The Analysis of Arabidopsis Nicotianamine Synthase Mutants Reveals Functions for Nicotianamine in Seed Iron Loading and Iron Deficiency Responses[1][C][W][OA]

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Nicotianamine chelates and transports micronutrient metal ions in plants. It has been speculated that nicotianamine is involved in seed loading with micronutrients. A tomato (*Solanum lycopersicum*) mutant (*chloronerva*) and a tobacco (*Nicotiana tabacum*) transgenic line have been utilized to analyze the effects of nicotianamine loss. These mutants showed early leaf chlorosis and had sterile flowers. Arabidopsis (*Arabidopsis thaliana*) has four *NICOTIANAMINE SYNTHASE* (*NAS*) genes. We constructed two quadruple *nas* mutants: one had full loss of *NAS* function, was sterile, and showed a *chloronerva*-like phenotype (*nas4x-2*); another mutant, with intermediate phenotype (*nas4x-1*), developed chlorotic leaves, which became severe upon transition from the vegetative to the reproductive phase and upon iron (Fe) deficiency. Residual nicotianamine levels were sufficient to sustain the life cycle. Therefore, the *nas4x-1* mutant enabled us to study late nicotianamine functions. This mutant had no detectable nicotianamine in rosette leaves of the reproductive stage but low nicotianamine levels in vegetative rosette leaves and seeds. Fe accumulated in the rosette leaves, while less Fe was present in flowers and seeds. Leaves, roots, and flowers showed symptoms of Fe deficiency, whereas leaves also showed signs of sufficient Fe supply, as revealed by molecular-physiological analysis. The mutant was not able to fully mobilize Fe to sustain Fe supply of flowers and seeds in the normal way. Thus, nicotianamine is needed for correct supply of seeds with Fe. These results are fundamental for plant manipulation approaches to modify Fe homeostasis regulation through alterations of *NAS* genes.

One of the breeding goals for high-quality nutrition food crops is the production of micronutrient (iron, zinc [Fe,Zn])-enriched plants. The relevance of Fe for human nutrition is evident from the most severe human micronutrient deficiency disease, which is Fe deficiency anemia (see the 2004 report of the World Health Organization; de Benoist et al., 2008). In plants, Fe deficiency is also the most widespread micronutrient deficiency; it is seen frequently on calcareous and alkaline soils, where Fe is almost unsoluble. On the other hand, beneficial biochemical properties can render this same metal potentially toxic. Free Fe is a catalyst in the formation of hydroxyl radicals, which can unspecifically damage biological molecules. To balance these effects, Fe uptake and homeostasis are tightly controlled.

Genetic and transgenic approaches targeting specific Fe homeostasis genes are under way to yield micronutrient-enriched crops (Goto et al., 1999; Takahashi et al., 2001; Sautter et al., 2006; Uauy et al., 2006). It is obvious that a precise breeding approach would be more efficient if more genetic components were known controlling Fe acquisition, transport, and storage.

Among the key elements for Fe homeostasis are small chelators of metals, both to render the metals soluble and to detoxify them (Colangelo and Guerinot, 2006; Briat et al., 2007). For example, organic acids such as citric acid can bind free metal ions. This mechanism is primarily used for the transport of Fe in the xylem, where it is considered that the majority of Fe is bound to citrate. On the other hand, phloem Fe is bound by nicotianamine or other amino acids such as part of proteins (von Wiren et al., 1999; Kruger et al., 2002).

The plant metal chelator nicotianamine is a free nonproteinogenic amino acid (Scholz et al., 1992; Stephan, 2002; Hell and Stephan, 2003). Nicotianamine is mobile in the plant and has been detected in root and leaf cells as well as in phloem sap. It can bind metal ions like Fe, Zn, copper (Cu), and nickel (Ni; Scholz

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et al., 1992; Schmiedeberg et al., 2003). Nicotianamine is synthesized by a one-step condensation reaction of three molecules of *S*-adenosyl-Met by the enzyme NICOTIANAMINE SYNTHASE (NAS; Shojima et al., 1989; Herbik et al., 1999; Higuchi et al., 1999). In graminaceous plants like barley (*Hordeum vulgare*), rice (*Oryza sativa*), and maize (*Zea mays*), nicotianamine has a prominent role in Fe acquisition from the soil. It is the direct precursor of phytosiderophores of the mugineic acid family (Mori, 1999). Phytosiderophores are strong Fe^{3+} chelators that are extruded to the rhizosphere, where they serve to chelate and solubilize Fe^{3+} (Römheld and Marschner, 1986a, 1986b). *NAS* genes form gene families in grasses that cover a range of expression patterns (Higuchi et al., 2001; Inoue et al., 2003; Mizuno et al., 2003). *NAS* genes involved in phytosiderophore production are induced by Fe deficiency in the root. Phytosiderophore-Fe^{3+} complexes are imported into the rhizodermis by the transporter of the type YELLOW STRIPE1 (YS1) from maize that is up-regulated by Fe deficiency in roots (Curie et al., 2001). *YELLOW STRIPE-LIKE* (*YSL*) genes are encoded by multigene families in grasses as well as in dicot plants (Curie et al., 2001; DiDonato et al., 2004). This indicates that *NAS* and *YSL* genes have diversified roles throughout plant development in addition to Fe uptake into the root.

In solanaceous plants, nicotianamine was shown to act in Fe homeostasis, which has been mostly found through analysis of the *nas* tomato (*Solanum lycopersicum*) mutant *chloronerva* and of a transgenic tobacco (*Nicotiana tabacum*) line with ectopic NICOTIANAMINE AMINOTRANSFERASE (NAAT) activity that consumed the available nicotianamine (Scholz et al., 1992; Ling et al., 1999; Takahashi et al., 2003). Tomato and tobacco showed leaf chlorosis as a result of reduced nicotianamine and sterility. Solanaceous plants and other dicots rely on a Fe reduction-based mechanism to solubilize and acquire Fe^{2+} (Römheld and Marschner, 1986a). Fe is reduced by a membrane-bound ferric chelate reductase (FRO2 in Arabidopsis [*Arabidopsis thaliana*]; Robinson et al., 1999). Fe^{2+} is taken up into the root epidermis by a membrane-bound divalent metal transporter (IRON-REGULATED TRANSPORTER1 [IRT1] in Arabidopsis; Eide et al., 1996; Vert et al., 2002). *FRO2* and *IRT1*-type genes are induced by an Fe deficiency-dependent basic helix-loop-helix transcription factor protein (FER in tomato, FIT in Arabidopsis; Ling et al., 2002; Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005). In tomato, it was found that *chloronerva* plants up-regulate *FER*, *IRT1*, and *FRO1* even upon sufficient Fe supply (Bereczky et al., 2003; Li et al., 2004; Brumbarova and Bauer, 2005). Since *chloronerva* plants can accumulate Fe in leaves but not Cu (Pich and Scholz, 1996), nicotianamine is not directly needed for Fe uptake and translocation into leaves in solanaceous plants (Scholz et al., 1992). Most likely, nicotianamine plays a role in intracellular and intercellular distribution of Fe, whereas it may be involved in Cu translocation to leaves. However, contrasting effects were found in tobacco *NAAT* plants, which had reduced Fe levels in leaves compared with the wild type, as well as reduced levels of Cu and Zn (Takahashi et al., 2003). Hence, there were clear distinctions between tomato and tobacco mutant nicotianamine lines. Another disadvantage of the two solanaceous plant studies was that only mutants with severe defects were available. Thus, it remains unknown which phenotypes were pleiotropic in nature and which phenotypes were directly caused by nicotianamine loss. For example, *chloronerva* and *NAAT* tobacco both had abnormal flowers.

A function of nicotianamine in seed metal loading has been inferred from studies of the presumptive nicotianamine-Fe transporter family YSL (Curie et al., 2001; Schaaf et al., 2004). Loss of YSL1 resulted in decreased nicotianamine and Fe levels in *ysl1* mutant seeds (Le Jean et al., 2005). *YSL* genes have clearly redundant functions. *ysl1ysl3* double mutants are chlorotic and have lower Fe levels in leaves than the wild type (Waters et al., 2006). Their seeds also contain less Fe, and fertility is reduced (Waters et al., 2006). *ysl3* and *ysl2* single mutants have no apparent phenotype (DiDonato et al., 2004; Waters et al., 2006). Expression patterns of the three *YSL* genes suggest that the encoded transporters are involved in exiting nicotianamine-Fe from the vascular system to leaves upon sufficient Fe supply, whereas reduced exit upon Fe deficiency may serve to translocate Fe toward the growing apex (DiDonato et al., 2004; Le Jean et al., 2005; Schaaf et al., 2005; Waters et al., 2006).

Indeed, various families of metal transporters and regulators were found and are to date best described in Arabidopsis. The Arabidopsis genome has four *NAS* genes (Suzuki et al., 1999; Bauer et al., 2004). Thanks to the reverse genetic resources, it was possible to identify mutant T-DNA alleles of all four genes. To be able to study nicotianamine function in a broad genetic network of known players in metal homeostasis, we decided to develop Arabidopsis as a model system for investigating nicotianamine function. Here, we present the construction and analysis of quadruple *nas* mutants, one of which had an intermediate phenotype, named *nas4x-1*. In contrast to *chloronerva*, the tobacco *NAAT* line, and *nas4x-2*, which is the second *nas* mutant we identified that had full loss of NAS function, *nas4x-1* had strongly reduced but not fully eliminated nicotianamine content. Due to its survival and maintained seed production, we could analyze nicotianamine involvement in Fe allocation to reproductive organs and seeds.

RESULTS

Generation and Initial Characterization of Multiple *nas* Mutants

The four members of the *NAS* gene family are distributed on both arms of chromosomes I and V. All *NAS* genes have a single exon of 963 bp (*NAS1*,

NAS2, and *NAS3*) and 975 bp (*NAS4*). With the aim of ultimately studying *nas* mutants, we identified and confirmed *nas* T-DNA insertion mutants for the four *NAS* genes (Fig. 1A). For the genes *NAS1*, *NAS3*, and *NAS4*, we could confirm that the positions of T-DNA insertions were in the exon-coding sequences (alleles *nas1-1*, *nas3-1*, and *nas4-1*, respectively). For the *NAS2* gene, we could confirm the mutant *nas2-1* allele harboring a T-DNA insertion 70 bp upstream of the start codon in the 5′ untranslated region of *NAS2* and the mutant *nas2-2* allele with an insertion 144 bp after the start codon in the exon.

Since single T-DNA insertion lines did not show any visible phenotype on soil (Bauer et al., 2004), we crossed together different *nas* mutants. First, we generated homozygous double mutants by crossing and selfing T-DNA insertion mutants of genes located on the same chromosome, namely *nas1-1nas2-1* and *nas3-1nas4-1*. Homozygous double mutants were confirmed by genotyping. We found that the crossing-over events took place with a recombination rate of approximately 5% on the two chromosomes. Homozygous *nas1-1nas2-1* double mutants were then crossed with *nas3-1nas4-1* to generate heterozygous quadruple mutants. Quadruple heterozygous plants were selfed. We used genomic PCR to identify and select the quadruple homozygous mutant as well as double and triple mutants. A quadruple homozygous mutant that was fertile was identified as *nas1-1nas2-1nas3-1nas4-1* and termed the *nas4x-1* mutant. We observed that the *nas4x-1* mutant had minor leaf chlorosis during vegetative growth that intensified during the transition to reproductive growth on soil (Fig. 1B). Nonchlorotic leaves from the vegetative stage of the *nas4x-1* mutant had darker green veins than wild-type leaves when observed at transmitted light conditions (Fig. 1C). The *nas4x-1* mutant was crossed with a homozygous *nas2-2* mutant. The resulting multiple heterozygous plant was selfed and its progeny genotyped. We identified a homozygous quadruple *nas1-1nas2-2nas3-1nas4-1* mutant that we termed *nas4x-2*. *nas4x-2* plants had strong leaf chlorosis already upon vegetative growth and were sterile (Fig. 1D). Therefore, we suspected that *nas4x-2* was a strong loss-of-function mutant similar to *chloronerva*, while *nas4x-1* had an intermediate *nas* phenotypic expression.

As a first experiment, we studied whether any full-length *NAS* gene products were made in the quadruple homozygous mutants (data not shown). We found that in *nas4x-1* and *nas4x-2* mutant plants, full-length coding transcripts were not produced for *nas1-1*, *nas2-2*, *nas3-1*, and *nas4-1*. However, in the case of the *nas2-1* allele present in the *nas4x-1* mutant, it was possible to amplify a DNA fragment that contained the full-length coding *NAS2* region after 35 PCR cycles, indicating that despite the insertion in the 5′ untranslated region a PCR product was made. Very interestingly, *nas2-1*-derived transcripts were found expressed not only in roots, as was the case in the wild type, but also in leaves (data not shown; see Fig. 3A below). This result indicated that the *NAS2* gene was aberrantly expressed from the *nas2-1* allele.

Next, we analyzed nicotianamine contents. We found that *nas4x-1* mutant rosette leaves harvested in the vegetative phase had 10% nicotianamine levels compared with the wild type (11-h light period; Fig. 2A). We were not able to detect any nicotianamine in *nas4x-1* rosette leaves of the reproductive stage when plants

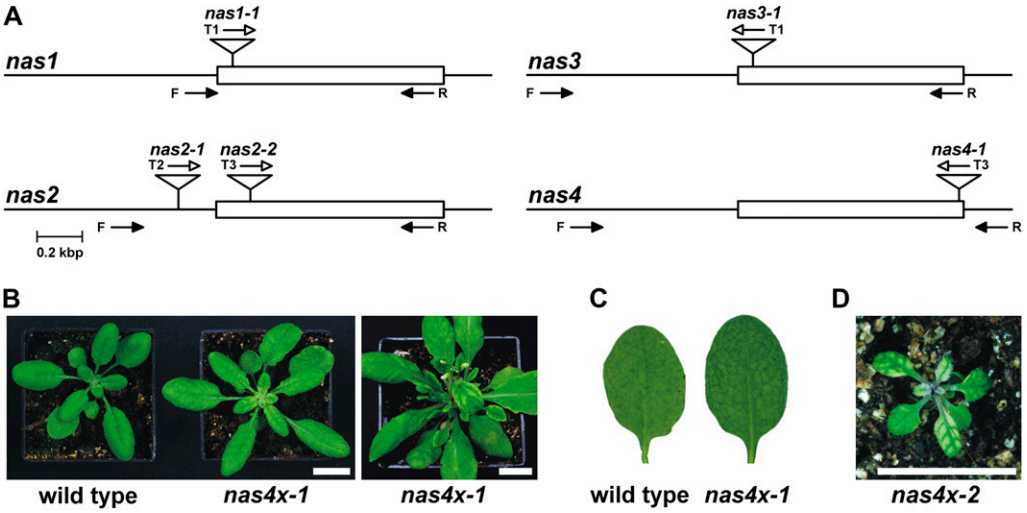

Figure 1. Genetic composition and phenotype of *nas4x-1* mutant plants. A, Location of T-DNA insertions in *nas* alleles: *nas1-1* (GABI_223A09), *nas2-1* (SAIL_156C08), *nas2-2* (SALK_066962), *nas3-1* (GABI_010A10), and *nas4-1* (SALK_135507). Arrows indicate the positions of primers used for genotyping by PCR. B, Morphological appearance of plants on soil; from left to right, Col-0 (wild type), *nas4x-1* (vegetative stage), *nas4x-1* (reproductive stage). C, Transmission light microscopic images of wild-type and *nas4x-1* rosette leaves. Note that *nas4x-1* rosette leaf veins are darker green than the intercostal leaf areas. D, Morphological appearance of *nas4x-2* (vegetative stage).

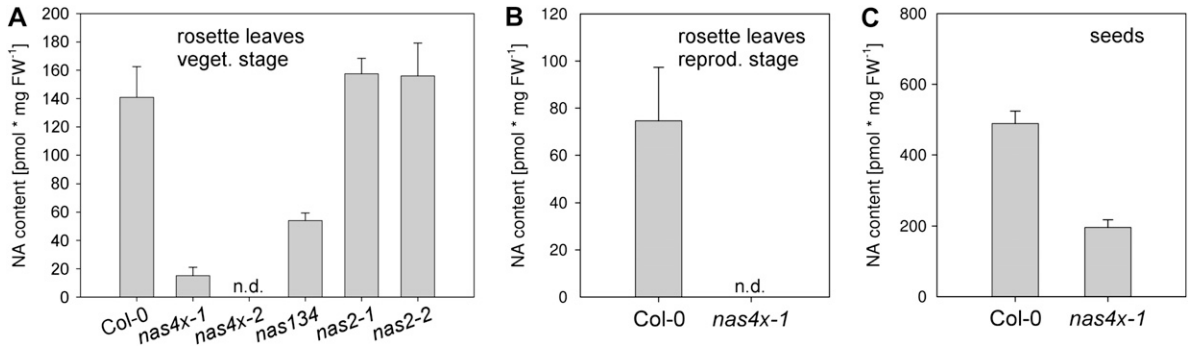

Figure 2. Nicotianamine (NA) contents. A, Rosette leaves harvested during the vegetative phase. Plants were grown in a hydroponic system with quarter-strength Hoagland medium and 50 μM Fe under an 11-h light period. Six-week-old plants were used for analysis (Col-0/*nas4x-1*, $n = 8$; other mutants, $n = 4$). FW, Fresh weight; n.d., nondetectable. B, Rosette leaves harvested during the reproductive phase. Plants were grown as in A but under a 16-h light period. Six-week-old plants were used for analysis ($n = 8$). C, Seeds harvested from plants grown in a hydroponic system as described in B ($n = 3$).

were grown with a 16-h light period (Fig. 2B). In *nas4x-1* seeds, nicotianamine levels were about 40% of those in the wild type (Fig. 2C).

Since vegetative growth resulted in the highest ratio of nicotianamine levels in *nas4x-1* mutant versus wild-type plants, we analyzed *nas4x-2* nicotianamine contents at the vegetative stage. We found that in the strong *nas4x-2* mutant, nicotianamine was not detectable in rosette leaves, demonstrating full loss of *NAS* function (Fig. 2A). This finding explained the strong *nas4x-2* phenotype.

Arabidopsis has four functional *NAS* genes that are differentially expressed (Bauer et al., 2004). Therefore, one might assume that *NAS* genes may have acquired specific functions during their evolution. The genetic data, however, indicate that *NAS* genes are functionally largely redundant. Therefore, we assessed the contribution of individual *nas* alleles for nicotianamine production by analyzing nicotianamine contents in the four single mutants with a single *nas* gene knockout and in triple mutants containing only a single viable *NAS* gene. We found that all *nas* single mutants had wild-type nicotianamine levels (Fig. 2A; Supplemental Fig. S1A). All triple mutants analyzed had about 30% to 50% of the nicotianamine content compared with wild-type plants (Fig. 2A; Supplemental Fig. S1B). No single *NAS* genes, therefore, may play any predominant role for overall nicotianamine synthesis. We also utilized these studies to refine the function of the *nas2-1* allele in the *nas4x-1* mutant. We found that *nas1-1nas3-1nas4-1* had 35% nicotianamine compared with the wild type. On the other hand, the *nas2-1* and *nas2-2* mutants had the same amount of nicotianamine as the wild type (Fig. 2A). Thus, *nas2-1* does not lead to increased nicotianamine production. Also, *NAS2* loss of function could be compensated by other functional *NAS* genes so that overall nicotianamine content remained constant.

To test whether the redundancy could be due to compensatory effects of *NAS* gene transcription levels, we tested *NAS* gene expression levels in the single or triple mutant background (Supplemental Fig. S2). We observed that the presence of other mutant *nas* alleles did not affect gene expression of *NAS2* and *NAS3*. *NAS1* expression levels did not seem to be influenced by other *nas* mutations; however, due to high variability of *NAS1* expression in these same plant samples, no clear conclusions were possible (see comment below on variable *NAS1* expression in response to Fe deficiency). The only case where *NAS* gene expression may have been influenced was that of *NAS4* wild-type allelic expression in the background of a *nas2-1* allele. These control experiments indicate that redundancy of the four *NAS* genes was not generally due to compensation of defective *NAS* genes by increased expression of intact *NAS* genes.

The reduced nicotianamine content of *nas4x-1* during the reproductive phase could be due to removal of nicotianamine through remobilization and transport and to reduced production. To test this, we grew plants in a hydroponic system for 6 weeks under either short-day conditions (8-h light period, harvest at the vegetative stage) or long-day conditions (16-h light period, harvest at the reproductive stage) and analyzed *NAS* gene expression (Supplemental Fig. S3). We found that *NAS1* and *NAS2* were expressed at 8- to 10-fold higher levels in roots at the vegetative stage than in the reproductive stage. *NAS3*, on the other hand, was expressed at about 4-fold higher levels in leaves at the reproductive stage than at the vegetative stage. *NAS4* was expressed at a similar level in the two stages. These results are in agreement with the microarray data available from public databases (data not shown). In the *nas4x-1* plants, *NAS2* was expressed 2-fold higher upon the vegetative stage than the reproductive stage, whereas all other *NAS* genes were down-regulated due to the mutations (Supplemental Fig. S3). Thus, *NAS* genes were differentially expressed with respect to the growth stage. At every stage, at least two *NAS* genes were expressed in the wild type. *nas4x-1* had *NAS* expression in roots at a higher level than in leaves, whereas in roots, it was

lower upon the reproductive phase than the vegetative stage. Therefore, reduced nicotianamine production along with increased transport away from leaves may explain the leaf chlorosis phenotype upon reproduction.

This prompted us to speculate that nicotianamine plays an important role in seed metal homeostasis at this growth phase. With the intermediate fertile *nas* mutant in hand, we could investigate this point further. To better understand the *nas4x-1* plants, we analyzed it in more detail at the molecular-physiological level.

Effect of Fe, Zn, and Cu Deficiency on *nas4x-1* Mutants

Nicotianamine chelates Fe, Cu, and Zn. Therefore, we tested first whether deficiency of these metals may play a role in the expression of the mutant phenotype.

We grew wild-type and *nas4x-1* plants in a hydroponic system and then exposed 5-week-old plants for 5 d to control medium (10 μM Fe) or Fe deficiency (0 Fe). We observed that *nas4x-1* mutants had developed leaf chlorosis after the first 5 weeks. The leaf chlorosis became severe when plants were transferred to 0 Fe (Supplemental Fig. S4).

When we tested 0 Cu and 0 Zn supply, we did not observe any strong leaf chlorosis in the mutant after 7 d of exposure to deficiency. Cu and Zn contents in leaves of 0 Cu-grown or 0 Zn-grown mutant and wild-type plants, however, were reduced, demonstrating that the plants had indeed been Cu and Zn deficient (Supplemental Fig. S5).

The results show that *nas4x-1* mutants were sensitive to Fe deficiency but not to deficiency of Zn and Cu. Hence, in *nas4x-1* plants, nicotianamine is present in insufficient amounts to properly distribute the low Fe, whereas no problem exists for distribution of low Cu and Zn.

Gene Expression of *nas4x-1* Plants in Response to Fe Deficiency

To analyze the susceptibility of *nas4x-1* plants to Fe deficiency further, we harvested roots and leaves from hydroponically grown *nas4x-1* and wild-type plants and studied gene expression in response to Fe deficiency by quantitative reverse transcription-PCR (Fig. 3).

As a control, *NAS* gene expression is first shown. *NAS1*, *NAS2*, and *NAS4* were expressed in roots, whereas *NAS3* was not expressed in roots (Fig. 3A). *NAS2* and *NAS4* were induced by Fe deficiency in roots to about three times. *NAS1* was not found to be generally induced by Fe deficiency in the hydroponic growth system, whereas on agar it was found induced (compare with Fig. 4A; note that in the various *nas* mutant backgrounds, *NAS1* also showed the most variable gene expression levels). In leaves, we found expression of *NAS1*, *NAS3*, and *NAS4*. *NAS4* was induced by Fe deficiency in leaves, whereas *NAS3* was repressed under Fe deficiency. *NAS1* was not Fe regulated in leaves. In the *nas4x-1* plants, *NAS1*, *NAS3*, and *NAS4* were down-regulated due to the respective *nas* mutations, whereas *NAS2* was found to be expressed in roots and leaves due to the *nas2-1* allele (Fig. 3A). Hence, in roots, *NAS* genes are regulated by Fe deficiency, suggesting a functional involvement of these genes in Fe deficiency responses. It was confirmed that *NAS* genes are deregulated in *nas4x-1* due to T-DNA insertions.

Next, we tested whether the susceptibility of the mutant to Fe deficiency was due to altered expression of Fe uptake genes (Fig. 3B). We investigated expression of the transcription factor genes *FIT*, encoding an essential regulator for Fe acquisition (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005), and *BHLH100*, which is induced by Fe deficiency in roots and leaves (Wang et al., 2007). Furthermore, we studied gene expression of the Fe acquisition genes *FRO2* (Fe reductase) and *IRT1* (Fe transporter), which are up-regulated by Fe deficiency dependent on FIT (Eide et al., 1996; Robinson et al., 1999; Vert et al., 2002). We found that *FIT*, *IRT1*, *FRO2*, and *BHLH100* were induced by Fe deficiency in roots of wild-type plants (Fig. 3B). In wild-type leaves, *BHLH100* was also induced by Fe deficiency. Therefore, the marker genes were up-regulated by Fe deficiency in the hydroponic system, as shown previously for the agar plate system (Jakoby et al., 2004; Wang et al., 2007). The levels of induction of *IRT1* and *FRO2* were lower in hydroponically grown plants than in agar plate-grown plants, due to the different growth conditions and ages of the plants. Interestingly, we found that in the *nas4x-1* mutant plants, Fe deficiency response genes were expressed at higher levels than in the wild-type plants upon sufficient Fe supply in roots and in leaves (Fig. 3B). Upon 0 Fe, the Fe deficiency response genes were expressed at lower or equal levels in *nas4x-1* plants than in wild-type plants. Hence, *nas4x-1* plants experienced Fe deficiency upon sufficient Fe supply, but the mutant phenotype was not explained by the inability to induce Fe deficiency response genes.

We then studied whether altered distribution of Fe might cause the phenotype. Therefore, we studied gene expression of *YSL* genes *YSL1*, *YSL2*, and *YSL3* encoding potential transporters for nicotianamine-metal complexes (DiDonato et al., 2004; Le Jean et al., 2005; Schaaf et al., 2005; Waters et al., 2006). We confirmed that in the wild type, all three *YSL* genes were down-regulated by Fe deficiency in roots and leaves compared with +Fe plants (Fig. 3C). In *nas4x-1* mutant roots grown at +Fe, the three genes were expressed at comparable levels as in wild-type –Fe. The same was found upon –Fe. In leaves of *nas4x-1* mutants, the three *YSL* genes were up-regulated or equally expressed upon sufficient Fe supply, whereas they were rather down-regulated upon Fe deficiency. Hence, *YSL* gene expression suggested that upon Fe sufficiency, *nas4x-1* mutant roots behaved like Fe-

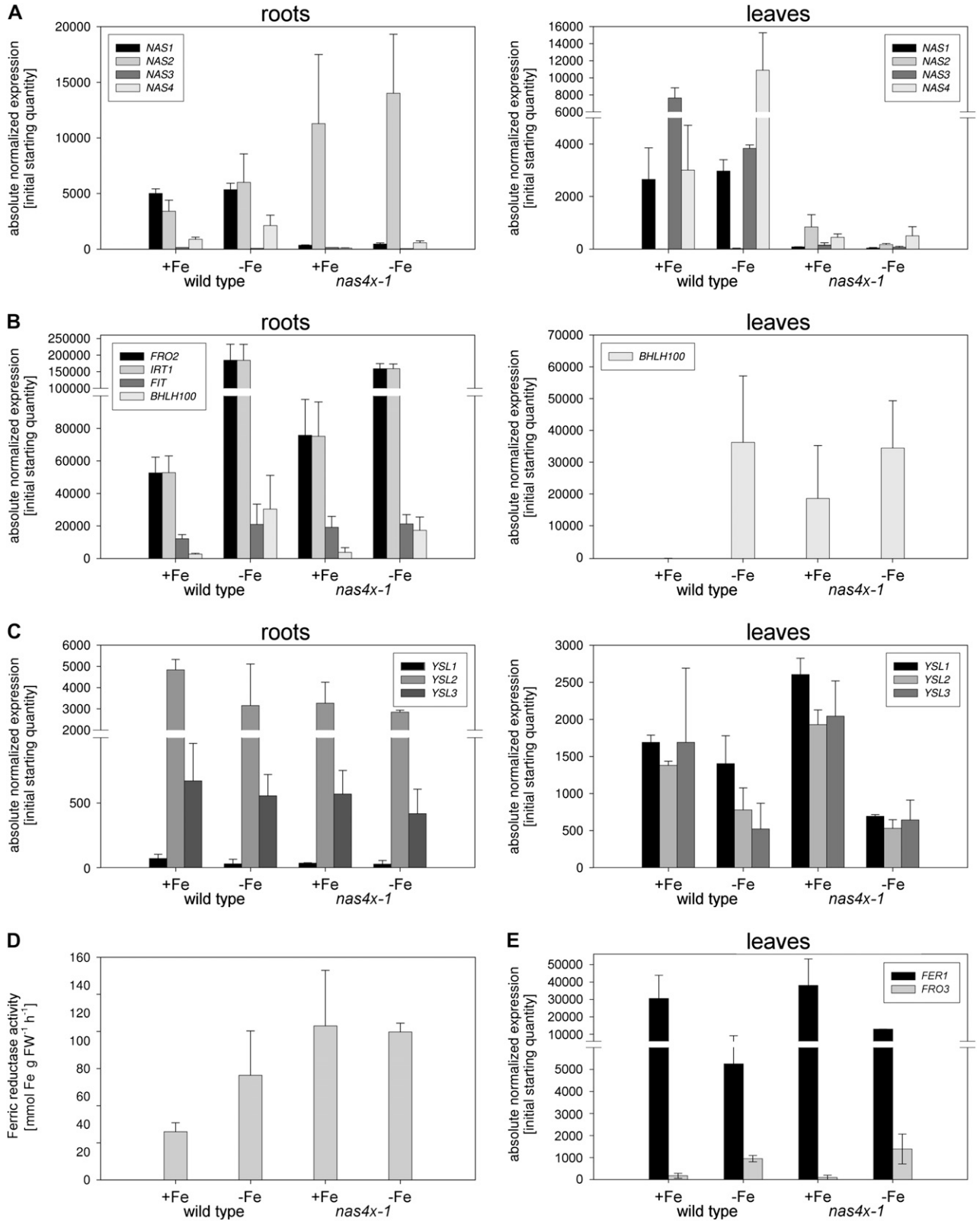

Figure 3. Molecular-physiological analysis of *nas4x-1* mutant and wild-type plants grown in a hydroponic system and exposed for 5 d to 0 μM Fe (−Fe) and control 50 μM Fe (+Fe). A to C and E, Quantitative reverse transcription-PCR analysis of root and leaf samples ($n = 2$). A, *NAS* gene expression. B, Fe deficiency response gene expression. C, *YSL* transporter gene expression in leaves and roots. E, *FER1* and *FRO3* gene expression. D, Ferric chelate reductase activity in roots ($n = 3$). FW, Fresh weight. Student's *t* test showed that significant differences ($P < 0.05$) were found for the comparisons wild-type +Fe versus −Fe, wild-type +Fe versus *nas4x-1* + Fe, and wild-type +Fe versus *nas4x-1* −Fe.

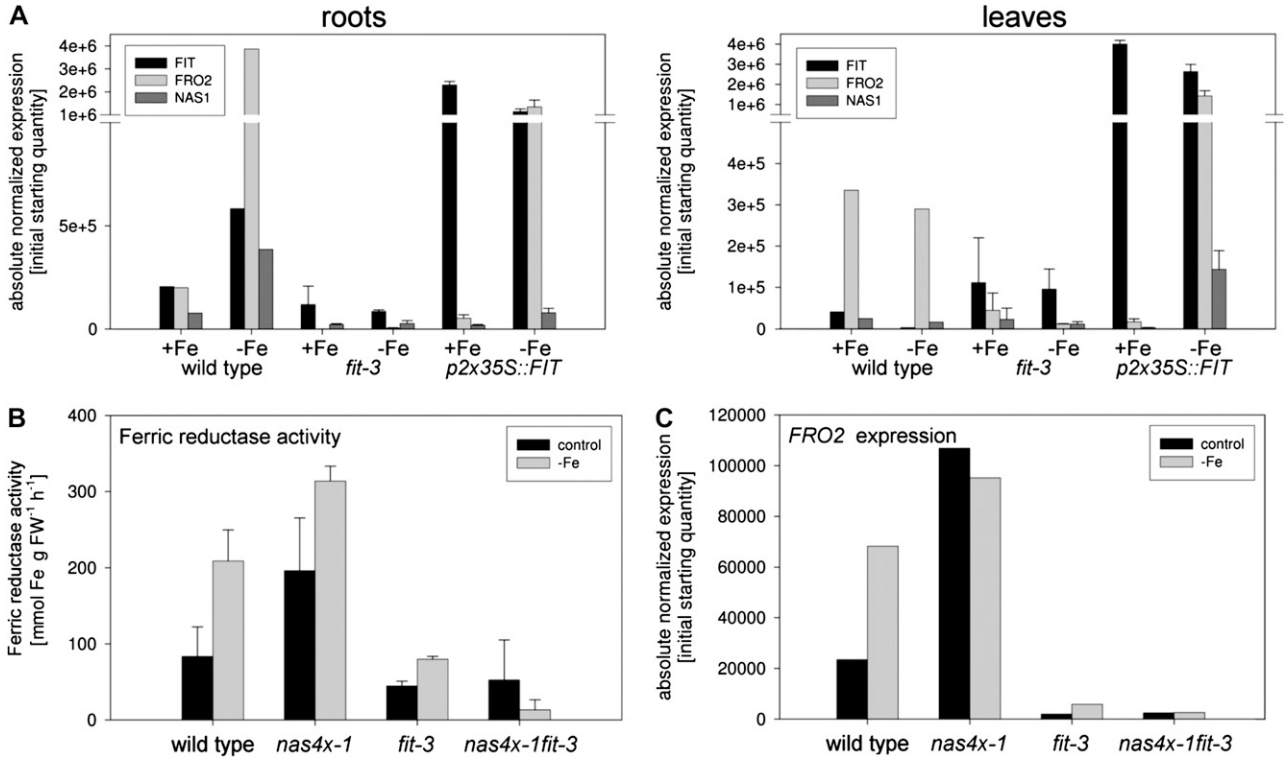

Figure 4. Analysis of *NAS* and *FIT* gene interactions. A, Quantitative reverse transcription-PCR analysis of *NAS1*, *FRO2*, and *FIT* gene expression in roots and leaves of *fit-3* mutant and *p2x35S*::*FIT* overexpression plants. B, Ferric chelate reductase activity in roots of *nas4x-1fit-3* plants. FW, Fresh weight. C, Quantitative reverse transcription-PCR analysis of *FRO2* gene expression in roots of *nas4x-1fit-3* mutant plants.

deficient roots, whereas leaves of *nas4x-1* plants had characteristics of Fe-sufficient leaves despite the weak leaf chlorosis.

The previous results were contradictory, as *nas4x-1* leaf cells were on the one hand Fe sufficient (as suggested by *YSL* up-regulation) and on the other hand Fe deficient (as revealed by *BHLH100* up-regulation). To further investigate this, we analyzed expression of the two leaf marker genes for either Fe sufficiency or Fe deficiency. One marker gene was the ferritin gene *FER1*, which encodes a Fe storage protein in leaf chloroplasts expressed upon sufficient Fe (Petit et al., 2001). The other was the Fe deficiency-inducible ferric reductase gene *FRO3* (Mukherjee et al., 2006). As expected, *FER1* was up-regulated in Fe-sufficient wild-type plants (Fig. 3E). *FRO3*, on the other hand, was up-regulated in wild-type leaves upon –Fe (Fig. 3E). However, in *nas4x-1* mutant plants, *FER1* and *FRO3* were surprisingly regulated as in the wild type upon both +Fe and –Fe, demonstrating that the leaf Fe regulatory system perceived Fe sufficiency in *nas4x-1* leaves upon +Fe (Fig. 3E). Up-regulation of *BHLH100* and up-regulation of Fe deficiency response genes in the root, therefore, must be linked with another signaling system that is defective in *nas4x-1*.

In order to assess a physiological marker for Fe deficiency, we determined Fe reductase activity of *nas4x-1* plants. We found that, as expected, root Fe reductase activity was induced in the wild type upon transfer to Fe deficiency in the hydroponic system. In *nas4x-1* roots, Fe reductase activity was increased compared with the wild type upon sufficient Fe supply (Fig. 3D).

Taken together, we conclude from these molecular and physiological results that *nas4x-1* mutants suffer from Fe deficiency when sufficient Fe is supplied. Roots showed symptoms of Fe deficiency (up-regulation of *FIT*, *IRT1*, *FRO2*, and *BHLH100*) upon sufficient Fe. Leaves show at the same time symptoms for Fe deficiency (*BHLH100* induction) and Fe sufficiency (*YSL* and *FER1* up-regulation and *FRO3* down-regulation), which can be explained by cellular or subcellular mislocalization of Fe. Obviously, low nicotianamine resulted in an altered regulation of Fe deficiency responses.

Genetic Interaction of *NAS* and *FIT* Genes

The gene expression analysis supports a role of nicotianamine in the regulation of Fe deficiency responses. We analyzed this further by studying the phenotypes of *nas4x-1* mutations in the background of a *fit-3* mutant. *nas4x-1fit-3* multiple mutants were not able to reduce Fe in the ferric reductase assay, as was

also the case for *fit-3* mutants (Fig. 4B), nor could they induce *FRO2* gene expression (Fig. 4C). *nas4x-1fit-3* multiple mutants showed a similar yellow leaf chlorosis phenotype as *fit-3* mutants (data not shown).

Since *NAS* genes were regulated by Fe supply, we investigated whether *NAS* gene expression in roots might require the Fe deficiency response regulator FIT. *fit* loss-of-function mutants are chlorotic and show down-regulation of *IRT1* and *FRO2*. We grew *fit-3* mutants and *p2x35S*::*FIT* overexpression plants for 2 weeks on sufficient Fe supply, transferred them for 3 d to Fe deficiency as described previously (Jakoby et al., 2004), and analyzed *NAS* gene expression. We found that among the four *NAS* genes tested, only *NAS1* was down-regulated in roots of *fit-3* mutant plants compared with wild-type plants. However, it was up-regulated in leaves of *FIT* overexpression plants exposed to Fe deficiency (Fig. 4A). From previous experiments, we know that in *FIT* overexpression plants, *FRO2* and *IRT1* are ectopically expressed (Jakoby et al., 2004), Hence, *NAS1* is partially under the control of the FIT transcription factor as well (Colangelo and Guerinot, 2004). The results also show that *NAS1* can be induced by –Fe in roots upon agar growth (Fig. 4A), whereas in the hydroponic system, this was not the case (Fig. 3A).

Taken together, these results show that *fit-3* is epistatic to *nas4x-1* and that *FIT* controls the expression of *NAS1*. Hence, *NAS* and *FIT* genes act in the same pathway of regulating Fe mobilization.

Gene Expression during Reproduction

Since *nas4x-1* leaf chlorosis phenotypes became severe upon transition to flowering, we reasoned that most likely increased nicotianamine levels were needed at this stage in the growing inflorescence.

We investigated whether in flowers the *nas4x-1* mutation had an influence on the expression of Fe-regulated genes (Fig. 5A). *IRT1* was found previously to be expressed in flowers, whereas loss of *irt1* function had no effect on reproduction (Vert et al., 2002). Interestingly, we found that *IRT1* and *BHLH100* were strongly up-regulated in *nas4x-1* flowers and siliques in contrast to the wild type. As expected, *FIT* and *FRO2* were not expressed significantly in these organs in either genetic background. Hence, this could be a hint that *IRT1* plays a role in Fe transport in flowers.

We studied the expression of *YSL* transporter genes (Fig. 5B). Whereas *YSL3* was expressed at similar levels between the mutant and the wild type, *YSL1* was up-regulated to about 30% in *nas4x-1* flowers and siliques. *YSL2* expression was drastically suppressed in *nas4x-1* mutant flowers and siliques to about 10- to 15-fold. Hence, *YSL1* and *YSL3* expression patterns behaved in flowers and siliques similar to the leaves in the wild type compared with *nas4x-1*. If *YSL1* and *YSL3* expression was indeed correlated with Fe sufficiency to promote lateral removal of Fe, this observation may indicate that despite Fe deficiency, flowers

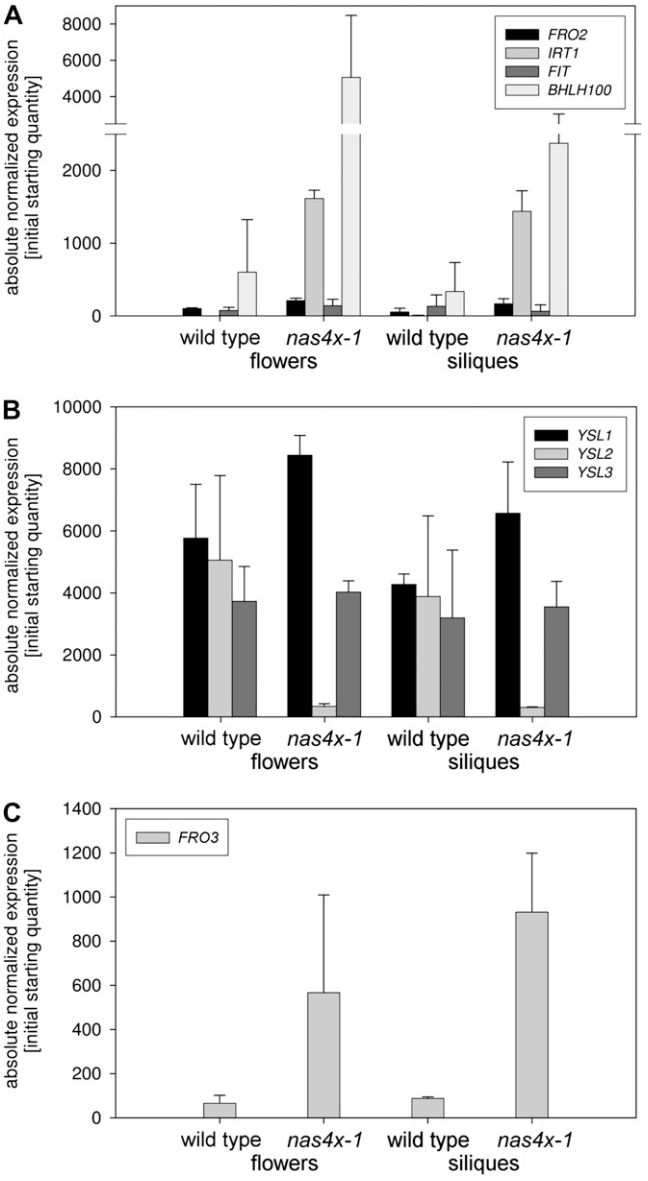

Figure 5. Quantitative reverse transcription-PCR analysis of *nas4x-1* mutant flowers and siliques. A, Fe deficiency response gene expression. B, *YSL* gene expression. C, *FRO3* gene expression.

and siliques were also Fe sufficient. *YSL2* down-regulation in *nas4x-1* flowers and siliques was very surprising, and in contrast to the expression in leaves. Perhaps down-regulation of *YSL2* in *nas4x-1* reproductive organs was advantageous for Fe supply, for example of seeds while nicotianamine was reduced.

Finally, we tested whether *FRO3* and *FER1* might also be regulated in reproductive organs. *FRO3* was found up-regulated in *nas4x-1* flowers and siliques compared with the wild type (Fig. 5C). This was an indication that in contrast to leaves, the reproductive organs were rather Fe deficient. *FER1* expression was highly variable in both the wild type and *nas4x-1*, so no conclusions were drawn (data not shown).

In summary, gene expression in *nas4x-1* mutant flowers and siliques indicated that these organs suffered from Fe deficiency and suggested that an alternative rescue pathway for Fe uptake of cells might have been switched on.

Metal Contents of *nas4x-1* Plants

It was then interesting to analyze whether low nicotianamine levels influenced the transport of metals (Fe, Cu, and Zn) into various organs. In roots, where metals are found in the vascular system, inside root cells, and in the apoplast, we did not detect changes between *nas4x-1* and the wild type (data not shown). Differences in metal contents were observed in aboveground organs. Vegetative leaves of *nas4x-1* plants contained about the same Fe as the wild type, whereas Zn was significantly reduced by about 38% (Fig. 6A). Cu contents were unchanged. In rosette leaves in the reproductive stage, Fe contents were significantly increased by 216% in *nas4x-1*, whereas Zn remained significantly decreased by half (Fig. 6B). Cu was if at all slightly reduced (approximately 11%) in these *nas4x-1* leaves. On the other hand, in flowers, siliques, and seeds, Fe contents were decreased by 32% (significantly), 13%, and 46% (significantly), respectively (Fig. 6C). Zn contents were also decreased in *nas4x-1* flowers (significantly) and siliques, whereas it was similar in seeds when compared with the wild type. Cu contents were slightly reduced in *nas4x-1* siliques but not in the other plant parts tested. Thus, significance tests of metal determination showed that nicotianamine was not needed to take up and transport Fe to leaves but it was required to efficiently mobilize Fe from leaves during the reproductive phase to flowers and seeds. Nicotianamine was also beneficial for Zn uptake and transport to leaves and flowers.

Phenotypes of *nas4x-1* Plants in Response to Toxic Metal Supply

Our results showed that reduced nicotianamine caused reduced Fe transport to reproductive plant parts and reduced Zn in the plant. Hence, we wondered whether the loss of the ability to translocate metals inside the plant could lead to an altered response to metal toxicity. To test this, we studied the responses of *nas4x-1* plants to supply of excess concentrations of Fe (up to 200 μM), Zn (up to 200 μM), and Cu (up to 28 μM). Fe, Zn, and Cu caused shorter roots and smaller shoots than control conditions, and Zn and Cu also caused yellow leaves (data not shown). In none of the three cases did we observed a significant phenotypic difference between *nas4x-1* and wild-type plants (data not shown). We conclude, therefore, that wild-type nicotianamine levels did not alleviate Fe, Zn, and Cu toxicity.

As a control, we tested the responses to Ni, which is known to be chelated by nicotianamine. Elevated nicotianamine levels have recently been correlated with tolerance to Ni (Becher et al., 2004; Weber et al., 2004; Douchkov et al., 2005; Kim et al., 2005; Pianelli et al., 2005). Interestingly, *nas4x-1* seedlings were highly sensitive to 60 μM Ni, in contrast to wild-type plants. *nas4x-1* mutants developed strongly chlorotic leaves and short roots at 2 weeks of age and older, whereas wild-type plants still remained green under

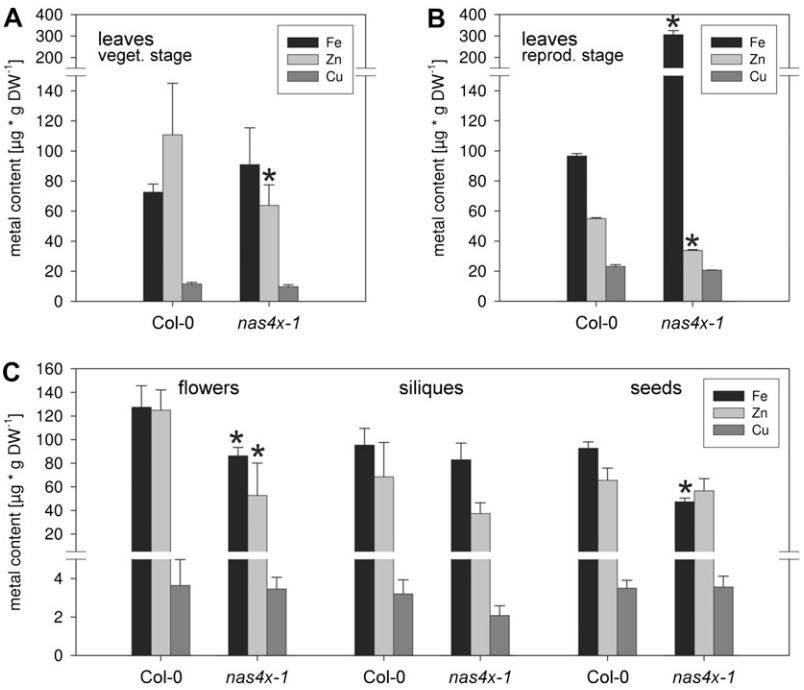

Figure 6. Metal contents of *nas4x-1* mutant and wild-type plants. A, Leaves harvested during the vegetative phase from plants grown under an 11-h light period. B, Leaves harvested during the reproductive phase from plants grown under a 16-h light period. C, Flowers, siliques, and seeds. Plants were grown in a regular hydroponic system. The indicated plant organs were dissected and freeze dried. Metal contents (Fe, Zn, and Cu) were determined by graphite furnace atomic absorption spectroscopy with direct solid sampling. For leaves, $n = 8$; for flowers/siliques, $n = 5$; for seeds, $n = 6$. DW, Dry weight. * $P < 0.01$ using the *t* test.

these conditions (Fig. 7A). Multiple mutant analysis showed epistatic interactions of *nas* mutant alleles. The single and multiple mutant combinations with *nas4-1* were most severely affected, the single and multiple mutants with *nas3-1* (but not *nas4-1*) were second most affected by Ni, whereas *nas1-1*, *nas2-1*, and *nas1-1nas2-1* double mutants were not affected more than the wild type (Fig. 7B).

We also tested whether *NAS* genes were affected by Ni treatment. *NAS1*, *NAS2*, and *NAS4* genes but not *NAS3* were induced by Ni treatment in roots, although only to a 2- to 3-fold level, whereas general transcript levels were low for *NAS4* compared with *NAS1* and *NAS2* (Supplemental Fig. S6). Interestingly, *FRO2* as a marker for Fe deficiency was also up-regulated in Ni-treated roots, suggesting that Ni-treated plants might experience Fe deficiency. In leaves, *NAS3* and *NAS4* were up-regulated 2- to 3-fold by Ni treatment (Supplemental Fig. S6). Hence, *NAS* gene expression was affected by Ni but induction was quite low. *NAS3* and *NAS4* responses to Ni were in agreement with Kim et al. (2005).

Hence, wild-type levels of nicotianamine were used for detoxification of Ni but not of Zn, Cu, and Fe. Moreover, *NAS4* and *NAS3* conferred higher Ni resistance than *NAS1* and *NAS2*, suggesting that at the level of Ni toxicity there was not full redundancy of *NAS* genes.

DISCUSSION

Our analysis of the Arabidopsis *nas* mutant may form the ground for targeted improvement of the Fe nutritional value of crops. Our results support that by way of altering expression of *NAS* genes using transgenic and classical breeding approaches, the seed and leaf Fe contents can be manipulated.

We have shown here that complete loss of nicotianamine resulted in a *chloronerva* phenotype in Arabidopsis (*nas4x-2* mutant), namely in severe leaf chlorosis and sterility. Since Arabidopsis and solanaceous plants belong to two different subclasses of the eudicots, namely Rosidae and Asteridae, we conclude that nicotianamine function is conserved in most if not all dicots.

Our study concentrated on the analysis of a knockdown mutant of nicotianamine synthase function, *nas4x-1*, that had residual nicotianamine. This mutant had *chloronerva*-like features, such as leaf chlorosis, susceptibility to Fe deficiency, increased Fe uptake and Fe levels in leaves, and *fit/nas* epistatic interactions. The mutant also was susceptible to Ni supply. Remaining nicotianamine contents in *nas4x-1* still allowed the plants to complete their life cycle and produce seeds. Mutants with intermediate phenotypes yielded interesting results in plant biology and were often discovered in forward genetic screens. Interpre-

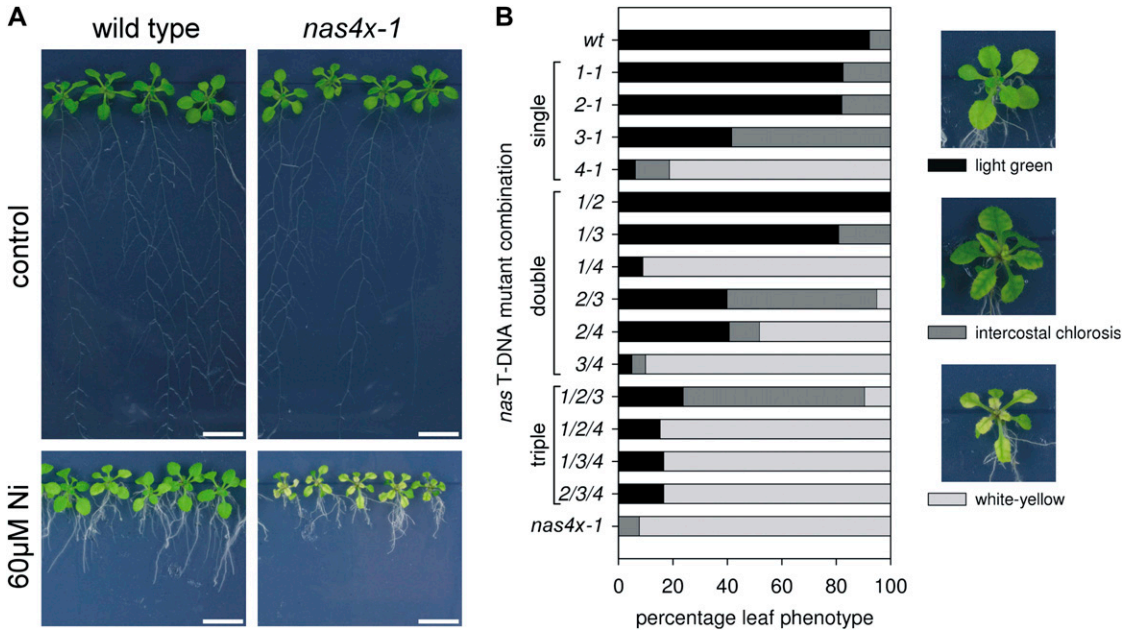

Figure 7. Phenotypes of *nas* mutants to Ni treatment. A, Growth assay showing 17-d-old seedlings grown on 60 μM Ni and control Hoagland agar medium. Note the short root phenotypes of Ni-treated plants and the severe white-yellow leaf chlorosis phenotype of *nas4x-1* mutants compared with the wild type on Ni. B, Distribution of leaf chlorosis phenotypes among multiple *nas* mutant lines grown for 17 d on 60 μM Ni and control Hoagland agar medium (n = 10–35). Three leaf phenotypic classes were defined: light green leaf appearance of mildly affected plants, intermediate intercostal leaf chlorosis of Ni-sensitive plants, and the most severe white-yellow leaf chlorosis of severely Ni-affected plants. The number of plants belonging to each phenotypic class was calculated in percentage. [See online article for color version of this figure.]

tation of null mutants is frequently limited by the onset of pleiotropic responses and/or a precocious block in development. Examples for intermediate phenotypes are found in many of the developmental mutants, and they have also been valuable in the Fe homeostasis field, where for most gene families only single mutant analysis was performed.

The four *NAS* genes act in a functionally redundant manner, since the enzyme product nicotianamine is a mobile compound in the plant. Despite the redundancy, individual NAS enzymes have gained specific properties, for example due to differential gene expression patterns such as in different tissues and by regulation through metal supply. Functional specification of *NAS1* can also explain the variable *NAS1* gene expression we have observed while analyzing multiple *nas* mutants and Fe deficiency responses. *NAS1* may have responded specifically to certain developmental or environmental cues that occurred in a variable manner in these latter growth conditions.

We have used this mutant to ask several questions.

Why have *nas4x-1* leaves been more chlorotic during the reproductive stage than the vegetative stage? The late leaf chlorosis was the first remarkable phenotype of *nas4x-1* plants. We found that rosette leaves of the reproductive stage of *nas4x-1* plants did not have any nicotianamine, whereas during the vegetative stage such leaves contained nicotianamine, although only at 10% of wild-type levels. We deduce from these observations that nicotianamine had disappeared from the rosette leaves during the reproductive phase and was no longer replenished through synthesis or transport. The removal of nicotianamine caused a Fe distribution problem in leaf cells, which was the cause for the leaf chlorosis. Clearly, some cells or compartments of *nas4x-1* plants were Fe starved, so that Fe deficiency signaling took place in the leaves. In support of this is the fact that leaves turned chlorotic and that expression of *BHLH100* was induced. The transcription factor gene *BHLH100* responds to local Fe deficiency, as shown by previous grafting studies (Wang et al., 2007). It is very interesting that in *nas4x-1* plants, *BHLH100* regulation was decoupled from the presence of Fe in the leaves. It could indeed have been that Fe deficiency-mediated stress responses that had occurred in *nas4x-1* leaves up-regulated *BHLH100*. Also in support of Fe deficiency of *nas4x-1* plants was the fact that root Fe acquisition responses had been switched on, such as *IRT1* and *FRO2* induction. On the other hand, *nas4x-1* plants contained Fe during the reproductive phase at even more than twice the amount compared with the wild type. Obviously, this Fe did not enter all target places. However, the Fe in the leaves was sensed, so that *FRO3*, a ferric reductase gene normally induced by −Fe in leaves (Mukherjee et al., 2006), was not induced. Therefore, *FRO3* was probably not required for uptake and transport of Fe into leaves of *nas4x-1* plants. Similarly, *nas4x-1* leaves had similar *FER1* expression levels as Fe-sufficient wild-type leaves. Therefore, the signaling machinery that regulated *FRO3* and *FER1* expression in response to Fe operated and sensed Fe in *nas4x-1* leaves.

The genetics of the *nas4x-1* mutant also support the idea that in leaves nicotianamine had disappeared during the reproductive phase. Gene expression studies and mutant T-DNA analysis had shown that only the *nas2-1* allele was responsible for residual nicotianamine production in the mutant. This allele was essentially expressed in roots, whereas it was expressed to a low level in leaves. Moreover, its gene expression levels were higher in the vegetative phase than in the reproductive phase. For example, in the reproductive phase, the total number of *NAS* transcripts of *nas4x-1* plants was 25% higher in roots compared with wild-type plants; on the other hand, it was 88% lower in leaves. This suggests that roots were able to continuously supply nicotianamine in *nas4x-1*. We can exclude that the higher and partially ectopic *NAS2* gene expression caused higher nicotianamine production from *nas2-1* than from the wild-type *NAS2*. In fact, we found that a *nas1-1nas3-1nas4-1* mutant with wild-type *NAS2* had three to four times higher nicotianamine levels than the mutant with the *nas2-1* allele (which was *nas4x-1*). Hence, *nas2-1* resulted in a reduced function of *NAS2*. The NAS leaf isoforms are normally encoded by *NAS1*, *NAS3*, and *NAS4*. Since *NAS3* and *NAS4* were mutated in *nas4x-1*, they could not replenish nicotianamine in leaves, especially during reproduction, when *NAS3* was induced in leaves.

The question remained, what happened to the nicotianamine that had been depleted from leaves? The answer led to a second striking phenotype of *nas4x-1* plants: reduced Fe contents in seeds. Our results show that wild-type nicotianamine levels were essential for normal Fe supply of seeds. Nicotianamine was present in wild-type seeds. *nas4x-1* seeds contained nicotianamine to a lower level than wild-type seeds, and they also contained Fe to a lower level. However, neither in flowers nor in developing siliques of the wild type or mutant could we detect *NAS* gene transcripts above background levels in our experiments (data not shown). In both the wild type and mutant, therefore, it seemed impossible that reproductive parts had their own nicotianamine synthase activity. Hence, any nicotianamine that was detected in seeds must have been produced elsewhere in the plant and transported to the flowers before its deposition into developing seeds. The disappearance of nicotianamine from leaves, accompanied by the onset of leaf chlorosis during reproduction, suggests an important function of nicotianamine at this stage. Therefore, this explains our results such that nicotianamine was transported away from leaves toward developing seeds. Presumably, nicotianamine was transported as Fe chelate. Since the *nas4x-1* mutant had less nicotianamine, a consequence was that less Fe reached the developing seeds and more Fe remained in leaves. In addition, due to diminished NAS activity in *nas4x-1* roots, we assume that less nicotianamine-Fe reached

the reproductive organs directly from the roots but may have ended up in leaves in a nicotianamine-independent manner.

ysl1 and *ysl1ysl3* mutants shared phenotypes of *nas4x-1*, such as low Fe content in seeds, Fe deficiency responses, and leaf chlorosis, which had suggested that *YSL* genes were involved in seed Fe partitioning (Le Jean et al., 2005; Waters et al., 2006; Waters and Grusak, 2008). We found that *YSL* gene expression was affected in *nas4x-1* plants. These results strongly suggest that *YSL* and *NAS* genes indeed acted in similar biological processes. The best explanation is that YSL proteins are indeed the transporters for nicotianamine-Fe.

Although seeds were clearly reduced in Fe, they were only slightly reduced in Zn content and not reduced in Cu. This shows that high amounts of nicotianamine were needed for Fe deposition but low amounts were sufficient for Zn and Cu transport into seeds. An interesting phenotype was observed in *naat* mutant rice plants. These mutants had increased nicotianamine levels due to dysfunctional phytosiderophore synthesis, which led to reduced Fe mobilization via strategy II. But in return, these plants could take up Fe through an IRT-like transporter, which may have been aided by the increased nicotianamine (Cheng et al., 2007). Since these plants had higher seed Fe contents, it may have been possible that in these rice plants increased nicotianamine was implicated in the loading of seeds with Fe, the opposite case of *nas4x-1*.

Why did the *nas4x-1* plants increase Fe acquisition during the reproductive phase while Fe accumulated in leaves? Previous systemic signaling studies of a number of mutants had suggested that systemic Fe deficiency signals must exist that stem from leaves and that regulate Fe acquisition in the root (Grusak and Pezeshgi, 1996; Vert et al., 2002; Enomoto et al., 2007; Wang et al., 2007). As discussed above, *nas4x-1* leaves showed signs of Fe sufficiency but also of Fe deficiency. It is possible that the *FER1/FRO3* regulatory system was simply not connected with the systemic signaling mechanism for root Fe acquisition responses, whereas *BHLH100* might have been. Another explanation for the induction of Fe acquisition in roots could be the high demand for Fe in the plants. Very interestingly, we have observed that *IRT1*, *BHLH100*, and *FRO3* were all induced in flowers and siliques, showing that these organs not only had low Fe levels but also responded to that. It is possible that due to reduced nicotianamine-Fe transport in flowers and siliques, alternative routes for Fe uptake were switched on based on *FRO3/IRT1*. Perhaps these reproductive organs with their Fe deficiency were capable of producing a systemic signal that overruled the potential Fe sufficiency of leaves so that they acted as sink organs for Fe.

Could the reduced Fe content of *nas4x-1* seeds result in a germination defect? The answer is that a 60% reduction of Fe in *nas4x-1* seeds did not result in any germination defect on regular medium. However, upon germination on Fe-deficient medium, *nas4x-1* seedlings had stronger chlorotic leaves than wild-type seedlings (M. Klatte and M. Schuler, unpublished data). Hence, nicotianamine helped in Fe mobilization during germination when external Fe supply was low. Interestingly, this susceptibility to Fe deficiency was also apparent later in development, when plants were transferred from regular to Fe-deficient medium for several days. These plants had been able to take up Fe during their postgermination growth, yet Fe deficiency was more severe than in the wild type. Since *NAS* genes and *FIT* acted in the same pathway of Fe acquisition (epistatic interactions of *FIT* over *NAS* in the multiple mutants), nicotianamine was needed during the Fe acquisition process. One possible explanation could be that nicotianamine is used to translocate Fe, such as from the epidermis to the vascular system in roots upon Fe deficiency.

The final question for discussion is, why have *nas4x-1* plants been sensitive to Ni but not to elevated Fe, Zn, or Cu? Obviously, the normal nicotianamine levels were not used in prevention of the Fenton reaction and other toxic effects of Fe, Cu, or Zn. If they were used for preventing toxic effects of these metals, we would have expected the mutant to react in an oversensitive manner to these metals. On the one hand, this finding is surprising. In fact, hyperaccumulators have mostly strongly up-regulated nicotianamine synthesis (Becher et al., 2004; Weber et al., 2004; Mari et al., 2006) and *NAS* overexpression can lead to increased tolerance to metals, including Zn (Kim et al., 2005). The function of high nicotianamine for Zn detoxification, therefore, remains dubious. Interestingly, overexpression of *Thlaspi TcNAS1* in Arabidopsis led to increased sensitivity to Fe deficiency (Cassin et al., 2009). This was also surprising, as Fe deficiency is normally conferred by a loss of *nas* function. As discussed by Cassin et al. (2009), the apparent contradictory results between *nas* loss of function and *NAS* overexpression could be explained by the altered location of nicotianamine. Our metal determination analyses have shown that *nas4x-1* leaves have lower Zn content than wild-type leaves. Thus, *nas4x-1* mutants translocate less Zn to leaves than the wild type, which may partially protect the mutants from Zn toxicity. On the other hand, less nicotianamine was available in *nas4x-1* leaves and roots to chelate Zn and prevent toxic effects in the cells, and in addition, stronger Fe deficiency was present. In turn, this may explain why *nas4x-1* mutants and the wild type reacted in the end similarly to high Zn, while only *NAS* overexpression was protective, as shown by Kim et al. (2005).

Indeed, we have confirmed that nicotianamine can be used for detoxification of Ni through induced Ni sensitivity in *nas4x-1*, whereas other groups showed Ni tolerance upon increased nicotianamine in Arabidopsis or tobacco (Douchkov et al., 2005; Kim et al., 2005; Pianelli et al., 2005). With respect to this heavy metal, we also found nonredundant functions of *NAS*

genes. *NAS4* and to a lower degree *NAS3* contributed essentially to Ni tolerance, whereas *NAS1* and *NAS2* could be neglected. Ni may directly affect NAS function, so perhaps NAS3 and NAS4 could be the Ni-tolerant NAS isoforms. Upon Ni treatment, increased nicotianamine might have been needed for Fe mobilization. Indeed, our results based on *FRO2* expression indicate that Ni treatment caused Fe deficiency, perhaps through competition of binding sites to nicotianamine and other compounds in the cells. Interestingly, previous studies have shown that increased nicotianamine in Arabidopsis not only resulted in tolerance to Ni but also in tolerance to Fe deficiency (Douchkov et al., 2005; Kim et al., 2005). Hence, the two processes could be physiologically linked.

MATERIALS AND METHODS

Plant Material and Genotyping

The Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) was used as the wild-type control. *nas* T-DNA insertion mutants were obtained from various stock centers (*nas1-1* = GABI-kat 223A09, *nas2-1* = SAIL_156_C08, *nas2-2* = SALK_066962, *nas3-1* = GABI-kat 010A10, and *nas4-1* = SALK_135507) and tested for the presence of the respective T-DNA insertions using genomic PCR (primer sequences are given in Supplemental Table S1). The inheritance of T-DNA insertions was analyzed by segregation analysis. Multiple mutants were obtained through crossing as indicated in the text. Genotypes of multiple mutants were confirmed by genomic PCR and subsequent genotyping in the progeny of the plants. *fit* mutants and the *p2x35S::FIT* overexpression line were described by Jakoby et al. (2004).

Plant Growth

Seeds were surface sterilized and stratified for 2 to 3 d at 4°C. The agar plate system using 1× Hoagland agar square plates was as described by Jakoby et al. (2004). Fe-deficient agar medium was devoid of Fe or supplemented with 50 μM ferrozine. For Ni stress conditions, Hoagland medium was modified to contain 60 μM Ni ($NiCl_2$). Zn stress medium contained 200 μM $ZnSO_4$ (instead of the regular 2 μM Zn), and Cu stress medium contained 28 μM $CuSO_4$ (instead of the regular 1.5 μM Cu). For the hydroponic system, seedlings were germinated on quarter-strength Hoagland agar medium in 500-μL support tubes containing a hole at the bottom for root growth. After 2 weeks, plants were placed into aerated quarter-strength Hoagland liquid medium for another 2 weeks. Medium was exchanged weekly. Quarter-strength Hoagland medium contained one-quarter of the Hoagland salts and 10 μM FeEDTA (control). Solid germination medium of the hydroponic system contained no Suc and 0.6% plant agar. Metal deficiency in the hydroponic solution was achieved using quarter-strength Hoagland medium lacking Fe, Cu, or Zn salts. Cultivation took place at 21°C/19°C and 16-h-light/8-h-dark cycles or as indicated in the text.

Physiological Plant Analysis

Leaf chlorosis phenotypes were determined by manual inspection as indicated in the text. Ferric reductase activity was measured as described by Jakoby et al. (2004). Metal contents were determined on dried plant samples using graphite furnace atomic absorption spectroscopy with direct solid sampling (AAS vario 6, graphite furnace and flame technique; Analytik Jena).

Gene Expression Analysis

Gene expression analysis was performed by reverse transcription-quantitative real-time PCR as described previously (Wang et al., 2007). Briefly, DNase-treated RNA was used for cDNA synthesis. SYBR Green I-based real-time PCR analysis was performed using ExTaq R-PCR (TaKaRa) in a Mx3000P real-time PCR cycler (Stratagene). For each gene, the absolute quantity of initial transcript was determined by standard curve analysis. Absolute expression data were normalized against the averaged expression values of the internal control genes *EF1BALPHA2* and *UBP6*. Primer sequences are provided in Supplemental Table S1. Specific PCR oligonucleotides were designed using Primer3 software. *NAS* primer specificity was thoroughly verified in test amplifications.

Determination of Nicotianamine Contents

Nicotianamine was extracted using a modified protocol (Neumann et al., 1999). The identity of nicotianamine was evidenced by spiking of chemically synthesized nicotianamine to plant samples. The nicotianamine-free tomato (*Solanum lycopersicum*) mutant *chloronerva* was used as a negative control. In leaves of Arabidopsis, a recovery rate of 98% ± 7% was achieved after addition of external standard to the plant sample prior to nicotianamine extraction (Supplemental Fig. S7). The detailed protocol of the modified method is available as Supplemental Materials S1.

Supplemental Data

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

Supplemental Figure S1. Nicotianamine contents of single and triple *nas* mutants.

Supplemental Figure S2. *NAS* gene expression in single and triple *nas* mutants.

Supplemental Figure S3. *NAS* gene expression during vegetative and reproductive growth.

Supplemental Figure S4. Effect of Fe deficiency treatment in *nas4x-1* mutants.

Supplemental Figure S5. Zn and Cu contents of *nas4x-1* and wild-type plants.

Supplemental Figure S6. *NAS* gene expression in response to Ni.

Supplemental Figure S7. Recovery rate of nicotianamine.

Supplemental Table S1. Gene-specific primers used for real-time quantitative PCR analysis.

Supplemental Materials S1. Determination of nicotianamine contents.

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