

FUNCTIONAL CHARACTERIZATION OF THE NICOTIANAMINE SYNTHASE (*NAS*) GENE FAMILY IN *ARABIDOPSIS THALIANA*

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ABSTRACT

Iron is an essential nutrient for all living things but it is toxic in excess. In addition, iron is often not readily available for uptake from the soil, so plants have evolved tightly regulated iron uptake strategies to ensure that the plant maintains appropriate levels of iron within cells. Strategy I plants utilize a combination of a ferric chelate reductase enzyme and a ferrous iron transporter to effectively take up iron, whereas Strategy II plants rely on the biosynthesis and secretion of phytosiderophores that chelate ferric iron in the rhizosphere and shuttle it back into the cells. Interestingly, many Strategy I plants synthesize the phytosiderophore precursor, nicotianamine (NA), even though they do not make the actual phytosiderophores. Previous studies have indicated that NA may play a role in long distance transport of metals. NA is made from three molecules of S-adenosyl methionine, through the action of the enzyme nicotianamine synthase (NAS). In this study, we examine the expression of the family of *NAS* genes in a strategy I plant, *Arabidopsis thaliana*, to try to elucidate the precise role(s) of NA in plants. Our results demonstrate that the *NAS* genes are expressed in a tissue specific manner and are differentially regulated by iron, copper, zinc and nickel. In addition, we have created transgenic lines in which the *NAS1* gene is overexpressed or its expression is knocked-down. Elemental analysis of the transgenic lines suggests that NA is necessary for the accumulation of copper. Transgenic lines with reduced *NAS1* expression accumulate reduced levels of copper whereas *35S::NAS1* (overexpression) lines accumulate increased levels of copper. Additionally, the *35S::NAS1* lines were resistant to iron deficiency stress, whereas *NAS1* knock-down lines were sensitive to iron deficiency stress.

INTRODUCTION

Iron is an important element for the proper growth and development of both plants and animals. It serves as an essential cofactor for many of the enzymes involved in photosynthesis and respiration, and in animals is required for the transport and delivery of oxygen by hemoglobin. However, if iron accumulates to very high levels in cells, it has the potential to become toxic. In the Haber-Weiss-Fenton sequence of reactions, ferrous iron reacts with oxygen to produce a series of reactive oxygen species. The most damaging of these is the hydroxyl radical, which can attack and destroy any of the macromolecular structures of the cell (Aisen et al 2001). Therefore, iron uptake, storage, and homeostasis are all controlled tightly by plant and animal cells to maintain healthy levels of iron.

Although iron is the fourth most abundant element in the earth's crust, it is often not readily available for uptake by plants. In neutral to basic pH soils, iron is found primarily as insoluble oxyhydroxide polymers. However, as the pH of the soil decreases, iron is released from these compounds and the solubility of iron increases (Guerinot and Yi 1994).

Since plants are essentially immobile and are unable to go in search of more nutrient rich soil or escape from potentially damaging levels of iron, they must have very tightly regulated mechanisms for taking up iron from the soil and storing it in the cells. In fact, plants have evolved two mechanisms

for iron acquisition under iron limited conditions: Strategy I and Strategy II. Strategy I plants utilize a series of enzymes that first pump protons into the rhizosphere to acidify the microenvironment, thereby making iron more soluble. Then, a ferric chelate reductase reduces Fe(III) to Fe(II) at the root surface and the Fe(II) is transported into cells via a specific, high affinity iron transporter (Buckhout et al. 1989). The Strategy II mechanism, however, depends on the biosynthesis of compounds called phytosiderophores that are pumped into the rhizosphere, where they chelate iron and bring it back into the cell through a specific transporter (Romheld and Marschner 1986).

The phytosiderophores are synthesized from the precursor nicotianamine (NA) through modifications by nicotianamine aminotransferase (NAAT) and deoxymugineic acid synthase (DMAS) (Mori and Nishizawa 1987; Shojima et al. 1990). Nicotianamine itself is synthesized from three molecules of S-adenosyl methionine through the action of the enzyme nicotianamine synthase (NAS). Interestingly, nicotianamine is synthesized in both Strategy I and Strategy II plants, even though phytosiderophores are synthesized only in Strategy II plants (Higuchi et al. 1999, Mizuno et al. 2003). This finding presents a puzzling question about the role of NA in strategy I plants.

In tomato, a mutant named *chloronerva* was identified that exhibits typical iron deficiency phenotypes and has its iron uptake mechanism constitutively expressed. However, the plants are not actually iron deficient. In fact, the *chloronerva* mutants accumulate more iron than wild type plants, suggesting that some component of the iron sensing pathway is defective. Additionally, grafting a wild type rootstock or wild type scion onto the mutant was sufficient to recover the phenotype, implying that the defect is in a transportable element. That transportable element is NA due to a mutation in the one NAS gene in tomato (Ling et al. 1999).

In this study, we attempt to identify other possible roles for the compound nicotianamine in the model plant, *Arabidopsis thaliana*. Based on the work with the *chloronerva* mutant, and other studies performed in rice (Inoue et al. 2003; Ling et al. 1999) it appears that NA is involved in the long distance transport of iron in the plant. However, it is not clear at what stage of transport NA is involved, and there may be other potential roles for this compound. We have used transgenic *Arabidopsis* lines to demonstrate that NA may play a role in the sensing of iron, copper, zinc and nickel levels in the plant in addition to its role in transport. In addition, we have been able identify specific parts of the plant where NA may be involved based on spatial expression patterns of the four NAS genes.

EXPERIMENTAL PROCEDURES

Arabidopsis lines and growing conditions. Wild type *Arabidopsis* seeds (ecotypes *Columbia gl-1* and *Landsberg erecta*), *35S::NAS1* lines, and *NAS1* RNAi lines were surface sterilized (25% bleach, 0.2% SDS), imbibed in the dark for 2-4 days and plated on Gamborg's B5 medium (Sigma-Aldrich, St. Louis) supplemented with 2% sucrose, 1mM MES, and 0.6% agar, pH 5.8. For metal experiments, plates were kept at 22°C under constant light for 14 days (4-6 true leaf stage) and then transferred to the appropriate metal condition (described below) for 1-3 days. Shoots and roots were collected separately and stored at -80°C.

Iron sufficient (50 mM FeIII-EDTA) medium was used as the basis for all other types, except iron deficient (300 mM Ferrozine) medium. For media containing excess iron (250 mM FeIII-EDTA), excess copper (100 mM CuSO₄), excess zinc (500 mM ZnSO₄), high nickel (500 mM Ni), or high cadmium (90 mM CdSO₄), the appropriate amounts of these metals were added to iron sufficient medium. Copper deficient and zinc deficient media was prepared in the same way as iron sufficient media, except that the copper or zinc was dropped out. To ensure that the media was as

copper or zinc free as possible, all glassware was acid washed in 0.2 N HCl for 24 hours prior to use to remove residual metals. However, it is possible that very small amounts of copper or zinc were still present in the medium.

Various tissues (silicles, flowers, rosette leaves, stems, and roots) were obtained from mature, soil grown plants grown at 22°C under constant light.

Construction of 35S::NAS1 and NAS1 IR/RNAi lines. *Arabidopsis NAS1* DNA was prepared by PCR amplification with wild type genomic DNA as the template, Taq polymerase and the following primers:

CCGGATCCCTTATCTTCTCCCCTGAAATAA
CCTCTAGAAACACTTAATAAGCGTAGCCAC

(The underlined sequences show the BamHI and XbaI recognition/cut sites added to the primers.) This DNA was first cloned into pBluescript replacing the small BamHI-XbaI fragment and the sequence of the insert was confirmed. The fragment was then subcloned into pCGN18, a derivative of pCGN1547 (McBride and Summerfield, 1990) that contains the CaMV 35S promoter and 3' NOS termination site.

For the RNAi construct, a fragment from the 3' UTR of *NAS1* was *chosen* for cloning. This region begins 35 bases downstream of the *NAS1* gene stop codon and extends to 135 bases downstream. The DNA was obtained by PCR amplification using wild type *Arabidopsis (Col)* genomic DNA as the template, Taq polymerase and the following primers:

CCTCGAGGATCCGATTGTGTTAATGTTTTTGTCTTATA
CGGTACCATCGATGGTATCTTTTTTCATTCATAACAAAG

Each end of the primers contains restriction sites for two restriction enzymes (Recognition /cut sequences are underlined). This allowed two nested DNA fragments to be cut from the PCR DNA product. The fragments were separately cloned into *pHannibal* (Wesley et al. 2001) in opposite directions using ClaI and BamHI sites for one fragment and XhoI and KpnI sites for the other. Transformants were selected on LB medium supplemented with ampicillin. The plasmid, *pHannibal*, contains the CaMV 35S promoter and OCS terminator that promotes transcription of an RNA that encompasses both DNA inserts. After both fragments were inserted into *pHannibal*, the entire region was cut out using the two NotI sites that flank the area and moved into the single NotI site of *pArt* (Gleave, AP, 1992). Transformants were selected on LB medium supplemented with spectinomycin.

Plant Transformation. The *pNAS1* vector DNA was transferred into *Agrobacterium tumefaciens* strain ASE. Transformants were selected on LB plates supplemented with kanamycin (50 ug/mL), gentamycin (50 ug/mL) and chloramphenicol (30 ug/mL). *Agrobacterium*-mediated transformation of wild type *Arabidopsis (Col and L-er)* was conducted using the floral dip method (Clough and Bent 1998). T1 seeds obtained from self-fertilized transformants were surface sterilized and plated on Gamborg's B5 medium supplemented with kanamycin for selection.

Agrobacterium tumefaciens GV3101 was transformed with *pNAS1-RNAi* vector DNA. The transformants were selected on LB medium supplemented with spectinomycin (100 ug/mL). The floral dip method was used to transform wild type *Arabidopsis (Col and L-er)* plants (Clough and Bent 1998). T1 seeds were plated on Gamborg's B5 medium supplemented with spectinomycin(100 ug/mL) and gentamycin (30 ug/mL).

Selected T1 plants were transferred to soil and T2 seeds were collected. The T2 seeds were surface sterilized and plated on Gamborg's B5 medium supplemented with spectinomycin(100 ug/mL) and gentamycin (30 ug/mL) to examine segregation ratios. Lines that exhibited a 3:1 ratio

(resistance:sensitivity) in the F2 generation were presumed to have a single copy of the transgene and T3 seeds were examined to look for homozygous lines.

Probe design. Probes were designed that would hybridize specifically to each *NAS* gene. For *NAS1*, a full length cDNA probe was used, but for *NAS2*, *NAS3* and *NAS4*, probes were designed in the 3' UTR. The specificity of all probes was confirmed by southern blotting. All of the UTR probes were amplified from genomic DNA and prepared as RNA probes using the MAXIScript and Lig'n Scribe kits (Ambion, Inc.).

RNA Isolation and RNA Gel Blot Analysis. Plants were grown as described above and total RNA was prepared (Verwoerd et al., 1989) from roots and shoots separately. RNA samples (15 mg) were modified covalently by treatment with glyoxal (McMaster and Carmichael, 1977), separated on a 1.2% agarose gel containing 10 mM NaPO₄, pH 6.5, transferred to a nylon membrane, and bound to the membrane by UV cross-linking (Stratalinker; Stratagene, La Jolla, CA). Hybridizations were performed in 50% formamide at 42°C using standard procedures (Ausubel et al., 2002). Membranes were washed twice for 15 min at room temperature in 1x SSC (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS, followed by two 15-min washes in 0.1x SSC and 0.1% SDS at 65°C.

Root Growth Assays. Plants were grown on Gamborg's B5 medium for 8 days and then transferred to square plates containing the metal supplemented media described above. Plants were placed on the plates such that their roots extended in as straight a line as possible across the surface of the agar. Plates were placed in the growth chamber in a vertical orientation so that the roots grew down along the surface of the agar. Root length was measured at days 0, 1, 2, 3, 4, 5, and 6.

Elemental Analysis. Plants were grown on B5 media and then transferred to iron sufficient or iron deficient media for three days as described above. Shoots were separated from roots and the roots were rinsed in distilled water. The tissues were dried for two days at 65°C and then analyzed by ICP-MS.

RESULTS

In *Arabidopsis*, there are four *NAS* genes, and three of these have been shown to have *NAS* activity in bacteria. We examined the expression of the four *NAS* genes in a variety of tissues to determine spatial expression patterns for these genes. *NAS1*, *NAS2*, and *NAS4* are all expressed in roots. *NAS1*, *NAS3* and *NAS4* are expressed in the non-reproductive, above ground tissues that were examined, and *NAS3* was expressed in some reproductive parts of the plant (flowers) as well (Figure 1). Since NA is known to bind metals (Benes et al. 1983; von Wiren et al. 1999), it stands to reason that fluctuations in metal levels in the growth media may induce changes in the expression of the *NAS* genes. *NAS1* expression appears to be downregulated by copper and zinc deficiency and *NAS3* expression is downregulated by iron and copper deficiency. *NAS4*, however, appears to be induced by iron deficiency and *NAS2* expression is not greatly affected by these changes, or may be mildly downregulated in response to the metal deficiencies (Figure 2a). In response to high metal levels, *NAS1* expression appears to be upregulated in shoots and roots in response to high levels of zinc and nickel. *NAS2* is slightly induced by high levels of copper, zinc and nickel, but *NAS3* expression is actually downregulated by high levels of copper and somewhat by high levels of zinc. *NAS4* is expressed at very low levels, so it is hard to detect its expression at all, much less changes in expression. However, it does appear to have increased expression in shoots when exposed to 100 mM copper (Figure 2B).

To gather more evidence for the functional role of NA, we generated transgenic lines that either

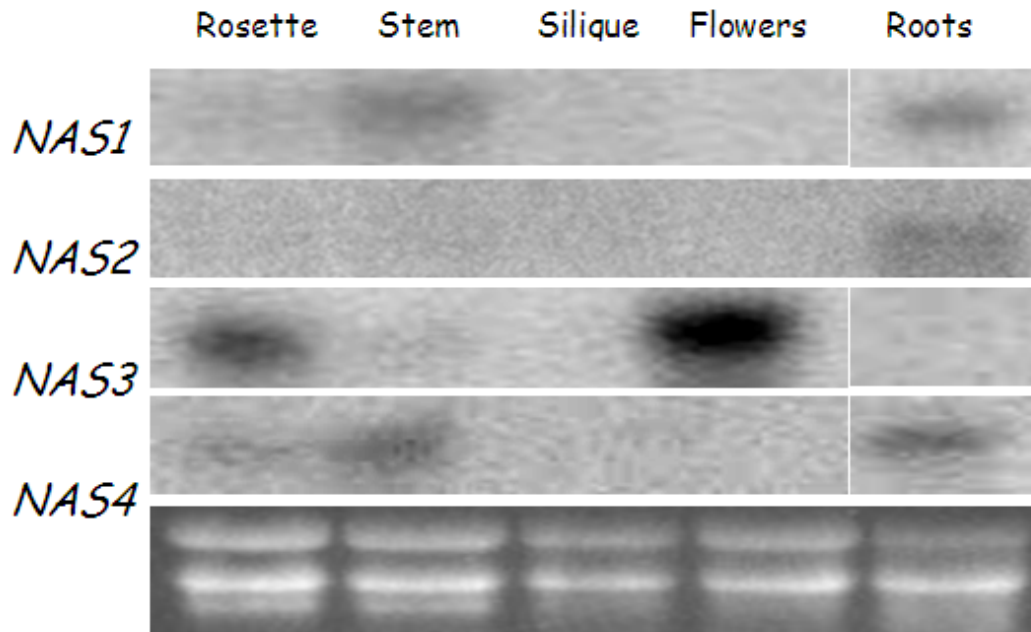


Figure 1. Spatial Expression Patterns of the four *NAS* genes in *Arabidopsis thaliana*. Tissues were collected from *Arabidopsis* plants (ecotype *Col gl-1*) and RNA was extracted. RNA gel blot analysis was performed with specific probes to determine where the genes were expressed.

overexpress *NAS1* by expressing behind the CaMV 35S promoter, or silence *NAS1* by creating an RNAi construct which produces a double stranded RNA that will induce the plants natural silencing mechanism (Figure 3A). Figure 3B shows that several 35S::*NAS1* lines and *NAS1* RNAi lines were generated that had confirmed changes in the expression of *NAS1*. An important thing to note here is that the overexpression lines and RNAi lines are in different wild type backgrounds. This means that we are able to compare the transgenic line to its respective wild type for all experiments, but unfortunately we are not able to directly compare the lines to each other.

Since NA is known to bind iron and some of the *NAS* genes appear to be regulated by changes in iron levels, the expression of other iron regulated genes was examined in the transgenic lines to determine if other aspects of iron homeostasis were altered in these plants. *FRO2* and *IRT1* are two genes involved in the Strategy I mechanism for iron uptake under iron deficient conditions. Both of these genes appear to be induced at the mRNA level in the RNAi lines (Figure 4). This suggests that a loss of *NAS1* function leads to a presumed state of iron deficiency by the plants. It is likely that the loss of *NAS* results in lower levels of NA in the plant which leads to a decreased efficiency of iron mobilization. Therefore the plant senses that it is iron deficient even though there are iron stores available.

FRO3 is a ferric chelate reductase present in the plasma membrane of leaf cells. We think that this enzyme is involved in the reduction of iron in leaf cells to aid in its transport through the plant (unpublished data). Although *FRO3* is highly induced in response to iron deficiency in wild type plants, we did not see induction of this gene in the RNAi lines (Figure 4).

FER1 is a gene encoding one of the ferritin proteins. Ferritin is a complex found in the chloroplasts of plant cells and stores iron to prevent the iron from causing damage to the cells. In the 35S::*NAS1* lines, we see slightly decreased levels of *FER1* mRNA accumulation when compared to wild type

Figure 2. Examination of changes in *NAS* gene expression in response to changes in metal levels. Wild type *Arabidopsis* plants were grown on standard media for 2 weeks and then transferred to conditions of iron, copper or zinc deficiency (A) or were transferred to conditions of 250 mM Fe, 100 mM Cu, 500 mM Zn or 500 mM Ni (B) for 3 days. Roots and shoots were collected separately, RNA was extracted and RNA gel blot analysis was performed with specific *NAS* probes.

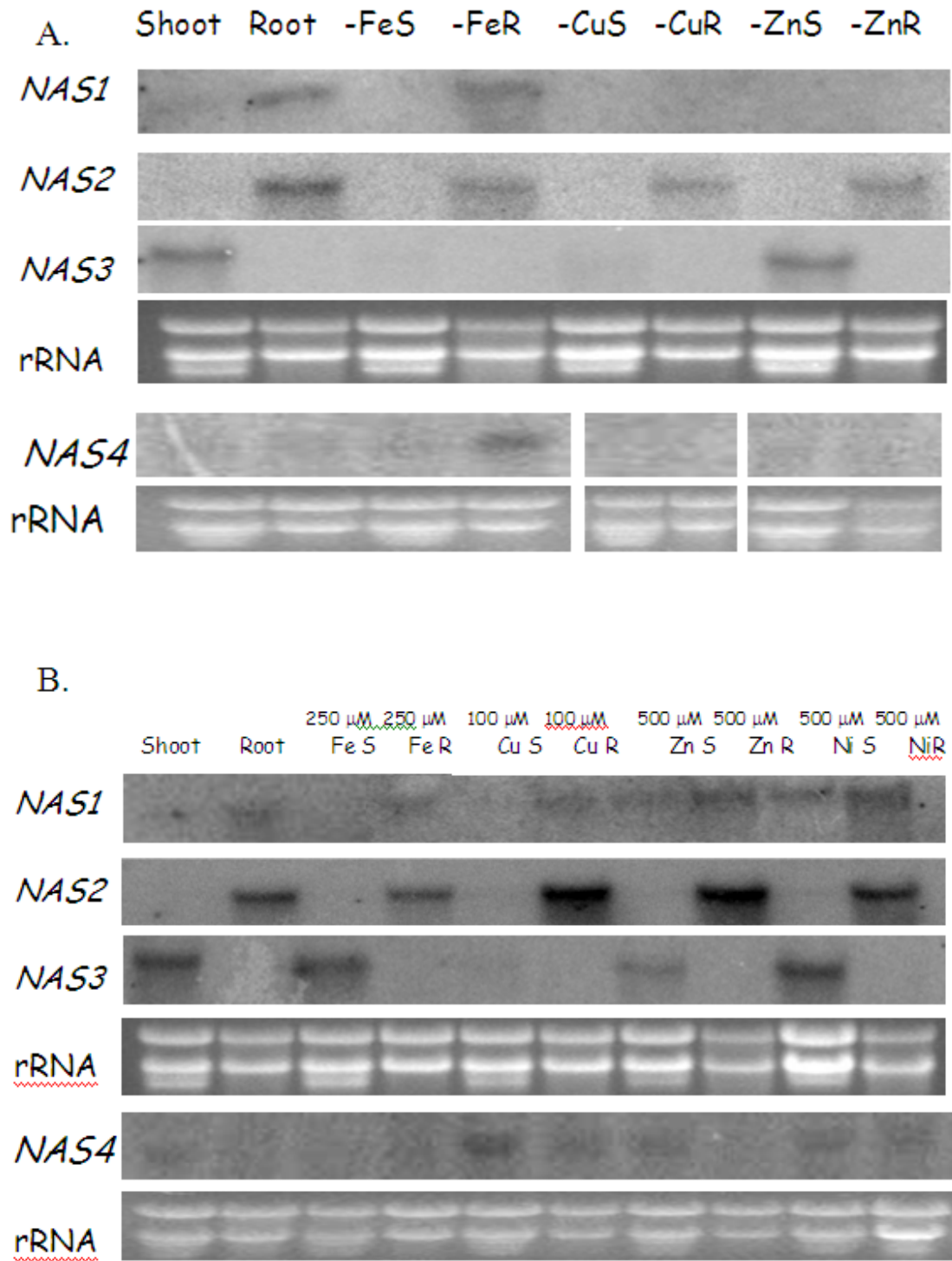


Figure 3. (A) Construction of plasmids used to overexpress or silence *NASI*. (B) RNA gel blot of seedlings from transgenic lines. Several overexpression lines and several RNAi lines were confirmed to have changes in *NASI* expression.

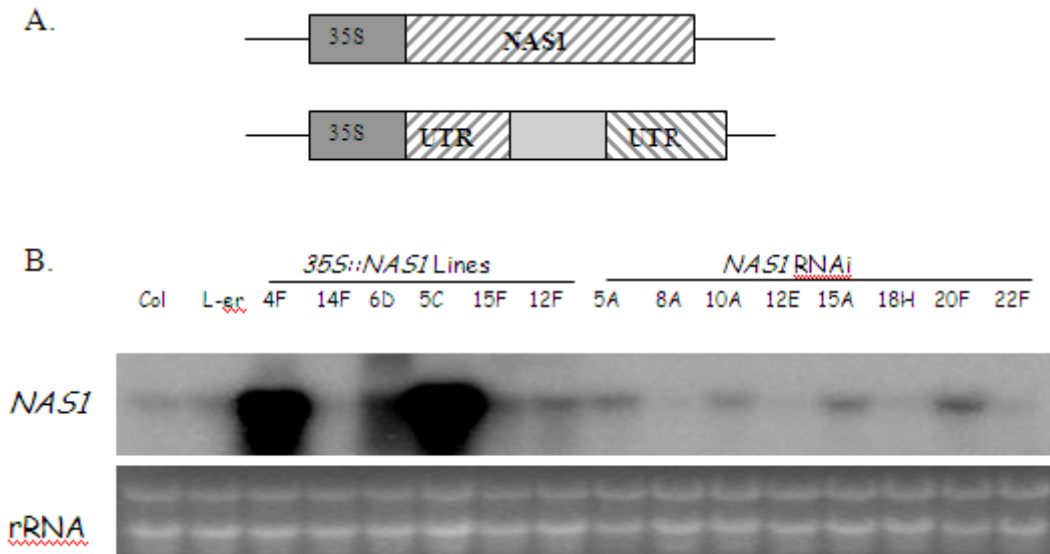
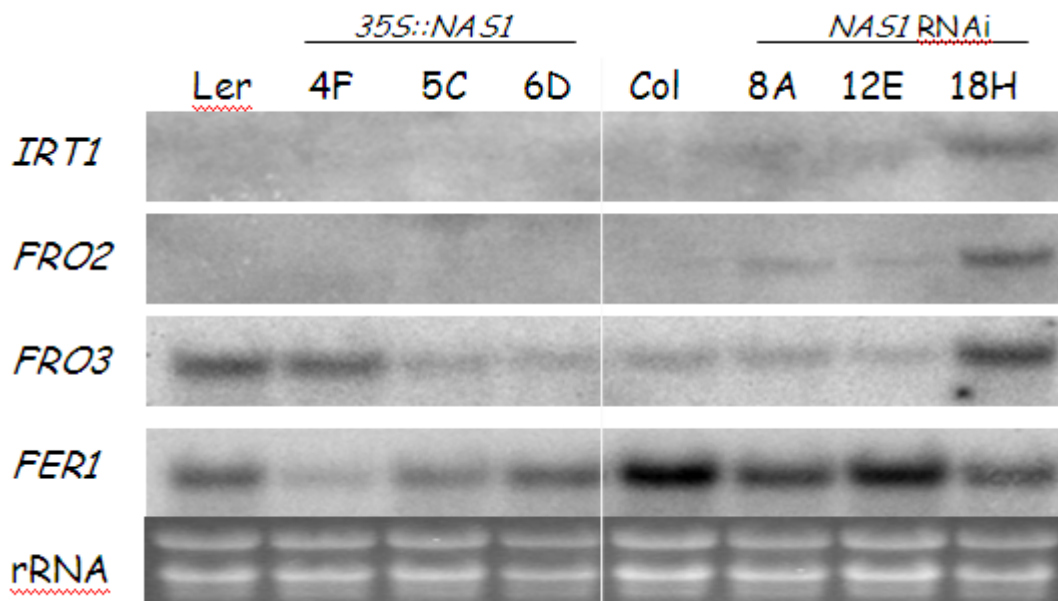


Figure 4. Changes in expression of other iron regulated genes. RNA gel blot analysis was performed on RNA extracted from seedlings of the transgenic lines using probes designed to hybridize to known iron regulated genes in *Arabidopsis*.

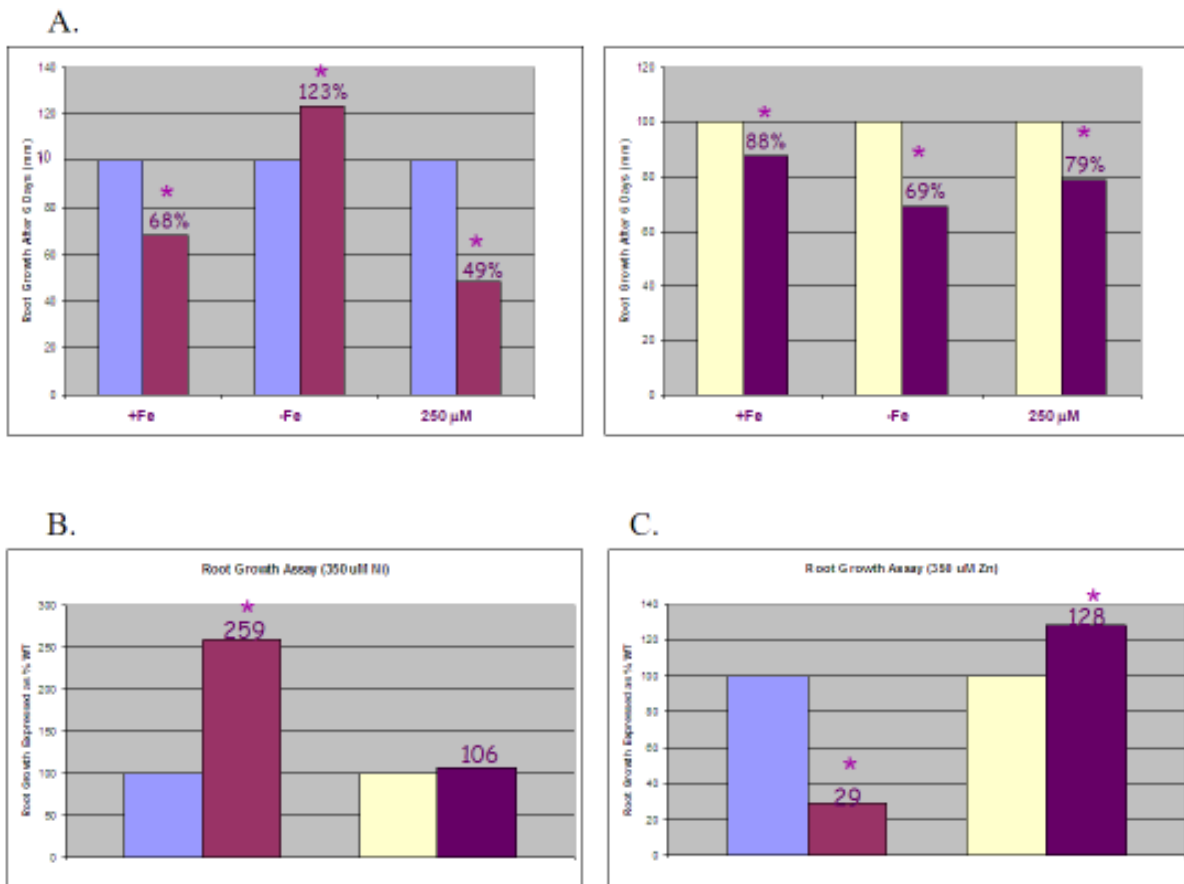


(Figure 4). This result would suggest that overexpression of *NAS1* results in presumably increased levels of NA making the plant more efficient at mobilizing iron and thereby reduces the necessity for large numbers of ferritin complexes.

To examine possible effects in the plant's physiology, root growth assays were performed in the transgenic lines. Root growth is often inhibited when plants experience stresses, including high levels of potentially toxic metals. The graphs display one representative overexpression line and one representative RNAi line. Root growth is expressed as percent wild type to clearly show the differences between standard media and other treatments. Both the overexpression lines and RNAi lines show significantly reduced root growth on standard media (+Fe). However, this sensitivity was enhanced in the *NAS1* RNAi line under both iron deficient and 250 mM iron conditions. The overexpression line also exhibited sensitivity to 250 mM iron, but displayed significant resistance to iron deficient conditions (Figure 5A). The overexpression line also displayed significant sensitivity to 350 mM Zinc and resistance to 350 mM Nickel. The knock-down line displayed significant resistance to 350 mM Zinc, but was not affected by the nickel treatment (Figure 5B and 5C).

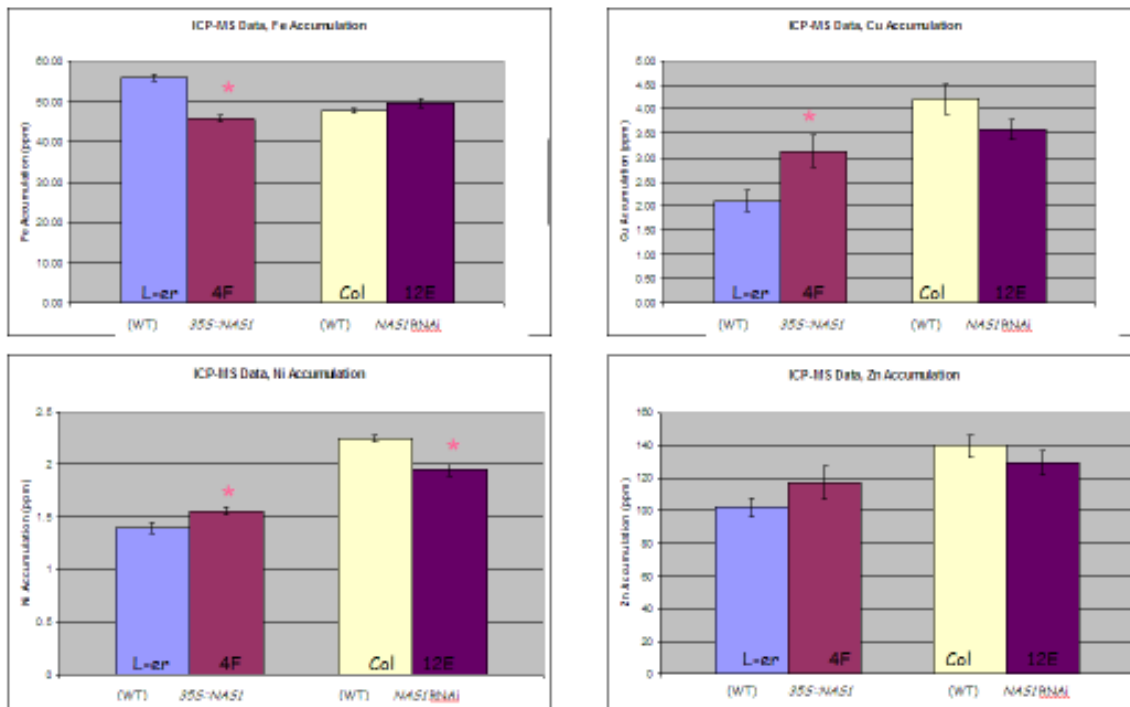
Since the *NAS* genes seem to respond to various metals in the plant, we wanted to determine if the transgenic lines actually accumulate metals to different levels. ICP-MS was used to determine

Figure 5. Root growth analysis of the transgenic lines. Plants were grown on standard media for 8 days and then transferred to media containing 250 μ M Fe, 350 μ M Zn or 350 μ M Ni. Standard media and iron deficient media were also tested for an effect on root growth. Graphs show one representative overexpression line and one representative RNAi line. Root growth is expressed as % wild type root growth.



how much of each metal was accumulated in these lines. The *NAS1* overexpression line accumulates lower than WT levels of Fe (Figure 6) yet has a significant resistance to iron deficiency as demonstrated with the root growth assays, so it seems that NA may be playing a role in binding and sensing iron levels in the plant. This would prevent the plant from initiating Fe deficiency responses

Figure 6. ICP-MS analysis of the transgenic lines was performed to determine if the transgenic lines accumulate metals to different levels compared to wild type.



when the plant is not actually iron deficient.

In addition, the overexpression line also accumulates higher levels of copper, and the RNAi lines accumulate lower than WT levels of copper, although this value is not statistically significant (Figure 6). This data suggests that NA is required for the accumulation of copper.

Although NA is not required for the accumulation of nickel, it does appear to play a role in protection from nickel. The *35S::NAS1* line accumulated significantly higher levels of nickel than wild type plants (Figure 6), yet exhibits a significant resistance to high levels of nickel (Figure 5B). This suggests that NA may play a protective role in the plant when bound to nickel, allowing the plant to accumulate more than would normally be allowed.

Although the *35S::NAS1* line was extremely sensitive to 350 mM Zn, and the *NAS1* RNAi line was significantly resistant to this condition (Figure 5C), there were no substantial changes in the accumulation of zinc in either transgenic line (Figure 6). This data suggests that overexpression or loss of the function of *NAS1* affects the plants ability to sense zinc levels. In other words, the presumably increased levels of NA in the overexpression line may allow the plants to sense higher levels of zinc than it normally would, causing it to turn on a stress response (inhibition of root elongation). However, the RNAi lines presumably produce lower levels of NA than wild type plants and may not sense as much intracellular zinc as a wild type plant, causing it to have a slower

response to the stress.

DISCUSSION

Recent studies in the *chloronerva* mutant in tomato have provided evidence that nicotianamine plays an important role in iron homeostasis in Strategy I plants (Ling et al. 1999). Specifically, NA appears to be involved in the transport of iron throughout the plant. However, it is still not clear at what stage of transport NA is involved. Also, NA has unique properties that make it an interesting candidate for other metal related roles in Strategy I plants. Because NA is able to bind metals other than iron, it likely plays a role in the homeostasis of those other metals, as well as iron. Also, when iron is bound to NA it is unable to participate in the Haber-Weiss-Fenton Sequence of reactions that lead to the production of hydroxyl radicals. Therefore, it is likely that NA plays a protective role when bound to iron. In the present study, we have attempted to gather supporting evidence for some of these roles in Strategy I plants.

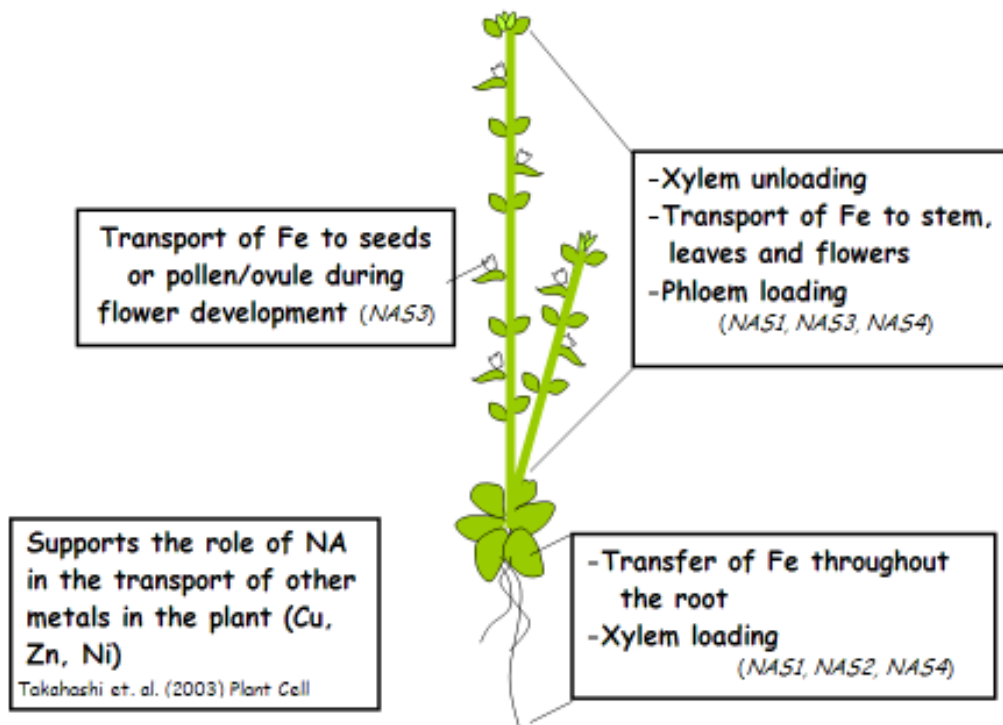
The four *NAS* genes in *Arabidopsis* have distinct spatial expression patterns, and all four genes are differentially regulated by metals at the mRNA level. This suggests that although the genes have some overlapping areas of expression, each gene individually caters to a different demand that the plant may have for nicotianamine, whether spatially or functionally.

Based on the expression patterns that we have identified, we have started to assign some very general roles for NA in Strategy I plants. First, based on the expression of *NAS1*, *NAS2* and *NAS4* in roots, it is likely that NA is involved in the lateral movement of iron in the root. It may also be involved in loading iron into the xylem where it gets transported to the aerial parts of the plant. Once in the xylem, iron is transported as Fe(III)-Citrate. However, *NAS1*, *NAS3* and *NAS4* are all expressed in the aerial parts of the plant, so it appears that nicotianamine plays a role in this part of the plant as well. NA may be involved in unloading iron from the xylem, transport of iron to the leaves, stem and flowers, or loading iron back into the vasculature (phloem) for transport to the shoot apex or back to the root. Since *NAS3* is expressed at such high levels in flowers, it is likely that NA is involved in loading iron into the seeds or possibly into the pollen and/or ovule during flower development (Figure 7). It will be necessary to perform in situ analysis for each of these genes to determine the specific cell types in each tissue that have *NAS* expression. This will allow us to make more conclusive statements about the role(s) of NA.

In addition, each of the four genes was differentially regulated by iron, copper, zinc, and/or nickel. This suggests that nicotianamine likely plays one or more of these roles for the other metals as well in *Arabidopsis*. This assertion is supported by work in tobacco in which the barley *NAAT* gene was overexpressed resulting in complete depletion of NA stores in the plant. The mutant was unable to transport iron, copper, zinc and manganese internally, and resulted in a range of severe phenotypes (Takahashi et. al. 2003).

Characterization of the transgenic lines makes it possible to gather more evidence for the proposed roles of nicotianamine in Strategy I plants. Since some typical iron regulated genes have altered mRNA expression in the transgenic lines but others don't, it appears that NA can affect iron homeostasis significantly in the plant, but may not be involved at all levels. Another possible explanation is that the changes in nicotianamine levels in the transgenic lines may not have been significant enough to have an affect on the *FRO3* pathway. Since we currently do not have a clear idea of how this mechanism is involved in iron homeostasis, it is impossible to fit the role of nicotianamine into the picture at this point. It will be necessary however, to measure the actual accumulation of NA in the transgenic lines before any of these conclusions can be validated.

Figure 7. Model outlining all of the potential roles of NA in strategy I plants, based on expression patterns and functional characterization of *NAS1*.



The root growth assays and ICP-MS analysis have provided some very useful information regarding the effect of NA levels on plant physiology and plant responses. Based on our data, we propose that NA is involved in not only transport of iron and other metals throughout the plant, but also in sensing metal levels and protection against high levels of metals by binding to them and making them less likely to cause damage. It is interesting that we see such different patterns in the resistance or sensitivity to metal stresses in the transgenic lines. All of the metals that we examined are divalent cations that are not easily distinguishable by transporters, reductases or other proteins. Therefore, we often do not see a lot of discrimination from the protein and all of these metals get transported, bound or modified. In this case, the overexpression lines appear to be sensitive to high levels of iron and zinc, which suggests that the metals are being mobilized too efficiently and normal levels of these metals is having an inhibitory effect on plant growth. However, the overexpression lines are also highly resistant to nickel, which suggests that NA plays a protective role. Accumulation of the various metals also varies in the transgenic lines compared to wild type. All of this suggests that NA acts at multiple stages of iron homeostasis in Strategy I plants, and each role appears to be very important for the overall health of the plant.

Notably, none of the RNAi experiments showed very significant differences when compared to wild type plants. Since there are four genes in this family, it is likely that the other three genes produce sufficient amounts of NA that the phenotype of the plants is not affected to extreme levels. However, mRNA accumulation of the other three *NAS* genes is not altered in the RNAi lines (data not shown). Therefore, loss of *NAS1* does not affect these genes at the transcriptional level. The activity of the enzymes may be upregulated, however, or the NA pool produced by the other *NAS*

genes may be sufficient. NA accumulation will have to be examined in all of the lines to determine which of these alternatives is more likely.

In conclusion, NA appears to be a very important compound in both Strategy I and Strategy II plants. In Strategy II plants, it serves as a precursor for the phytosiderophore iron chelators. However, in Strategy I plants, NA seems to play a variety of other roles in internal transport of iron and in maintaining proper levels of iron and other metals throughout the plant. It is likely that NA also serves these functions in Strategy II plants, in addition to serving the phytosiderophore biosynthetic pathway.

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