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Heavy metals need assistance: the contribution of nicotianamine to metal circulation throughout the plant and the *Arabidopsis NAS* gene family

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Understanding the regulated inter- and intra-cellular metal circulation is one of the challenges in the field of metal homeostasis. Inside organisms metal ions are bound to organic ligands to prevent their uncontrolled reactivity and to increase their solubility. Nicotianamine (NA) is one of the important ligands. This non-proteinogenic amino acid is synthesized by nicotianamine synthase (NAS). NA is involved in mobilization, uptake, transport, storage, and detoxification of metals. Much of the progress in understanding NA function has been achieved by studying mutants with altered nicotianamine levels. Mild and strong *Arabidopsis* mutants impaired in nicotianamine synthesis have been identified and characterized, namely *nas4x-1* and *nas4x-2*. *Arabidopsis thaliana* has four *NAS* genes. In this review, we summarize the structure and evolution of the *NAS* genes in the *Arabidopsis* genome. We summarize previous results and present novel evidence that the four *NAS* genes have partially overlapping functions when plants are exposed to Fe deficiency and nickel supply. We compare the phenotypes of *nas4x-1* and *nas4x-2* and summarize the functions of *NAS* genes and NA as deduced from the studies of mutant phenotypes.

Keywords: nicotianamine, metal binding, chelation, gene family, multiple mutant

INTRODUCTION

Iron (Fe) and copper (Cu) are essential elements for all living organisms because of their unique property of being able to catalyze oxidation/reduction reactions. Conversely, an excess of Fe, especially Fe^{2+}, is detrimental since it catalyzes the production of reactive oxygen species (ROS) in the Fenton reaction (Fenton, 1894; Hell and Stephan, 2003). For this reason, free metal ions are not likely to exist in large amounts in cells. Indeed, Fe and other metals are mainly present in stable complexes with organic ligands or inorganic phosphates (Haydon and Cobbett, 2007).

Nicotianamine (NA) is one of the most investigated metal chelator molecules in plants. NA is a non-proteinogenic amino acid and it results from the enzymatic condensation of three S-adenosyl methionine molecules (SAM) catalyzed by nicotianamine synthase (NAS; Herbik et al., 1999; Ling et al., 1999; Takahashi et al., 1999). NA is able to form stable complexes with Mn^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, and Cu^{2+} (Benes et al., 1983; Anderegg and Ripperger, 1989). Moreover, NA has a high capacity to chelate Fe^{3+} (von Wiren et al., 1999; Weber et al., 2006; Rellan-Alvarez et al., 2008). For all the metals considered, the stability of the NA–metal complexes had its maximum at pH values above 6.5, suggesting that NA is more likely a symplastic chelator of metals and therefore would bind metals predominately within cells and the phloem (von Wiren et al., 1999). Cu^{2+} is an exception among the essential metals, since the Cu^{2+}–NA complex is very stable in mild acidic conditions, which is a strong argument in favor of the possible occurrence of Cu^{2+}–NA complexes in the apoplastic environment as prevailing in the xylem sap (von Wiren et al., 1999). Nicotianamine can be transported to the various organs and tissues via oligopeptide transporters, such as yellowstripe1-like (YSL) proteins (Curie et al., 2001, 2009). Rice ENA1 and ENA2 transporters were just recently described to mediate NA export from cells (Nozoye et al., 2011).

Studies of solanaceous and graminaceous plants as well as of hyperaccumulators showed that NA functions in long-distance transport of Cu (Pich and Scholz, 1996), short-distance and intracellular transport of Fe (Becker et al., 1995; Curie and Briat, 2003), plant reproduction (Takahashi et al., 2006), detoxification of heavy metals like Ni (Douchkov et al., 2005; Kim et al., 2005; Pianelli et al., 2005; Mari et al., 2006; Ouerdane et al., 2006; van de Mortel et al., 2006; Callahan et al., 2007) and Zn (Becher et al., 2004; Weber et al., 2004; Talke et al., 2006; van de Mortel et al., 2006), and in grasses as a precursor in the biosynthesis of phytosiderophores (Mori and Nishizawa, 1987). Several studies suggested that NA could be involved in iron mobilization and accumulation in plant roots and seeds (Douchkov et al., 2001, 2005; Cheng et al., 2007; Lee et al., 2009). NA is beneficial for increased bioavailability of Fe in foods (Maurer et al., 2010; Zheng et al., 2010).

Taken together, NA is a key compound of metal homeostasis in plants contributing to mobilization, uptake, transport, storage, and detoxification of metals. Since NA was found to be an important biofortification factor for essential nutrients like Fe and Zn in edible portions of crop plants (Zheng et al., 2010), further knowledge about the functions of NA and the

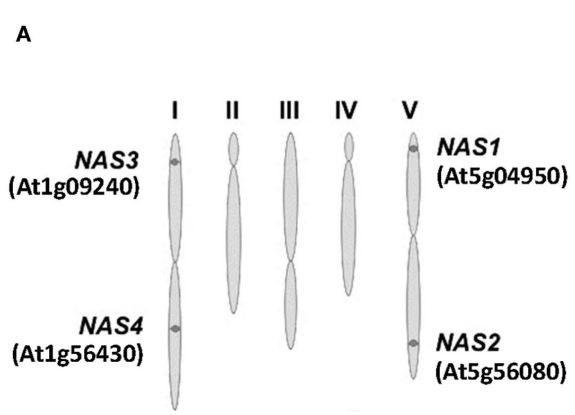
Abbreviations: NA, nicotianamine; NAS, nicotianamine synthase.

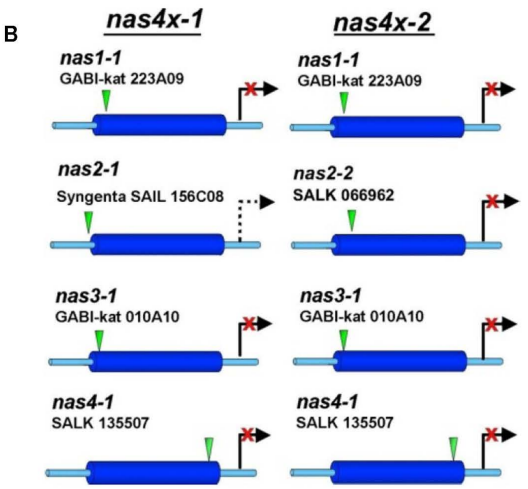
characterization of the essential genes for its production are of high relevance.

NAS GENE FAMILY OF *A. THALIANA*

The *Arabidopsis* system provides all tools that allow combining genetic studies with physiological analyzes and global gene expression experiments. This species was therefore utilized to investigate NA function. While not all plant species have multiple *NAS* genes, the *Arabidopsis* genome harbors a *NAS* gene family comprising four members (Bauer et al., 2004). *NAS1* and *NAS2* are located on chromosome V, while *NAS3* and *NAS4* are located on chromosome I (**Figure 1A**). Multiple alignment (CLUSTALW) showed a close relation between *NAS* genes located on the same chromosome with more than 80% identity while alignment of genes belonging to separate chromosomes showed an identity of about 70% (Bauer et al., 2004). Gene mapping between *Solanum esculentum* and *A. thaliana* suggested that the four *Arabidopsis* genes, as well as the single tomato *NAS* gene originated from a common ancestor *NAS* gene. This finding is in agreement with a first genome duplication event in the evolution of *Arabidopsis*, followed later by two independent duplication events (Bauer et al., 2004).

T-DNA insertion lines of all four *NAS* genes were identified and crossed to each other (**Figure 1B**; primer sequences for genotyping are found in **Figure 1C**). Under regular growth conditions single, double, and triple mutants did not show any obvious phenotypes suggesting functional redundancy (Klatte et al., 2009).





C

	Primer name	*NAS1*	*NAS2*	*NAS3*	*NAS4*
genotyping WT allele	Gene primer 1	GCCAAGAATTGAC CCCACG	TGGAACATATCGCGA TTTTTAGTTG	TGTCGCAAGTCATGCA CATTTC	AACTATGCACCCCGTAG AGCC
	Gene primer 2	ACGGTTGTTCATGA TCGCG	TCGATGGCACTATAC TCCTCGATC	TTCAGCCCAACAGTGT CGC	CCCACCCTTTGCAAATT GC
genotyping T-DNA allele	Gene primer	Gene primer 2 *NAS1*	Gene primer 2 *NAS2*	Gene primer 1 *NAS3*	Gene primer 1 *NAS4*
	T-DNA primer	*nas1-1* CCCATTTGGACGTG AATGTAGACAC	*nas2-1* TAGCATCTGAATTTC ATAACCAATCT *nas2-2* GCGTGGACCGCTTG CTGCAACT	*nas3-1* CCCATTTGGACGTGAA TGTAGACAC	*nas4-1* GCGTGGACCGCTTGCTG CAACT
Gene RT-qPCR	5′ primer	ATCTTCCACACAAC GGACG	AGATCGGACGGTGT GTGG	CAATTGGGAATGTTGG TGG	TGTAATCTCAAGGAAGC TAGGTG
	3′ primer	AACGACGTCATATT GGTCAAG	CCTCGATCAAATTCT TCTCCAT	TGTTCCTCCCTAGCTCC G	CAGTTACACGCGAGATC CG
Mass standard for RT-qPCR	5′ primer	ATGGCTTGCCAAAA CAATCTC	ATGGCTTGCGAAAA CAACCTC	ATGGGTTGCCAAGACG AACA	ATGGGTTATTGCCAAGA CGACC
	3′ primer	TCTAGACTCGATGG CACTAAACTCCTC	TCTAGACTCGATGGC ACTATACTCCTCGAT	TCTAGAAGACAACTGT TCCTCCCTAGCTCC	TCTAGAGGTAAGTTGTT CTTCATTAGCACCTGC

Primer sequences (each in 5′-3′ direction) for genotyping of *nas* mutants according to Klatte et al. (2009) and for reverse transcription-qPCR of *NAS* genes according to Klatte and Bauer (2009).

FIGURE 1 | Genomic organization of *NAS* genes and tools for analyzing *NAS* gene function. (A) Scheme representing the five chromosomes of *Arabidopsis*, indicating the location of the four *NAS* genes and their gene identity numbers (Bauer et al., 2004). **(B)** Scheme representing the location of T-DNA insertions, indicating the allele names. It is shown which alleles are combined in the quadruple mutants *nas4x-1* and *nas4x-2* (Klatte et al., 2009). **(C)** Table listing the primers used for genotyping as described in (Klatte et al., 2009) and the primers used for gene expression analysis by reverse transcription-qPCR according to (Klatte and Bauer, 2009).

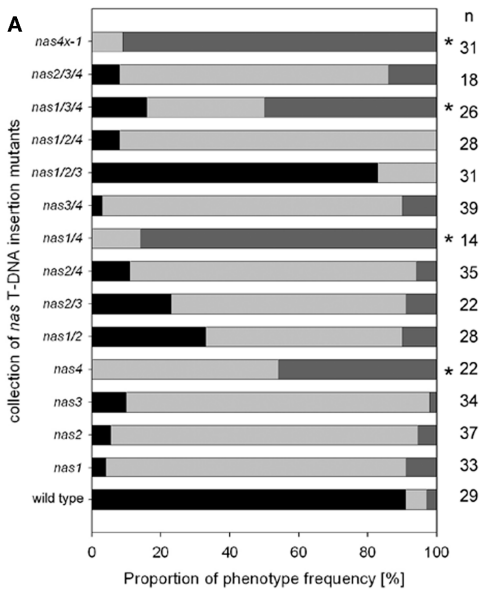
Single mutants had similar NA contents as wild type. Triple *nas* mutants had NA levels that were reduced to 30–40% of wild type levels (Klatte et al., 2009). Since NA can be transported short and long-distance in plants, severe metal homeostasis phenotypes are not expected in the presence of a functional *NAS* gene. Interestingly, however, upon exposure to modestly toxic Ni supply, *nas4-1* had a more chlorotic phenotype than *nas3-1*, while *nas1-1* and *nas2-1* had mild phenotypes like the wild type (Klatte et al., 2009). With increasing number of *NAS* knockout alleles, the NA contents decreased in the mutants while the severity of the leaf chlorosis was enhanced (Klatte et al., 2009). This suggests that NA contents correlate with Ni tolerance. Here, we show the seedling growth responses of single and multiple mutants in response to Fe deficiency. We found that all single *nas* mutants tested had a stronger leaf chlorosis than wild type plants upon Fe deficiency (**Figure 2A**). *nas4-1* Mutants had the strongest leaf chlorosis among the tested single mutants (**Figure 2A**). It can therefore be concluded that the *NAS* gene functions are partially non-overlapping. Perhaps the location of NA production is important. Alternatively, the NAS isoforms might have different enzyme activities, perhaps under specific conditions like Fe deficiency and Ni supply.

Partial non-redundancy is further confirmed by the fact that the *NAS* genes are differentially regulated in plants (Bauer et al., 2004; Klatte et al., 2009; summarized in **Figure 2B**; primers for gene expression analysis in **Figure 1C**). *NAS1*, *NAS2*, and *NAS4* were found expressed in roots, where they were induced by Ni supply. *NAS2* was also up-regulated by Fe deficiency. *NAS1* and *NAS4* were expressed in leaves, and *NAS4* could be induced by Fe deficiency and Ni in leaves. *NAS2* was not expressed in leaves. On the other hand, *NAS3* was expressed in leaves where it was repressed by Fe deficiency but strongly induced by Ni supply. In flowers, *NAS3* was expressed in sepals and petals while *NAS1*, *NAS2*, and *NAS4* were not expressed (Schuler et al., in preparation).

Taken together, *NAS* genes evolved as a gene family in *Arabidopsis* where they acquired overlapping and specific functions in metal homeostasis as well as differential gene regulation in response to metals. *NAS3* seems important for leaf and flower nicotianamine production upon Fe supply as well as Ni tolerance, while *NAS4* was more important for Fe deficiency in leaves and perhaps in roots. *NAS2* might be especially relevant for Fe deficiency responses in roots.

PHYSIOLOGICAL ANALYSIS OF QUADRUPLE *NAS* MUTANTS

Mutant analysis showed that all four *NAS* genes are functional, so that quadruple mutant analysis was needed to study NA function. The leaf chlorosis phenotypes of quadruple *nas* mutants were more severe than those of single mutants. Quadruple *nas1-1 nas2-1 nas3-1 nas4-1* mutants (termed *nas4x-1*) were found to have a stronger reduction of NA levels than all triple mutant combinations analyzed, namely to approximately 15% in vegetative leaves and 30% in seeds compared to wild type (Klatte et al., 2009). While *nas4x-1* plants had a residual NA level in leaves at the vegetative stage, this was not the case in the reproductive stage in leaves. Full loss of function *nas1-1 nas2-2 nas3-1 nas4-1* mutants (termed *nas4x-2*) did not contain any NA (Klatte et al., 2009). The morphological phenotypes of *nas4x-1* and *nas4x-2* were compared (summarized in **Table 1**).



B

	NAS1	NAS2	NAS3	NAS4
roots				
leaves				
petals				
sepals				
carpels				
stamen				
- Fe, roots				
- Fe, leaves				
Ni, roots				
Ni, leaves				

Gene expression pattern: black = expressed; white = not expressed; dark grey = induced vs. + Fe/- Ni; light grey = repressed vs. + Fe/- Ni

FIGURE 2 | Analysis of multiple *nas mutants*. (A) Percentage of leaf chlorosis phenotypes of multiple *nas* mutants, combining the alleles *nas1-1*, *nas2-1*, *nas3-1*, and *nas4-1*, germinated for 2 weeks on Hoagland agar medium devoid of Fe; the medium is described in (Jakoby et al., 2004). The colors indicate the percentage of plants with light green leaves (weak leaf chlorosis, black), light green intercostal areas (intermediate degree of leaf chlorosis, light gray), yellow intercostal areas (strong leaf chlorosis, dark gray). The numbers on the right side indicate the number of seedlings examined; * indicates a strong phenotype. **(B)** Table summarizing the gene expression results from (Bauer et al., 2004; Klatte et al., 2009) and Schuler et al. (in preparation).

Table 1 | Comparison of *nas4x-1* and *nas4x-2*.

	nas4x-1	***nas4x-2****	**Reference**
Genotype	*nas1-1 nas2-1 nas3-1 nas4-1*	*nas1-1 nas2-2 nas3-1 nas4-1*	Klatte et al. (2009)
Full-length *NAS* transcripts	*NAS2*	none	Klatte et al. (2009)
NA content, versus WT	10% in leaves, vegetative stage; 0% in leaves, reproductive stage; 40% in seeds	0% in all tested leaves; no seeds produced	Klatte et al. (2009)
Interveinal leaf chlorosis	Starts during reproductive phase	Strong during vegetative and reproductive phase; more severe in young leaves than in older leaves	Klatte et al. (2009)
Numerous small rosette leaves	Yes	Yes	Klatte et al. (2009)
Flowers	Normal	Sterile	Klatte et al. (2009)
Flowering time and senescence	Normal	Delayed	Klatte et al. (2009)
Global gene expression	Categories affected: metal homeostasis, biotic stress responses, leaf photosystem and root carbohydrate metabolism	n.a.	Schuler et al. (2011)

**A more detailed investigation of nas4x-2 phenotypes will be presented in Schuler et al. (in preparation); n.a. = not analyzed.*

nas4x-1 plants appeared nearly normal during the vegetative stage, unless they were grown under Fe deficiency or Ni supply. However, *nas4x-1* plants showed an interveinal leaf chlorosis upon transition to the reproductive growth stage, and Fe contents were increased in leaves at this stage compared to wild type (Klatte et al., 2009). Mobilization of Fe by *nas4x-1* roots was up-regulated at this stage which accounts for the increased Fe contents (Klatte et al., 2009). Presumably, intercostal leaf areas with mesophyll cells did not acquire Fe in sufficient amounts and may have emitted a long-distance Fe deficiency signal that stimulated root Fe uptake. *nas4x-1* plants are still fertile, yet flowers and seeds were found to contain less Fe than in the wild type (Klatte et al., 2009). *nas4x-1* mutant plants are valuable models to study NA function in late phases of plant development. These plants also served to perform a transcriptome analysis of roots and leaves upon Fe supply and Fe deficiency (Schuler et al., 2011). A comparison with the wild type transcriptomes confirmed that *nas4x-1* was affected in metal homeostasis since a high number of genes of this category was hit by differential expression. Besides this category, the mutant was also affected in biotic stress responses, leaf photosystem organization, and root carbohydrate metabolism (Schuler et al., 2011). Significantly more genes of these four biological categories were affected by differential expression between mutant and wild type compared to all genes analyzed in the microarray study. A change of expression of genes from these categories can be explained as an adaptation response to altered Fe levels.

Nas4x-2, on the other hand, is a severely affected mutant. Leaf chlorosis started during the vegetative phase (Klatte et al., 2009). Closer inspection of this mutant showed that NA was involved in the long-distance transport of Fe to young leaves presumably using the phloem, while older leaves received Fe from citrate-mediated transport in the xylem (Schuler et al., in preparation). The leaf chlorosis was due to Fe accumulation in the vascular system suggesting that NA is involved in lateral transport of Fe from vascular tissues to mesophyll (Schuler et al., in preparation). Furthermore, *nas4x-2* mutants were affected in pollination (Schuler et al., in preparation).

CONCLUDING REMARKS

Arabidopsis served as a model for the study of nicotianamine function in plants. The split of a *NAS* locus to four *NAS* genes in *Arabidopsis* resulted in a partial non-redundant specialization of *NAS* gene functions. These are conferred at least partly by differential gene expression of the *NAS* genes in response to developmental cues, tissue specificity, and metals. It has not been investigated yet whether the enzyme activities of the NAS isoforms are differentially regulated by metal supply. Using the mild and severe *nas4x-1* and *nas4x-2* mutants novel nicotianamine functions were uncovered, such as seed Fe loading, long-distance Fe transport to leaves, short-distance transport from vascular tissues to mesophyll, and in pollination.

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