Elevated Nicotiamine Levels in Arabidopsis halleri Roots Play a Key Role in Zinc Hyperaccumulation

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Zn deficiency is among the leading health risk factors in developing countries. Breeding of Zn-enriched crops is expected to be facilitated by molecular dissection of plant Zn hyperaccumulation (i.e., the ability of certain plants to accumulate Zn to levels >100-fold higher than normal plants). The model hyperaccumulators Arabidopsis halleri and Noccaea caerulescens share elevated nicotiamine synthase (NAS) expression relative to nonaccumulators among a core of alterations in metal homeostasis. Suppression of Ah-NAS2 by RNA interference (RNAi) resulted in strongly reduced root nicotiamine (NA) accumulation and a concomitant decrease in root-to-shoot translocation of Zn. Speciation analysis by size-exclusion chromatography coupled to inductively coupled plasma mass spectrometry showed that the dominating Zn ligands in roots were NA and thiols. In NAS2-RNAI plants, a marked increase in Zn-thiol species was observed. Wild-type A. halleri plants cultivated on their native soil showed elemental profiles very similar to those found in field samples. Leaf Zn concentrations in NAS2-RNAI lines, however, did not reach the Zn hyperaccumulation threshold. Leaf Cd accumulation was also significantly reduced. These results demonstrate a role for NAS2 in Zn hyperaccumulation also under near-natural conditions. We propose that NA forms complexes with Zn(II) in root cells and facilitates symplastic passage of Zn(II) toward the xylem.

INTRODUCTION

Aside from Fe, Zn is the most widely used transition metal in living systems. During evolution, it has been recruited for a myriad of catalytic and structural functions. In most prokaryotes, 5 to 6% of all proteins are Zn dependent, and in eukaryotes, the figure is 8 to 9% (Andreini et al., 2006). Accordingly, Arabidopsis thaliana is estimated to express ~2400 different Zn proteins (Broadley et al., 2007). Across nature, the requirements for Zn result in a Zn quota of ~0.1 to 0.5 mM (Eide, 2006) in bacteria, yeast, and mammalian cells and 0.3 to 3 mM in plant cells (Broadley et al., 2007; Blindauer and Schmid, 2010). Zn deficiency is globally the most widespread crop micronutrient deficiency, particularly occurring on alkaline soils and leached sandy soils (Cakmak, 2002; Alloway, 2009). Deficiency symptoms are apparent at leaf concentrations below 5 to 20 μg g⁻¹ dry biomass in most crops (Marschner, 1995). Shoot Zn concentrations vary considerably but are usually in the range of 50 to 100 μg g⁻¹ dry biomass (Broadley et al., 2007). An extreme exception are Zn-hyperaccumulating plants that can show leaf Zn levels of >10,000 μg g⁻¹ dry biomass (Baker and Brooks, 1989; Krämer, 2010) (i.e., >100-fold higher than normal plants or most other organisms). Such accumulation rates are remarkable because Zn ions are very reactive and an excess of Zn results in toxicity. In most plants, toxicity symptoms, such as leaf chlorosis, are observed at leaf Zn concentrations exceeding 300 μg g⁻¹ dry biomass (Marschner, 1995). Generally, transition metals are under tight homeostatic regulation once taken up by an organism because of the same biochemical activities that resulted in their recruitment during evolution. Reactivity and the ability to compete with other cations for binding sites demands that cytosolic pools of labile free hydrated ions are either nonexistent, as is the case for Cu ions (Kim et al., 2008), or represent only a minute fraction of total metal, as is hypothesized for Zn ions (Eide, 2006). Acquisition, intracellular trafficking, storage, mobilization, and long-distance transport of transition metals require a complex network of transporters, low molecular mass chelators, and binding proteins that are regulated according to micronutrient availability in the soil solution and internal metal status (Palmer and Guerinot, 2009). Most steps in the pathways of Zn ions to their target sites are not yet understood (Blindauer and Schmid, 2010).

Interest in the mechanisms of plant Zn homeostasis has been fueled in recent years by the concept of biofortification (Palmgren et al., 2008; Zhao and McGrath, 2009). According to World Health Organization estimates, up to 2 billion people worldwide are at risk of or acutely affected by Zn deficiency (Stein, 2010). The most sustainable and cost-effective way of alleviating...
this deficiency would be to increase the amount of bioavailable Zn in edible parts of crop plants. Both molecular breeding and genetic engineering of Zn-enriched crops, however, necessitate an understanding of the molecular mechanisms underlying Zn accumulation. One potentially powerful approach toward this goal is the dissection of Zn hyperaccumulation (Palmgren et al., 2008). Hundreds of plant species, termed metallophytes, evolved metal hypertolerance, which enables them to thrive on metal-rich soils free of competition from plants that show only basal tolerance levels. Ecologists and evolutionary biologists have long considered this phenomenon a particularly exciting case of evolution in action (Antonovics et al., 1971; Roosens et al., 2008). About 500 of these metallophytes also hyperaccumulate certain metals, with the vast majority hyperaccumulating Ni. Zn hyperaccumulation has to date been found in 15 taxa (Krämer, 2010). Several Zn-hyperaccumulating species also show Cd hyperaccumulation (i.e., shoot Cd levels of >100 μg g⁻¹ dry biomass). Mechanistic studies of Zn and Cd hyperaccumulation and hypertolerance have largely focused on two A. thaliana relatives, Noccaea caerulescens (formerly Thlaspi caerulescens) and Arabidopsis halleri (Verbruggen et al., 2009; Krämer, 2010). Genetic analyses revealed that in both species Zn hypertolerance and Zn hyperaccumulation are at least partially independent traits determined by several quantitative trait loci (QTL) (Verbruggen et al., 2009). In the backcross 1 population of an A. lyrata × A. halleri interspecies cross, three major-effect loci for Zn tolerance were found (Willems et al., 2007). In an F2 population of the same cross, Zn accumulation was found to be affected by a minimum of five QTLs, one of which overlaps with a major Zn tolerance QTL (Frérot et al., 2010).

Candidate genes that might underlie the identified QTLs were revealed by cross-species transcriptomics studies that used the close relatedness of A. halleri and N. caerulescens to A. thaliana. A. halleri is a pseudometallophyte (i.e., it occurs both on metal-contaminated and noncontaminated sites). Regardless of soil Zn content, A. halleri shows Zn hyperaccumulation (Bert et al., 2000). Thus, a comparison of transcriptomes at Zn levels common for cultivation of nonmetallophtytes plants can be informative with respect to mechanisms involved in hyperaccumulation. Indeed, a series of studies demonstrated that components of plant Zn homeostasis are constitutively expressed at a much higher level in A. halleri and N. caerulescens relative to A. thaliana (Broadley et al., 2007; Verbruggen et al., 2009; Krämer, 2010). These findings suggested that an altered regulation of metal homeostasis arose during the evolution of hyperaccumulation (Hankenne and Nouet, 2011). The metal homeostasis genes more highly expressed encode predominantly metal transporters (Heavy Metal ATPases [HMAs], Metal Tolerance Proteins [MTPs], ZRT, IRT-like Proteins [ZIPs], and Natural Resistance Associated Macrophage Proteins) (Krämer et al., 2007). For one of them, Ah-HMA4, it could be demonstrated that elevated expression is indeed essential for Zn hyperaccumulation and Zn hypertolerance (Hankenne et al., 2008). A second group of genes implicated in metal homeostasis and expressed at higher levels in Zn hyperaccumulators encode nicotianamine synthases (NASs) (Becher et al., 2004; Weber et al., 2004; van de Mortel et al., 2006; Talke et al., 2006). They synthesize the nonproteinogenic amino acid nicotianamine (NA) through condensation of three molecules of S-adenosylmethionine (Higuchi et al., 1999). NA can bind several transition metals with high affinity in vitro (Benes et al., 1983; Callahan et al., 2006). Numerous observations reported for NA-deficient tomato (Solanum lycopersicum), tobacco (Nicotiana tabacum), and A. thaliana mutants have implicated NA in Fe, Cu, Zn, and Mn homeostasis (Curie et al., 2009). Phenotypes of NAS mutants are predominantly attributable to defects in the distribution (cell-to-cell movement and long-distance transport) of Fe. For Zn hyperaccumulators, however, it was hypothesized that NA could contribute to Zn translocation, for instance, by increasing the symplastic mobility of Zn(II) (Weber et al., 2004). Synchrotron experiments with NAS-expressing Schizosaccharomyces pombe cells demonstrated recently that Zn(II)-NA complexes can indeed be formed intracellularly (Trampczynska et al., 2010). However, a role of NA in metal hyperaccumulation has not been demonstrated yet.

Here, we show that suppression of constitutively high NA accumulation in A. halleri roots through RNA interference (RNAi) targeting NAS2 results in a significant reduction in Zn hyperaccumulation. Importantly, we show that this phenotype is present also under near-natural conditions in native soil. Furthermore, we demonstrate by speciation analysis that NA is a major Zn(II) ligand in A. halleri roots besides thiols. Taken together, our data support the hypothesis that the low molecular mass metal chelator NA enhances symplastic mobility of Zn(II) and is therefore a key molecule for the translocation of Zn within plants. These findings are relevant for understanding plant metal homeostasis and the development of biofortification strategies.

RESULTS

Elevated Root NA Concentrations Are Found in A. halleri Individuals from Metaliferous and Nonmetaliferous Sites

Our earlier study indicated that the NA content is ∼3.5-fold higher in roots of hydroponically grown A. halleri plants compared with A. thaliana (Weber et al., 2004). This observation was made in plants originating from the population Langelsheim, which grows on a highly metal-contaminated soil. Since A. halleri is a pseudometallophyte found also on noncontaminated soil, we asked whether constitutively higher root NAS2 transcript and NA levels are a common characteristic of A. halleri that is independent of soil metal content found at the natural site of origin. Six geographically distant populations from Poland and Germany growing on either metaliferous or nonmetaliferous sites (Table 1) were chosen. Extractable soil Zn concentrations varied between 3.4 and 1178 μg g⁻¹. All populations showed strong Zn accumulation in the field with leaf Zn concentrations between 2800 and 30,500 μg g⁻¹ dry biomass. Individuals from each population were transferred to the laboratory and propagated vegetatively. In addition, we included in our further analysis one individual of A. halleri ssp gemmifera from a metaliferous site in Japan. We first analyzed NAS2 transcript abundance in hydroponically grown plants. In roots of all A. halleri plants, NAS2 root transcript abundance was between 7- and 31-fold higher than in A. thaliana Columbia-0 (Col-0) (Figure 1A). Next, we determined NA levels using stable isotope dilution analysis that
allows more accurate quantification based on a recombinantly produced $^{15}$N-labeled internal standard (Schmidt et al., 2011). Root NA levels ranged from 60.4 to 156.6 μg g$^{-1}$ fresh biomass in A. halleri (i.e., levels that were 2.7- to 6.9-fold higher than in A. thaliana Col-0) (Figure 1B). By contrast, leaf NA levels were comparable in all plants tested, with on average 55.3 μg g$^{-1}$ fresh biomass in A. thaliana Col-0 and between 37.7 and 57.6 μg g$^{-1}$ fresh biomass in A. halleri, with the exception of the population from Miasteczko Slaskie, which contained 95.1 μg g$^{-1}$ fresh biomass.

### Generation of A. halleri NAS2 Knockdown Lines

To test directly the hypothesis that higher root NA levels contribute to Zn hyperaccumulation in A. halleri, individuals of the Langelsheim population were transformed with an RNAi construct targeting NAS2. Nine independent lines were isolated. Five lines were chosen for a detailed characterization after a prescreening for NAS2 suppression. Lines 1-2, 7-12, and 11-1 showed strong reduction in root NAS2 transcript abundance by 81 to 85% (Figure 2A), whereas NAS2 transcript levels in lines 0-7 and 3-3 were not significantly different from the Langelsheim wild-type plants. Nonetheless, these latter two lines were included as control transformants in subsequent experiments because they had been subjected to the transformation procedure including in vitro tissue culture. NAS2 suppression remained stable in vegetatively propagated lines 1-2, 7-12, and 11-1 for at least 3 years.

A. halleri expresses orthologs of the four NAS genes present in A. thaliana (Weber et al., 2004; Talke et al., 2006). Full-length cDNAs were cloned and all found to encode functional NAS proteins as they confer NA synthesis capacity and an increase in Zn$^{2+}$ tolerance to Zn$^{2+}$ hypersensitive S. pombe zhfΔ cells (see Supplemental Figure 1 online). To test the specificity of the construct targeting NAS2, transcript accumulation of NAS1, NAS2, NAS3, and NAS4 was assayed by quantitative real-time RT-PCR in roots and leaves of hydroponically grown plants. The expression pattern of these isoforms in wild-type A. halleri is qualitatively comparable to that in A. thaliana (Klatte et al., 2009).

### NAS1 and NAS4 are expressed in both roots and leaves, while NAS2 is expressed predominantly in roots and NAS3 almost exclusively in leaves (see Supplemental Figure 2 online). When roots and leaves of Ah-NAS2-RNAi lines were analyzed, no significant change in transcript abundance relative to the wild type was found for NAS3 and NAS4. Coding sequences of both genes share only around 68% identity with NAS2. Real-time RT-PCR suggested that transcript abundance of NAS1 was not affected in leaves and roots of the three strongest RNAi lines. NAS1 is the closest homolog of NAS2, sharing 84.2% nucleotide sequence identity within the coding region and 85.2% within the fragment used for the RNAi construct. In the two control transformant lines, NAS1 transcript levels were between 1.7- and 2.1-fold higher than in wild-type plants and strong RNAi lines, albeit not significantly (see Supplemental Figure 3 online).

### NAS2 Knockdown Results in Strongly Reduced Root NA Concentrations

NAS2 suppression resulted in a strong reduction in root NA levels of hydroponically grown plants (control medium, 0.077 μM ZnSO$_4$). As shown in Figure 2B, RNAi lines 1-2, 7-12, and 11-1 contained only 21 to 25% of the NA found in Langelsheim plants, whereas control transformant lines exhibiting wild-type NAS2 transcript abundance were not significantly different from the wild type. Overall, a linear correlation was detected between NAS2 transcript level and root NA concentration across all genotypes (Figure 2C, $r = 0.81$, $P < 0.001$). Similar observations ($r = 0.75$, $P < 0.001$) were made in 1-2, 7-12, and 11-1 plants cultivated in a medium supplemented with additional 10 μM ZnSO$_4$ (see Supplemental Figure 4 online; $r = 0.75$, $P < 0.001$). It is noteworthy, however, that we consistently found approximately twofold higher NA concentrations in roots of wild-type plants grown at this slightly elevated Zn$^{2+}$ concentration compared with control conditions ($130.9 \pm 24.5$ μg g$^{-1}$ fresh biomass versus 66.34 ± 7.41 μg g$^{-1}$ fresh biomass, respectively). Thus, the relative suppression of NA accumulation in roots of RNAi lines 1-2,
7-12, and 11-1 compared with the wild type was even stronger at 10 μM Zn\(^{2+}\), ranging from 91.3 to 94.3% instead of 75.6 to 78.8% when cultivated in low Zn\(^{2+}\) (control) medium (Figures 2B and 2C; see Supplemental Figure 4 online). Leaf NA concentrations were not affected by externally provided Zn\(^{2+}\) concentrations in wild-type plants. In control medium, all genotypes displayed leaf NA

![Figure 1](image1.png)

**Figure 1.** Steady State NAS2 Transcript Levels and NA Concentrations in Roots of Hydroponically Grown *A. halleri* ssp *halleri* Individuals Representing Six Different European Populations. The individuals analyzed were clones of individuals collected from German and Polish populations at either metalliferous (yellow) or non-metalliferous (green) sites in Oker (Ok), Langelsheim (Lan), Rodacherbrunn (Rod), Miasteczko Slaskie (MS), Bibiela (Bib), and Muchowiec (Much) (Table 1). *A. halleri* ssp *gemmifera* from Japan (white) and *A. thaliana* Col-0 (black) were included for comparison. Plants were grown in control medium for 5 weeks.

(A) NAS2 transcript abundance in roots, expressed relative to *EF1\(a\)*, as determined by real-time RT-PCR. Values are arithmetic means ± SD of three independent experiments (three plants of each genotype were pooled for each data point).

(B) Root NA concentrations. NA was extracted with water, derivatized with Fmoc-Cl, and quantified by UPLC-ESI-QTOF-MS using a 15N-labeled standard. Values in (A) and (B) are arithmetic means ± SD of three independent experiments (three plants of each genotype were pooled for each data point). Statistical significance was determined using one-way analysis of variance followed by a Tukey test. Asterisks denote significant differences compared with the wild-type mean: *P < 0.05, **P < 0.01, and ***P < 0.001. f.w., fresh weight.

![Figure 2](image2.png)

**Figure 2.** Steady State NAS2 Transcript Levels and NA Concentrations in Roots of Hydroponically Grown *A. halleri* Wild-Type (Langelsheim) and *Ah-NAS2-RNAi* Plants.

Plants were grown in control medium for 5 weeks.

(A) NAS2 transcript abundance in roots, expressed relative to *EF1\(a\)*, as determined by real-time RT-PCR. WT, wild type.

(B) Root NA concentrations. NA was extracted with water, derivatized with Fmoc-Cl, and quantified by UPLC-ESI-QTOF-MS using a 15N-labeled standard. Values in (A) and (B) are arithmetic means ± SD of three independent experiments (three plants of each genotype were pooled for each data point). Statistical significance was determined using one-way analysis of variance followed by a Tukey test. Asterisks denote significant differences compared with the wild-type mean: *P < 0.05 and ***P < 0.001. f.w., fresh weight.

(C) Root NA concentrations shown as a function of NAS2 transcript levels.
levels in a range between 20 and 39 μg g⁻¹ fresh biomass, irrespective of NAS2 expression (Figure 2A; see Supplemental Figures 4 and 5 online). Upon cultivation in the presence of 10 μM Zn²⁺ we detected a slight, yet significant (P < 0.05) reduction (by a maximum of 41%) of NA levels in two of the strong RNAi lines (1-2 and 11-1; see Supplemental Figure 5 online).

**Lowered Root NA Concentrations Result in Less Efficient Root-to-Shoot Translocation Specifically of Zn**

To assess the consequences of lower root NA production for Zn accumulation, the selected NAS2-suppressed lines, Langelsheim wild-type plants, and the control transformants were grown hydroponically in the presence of 10 μM Zn²⁺, a moderately elevated concentration identical or close to those applied in previous studies on A. halleri metal hyperaccumulation to ensure Zn sufficiency (Mirouze et al., 2006; Talke et al., 2006; Hanikenne et al., 2008). Root and leaf material was subjected to inductively coupled plasma–optical emission spectroscopy (ICP-OES) analysis. Root Zn concentrations of the three NAS2-suppressed lines were significantly (P < 0.05) higher than those of wild-type plants and of the two control transformants (Figure 3A). The opposite effect was observed in leaves (Figure 3B), with an overall reduction of leaf Zn concentrations in strong Ah-NAS2-RNAi lines by 26 to 48% when compared with the control genotypes. As an indicator of Zn hyperaccumulation, shoot:root concentration ratios were approximately fivefold lower in the lines with reduced NAS2 transcript accumulation when compared with wild-type plants and line 0-7 (Figure 3C). These differences were highly significant (P < 0.01). Shoot:root ratios of Zn concentrations correlated well (r = 0.82, P < 0.001) with root NA concentrations (Figure 3D) across all genotypes tested. By contrast, Zn concentrations in plants grown in the presence of low external Zn levels (0.077 μM ZnSO₄) were not different between any of the five tested transgenic lines and wild-type plants (see Supplemental Figure 6 online). In the presence of low Zn²⁺, all A. halleri plants barely hyperaccumulated Zn. Shoot:root ratios were ~10-fold lower than after growth in the presence of micromolar Zn²⁺ concentrations (Figure 3; see Supplemental Figure 6 online). Please note, also, that in low Zn (control) medium, the difference between strong Ah-NAS2-RNAi and control genotypes may be partly diminished due to twofold lower NA concentrations in roots of control genotypes in control medium compared with 10 μM Zn²⁺, an observation described above.

Besides Zn, Fe and Mn are micronutrients apparently not regulated by designated metallochaperones in vivo and are known to form complexes with NA in vitro. In both control and Zn-supplemented medium, the strong Ah-NAS2-RNAi lines showed a slight reduction in shoot:root ratios of Mn concentrations compared with the wild type, which was statistically significant (P < 0.05) only in line 11-1 in the presence of 10 μM Zn²⁺ (Figure 4A). Shoot:root ratios of Fe concentrations were generally lower in plants cultivated at elevated Zn²⁺ levels than in plants cultivated under control conditions, but there were no differences between genotypes (Figure 4B). There were also no genotype-dependent differences in shoot:root ratios of Cu concentrations.

The results reported above suggest a facilitating effect of root NA for root-to-shoot Zn translocation in A. halleri. Possible effects of NAS2 suppression on the distribution of loosely bound Zn inside A. halleri roots were determined by Zinpyr staining (Sinclair et al., 2007). As shown in Figure 5A for roots cultivated in 1 μM Zn²⁺, the signal was largely confined to the pericycle and inward cell layers of the root. No qualitative difference in distribution was apparent between the genotypes. However, relative quantification of Zinpyr staining intensity compared with propidium iodide revealed a 1.7-fold stronger signal for the Ah-NAS2-RNAi line 1-2 (Figure 5B). This confirms the more pronounced retention of Zn in roots of plants with reduced NA levels.

**Zn(II) Speciation Analysis of Root Tissue**

To investigate if NA indeed affects the speciation of Zn and forms complexes with Zn(II) in A. halleri cells, Tris/NaCl extracts of leaf and root tissues of wild-type plants and Ah-NAS2-RNAi line 1-2 grown in medium with 10 μM Zn²⁺ were subjected to size-exclusion chromatography coupled to ICP mass spectrometry (SEC-ICP-MS). The data for extractable Zn mirrored the results obtained when total Zn was analyzed. About 40% more extractable Zn was found in root tissue of the RNAi line compared with wild-type plants (54 ± 4 μg g⁻¹ fresh biomass for line 1-2 versus 39 ± 5 μg g⁻¹ fresh biomass for the wild type; Table 2), while the opposite was the case in the shoots (Ah-NAS2-RNAi line 1-2 showed only ~60% of the wild-type level; Table 2). Very little free Zn²⁺ was present in extracts from roots of both wild-type and Ah-NAS2-RNAi plants. Speciation analysis by SEC-ICP-MS showed that >97% of all extractable Zn consisted of low molecular mass species with apparent masses below 1000 D (Table 2; LMM Zn species versus free Zn²⁺; Figure 6A). Ligand identification by electrospray ionization time-of-flight MS (ESI-TOF-MS) of the heart-cut fraction of low molecular mass Zn species confirmed that NA was a dominating ligand together with GSH and the phytochelatin derivative des-γGlu-PC (see Supplemental Figure 7 online). Interestingly, the S signal coeluting with Zn was almost twice as strong in the roots of RNAi plants when compared with wild-type roots (Figure 6C; quantification in Table 2, LMM S species in roots). This indicates a marked ligand exchange toward a higher proportion of thiol-bound Zn. By contrast, most of the extractable Zn in the shoot of both wild-type and RNAi plants existed as free Zn²⁺, with <2% occurring in coordination complexes with NA and PC (Table 2, Figure 6B).

**Microarray Analysis of NAS2 Knockdown Effects**

Besides HMA4 and NAS2, a number of other genes are more strongly expressed in A. halleri relative to A. thaliana (Becher et al., 2004; Weber et al., 2004, 2006; Talke et al., 2006), including several metal homeostasis genes (Verbruggen et al., 2009; Hanikenne and Nouet, 2011). Based on the effects observed upon HMA4 suppression, it was proposed that the elevated expression of at least some of these genes in A. halleri roots is caused by efficient HMA4-dependent xylem loading of Zn, which results in Zn deficiency responses of root cells (Hanikenne et al., 2008). We wanted to investigate on a transcriptome-wide level whether the high expression of NAS2 in A. halleri roots has similar secondary effects on the expression of other metal homeostasis candidate genes, such as putative Zn²⁺ uptake transporters.
Therefore, we compared the root transcriptomes of Ah-NAS2-RNAi and wild-type A. halleri plants. RNA extracted from A. halleri wild-type plants and the Ah-NAS2-RNAi line 1-2, grown hydroponically in either low Zn2+ control conditions or in the presence of 10 μM Zn2+, was analyzed with the ATH1 microarray (three independent experiments). As indicated in Table 3, only a small number of genes showed significant (adjusted P < 0.05) differences in transcript levels between the two genotypes. Overall, more changes associated with NAS2 suppression were detected under conditions of strongly reduced root-to-shoot translocation of Zn in Ah-NAS2-RNAi plants (i.e., at 10 μM external Zn2+). Most of the differentially expressed genes showed consistent changes under both tested conditions, albeit not always to the same extent. As expected, microarray hybridization confirmed the decrease in NAS2 transcript levels in roots, as determined by real-time RT-PCR (Figure 2A). While transcript levels of NAS3 and NAS4 were not affected, transcription abundance of NAS2 was strongly reduced in RNAi line 1-2 (77% under control conditions and 87% at 10 μM external Zn2+ compared with the wild type). NAS1 transcript abundance appeared to be reduced by ~50%. However, this is likely to result from cross-hybridization of NAS2 transcript to the NAS1 probe set, which would be reduced in the RNAi line compared with the wild type (cf. real-time RT-PCR data; see Supplemental Figure 3 online). There were no reductions in transcript levels of Zn deficiency marker genes that encode putative Zn2+ uptake transporters and are known to be highly expressed in A. halleri roots (i.e., IRT3, ZIP4, ZIP9, or ZIP10) (Weber et al., 2004; Talke et al., 2006; Lin et al., 2009) in the Ah-NAS2-RNAi line compared with the wild type. Also, no other known metal homeostasis genes appeared to be affected by NAS2 suppression. The lack of changes in transcript abundance of the Zn2+ deficiency marker gene ZIP9 in RNAi line 1-2 was independently confirmed by real-time RT-PCR (data not shown). The gene showing strongest upregulation in the Ah-NAS2-RNAi plants at both Zn supply levels encodes a putative dual specificity protein phosphatase-related protein (AT4G18593). Another gene showing similar upregulation encodes a peroxidase (Table 3). Most pronounced downregulation in the NAS2 RNAi plants was found for genes encoding seed storage/lipid transfer proteins (AT5G46890/AT5G46900 and AT1G48750) (Table 3). Among the ~100 genes more highly expressed (more than fivefold, P < 0.05) in A. halleri roots relative to A. thaliana roots (Weber et al., 2004, 2006; Talke et al., 2006), only NAS2 was significantly affected in line 1-2. In summary, these data demonstrate that unlike HMA4 (Hanikenne et al., 2008), higher NAS2 expression in wild-type A. halleri roots and its effects on Zn homeostasis do not cause pronounced secondary effects on other metal homeostasis genes at the transcript level.

**Figure 3.** Zn Accumulation in Hydroponically Grown A. halleri Wild-Type (Langelsheim) and Ah-NAS2-RNAi Plants.

Plants were cultivated in a medium containing 10 μM ZnSO4. Tissues were harvested after 5 weeks, digested, and analyzed by ICP-OES. Shown in (A) and (B) are values for roots and leaves, respectively. For (C), Zn shoot/root ratios of Zn concentrations were calculated from data shown above. All values are arithmetic means ± SD, n = 4 to 6 with three replicate clones per genotype pooled per experiment. Asterisks denote significant differences compared with the wild-type (WT) mean: *P < 0.05 and **P < 0.01. In (D), shoot/root ratios of Zn concentrations are shown as a function of NA concentrations. d.w., dry weight.
A. halleri contamination; Table 4). These soils represent the natural range of Harz 2, intermediate Zn contamination; Harz 3, heavy Zn contamination in the Harz Mountains in Germany (Harz 1, no Zn contamination; DTPA)-exchangeable Zn as an indicator of bioavailable Zn obtained from three differentially contaminated sites.

Root and leaf tissues were harvested after 5 weeks, digested, and analyzed by ICP-OES for Mn (A) and Fe (B). Values are arithmetic means ± SD (n = 3, with three replicate clones per genotype pooled per experiment) of shoot:root concentration ratios for plants grown either in control medium (white) or in the presence of 10 μM Zn2+ (black). Asterisks denote significant differences compared with the wild-type (WT) mean: *P < 0.05.

**Figure 4.** Shoot:Root Ratios of Mn and Fe Concentrations in Hydroponically Grown A. halleri Wild-Type (Langelsheim) and Ah-NAS2-RNAi Plants.

Root and leaf tissues were harvested after 5 weeks, digested, and analyzed by ICP-OES for Mn (A) and Fe (B). Values are arithmetic means ± SD (n = 3, with three replicate clones per genotype pooled per experiment) of shoot:root concentration ratios for plants grown either in control medium (white) or in the presence of 10 μM Zn2+ (black). Asterisks denote significant differences compared with the wild-type (WT) mean: *P < 0.05.

**Lowered Root NA Concentrations Cause a Reduction in Leaf Zn and Cd Accumulation in Plants Grown on Native A. halleri Soil**

Finally, we asked whether results obtained from controlled hydroponic experiments are transferable to near-natural conditions. Therefore, we cultivated plants in soils collected at sites hosting natural A. halleri populations and tested the effect of NAS2 suppression on leaf metal accumulation. Soils were obtained from three differentially contaminated A. halleri sites in the Harz Mountains in Germany (Harz 1, no Zn contamination; Harz 2, intermediate Zn contamination; Harz 3, heavy Zn contamination; Table 4). These soils represent the natural range of A. halleri occurrence from virtually absent to heavy Zn contamination (Table 4). Values for diethylenetriaminepentaaetic acid (DTPA)-exchangeable Zn as an indicator of bioavailable Zn suggested that Zn2+ concentrations in soil solution were in the micromolar range for all soil types (Table 4; i.e., similar to the 10 μM Zn2+ conditions in hydroponic culture). The three strong Ah-NAS2-RNAi lines were compared with wild-type plants and control RNAi line 0-7 in three independent experiments. Plants were grown for 4 weeks in the three soil types. No visible toxicity symptoms were observed in any of the plants upon cultivation in Zn-contaminated soil, suggesting that silencing of NAS2 did not affect metal hypertolerance (Figure 7A). Leaf elemental profiles were analyzed by ICP-OES. Langelsheim wild-type plants and the control transformant line 0-7 hyperaccumulated Zn to levels above 10,000 μg g⁻¹ dry biomass on all soil types, which is consistent with field observations. NAS2 suppression resulted in a pronounced reduction of up to 63% in leaf Zn concentrations on noncontaminated soil in all three lines both compared with wild-type plants (P < 0.001 for 1-2 and P < 0.01 for 7-12 and 11-1) and to the control RNAi line 0-7 (P < 0.001 for 1-2 and P < 0.01 for 7-12 and 11-1) (Figure 7B). Reduced Zn accumulation by the three NAS2-suppressed lines was also found on soil with an intermediate Zn contamination level. Differences were significant for all lines when compared with the control line 0-7 and for 1-2 when compared with wild type (P values of 0.093 and 0.105 for 7-12 and 11-1, respectively) (Figure 7B). On heavily Zn-contaminated soil, however, Zn accumulation levels were generally lower for all the lines and comparable between the genotypes. For the micronutrients Fe, Mn, and Cu, no consistent patterns or statistically significant differences in leaf concentrations were found among wild-type plants, the control transformant, and the three strong RNAi lines, with the sole exception being a small difference between wild-type plants and line 0-7 in leaf Cu concentration (see Supplemental Figure 8 online).

Cd is the second metal that A. halleri is known to hyperaccumulate. Leaf Cd concentrations of control plants in our soil experiments were again consistent with field observations in the Langelsheim population. Wild-type plants and the control transformant line 0-7 accumulated Cd to levels above 80 μg g⁻¹ dry biomass. Plants with reduced root NA concentrations showed a significant reduction in leaf Cd accumulation by up to 50% upon cultivation on Harz 1 soil with normal Zn levels and only marginal Cd contamination (Figure 7C). Differences were significant for all pairwise comparisons of lines 1-2, 7-12, and 11-1 with the control RNAi line 0-7 and for wild-type plants compared with line 1-2 and line 11-1 (P < 0.001 and P < 0.01, respectively; P value for the comparison between the wild type and line 7-12 was 0.057). On both types of contaminated soil (Harz 2 and Harz 3), all A. halleri genotypes accumulated similar concentrations of Cd within a soil type. Between the two contaminated soil types, lower leaf Cd concentrations were accumulated on the soil with heavy Zn contamination.

Taken together, the results described above demonstrate that high expression of NAS2 in roots is necessary for A. halleri plants to reach levels of Zn and Cd accumulation in leaves that are displayed by wild-type plants both in the field and on non-contaminated native soil under greenhouse conditions.

**DISCUSSION**

Hyperaccumulation evolved several times independently and is particularly prevalent in the Brassicaceae (Krämer, 2010). Its
molecular dissection is likely to facilitate crop breeding for micronutrient-enriched varieties (Palmgren et al., 2008). Key to advancing our understanding of this extreme trait is to determine the contribution of putative metal homeostasis genes that have been identified in both comparative transcriptomic studies and genetic analyses addressing metal hyperaccumulation or hyper-tolerance in the model species *A. halleri* and *N. caerulescens* (Krämer et al., 2007; Verbruggen et al., 2009).

Elevated transcript levels of NAS genes relative to *A. thaliana* belong to the core group of alterations in the metal homeostasis network that are shared by *N. caerulescens* and *A. halleri* (Krämer et al., 2007; Verbruggen et al., 2009). Our analysis of individuals representing geographically distant populations from both metaliferous and nonmetaliferous soils indicates that high NAS2 expression relative to *A. thaliana* and concomitant elevation of NA levels specifically in roots are constitutive in *A. halleri* (Figure 1). Our data are consistent with the observation that Zn hyperaccumulation is a species-wide trait in *A. halleri*, with NAS2 having a species-wide role in this trait.

We achieved strong and stable suppression of NAS2 transcript accumulation in three independent transgenic *A. halleri* lines via transformation with an Ah-NAS2-RNAi construct. Across all plants and tested conditions, there was a very good correlation of NAS2 transcript abundance with root NA levels (Figure 2C), suggesting that (1) NAS represents the rate-limiting step for NA biosynthesis and is predominantly regulated at the level of transcript abundance, and (2) total root NA concentrations are mostly determined by NAS2.

The NAS gene family appears to be conserved in the genus *Arabidopsis*. We cloned functional orthologs of all four *A. thaliana* NAS genes from *A. halleri* and found a pattern of expression comparable to *A. thaliana* with respect to tissue specificity. The combination of microarray and real-time RT-PCR data suggested that the strong reduction in root NA levels of RNAi lines 1-2, 7-12, and 11-1 is clearly attributable to the RNAi effect on Ah-NAS2 transcript abundance (Figure 2C). Expectedly, no effects were observed on transcript abundance of NAS3 and NAS4.

A major finding of our study is that *A. halleri* Ah-NAS2-RNAi transformants with strongly reduced root NA levels exhibited a

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### Table 2. Quantitative Analysis of Low Molecular Mass Zn and S Species with Apparent Masses below 1000 D in Roots and Shoots of the Wild Type and Ah-NAS2-RNAi Line 1-2

<table>
<thead>
<tr>
<th>Element</th>
<th>A. halleri Wild Type</th>
<th>Ah-NAS2-RNAi Line 1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root</td>
<td>Shoot</td>
</tr>
<tr>
<td>Extracted Zn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMM Zn species</td>
<td>39 ± 5</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>Free Zn$^{2+}$</td>
<td>1 ± 1</td>
<td>216 ± 24</td>
</tr>
<tr>
<td>Extracted S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMM S species</td>
<td>409 ± 70</td>
<td>835 ± 351</td>
</tr>
</tbody>
</table>

Quantification was performed by peak integration (Figure 6). Zn eluted with five injections of EDTA is defined as free Zn$^{2+}$. Data are presented as mean values (µg g$^{-1}$ fresh biomass) ± SD (n = 3 independent experiments; three plants were pooled for one sample). LMM, low molecular mass.
Figure 6. Speciation of Zn in Root and Shoot Tissue for the Wild Type (Langelsheim) and the Ah-NAS2-RNAi Line 1-2.

Tris/NaCl extracts of root (left) and shoot (right) tissue of the wild type (black lines) and the Ah-NAS2-RNAi line 1-2 (red lines) were subjected to SEC-ICP-MS analysis. Data for Zn ([A] and [B]) and S ([C] and [D]) are shown. Note that the figure shows low molecular mass Zn fractions that in shoots represent only a very small fraction of extractable Zn, most of which is present as free Zn\(^{2+}\) (Table 4). Day-to-day variability in ion intensity was normalized using an external standard. Shown here are data from one experiment representative of three independent experiments (Table 2). WT, wild type.

reduction in Zn hyperaccumulation. To assess hyperaccumulation status for plants cultivated in hydroponics, where both root and shoot tissue are accessible, a shoot:root ratio of Zn concentrations above unity was used as a criterion (Talke et al., 2006). We observed a Zn shoot:root ratio of ~15 for wild-type plants in the presence of 10 \(\mu\)M Zn\(^{2+}\) (Figure 3). Strong suppression of NAS2 resulted in a fivefold reduction of the Zn shoot:root ratio at 10 \(\mu\)M Zn\(^{2+}\) (Figure 3; i.e., at a concentration that can be tentatively assumed to be in the range of bioavailable Zn\(^{2+}\) concentrations in soils where \textit{A. halleri} grows naturally) (Table 4, soil metal levels). At a low external Zn\(^{2+}\) concentration of 0.077 \(\mu\)M, hyperaccumulation was barely apparent even in wild-type plants. One can assume that with less Zn available to the plant, the wild type may reduce root-to-shoot Zn transport, possibly to protect roots from physiological Zn deficiency. Furthermore, the absence of an effect of NAS2 suppression on root-to-shoot Zn partitioning in plants grown at a low external Zn\(^{2+}\) concentration might be partly attributable to the fact that root NA concentration might be approximately twofold lower in roots of wild-type plants under these conditions. The mechanism underlying the increase of root NA levels at higher Zn\(^{2+}\) is not clear as no corresponding upregulation of NAS2 was observed (see Supplemental Figure 2 online). We propose that NA chelation contributes to the symplastic mobility of Zn\(^{2+}\) in \textit{A. halleri} roots toward the xylem. Suppression of vacuolar sequestration in root cells has been hypothesized as a mechanism important for metal hyperaccumulation (Clemens et al., 2002; Verbruggen et al., 2009), and chelation of Zn by NA might suppress vacuolar Zn sequestration in roots. Through grafting experiments it was shown that Zn hyperaccumulation in \textit{N. caerulescens} is largely governed by root processes (de Guimarães et al., 2009). Similar to the effects of NA in \textit{A. halleri} described here, elevated His levels specifically in roots appear to be important for Ni hyperaccumulation in \textit{N. caerulescens} (Richau et al., 2009). Analysis of Zn distribution in \textit{A. halleri} roots by Zinpyr staining and confocal microscopy supports a reduction in Zn xylem loading upon downregulation of NA biosynthesis. Whereas no change in Zn distribution was apparent between wild-type and RNAi plants, relative quantification of Zinpyr staining indicated enhanced retention of Zn in pericycle and inward cells of the tested Ah-NAS2-RNAi line (Figure 5).

The most important criterion for Zn hyperaccumulation is a Zn content >10,000 \(\mu\)g g\(^{-1}\) dry biomass in leaves of plants grown under natural conditions. To address this, we cultivated the wild type, a control transformant, and Ah-NAS2-RNAi plants in soil taken from native \textit{A. halleri} sites. The metal accumulation we observed in individuals from the Langelsheim population on the contaminated soil originating from this site was highly congruent with field data (cf. Table 1, Figure 7). We determined leaf Zn and Cd concentrations of ~12,000 and 40 \(\mu\)g g\(^{-1}\) dry biomass, respectively, upon cultivation on heavily contaminated soil from Langelsheim in growth chamber experiments (Figure 7, Harz 3). \textit{A. halleri} plants sampled in the field at the Langelsheim site contained leaf Zn and Cd concentrations of ~11,500 and 40 \(\mu\)g g\(^{-1}\) dry biomass, respectively (Table 1). Thus, with respect to metal accumulation, we were able to closely mimic the natural conditions in growth chamber experiments. Our results from growth chamber experiments on soil also confirmed that wild-type \textit{A. halleri} hyperaccumulate Zn on soils with very different levels of metal contamination (Figure 7, Table 4, extractable Zn). Wild-type and control RNAi plants accumulated leaf Zn concentrations averaging above the 10,000 \(\mu\)g g\(^{-1}\) dry biomass threshold on all soil types, whereas Zn concentrations in leaves of all strongly NAS2-suppressed plants were significantly lower than those of the control genotypes on soil with background and intermediate contamination level (Figure 7, Harz 1 and Harz 2 soils). At this point, we cannot explain why plants grown in the most highly Zn-contaminated soil (Harz 3) accumulated lower concentrations of Zn than plants grown in the soil containing intermediate Zn contamination and why NAS2 suppression had progressively lower effects on root-shoot Zn partitioning with increasing soil Zn contamination. The former may be attributable to the soil and its composition or to root–soil interactions and concurrent changes in root metabolite or mRNA levels that cannot be reliably monitored in soil-grown plants but that may affect metal homeostasis mechanisms. The latter might be taken to suggest that high expression of NAS2 is more important for
A. halleri when growing on noncontaminated or intermediate-level contaminated soils than on contaminated soils. Because of its biochemical properties, NA can effectively function as a symplastic metal chelator (Curie et al., 2009). At neutral pH, it forms very stable complexes with various divalent cations, including Zn(II) (von Wiren et al., 1999; Rellán-Alvarez et al., 2008). Direct demonstration of in vivo formation of Zn(II)-NA complexes was achieved in S. pombe cells by extended x-ray absorption fine structure analysis (Trampczynska et al., 2010). Given the high degree of conservation in eukaryotic metal transporters and chelators as well as Zn-requiring proteins and protein domains, this finding was interpreted as evidence for a role of NA in plant Zn trafficking via the formation of Zn(II)-NA complexes (Blindauer and Schmid, 2010). Indeed, speciation

Table 3. A. halleri Genes Showing Changes in Transcript Abundance in Roots of Ah-NAS2-RNAi Line 1-2 Compared to Wild-Type Plants

<table>
<thead>
<tr>
<th>AGI Code</th>
<th>Annotation</th>
<th>Control Zn^{2+} (0.077 μM)</th>
<th>Elevated Zn^{2+} (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Higher transcript abundance in Ah-NAS2-RNAi line 1-2 than in the wild type</td>
<td>Fold Change</td>
<td>Adjusted P Value</td>
</tr>
<tr>
<td>AT4G18593</td>
<td>Dual specificity protein phosphatase-related</td>
<td>7.28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AT1G71695</td>
<td>Peroxidase 12</td>
<td>2.02</td>
<td>0.1134</td>
</tr>
<tr>
<td>AT2G02020</td>
<td>Proton-dependent oligopeptide transport family protein</td>
<td>1.90</td>
<td>0.0282</td>
</tr>
<tr>
<td>AT5G02240</td>
<td>Catalytic/coenzyme binding; identical to protein At5g02240</td>
<td>1.52</td>
<td>0.0386</td>
</tr>
<tr>
<td>AT3G44280</td>
<td>Similar to unknown protein (A. thaliana)</td>
<td>1.49</td>
<td>0.1241</td>
</tr>
<tr>
<td>AT1G57870</td>
<td>Shaggy-related protein kinase kappa</td>
<td>1.34</td>
<td>0.3124</td>
</tr>
<tr>
<td></td>
<td>Lower transcript abundance in Ah-NAS2-RNAi line 1-2 than in the wild type</td>
<td>Fold Change</td>
<td>Adjusted P Value</td>
</tr>
<tr>
<td>AT5G46890</td>
<td>Bifunctional inhibitor lipid transfer protein/seed storage 2S albumin superfamily protein</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>AT5G56080</td>
<td>NAS2</td>
<td>0.23</td>
<td>0.0852</td>
</tr>
<tr>
<td>AT1G21010</td>
<td>Similar to 1-amino-cyclopropane-1-carboxylic acid oxidase</td>
<td>0.29</td>
<td>0.0582</td>
</tr>
<tr>
<td>AT1G48750</td>
<td>Protease inhibitor/seed storage/lipid transfer protein family protein</td>
<td>0.36</td>
<td>0.1901</td>
</tr>
<tr>
<td>AT5G47240</td>
<td>A. thaliana Nudix hydrolase homolog8</td>
<td>0.47</td>
<td>0.0586</td>
</tr>
<tr>
<td>AT1G09100</td>
<td>Subunit shown to interact with gene product of hexokinase 1</td>
<td>0.53</td>
<td>0.0152</td>
</tr>
<tr>
<td>AT5G04950</td>
<td>NAS1</td>
<td>0.54</td>
<td>0.1707</td>
</tr>
<tr>
<td>AT5G49560</td>
<td>Similar to unknown protein (A. thaliana)</td>
<td>0.59</td>
<td>0.0245</td>
</tr>
<tr>
<td>AT4G19750</td>
<td>Glycosyl hydrolase family protein with chitinase insertion domain</td>
<td>0.75</td>
<td>0.5390</td>
</tr>
</tbody>
</table>

RNA of plants grown hydroponically either in control medium or in the presence of 10 μM Zn^{2+} was extracted for the transcriptome analysis using ATH1 chips. Shown are fold changes, defined as the average ratio of normalized signals in Ah-NAS2-RNAi plants relative to the wild type (three independent experiments). P values listed are adjusted to a false discovery rate of 5% (Benjamini and Hochberg, 1995). Numbers in bold highlight significant changes (adjusted P < 0.05). The Arabidopsis Genome Initiative (AGI) codes and annotations of the putative A. thaliana orthologs are given.

Table 4. Zn and Cd Content of Soil from Three Native A. halleri Sites Representing the Natural Range of Habitat Conditions

<table>
<thead>
<tr>
<th>Soil</th>
<th>GPS Coordinates</th>
<th>Extractable (μg g⁻¹)</th>
<th>Exchangeable (μg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn</td>
<td>Cd</td>
<td></td>
</tr>
<tr>
<td>Harz 1: No Zn contamination</td>
<td>N51 53.752</td>
<td>93.5</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>E10 29.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harz 2: Intermediate Zn contamination</td>
<td>N51 56.563</td>
<td>2075</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>E10 20.962</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harz 3: Heavy Zn contamination</td>
<td>N51 56.574</td>
<td>15,150</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>E10 20.954</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These soils were used in metal accumulation experiments presented in Figure 7. Harz 1 was taken at the Oker population site, and Harz 2 and Harz 3 were taken at the Langelsheim population site, respectively. Please note that areas with different metal contamination levels lie in close proximity at both Oker and Langelsheim. This is reflected in only small differences of GPS coordinates. Both extractable (HCl) and exchangeable (DTPA) metal were determined.
analysis of A. halleri tissues by SEC-ICP-MS strongly supports the existence of such complexes. Most of the Tris/NaCl-extractable Zn in roots was associated with low molecular mass complexes (Figure 6). NA was unequivocally identified by ESI-MS in the respective fractions (see Supplemental Figure 7 online), thus providing evidence for co-migration of Zn(II) and NA in one complex. Overall, the higher total Zn concentration in roots of Ah-NAS2-RNAi plants as determined by ICP-OES (Figure 3) resulted in elevated levels also of chelated Zn(II) (Table 2). Thus, the three independent methods employed to determine effects of NAS2 suppression on root Zn showed very good agreement. ICP data on total root Zn, speciation analysis of Tris/NaCl extractable Zn, and Zinpyr staining all revealed an elevation of root Zn by a factor of 1.5 to 2 in plants with reduced root NA levels.

The concomitant increase in S coeluting with Zn in root extracts of Ah-NAS2-RNAi plants together with the detection of GSH and des-γGlu-PC2 in the low molecular mass Zn fraction by ESI-MS suggests upregulation of the levels of thiol ligands as a compensatory response to the reduced NA biosynthesis. Given the reduced root-shoot translocation in RNAi plants, we hypothesize that thiol ligands are less effective in enabling xylem loading of Zn. It will now be important to identify all relevant thiols, including GSH and various possible PC variants in A. halleri roots, and to quantify them accurately in wild-type and Ah-NAS2-RNAi plants. The hydrophilic interaction liquid chromatography (HILIC)-ESI-TOF-MS analysis of low molecular mass Zn fractions performed here does not allow this kind of in-depth analysis and quantification of individual thiol ligands.

Our data for a species at an extreme end of natural variation in metal homeostasis (Hanikenne and Nouet, 2011) add strong evidence to a number of observations that have accumulated in the past years, all indicating an involvement of NA in plant Zn homeostasis. NA-deficient tobacco plants not only contain less Fe in their leaves but also less Zn (Takahashi et al., 2003). Corresponding findings have been reported for an A. thaliana nas quadruple mutant (Klatte et al., 2009). Similarly, in monocots such as rice (Oryza sativa), modulation of NA levels was shown to affect Zn content of grains as much as Fe content (Lee et al., 2009; Lee et al., 2011). Rice plants overexpressing At-NAS1 together with the Fe storage protein ferritin and a phytase accumulate more Zn in the grain than do wild-type plants (Wirth et al., 2009).

Because NA can form complexes with different essential transition metal cations, high NA levels alternatively may contribute to Zn hyperaccumulation by ensuring mobility of non-hyperaccumulated micronutrient cations in the presence of high Zn fluxes (Krämer, 2010). In accordance with the Irving-Williams...
series, Cu(II)-NA complexes are the most stable NA complexes with divalent cations in vitro. However, Cu(II) is unlikely to be available for binding as its symplastic distribution is determined by designated metallochaperones (Pilon et al., 2009). Correspondingly, we did not observe any changes in Cu partitioning upon NAS2 suppression. Complexes of NA with Fe(II), the oxidation state of Fe expected to predominate in the cytoplasm, and Mn(II) are less stable in vitro than those formed with Zn(II). In silico calculations predict exchange reactions at cytosolic pH when Zn(II) is added to Fe(II)-NA (Relían-Alvarez et al., 2008). Elemental profiles obtained for A. halleri plants grown in soil or hydroponically did not reveal any Ah-NAS2-RNAi-dependent changes in root-to-shoot partitioning of Fe and only a minor effect for Mn in hydroponically grown plants (Figure 4; see Supplemental Figure 8 online). Together with our speciation analysis, these observations strongly suggest that NA is contributing to Zn hyperaccumulation by directly forming complexes with Zn(II).

Analysis of AhHMA4-RNAi lines indicated that high steady state transcript levels of some Zn deficiency response marker genes and hyperaccumulation candidate genes (e.g., IRT3 and ZIP4 encoding Zn$^{2+}$ transporters) are a consequence of efficient root-to-shoot Zn translocation (Hanikenne et al., 2008). When root Zn levels are higher due to Ah-HMA4 suppression, expression of these genes is repressed and approaches levels found in the nonhyperaccumulator A. thaliana. By contrast, our microarray data showed very few secondary effects of Ah-NAS2 suppression on the transcript level of other genes. This means that the reduced root-to-shoot Zn translocation is a direct consequence of the NAS2 knockdown alone. The lack of secondary effects of NAS2 suppression on gene expression may have different reasons. The elevation of Zn levels in root cells of strong RNAi plants may not have exceeded a threshold level required to suppress expression of Zn transporter genes. Alternatively, the effect of NAS2 suppression could be restricted to fewer cell types than HMA4 knockdown, as suggested by the Zinpyr staining. Complex formation is not only a function of stability constants, pool sizes of binding partners, and pH but also of the availability of other ligands and transport pathways. Metal hyperaccumulation likely depends on fundamentally altered partitioning of metals not only at the tissue level but also at the cellular and subcellular level (Krämer, 2010). We hypothesize that this altered partitioning is partly facilitated by the upregulation of NA biosynthesis specifically in roots of Zn hyperaccumulators. However, dissection of the underlying network of chelators and transporters that mediate efficient hyperaccumulation of Zn as well as the adjustment of Cu, Fe, and Mn homeostasis in the presence of high Zn fluxes (Hanikenne and Nouet, 2011) at the cellular level will require molecular techniques with the corresponding resolution (Dinneny et al., 2008; Long et al., 2010). These are not yet available for hyperaccumulating plants.

Zn enrichment or biofortification of crops faces the challenge of increasing Zn fluxes into edible parts without concomitant accumulation of Cd (Palmgren et al., 2008; Zhao and McGrath, 2009). Cd is a highly toxic pollutant present in agricultural soil, for instance, as a consequence of fertilizer or sewage sludge application (Grant et al., 2008). The available evidence suggests that Cd$^{2+}$ ions are distributed through homeostatic pathways for essential metals (Clemens, 2006). To assess whether modulation of NA levels offers a chance to separate Zn and Cd accumulation, we also analyzed Cd levels in plants grown on native A. halleri soils. Under conditions of low levels of Cd in the soil, which of course are the most relevant in the context of biofortification, leaf Cd accumulation was significantly reduced in strong Ah-NAS2-RNAi plants (Figure 7C). Thus, the contribution of NAS2 to translocation mechanisms into aboveground tissues affects Cd movement in a comparable fashion as HMA4, enhancing Cd root-to-shoot mobility in A. halleri.

Elevated NA accumulation has been discussed also as a possible factor contributing to Zn$^{2+}$ hypertolerance of metallophytes (i.e., their naturally selected ability to grow and thrive in the presence of metal ion concentrations far exceeding the levels that nonmetallophytes with basal metal tolerance can cope with) (Becher et al., 2004; Weber et al., 2004; Krämer, 2005). This hypothesis was based on Zn$^{2+}$ tolerance effects upon heterologous expression of NAS genes in yeast cells (see Supplemental Figure 1 online). However, we did not observe Zn toxicity symptoms in Ah-NAS2-RNAi plants under any of the tested conditions. Root elongation was not affected in hydroponic medium containing up to 300 μM Zn$^{2+}$ (data not shown). Leaf growth and coloration appeared normal even in plants cultivated on highly metal-contaminated soil (Figure 7A). Thus, unlike increased cellular Zn efflux activity through HMA4, elevated NA content appears to be important for Zn hyperaccumulation only and not for hypertolerance. This is consistent with findings in A. thaliana. A nas quadruple mutant did not show a phenotype under excess Zn (Klatte et al., 2009). Conversely, NAS overexpression strongly enhanced Ni tolerance in A. thaliana (Pianelli et al., 2005) and tobacco, but showed little effect on Zn tolerance (Kim et al., 2005). Overall, this is in agreement with a role of NA in intercellular Zn flux rather than sequestration.

Interspecies crosses between A. halleri ssp halleri and A. lyrata provided insights into the genetic architecture of metal hypertolerance and hyperaccumulation. Three major QTLs were detected each for Zn and Cd hypertolerance (Courbot et al., 2007; Willems et al., 2007). Ah-HMA4 most likely underlies the one overlapping QTL for both. Zn hyperaccumulation appears to be genetically more complex. Five significant QTLs plus several candidate QTLs were found when progeny of the interspecies crosses was cultivated in low Zn (Frérot et al., 2010). Again, HMA4 is the candidate gene located in the interval which accounts for the largest contribution to the hyperaccumulation phenotype, but contributions from additional genes are clearly required. Efficient xylem loading as mediated by HMA4 depends on efficient radial symplastic passage. In agreement with this, strong HMA4 expression has been demonstrated to be necessary but not sufficient for Zn hyperaccumulation. A. thaliana plants expressing Ah-HMA4 under the control of its native promoter accumulated only minimally higher concentrations of Zn in their leaves than wild-type plants (Hanikenne et al., 2008). Future studies will aim at determining whether Zn translocation in nonhyperaccumulator plants can be further enhanced by combining strong expression of both Ah-HMA4 and Ah-NAS2.

To date, none of the mapped QTLs for Zn hyperaccumulation have been shown to contain a NAS2 gene (Filatov et al., 2007;
Frérot et al., 2010). There are several possible explanations for this apparent discrepancy. First, the demonstrated quantitative contribution of elevated \(NAS2\) expression may not be strong enough to be detected in QTL analysis. Second, \(NAS2\) expression could be controlled by a regulatory gene located elsewhere in the genome, as discussed for \(MTP1\) (Frérot et al., 2010). Third, higher activity of certain metal homeostasis genes in \(A. halleri\) appears to be at least partly due to gene copy number expansion. Examples are three tandem \(HMA4\) copies (Hanikenne et al., 2008) and a total of up to five \(MTP1\) paralogs present either in tandem or dispersed in the genome (Shahzad et al., 2010). Not all of these \(MTP1\) paralogs contribute to higher transcript abundance in \(A. halleri\) (Dräger et al., 2004; Shahzad et al., 2010) and thus have not all been detected by QTL analysis. Similarly, there could be as yet undiscovered \(NAS2\) paralogs in the \(A. halleri\) genome.

In summary, we elucidated the role of NA in root-to-shoot translocation of Zn. We also demonstrated the quantitative contribution of elevated \(NAS2\) expression in \(A. halleri\) roots to Zn hyperaccumulation both in a hydroponic system and in natural conditions on native soil. It will be important to uncover the molecular basis for strong constitutive \(NAS2\) expression, to map the position of additional, as yet hypothetical, \(NAS2\) gene copies, and to study their evolutionary origin. Our findings point toward possible solutions for enhancing plant Zn accumulation in biofortification approaches.

**METHODS**

**Plant Material, Arabidopsis halleri Transformation, and Plant Cultivation**

Leaf tissue from different \(A. halleri\) ssp \(halleri\) populations (listed in Table 1) was collected in the field for elemental profiling. Individual plants were transferred from the field into growth chambers (Percival Scientific) and vegetatively propagated on soil through cuttings. Plants were cultivated with a temperature cycle of 23°C (day)/18°C (night) and a photoperiod of 16 h light (110 \(\mu\)E m \(^{-2}\) s \(^{-1}\)) and 8 h dark. \(A. halleri\) ssp \(gemmifera\) was grown from seed and propagated through cuttings, and \(Arabidopsis\) thaliana (accession Col) plants were grown from seed.

For the generation of the \(Ah-NAS2\)-RNAi construct, a 392-bp fragment of the \(NAS2\) cDNA was PCR amplified using the following primers: RNAi-fw, 5’-TTGGCCAAGTTCCATCTTCC-3’; RNAi-rev, 5’-GGATGAC-CACCGAATTAACC-3’. The fragment was cloned into pENTR/D-Topo (Invitrogen). Using the Gateway LR Clonase enzyme mix (Invitrogen) the fragment was subcloned into pHELLSGATE8 (Hellwell et al., 2002). The final plasmid was transformed into \(Agrobacterium\) \(tumefaciens\) (GV3101). \(Agrobacterium\)-mediated stable transformation of \(A. halleri\) ssp \(halleri\) (accession Langelsheim) was performed using a tissue culture-based procedure as described (Hanikenne et al., 2008). Sterile roots were cut and incubated for 7 to 8 d on callus induction medium. The root pieces were then dipped into \(Agrobacterium\) suspension and cocultivated for 2 to 3 d. Afterwards, shoots were induced on shoot induction medium containing kanamycin for selection of transgenic shoots. After the induction of roots, the plants were transferred to soil.

For initial characterization of genotypes with respect to transcript and NA analysis as well as metal partitioning, plants were grown hydroponically in a modified 1/10 Hoagland medium (0.0871 \(\text{mM} (\text{NH}_4)_2\text{HPO}_4\), 0.4 \(\text{mM} \text{Ca(NO}_3\)\)\(_2\), 0.6 \(\text{mM} \text{KNO}_3\), 0.2 \(\text{mM} \text{MgSO}_4\), 5 \(\mu\)M of a complex of Fe(III) and N,N’-di-(2-hydroxybenzoyl)-ethylenediamine-N,N’-dicacetate (HBED; ABCR GmbH), 4.63 \(\mu\)M \(\text{H}_2\text{BO}_3\), 0.032 \(\mu\)M \(\text{CuSO}_4\), 0.915 \(\mu\)M \(\text{MnO}_2\), 0.011 \(\mu\)M \(\text{MoO}_3\), and 0.077 \(\mu\)M \(\text{ZnSO}_4\)). Medium was changed weekly. For experiments assessing Zn hyperaccumulation in hydroponic culture, Zn\(^{2+}\) levels were elevated to 10 \(\mu\)M ZnSO4.

Elemental profiling of leaf tissue was in addition performed on \(A. halleri\) ssp \(halleri\) wild-type and \(Ah-NAS2\)-RNAi plants cultivated in untreated soil from native \(A. halleri\) sites in the Harz Mountains in Germany (Table 4).

**Transcript Analysis**

For transcript quantification by real-time RT-PCR and microarray analysis, RNA was extracted from homogenized \(A. halleri\) leaf or root tissue with Trizol (Invitrogen Life Technologies) according to the manufacturer’s instructions. RNA quality was checked with a nanodrop photometer. DNasel-treated RNA (0.75 \(\mu\)g) was used for cDNA synthesis with an Invitrogen SuperScript II kit. Real-time RT-PCR reactions were performed in 96-well plates in a Bio-Rad Cycler with a MyQ real-time PCR detection system using SYBR Green (Eurogentec) to monitor cDNA amplification. Five microliters of 1:50 diluted cDNA and 5 pmol of forward and reverse primers were added to 10 \(\mu\)L SYBR Green mix in a total volume of 20 \(\mu\)L. The standard thermal profile was 95°C for 8 s, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Data were analyzed using iQ5 Optical System software version 2.0 (Bio-Rad). Relative expression values were determined calculating the difference between the cycle threshold (Ct) value of the target gene and the Ct value of the reference gene \(EF1\alpha\) (relative transcript level = 1000 \times 2 \(^{-\Delta\Delta Ct}\)). The mean Ct for \(EF1\alpha\) of all leaf cDNAs had a standard deviation of 5.98% (\(\pm\)1.37 amplification cycles, \(n = 18\)). The mean Ct for \(EF1\alpha\) of all root cDNAs had a standard deviation variation of 8.44% (\(\pm\)1.61 amplification cycles, \(n = 21\)). Primers were designed using the Primer3 software (http://primer3.sourceforge.net/). Sequences are listed in Supplemental Table 1 online. Primers and amplicons were checked for potential secondary structures with the program mfold (Zuker, 2003). Prior to the analysis of \(NAS2\) transcript levels in individuals from different \(A. halleri\) populations, the intron-free genomic \(NAS2\) coding sequence was amplified, sequenced, and checked for polymorphisms in primer binding sites to ensure equal amplification efficiency.

Microarray hybridization to Affymetrix ATH1 chips was performed at the Affymetrix Service Provider and core facility KFB Center of Excellence for Fluorescent Bioanalytics (Regensburg, Germany) in accordance with the manufacturer’s manual. Total RNA (250 ng), purified with RNAeasy Mini columns (Qiagen) according to the manufacturer’s directions, was used to generate double-stranded cDNA and subsequently biotin-labeled cRNA. The length of the cRNA products was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies). Following fragmentation, cRNA products were hybridized to the array for 16 h at 45°C in a rotating chamber. Hybridized arrays were washed and stained in an Affymetrix Fluidics Station FS450, and the fluorescent signals were measured with an Affymetrix GeneChip Scanner 3000.

CEL files from the Affymetrix microarray hybridization were processed using the R program and Bioconductor packages (Gentleman et al., 2004). The robust multichip average normalization was performed using the default settings of the corresponding R function (Irizarry et al., 2003). To estimate the amount of expressed mRNAs, the present call information of the nonparametric Wilcoxon signed rank test (PMA values) was computed with the “affy” package (Gautier et al., 2004). Hybridization data from three biological replicates were generated each for Langelsheim wild-type and RNAi line 1-2. Analysis of differentially expressed genes was performed with the LIMMA package using the robust multichip average normalized expression values (Smyth, 2004). P values were corrected for multiple testing and adjusted to a 5% false discovery rate (Benjamini and Hochberg, 1995). The confidence threshold for up- or downregulated genes was set to an adjusted P value of <0.05.

**NA Analysis**

Quantification of Fmoc-derivatized NA was performed via ultraperformance liquid chromatography (UPLC)-ESI-QTOF-MS and stable isotope dilution analysis as described (Schmidt et al., 2011). In brief, 100 mg plant
tissue powder was extracted with 100 \mu L water. After addition of internal standard [\textsuperscript{15}N\textsubscript{3}-NA], 10 \mu L of the extract were derivatized with Fmoc-Cl at room temperature. The 120-\mu L reaction was terminated after 1 min by addition of 80 \mu L aminopropanol solution. For separation and detection, an Agilent UPLC system connected to a Q-TOF Premier mass spectrometer (Waters) was employed. Two microliters of sample was separated at 40°C on a BEH C18 column (2.1 \times 100 mm, 1.7 \mu m; Waters) with a flow rate of 0.5 mL/min. Solvents were water (A) and acetonitrile (B), both acidified with 0.1% (v/v) formic acid. The binary gradient used was as follows: 0.5 min 20% (v/v) B, from 0.5 to 4.5 min a linear gradient to 95% B, 0.9 min 95% B, a linear gradient to initial conditions in 1.6 min and equilibration (20% B) for 1 min. The mass spectrometer was operated in the ESI V+ mode. Quantification was performed on the respective reconstructed ion traces of the protonated molecular ions using the QuanLynx module of the MarkerLynx software.

Speciation Analysis by SEC-ICP-MS and Ligand Identification by ESI-MS

A. halleri root tissue was extracted with Tris/NaCl and subjected to speciation analysis essentially as described (Persson et al., 2009). The water-soluble fraction of a sample, consisting of 100 mg fresh plant material stored at \(-80^\circ\text{C}\), was extracted in 2 mL 50 mM ice-cold Tris buffer, pH 7.5, following grinding to homogeneity with pestle and mortar in liquid nitrogen together with 500 mg acid-washed quartz sand. The suspension was centrifuged for 5 min at 11,000g and 4°C whereupon the supernatant was transferred to a centrifugal filter (Micronex Ultracel YM-100) and spun for 20 min at 11,000g (4°C) to obtain filtrate with a cutoff below 100 kD. The filtrate was kept on ice until analysis.

SEC-ICP-MS analysis was based on the procedures described by Persson et al. (2009), allowing reproducible quantitative determination of mineral elements. Briefly, 50 mM Tris, pH 7.5, at a flow rate of 1 mL min\(^{-1}\) was used as eluent for SEC (Superdex Peptide 10/300 GL; Amersham Biosciences). The ICP-MS (Agilent 7700; Agilent Technologies) was operated using oxygen in the reaction cell to increase the sensitivity for S, P, S, Mn, Co, Ni, Fe, and Cd were recorded in addition to Zn. All solvents were degassed prior to analysis. Peaks below 1 kD were heart-cut, and ligand identification was performed using direct injection in an ESI-TOF-MS (Micromass LCT; Waters). Hydrophilic ligands were further separated by HILIC to reduce salt signal suppression (Lee et al., 2011). The heart-cut peaks were lyophilized and resuspended in a methanol solution before being injected on the HILIC column (2.1 \times 100 mm, 3-\mu m particle size) and eluted with an acetonitrile-formic acid gradient.

Elemental Analysis

For elemental analysis, leaves were harvested, rinsed with Millipore water, and blotted dry. Roots of hydropically grown samples were desorbed for 10 min each in ice-cold solutions of 0.1 M CaCl\(_2\) and 10 mM EDTA, then rinsed and blotted dry. Plant material was digested in a 2:1 mixture of HNO\(_3\) (65%, v/v) and H\(_2\)O\(_2\) (30%, v/v) in a microwave oven using a temperature step gradient (maximum of 210°C). Digests were, if necessary, diluted with Millipore water, filtered, and analyzed by ICP-OES on an iCAP 6000 Series spectrometer (Thermo, Fisher). To determine extractable metal content in soil samples, \(-3.0\) g of dried and sieved (1-mm mesh) soil was extracted with 25 mL of 0.1 M HCl for 30 min at 25°C with gentle shaking. Exchangeable metal was determined with a solution containing 5 mM DTPA, 100 mM triethanolamine, and 10 mM CaCl\(_2\), pH 7.3.

Zinpyr Staining

Working solutions of Zinpyr-1 in 0.9% saline were diluted from 1 mM stock made up in DMSO and stored at \(-20^\circ\text{C}\). Clones of wild-type and Ah-NAS2-RNAi 1-2 plants were cultivated in hydroponic medium containing 1 \mu M ZnSO\(_4\). Roots were completely cut off and washed with deionized water three times before immersion in a working solution of 20 \mu M Zinpyr-1 and incubation in darkness at room temperature for 3 h. After incubation, the roots were washed in deionized water and immersed in 75 \mu M propidium iodide for 5 min to stain cell walls. Before being transferred to 0.9% saline, samples were again rinsed in deionized water. Images were taken with a Leica SP5 laser confocal microscope with 488-nm excitation using fluorescein isothiocyanate and Texas Red filters. To compare the Zinpyr-1 signals in wild-type and mutant roots, fixed settings of the confocal microscope were used. Quantification was performed using the ImageJ software (V 1.43u, Rasband, 1997–2006). Green and red fluorescence intensities were measured as a pixel count within a defined area. The results were subsequently normalized using the ratio of green-to-red fluorescence.

Statistical Analysis

One-way analysis of variance and Tukey tests were performed with SigmaPlot 11.0.

Accession Numbers

Microarray data presented here are deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE31778. GenBank accession numbers of A. halleri NAS1, NAS2, NAS3, and NAS4 genes are JQ619641, JQ619642, AJ580399, and AJ580400, respectively.

Supplemental Data

The following materials are available in the online version of this article.

- Supplemental Figure 1. Functional Characterization of A. halleri NAS Genes in Zn\(^{2+}\) Hypersensitive S. pombe \(\Delta Mf\) Mutant Cells.
- Supplemental Figure 2. Expression of NAS Genes in A. halleri Roots and Leaves.
- Supplemental Figure 3. Effects of Ah-NAS2-RNAi on NAS1, NAS3, and NAS4 Transcript Abundance.
- Supplemental Figure 4. Correlation of NAS2 Transcript Level and Root NA Concentrations in A. halleri Wild-Type and Ah-NAS2-RNAi Plants Grown in the Presence of 10 \mu M Zn\(^{2+}\).
- Supplemental Figure 5. NA Concentration in Leaves of A. halleri Wild-Type and Ah-NAS2-RNAi Plants.
- Supplemental Figure 6. Zn Concentrations in Roots and Leaves of A. halleri Wild-Type and Ah-NAS2-RNAi Plants Grown in Control Medium.
- Supplemental Figure 7. HILIC-ESI-TOF-MS Analysis of SEC Low Molecular Mass Zn Fractions.
- Supplemental Figure 8. Leaf Mn, Cu, and Fe Concentrations of A. halleri Wild-Type and Ah-NAS2-RNAi Plants Grown in Native A. halleri Soils.
- Supplemental Table 1. Sequences of Primers Used for Transcript Analysis by Real-Time RT-PCR.

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AUTHOR CONTRIBUTIONS


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Elevated Nicotianamine Levels in *Arabidopsis halleri* Roots Play a Key Role in Zinc Hyperaccumulation

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