx values. The statistical significance of differences in Cd fluxes in Nramp1 expressing yeast compared with fluxes measured in yeast expressing the empty vector (control) was determined using Student's *t*-test. A *P*-value of <0.05 was deemed significant.

Plant transformation

The plant expression vector pBAR was used to over express either *NcNramp1* ortholog in Arabidopsis. The *NcNramp1* constructs were subcloned into pBAR using *Bam*HI and *Xba*I restriction sites added to the 5′ and 3′ ends, respectively, via PCR. The two different pBAR constructs containing the different *Nramp1* genes were transformed into *Agrobacterium tumefaciens* line C58 and selected for on kanamycin plates. *Agrobacterium*-mediated transformation of Arabidopsis ecotype Columbia (Col-0) was performed using the floral dip method (Clough and Bent, 1998; modified from Bechtold *et al.*, 1993).

Metal tolerance and accumulation in Arabidopsis over-expression lines

The seed of over-expression lines were surface sterilized by first exposing the seed to dilute bleach (0.5% NaOCl) and then 50% ethanol. Subsequently the seed was washed five times with ultrapure water before being imbibed in 0.1% (w/v) low melting point agarose at 4°C for 5 days. Homozygous lines were then grown on MS media with 1% sucrose, or high metal treatments which were final concentrations of: high Zn (300 μM $ZnSO_4$), high Cu (10 μM $CuSO_4$), high Fe (250 μM Fe:EDDHA), high Mn (1500 μM $MnSO_4$) and Cd (25 μM $CdSO_4$) medium for 10 days and digital images of the roots were taken using a Nikon D200 camera with a 60 mm lens. Total root length of each plant was determined using ROOT READER 2D software (Clark *et al.*, 2013; www.plantmineralnutrition.net). Relative root length was then calculated by dividing the mean total root length of each high metal-treated plant by the mean total root length of the control plants. The data were compared using a one-way analysis of variance (ANOVA) with Tukey's

test for post hoc analysis. Each line was grown three separate times and compared with wild type Columbia for sensitivity to each metal. To study plant mineral content, true breeding overexpression lines were grown for 10 days on MS medium plus 1% sucrose with 25 μM $CdSO_4$. Roots were desorbed for 15 min in 5 mM $CaCl_2$ and plants were then separated into roots and shoots for mineral content analysis. Elemental analysis was carried out using inductively-coupled plasma atomic emission spectroscopy (ICP-AES; model ICAP 61E trace analyzer, Thermo Electron, Waltham, MA, USA) to determine root and shoot mineral content.

Immunohistological staining

An antibody against NcNramp1 from Ganges and Prayon was prepared by immunizing rabbits with the synthetic peptide C-DIVDMQLHGRVSTTDVN (positions 516–532 of NcNramp1). The obtained antiserum was purified through a peptide affinity column before use. The roots and rosette leaves of both Ganges and Prayon were used for immunostaining of NcNramp1 protein with a 1:300 dilution of the antibody according to Ueno *et al.* (2011). Fluorescence of secondary antibody (Alexa Fluor 555 goat anti-rabbit IgG; Molecular Probes) was observed with a confocal laser scanning microscopy (LSM700; Carl Zeiss).

Western blot analysis

Roots of both Ganges and Prayon (2-month-old) were homogenized in ice-cold homogenizing buffer consisting of 100 mM Tris–HCl (pH 8.0), 150 mM KCl, 0.5% (w/v) polyvinylpolypyrrolidone, 5 mM EDTA, 3.3 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% (v/v) glycerol mainly in accordance with Sugiyama *et al.* (2007). After filtration, the homogenates were centrifuged at 8000 ***g*** for 10 min twice. The supernatants were then ultracentrifuged at 100 000 ***g*** for 40 min. The pellets (microsomal fraction) were resuspended in a small volume of resuspension buffer that contained 10 mM Tris–HCl (pH7.6), 10% (v/v) glycerol and 1 mM EDTA. For the determination of the subcellular localization of NcNramp1, the microsomal fraction from ecotype Ganges was fractionated through a 20, 30, 40, 50, and 60% (w/v) discontinuous sucrose gradient in 10 mM Tris–HCl (pH 7.6), 1 mM EDTA, and 1 mM DTT by ultracentrifugation at 100 000 ***g*** for 18 h. The fractionated membranes were recovered by ultracentrifugation at 100 000 ***g*** for 40 min. Each pellet was resuspended in small volume of resuspension buffer. Protein concentration was measured by the Bradford assay (Biorad, Hercules, CA, USA). Then, 15 μg of microsomal fraction and 5 μg of each fraction for NcNramp1 detection, and 1.5 μg of microsomal fraction and 0.5 μg of each fraction for membrane marker protein detection were mixed with the same volume of sample buffer that contained 250 mM Tris–HCl (pH6.8), 8% (w/v) SDS, 40% (v/v) glycerol, 0.01% (w/v) bromophenol blue (BPB) and 200 mM β-mercaptoethanol. The mixtures were denatured for 10 min at 65°C for all samples. SDS-PAGE was then performed using 5–20% gradient polyacrylamide gels (ATTO, Japan). The transfer to PVDF membrane was performed with a semi-dry blotting system and the membrane was treated with the purified primary rabbit anti-NcNramp1 (1:500) as described above, V-type ATPase (TP marker; 1:5000, Agrisera), H^+-ATPase (PM marker; 1:2000, Agrisera), and ER marker Bip (1:2000, Cosmo Bio). Anti-rabbit IgG (H+L), horseradish peroxidase (HRP) conjugate (1:10 000; Promega) was used as a secondary antibody, and an ECL Plus western blotting detection system (GE Healthcare, Piscataway, NJ, USA) was used for detection via chemiluminescence. Western blot analysis using samples isolated from plants exposed to –Fe conditions for 1 week was also performed.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Concentration of Cd in the shoots of seedlings of the Prayon and Ganges ecotypes of *N. caerulescens* following a 7 d exposure to Cd.
Figure S2. Phylogenetic tree of Nramp family members from *Arabidopsis thaliana* and *Noccaea caerulescens*.
Figure S3. Alignment of the isolated DNA sequences of *NcNramp1* from both ecotypes of *N. caerulescens*, along with the DNA sequence for *AtNramp1*.
Figure S4. Alignment of the amino acid sequences of NcNramp1 from both orthologs along with the amino acid sequence for AtNramp1.
Figure S5. Plant Zn, Fe and Mn concentrations for transgenic *NcNramp1* overexpressing Arabidopsis lines.
Table S1. Transporter genes that show higher expression in the roots of Prayon versus Ganges in response to Cd exposure.
Table S2. Transporter genes that show higher expression in the shoots of Ganges versus Prayon in response to Cd exposure.
Table S3. Transporter genes that show higher expression in the shoots of Prayon versus Ganges in response to Cd exposure.

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