Differential Regulation of nramp and irt Metal Transporter Genes in Wild Type and Iron Uptake Mutants of Tomato*

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Metal transporters regulated by iron can transport a variety of divalent metals, suggesting that iron regulation is important for specificity of iron transport. In plants, the iron-regulated broad-range metal transporter IRT1 is required for uptake of iron into the root epidermis. Functions of other iron-regulated plant metal transporters are not yet established. To deduce novel plant iron transport functions we studied the regulation of four tomato metal transporter genes belonging to the nramp and irt families with respect to environmental and genetic factors influencing iron uptake. We isolated Lenramp1 and Lenramp3 from tomato and demonstrate that these genes encode functional NRAMP metal transporters in yeast, where they were iron-regulated and localized mainly to intracellular vesicles. Lenramp1 and Leirt1 revealed both root-specific expression and up-regulation by iron deficiency, respectively, in contrast to Leirt2 and Lenramp2. Lenramp1 and Leirt1, but not Lenramp3 and Leirt2, were down-regulated in the roots of fer mutant plants deficient in a bHLH gene regulating iron uptake. In chloronerva mutant plants lacking the functional enzyme for synthesis of the plant-specific metal chelator nicotianamine Leirt1 and Lenramp1 were up-regulated despite sufficient iron supply independent of a functional fer gene. Lenramp1 was expressed in the vascular root parenchyma in a similar cellular pattern as the fer gene. However, the fer gene was not sufficient for inducing Lenramp1 and Leirt1 when ectopically expressed. Based on our results, we suggest a novel function for NRAMP1 in mobilizing iron in the vascular parenchyma upon iron deficiency in plants. We discuss fer/nicotianamine synthase-dependent and -independent regulatory pathways for metal transporter gene regulation.

The transition metal iron is essential for many cellular electron transfer reactions. Because of its redox potential free iron may become toxic and produce radicals. In addition, uptake of various divalent metals can interfere with that of iron and vice versa, as iron transport mechanisms often do not discriminate metals. These characteristics may explain the presence of multiple transport and regulatory mechanisms for storage and mobilization of iron in all cells and organisms. Molecular characterization of iron mobilization in plants exposed to iron deficiency has been a recent subject of investigation. In dicotyledon plants, iron is mobilized by reduction and taken up across the plasmalemma into the root epidermis via IRT1 (1–3). IRT1 is a member of the ZIP family of broad range metal transport proteins identified from eukaryotes and, recently, also bacteria (4–6). Atirt1 transcription is predominant in roots and induced upon iron deficiency (2). Loss of function of irt1 leads to reduced viability in Arabidopsis unless excess iron is supplied, suggesting that IRT1 is a major structural component of iron uptake in plants (3, 7). Grasses may take up iron differently because they mobilize iron by secreting potent phytosiderophores from the root and subsequently take up the iron-siderophore complexes (1). Internal transport and mobilization of iron inside the plant root is still unclear. Iron is transported via the xylem to the shoot essentially as iron citrate (for an example, see Ref. 8). Furthermore, iron can be translocated in the phloem, for example as complex with nicotianamine, a diffusible plant-specific small non-proteinogenic tripeptide derived from methionine (for review, see Ref. 9), or iron-binding proteins (10). Because plants play a major role for human nutrition and because iron deficiency is a worldwide nutritional problem, one challenge is to produce iron-rich crops (for examples, see Refs. 11 and 12). In this context, it is of great interest to study internal iron mobilization and transport in plants. The specific roles of iron transporters other than IRT1 are not known yet. Atirt2, although in sequence and expression pattern similar to Atirt1, is not capable of functionally complementing irt1 mutants, and loss of function causes no obvious phenotype upon low iron supply (7, 13). To date six members of the Arabidopsis nramp gene family have been assigned and partially characterized. NRAMP proteins are broad range membrane-bound metal transporters found in all eukaryotes (for review, see Ref. 14). Their name was derived from phagosomal NRAMP1 (natural resistance-associated macrophage protein 1), which functions as an efflux pump in the membrane and in this way enhances resistance against intracellular bacteria by reducing metal availability (for review, see Ref. 15). Mammalian NRAMP2 is an important factor for iron transport from the duodenum lumen into epithelial cells (16, 17). The three yeast NRAMP proteins, SMP1, SMP2, and SMP3, are differentially regulated by iron and manganese and may serve to release these metals from intracellular stores (18, 19). It was found that Atnramp1, Atnramp3, and Atnramp4 encode functional plant metal transporters and are expressed at higher levels upon low iron supply in plants (20, 21). Specificity of plant transporters may be achieved essentially by differential

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The abbreviations used are: IRT, iron-regulated transporter; NRAMP, natural resistance-associated macrophage protein 1; ZIP, zinc- and iron-regulated transporter; bHLH, basic helix-loop-helix; BPDS, bathophenanthroline disulfonic acid disodium salt; GFP, green fluorescent protein; DIC, differential interference contrast; MES, 4-morpholineethanesulfonic acid; BAC, bacterial artificial chromosome.
regulation at transcriptional and post-transcriptional levels as shown for IRT1 and metal transporters in other eukaryotes (2, 22–24).

In plants, iron acquisition is controlled by yet unknown iron signals. A promising clue about signaling components involved in iron acquisition was recently derived from the molecular identification of the tomato fer gene encoding a root-specific hHIL protein (25). The tomato fer mutant is unable to develop physiological and morphological iron-deficiency responses such as iron reduction and induced Leit1 transporter gene expression in roots and accumulates less iron than wild type, leading to severe chlorosis (25–27). The fer gene seems to be required for sensing iron availability in the root tip and subsequently regulating the appropriate physiological and morphological responses (25). The regulatory mechanism and concrete molecular responses affected by the fer mutation are not known.

An opposite effect on strategy I is observed in the chloronerva mutant from tomato, which lacks nicotianamine due to a mutation in nicotianamine synthase (28). chloronerva mutant plants display increased iron reductase activity at sufficient iron supply and over-accumulate metals compared with the wild type, suggesting that nicotianamine synthase is required for adequate uptake and distribution of iron in plants (for review, see Ref. 9).

We analyzed the possible role and regulation of two nramp genes and two irt genes from tomato in response to environmental and genetic factors related to iron nutrition. We could establish a model of gene interactions and regulation involved in iron uptake in plants. From our results we propose a novel role for nramp1 in plants.

**EXPERIMENTAL PROCEDURES**

**Plant Growth—**10-Day-old tomato seedlings were transferred into moderately aerated hydroponic culture with Hoagland solution containing 10 μM FeNaEDTA (normal iron supply) (according to Stephan and Procházková (29)). After 2 days, the experiment was started by transferring the seedlings into Hoagland solution with varying concentrations of iron for up to 8 days. The limiting supply was 0.1 μM FeNaEDTA; excess supply was 100 μM FeNaEDTA.

**Plant Material—**Seeds were derived from the lines Lycopersicon esculentum cv. Moneymaker (wild type), T3238f8 (mutant fer), and chloronerva (mutant chloronerva). Homozygous fer or chloronerva mutant seeds were obtained after grafting mutant shoots onto wild type root stocks, which rescued the phenotypes. Double homozygous mutants for fer and chloronerva (fer; chlor1) were obtained in the following way. Among the 1:8 segregating progeny of a double heterozygous plant (fer+/fer; chlor1+/chlor1) chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background (yellow leaves, green veins, green cotyledons = fer; chlor1) in a chloronerva background.
(20 cycles) (36). Amplification products were analyzed by agarose gel electrophoresis and Southern blot hybridization according to standard procedures.

**In Situ Hybridization**—Roots tips from plants grown for 8 days in Hoagland medium with 0.1 μM FeNaEDTA were fixed and embedded in paraplast according to Kyozuka et al. (37). 10-μm transverse root sections were hybridized using digoxigenin-labeled RNA probes according to Jackson (38). RNA probes were prepared from 500–600 bp of amplified cDNA fragments subcloned into pCRII (Invitrogen) using either T7 or SP6 RNA polymerase for in vitro transcription. Hybridization signals were detected using primary anti-Dig antibodies coupled to alkaline phosphatase (violet staining, according to Jackson (38). Probe cDNA fragments were obtained by using the following primer pairs: 5' - ggcatacgtcgaattcc-3' and 5' - ggatcagcaccaccagttt-3' for Lenramp1; 5' - aatcagatgaaaagttaaagatgccc-3' and 5' - addtgagcaccattctctcttc-3' for LeNRAMP1; 5' - atctggtggtggaaggctcaaa-3' for Atnramp1; 5' - ggggatctgatcagcaccaccaccatc-3' for AtNRAMP3; 5' - tatcagcaccattctctcttc-3' for LeNRAMP1 (35).

**RESULTS**

Identification of nramp Metal Transporter Genes from Tomato—

NRAMP proteins are encoded by evolutionarily conserved genes. By performing a BLAST search of plant NRAMP peptide sequences against the tomato EST data base we could identify several EST clones covering four non-overlapping regions of the same gene. Because of its similarity with the tomato EST clones (Leirt1 and Leirt2) for Leirt2 for Leirt2 (35). Leirt2a and Leirt2b probe fragments covered Leirt2 cDNA and were mixed to yield the Leirt2 probe. The Leirt2 probe was specific for Leirt2. Probe fragments covering Leirt1 did not result in any hybridization signals, indicating that there was no cross-hybridization of Leirt1 and Leirt2 RNA probes with the respective mRNAs (not shown). In control reactions labeled sense probes were used that resulted in no signals (Fig. 5, G–I).

To determine in which compartments LeNRAMP1 and LeNRAMP3 both encode functional metal transporters able to restore growth of a yeast metal uptake mutant.

**Functional Analysis of Lenramp1 and Lenramp3 in Yeast**—To determine whether Lenramp1 and Lenramp3 might encode functional metal NRAMP transporters, full-length coding sequences have been cloned into the yeast expression vector pYES2 behind the galactose-inducible GAL1 yeast promoter. These constructs were expressed in the yeast smf1 mutant, deficient in a broad range metal transporter nramp homolog (40, 41). To test for functional complementation, smf1 mutants transformed with pYES2 containing either Lenramp1, Lenramp3, or empty control vector and, as positive control, wild type yeast were spotted in different dilutions on minimal medium in the presence of 5, 10, 20, and 50 mM divalent metal chelator EGTA. Under these conditions, Lenramp1- and Lenramp3-transformed smf1 strains grew significantly better than control mutant smf1 strain transformed with empty vector (Fig. 2A). Growth of LeNRAMP1- and LeNRAMP3-transformed yeast cells was comparable with that of wild type if the medium was supplemented with up to 20 mM EGTA (Fig. 2A). At 50 mM EGTA, growth of LeNRAMP1- and LeNRAMP3-transformed cells was retarded compared with that of wild type but significantly better than that of control pYES2-smf1 mutant cells (Fig. 2A). Growth of wild type was also reduced at 50 mM EGTA compared with lower EGTA concentrations (Fig. 2A).

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By performing a BLAST search of plant NRAMP peptide sequences against the tomato EST data base we could identify several EST clones covering four non-overlapping regions of the same gene. Because of its similarity with the tomato EST clones (Atnramp1 and Atnramp3) for Atnramp1 (21) we have termed this gene Lenramp3 (accession number AY196092). The Lenramp3 coding sequence was fully contained in EST clone cLET21E13.

In addition, degenerate primers were designed recognizing two conserved regions between Atnramp1 and OsNRAMP1, one of them representing the conserved transport motif (20, 39). Using the degenerate primers, a 334-bp nramp cDNA fragment was amplified from iron-starved root cDNA. Sequencing revealed best sequence similarity with Atnramp1 so that this gene was termed Lenramp1. Full-length Lenramp1 sequence was obtained from a tomato BAC clone containing the 334-bp DNA fragment, namely 24I11. Full-length Lenramp1 cDNA was amplified from root cDNA, subcloned, and sequenced (accession number AY196091). Lenramp1 cDNA sequence was identical to the exon genomic sequence obtained from the BAC clone.

The deduced protein sequences of LeNRAMP1 and LeNRAMP3 were compared with those of the six Arabidopsis and three rice NRAMP protein sequences (20, 21, 30). Sequence comparison showed that plant NRAMP sequences can be divided into two subgroups and that LeNRAMP1 and LeNRAMP3 fall into distinct subgroups (Fig. 1). Comparing Arabidopsis and tomato NRAMP sequences showed that LeNRAMP1 is most similar with AtNRAMP1 and AtNRAMP6. LeNRAMP3 shows the highest sequence similarity with AtNRAMP3 and AtNRAMP4. This finding indicates that distinct NRAMP functions might be conserved in plants.

**Comparison of plant NRAMP amino acid sequences.** Sequences were aligned using ClustalW. The numbers indicate percent changes in amino acid composition. Accession numbers or Arabidopsis gene numbers are as follows: AtNRAMP1 (At1g08590), AtNRAMP2 (At1g7240), AtNRAMP3 (At2g25150), AtNRAMP4 (At5g8730), AtNRAMP5 (At4g18790), AtNRAMP6 (At1g15960) (20, 21), OsNRAMP1 (L41217), OsNRAMP2 (L81152), OsNRAMP3 (U60767) (30), LeNRAMP1 (AY196091), LeNRAMP3 (AY196092).

![Fig. 1. Comparison of plant NRAMP amino acid sequences.](image-url)
of an intact root hair zone of wild type roots (Fig. 5 A). Expression of Leirt1 transcripts were also found in green parts of the shoot (Fig. 3 A). Expression of Leirt2 transcripts were only found in roots but not leaves and cotyledons (Fig. 3 A). Thus, Leirt1, Leirt2, Lenramp1, and Lenramp3 show differential expression with respect to tissue specificity and regulation by iron supply.

Expression Analysis of Metal Transporter Genes in fer Mutant Plants—We tested whether the fer defect affected the expression of metal transporter genes in fer plants grown in the presence of limiting (0.1 μM iron), sufficient (10 μM iron), or excess amounts of iron (100 μM iron). We found that Lenramp1 expression was reduced in fer roots compared with wild type, however, to a lower extent than that of Lenramp1 (Fig. 4, also see Ref. 25). In contrast, expression of Lenramp3 and Leirt2 were not significantly altered in the fer mutant (Fig. 4). Lenramp3 was found to be induced in roots after iron starvation even in the absence of an intact fer gene (Fig. 4). Therefore, expression of Leirt1 and Lenramp1 but not Leirt2 and Lenramp3 is dependent on fer.

To investigate in which tissues Lenramp1 may be active, the expression pattern of the fer-dependent Lenramp1 gene was studied using in situ hybridization on transverse sections of iron-starved roots. We detected hybridization signals in the root epidermis and cortex behind the root tip (not shown) as well as in the vascular parenchyma between xylem and phloem in the root hair zone of wild type roots (Fig. 5 A). In fer mutant

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vacular membrane was stained (Fig. 2F), or staining was found in the cytoplasm (not shown) or, partially, in the plasma membrane (Fig. 2H). Vesicles showing Lenramp1 or Lenramp3-GFP staining were presumably of similar origin. These vesicles were in distinct location from the nucleus (Fig. 2D, shown for Lenramp1-GFP) and located underneath the plasma membrane outside of the vacuole (Fig. 2, C and H). In summary, Lenramp1 and Lenramp3 protein expression and localization patterns are regulated by iron and to a lower extent manganese supply in yeast. Lenramp1 was predomi-
roots, *Lenramp1* hybridization signals were not visible in the vascular parenchyma (Fig. 5D) but were still apparent in the outer cell layers close to the root tip (not shown). As a control we utilized *Leirt2* as an *in situ* hybridization probe. We found that *Leirt2* was expressed in the epidermis and the vascular parenchyma (Fig. 5B). *Leirt2* expression in the vascular parenchyma cells of the root hair zone was apparent in wild type as well as in mutant *fer* roots (Fig. 5E). *fer* expression was localized to the vascular cylinder of the root hair zone and in the outer cells close to the root tip (Fig. 5, C and F; see also Ref. 25). Thus, *Lenramp1* and *Leirt2* expression co-localize with that of *fer* in the vascular parenchyma. Only the cellular expression pattern of *Lenramp1* but not that of *Leirt2* was affected by the *fer* mutation and only within the vascular cylinder.

To analyze whether *fer* may be able to directly activate metal transporter genes, we examined *Lenramp1* and *Leirt1* gene expression in roots and leaves of transgenic tomato plants expressing an intact *fer* cDNA behind the constitutive cauliflower mosaic virus 35S promoter in a *fer* mutant background. *nt1* and *nt2* are two non-transgenic siblings devoid of a functional *fer* gene. The *fer* cDNA was amplified using primers that surrounded the presumptive insertion site in the mutant *fer* allele (25) so that only transcripts from an intact *fer* open reading frame were detectable. Elongation factor 1α (*Leaf-1a*) expression, which occurs in all tissues, growth conditions, and tomato lines, was used as the control.

irrespective of iron supply (Fig. 6, *Leaves −Fe* and +Fe, 35s1 and 35s2). Therefore, we concluded that *fer* is required but not sufficient to direct expression of *Leirt1* and *Lenramp1*.

Expression Analysis of Metal Transporter Genes in *chloronerva* and ferclorornerva Double Mutants—The mutant *chloronerva*, to date the only nicotianamine-free plant mutant, over-accumulates metals and displays up-regulation of iron reduction upon sufficient iron supply (9). We examined whether *chloronerva* mutant plants iron transporter gene regulation was affected. We found that in *chloronerva* roots low iron supply did not significantly induce expression of *Leirt1* and *Lenramp1* as was the case in wild type plants (Fig. 7). However, upon sufficient and excess iron supply, the expression level of these two genes was significantly increased in *chloronerva* plants compared with wild type (Fig. 7). Expression of *Leirt2* appeared unaltered in *chloronerva* mutant versus wild type plants regardless of available iron in the medium. *Lenramp3* appeared to be expressed at comparable or slightly increased level in *chloronerva* mutant versus wild type plants (Fig. 7). Thus, nicotianamine synthase is required for proper regulation of *Lenramp1* and *Leirt1*.

We tested whether a functional *fer* gene was needed for increased expression of *Leirt1* and *Lenramp1* in *chloronerva* mutants. Therefore, we constructed non-segregating double homozygous mutant lines (ferfer; *chloronerva*/chloronerva) as well as lines segregating 1:3 for the double mutant in an otherwise *fer* or *chloronerva* mutant background, respectively. We found that double homozygous mutants could be recognized morphologically by their shoot appearance. *ferchloronerva* double mutant plants had yellow-green cotyledons and very small yellow leaves with yellow veins at both low and sufficient

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**Fig. 4.** Expression analysis of metal transporter genes in roots of *fer* mutant and wild type (WT) plants. Plants were grown in the presence of 0.1, 10.0, and 100.0 μM iron, respectively. As the control elongation factor 1α (*Leaf-1a*), which occurs in all growth conditions and tomato lines, was used.

**Fig. 5.** Localization of gene expression by *in situ* hybridization in transverse sections of iron-starved roots of wild type and *fer* mutant plants. A, wild type tomato, *Lenramp1* antisense probe. B, wild type tomato, *Leirt2* antisense probe. C, wild type tomato, *fer* gene antisense probe. D, *fer* mutant, *Lenramp1* antisense probe. E, *fer* mutant, *Leirt2* antisense probe. F, *fer* mutant, *fer* gene antisense probe. G, wild type tomato, *Lenramp1* sense probe. H, wild type tomato, *Leirt2* sense probe. I, wild type tomato, *fer* gene sense probe. ep, epidermis; co, cortex; xy, xylem. Red arrowheads indicate positive hybridization signals.

**Fig. 6.** Expression analysis in roots and leaves of 35S transgenic plants grown in iron-limiting and iron-sufficient conditions. 35s1 and 35s2 plants express a functional *fer* cDNA behind the constitutive cauliflower mosaic virus 35S promoter in a *fer* mutant background. *nt1* and *nt2* are two non-transgenic siblings devoid of a functional *fer* gene. The *fer* cDNA was amplified using primers that surrounded the presumptive insertion site in the mutant *fer* allele (25) so that only transcripts from an intact *fer* open reading frame were detectable. Elongation factor 1α (*Leaf-1a*) expression, which occurs in all tissues, growth conditions, and tomato lines, was used as the control.

**Fig. 7.** Expression analysis in roots and leaves of 35S transgenic plants grown in iron-limiting and iron-sufficient conditions. 35s1 and 35s2 plants express a functional *fer* cDNA behind the constitutive cauliflower mosaic virus 35S promoter in a *fer* mutant background. *nt1* and *nt2* are two non-transgenic siblings devoid of a functional *fer* gene. The *fer* cDNA was amplified using primers that surrounded the presumptive insertion site in the mutant *fer* allele (25) so that only transcripts from an intact *fer* open reading frame were detectable. Elongation factor 1α (*Leaf-1a*) expression, which occurs in all tissues, growth conditions, and tomato lines, was used as the control.

WT, wild type.
iron supply (Fig. 8A, only – Fe shown). Shoots of double mutant plants were significantly smaller at iron-limiting and sufficient iron supply conditions than shoots of either fer or chloronerva single mutants (Fig. 8B). These observations indicate that double mutants were more severely affected than the single mutants and could not be partially rescued by increasing iron concentrations in the growth medium as was the case for single mutants. Double mutants like the single fer mutant plants did not show iron reductase activity (data not shown). CHLORONERVA transcripts were detectable in fer mutant roots, and FER transcripts were found in chloronerva mutant roots (Fig. 8, C and D), indicating that fer and chloronerva genes are not involved in regulating gene expression of each other.

We found that in the double mutant roots, expression of Leirt1 and Lenramp1 was significantly decreased at the three iron supply conditions tested, as was the case for fer mutant roots (Fig. 7). Expression of Lenramp3 and Leirt2 was not significantly affected in the double mutants (Fig. 7). Therefore, double fer chloronerva mutants had a similar expression pattern of metal transporter genes as the single fer mutant plants showing that the fer gene was required for increased Leirt1 and Lenramp1 expression observed in single chloronerva mutant plants.

**DISCUSSION**

Regarding plants, only for IRT1 a well determined role as membrane-bound transporter mediating iron uptake into roots was proposed to date (2, 3, 7). In the present study we characterized two novel nramp genes from tomato, and we investigated the regulation of four different metal transporter genes belonging to the irt and nramp gene families. Based on our results we suggest a novel function for nramp1 in mobilizing iron upon iron deficiency in the vascular parenchyma in plants. We could establish that a fer/nicotianamine synthase-dependent signal transduction pathway exists that controls expression of iron-regulated genes Leirt1 and Lenramp1. Another regulatory mechanism not dependent on fer and nicotianamine synthase involves Leirt2 and Lenramp3.

**Differential Functions for Metal Transporter Genes**—Sequence comparisons between plant NRAMP amino acid sequences indicate that NRAMP1-type proteins form a conserved subfamily of NRAMP metal transporters in the plant kingdom.

**Fig. 7.** Expression analysis of metal transporter genes in *chloronerva* (*chln*) and *fer chloronerva* double mutants (*fer chln*). Expression was monitored before the start of the experiment (0) and after 8 days of different iron supply treatments, fer mutant plants and wild type tomato serve as controls. Elongation factor 1a (Leef-1a), which occurs in all tissues, growth conditions, and tomato lines, is the control. WT, wild type.
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ior factor in iron uptake from the soil (3). Three observations support that this could be also the case for Leirt1. First, among tomato EST sequences representing putative zip genes, Leirt1 showed the highest sequence similarity with Atirt1. Second, Leirt1 was induced in response to iron starvation. Finally, Leirt1 expression was altered in mutants with well defined defects in iron uptake.

Leirt1 and Lenramp1 were down-regulated in the fer mutant irrespective of iron supply conditions. It was demonstrated by Ling et al. (25) that the fer gene acts upon iron limitation and sufficient iron supply. Only excessive amounts of iron (100 μM) can rescue fer mutant plants. Therefore, Leirt1 and Lenramp1 are presumably not only involved in transporting iron upon iron limitation but also function perhaps to a lower extent in iron transport upon sufficient iron supply. Interestingly, Arabidopsis irt1 mutant plants also require high iron concentrations for rescue (more than 100 μM iron), indicating that Atirt1 functions at sufficient iron supply conditions (10–50 μM iron) where wild type plants would show no obvious symptoms of iron deficiency (3, 7).

Different expression behaviors were observed for Leirt2 and Lenramp3. Leirt2 was found constitutively expressed in roots irrespective of iron supply. Lenramp3, which also encodes a functional metal transporter, was found induced by iron deficiency in roots and is expressed in roots and leaves. Lenramp3 and Leirt2 do not require functional fer and nicotianamine synthase genes for regulation of their expression. Apparently, Leirt2 and Lenramp3 cannot functionally compensate the reduced Leirt1 and Lenramp1 expression in fer mutant plants. Therefore, their function remains rather speculative. It is possible that Leirt2 and Lenramp3 mediate iron uptake only in the absence of Leirt1 and Lenramp1 provided that excessive iron is available. This may also explain why in Arabidopsis neither Atnramp3 nor Atirt2 loss of function leads to an obvious chlorotic phenotype (7, 21). Alternatively, LeIRT2 or LeNRAMP3 may supply iron to particular compartments after iron has been mobilized, function upstream of the fer gene, or transport predominantly other metals than iron.

Distinct functions for nramp genes have also been found in yeast. SMF1 was reported to be present in portions of the plasma membrane, where it may serve metal uptake (42). SMF2 was found in intracellular vesicles of an unknown nature, where it may mobilize metals from intracellular stores (18). Upon metal replete conditions, SMF1 and SMF2 become targeted to the vacuole for degradation (18, 42). SMF2 was only detected in the vacuolar membrane, where it may mobilize iron from vacuolar stores (18). LeNRAMP1 and LeNRAMP3 could complement the smf1 defect. Because LeNRAMP1 and LeNRAMP3 were mainly localized to vesicles upon manganese and iron deficiency in yeast, smf1 complementation may work essentially by release of internal metals, as was proposed for SMF2 function (18). Both NRAMP gene products may be subject to post-transcriptional or post-translational control by iron and, to a lower degree, manganese. GFP fusion protein signals were detected in more cells and localized less frequently to the vacuole upon iron-deficient growth conditions than upon sufficient iron supply. This can be explained by increased protein degradation in the vacuole or lower mRNA stability upon iron supply. Therefore, LeNRAMP1 and LeNRAMP3 may contain signals, allowing mRNA and/or protein regulation in response to iron in yeast. We are currently generating transgenic plants expressing LeNRAMP1- and LeNRAMP3-GFP fusion proteins to test iron-mediated post-transcriptional or post-translational regulation in the plant system. Iron-regulated and ubiquitin-mediated protein degradation was suggested for IRT1 in plants (24).

As a working model we propose the following new function for NRAMP1 in plants. Upon iron deficiency FER may up-regulate Lenramp1 transcription. In addition, LeNRAMP1 may become stabilized at the protein level and mobilize iron in the vascular parenchyma cells. This iron may be utilized for transport toward the shoot to meet rapid iron requirements. LeNRAMP1 may also be involved in further signaling of the iron status. Upon sufficient iron supply, Lenramp1 expression is down-regulated to a low level. This system may allow the plant to rapidly respond to even small changes in iron requirements.

Regulation of Iron Uptake by Nicotianamine and fer—Our expression analysis of metal transporter genes in fer and chloronerva mutant plants suggests that the mutant phenotypes can be explained by inadequate iron transporter regulation. Lenramp1 and fer genes were expressed in similar tissues, opening the possibility that FER might directly activate Lenramp1. However, we failed to demonstrate Leirt1 or Lenramp1 induction when ectopically expressing the fer gene. We have been unsuccessful so far in obtaining binding of FER to E-box sequences contained in the Lenramp1 promoter. BHLH factors usually bind E-box target sequences (CANNTG) as homo- or heterodimers. Several bHLH proteins bind their targets when activated by another protein partner (for example, see Refs. 43–45). If there is direct interaction between FER and the Lenramp1 promoter we predict that FER requires for transcriptional activation the interaction with a root-specific binding partner.

chloronerva (nicotianamine synthase) mutant plants showed decreased expression of Leirt1 and Lenramp1 upon iron starvation and increased expression upon sufficient iron supply compared with wild type. Iron over-accumulation in chloronerva mutant plants, thus, can be explained by increased metal transporter activity despite sufficient iron supply. Altered regulation of metal transporter genes due to the nicotianamine synthase mutation in the chloronerva mutant plants was dependent on a functional fer gene. Regarding iron transporter gene expression and iron reduction in the double mutant, the fer mutant allele acted in an epistatic way to the chloronerva mutant allele. This finding suggests that the fer gene would act before the chloronerva nicotianamine synthase gene in a same pathway. Regarding shoot chlorosis, the two mutations clearly behaved in a synergistic way as the double mutant shoot phenotypes were more severe than those of either single mutant. It can be interpreted that fer and chloronerva genes act together in the same pathway. We could exclude that the fer gene acted upstream of nicotianamine synthase by regulating nicotianamine synthase gene expression and vice versa. To explain our results, we propose the following model. At low iron supply nicotianamine produced by nicotianamine synthase may be required for the fer gene-mediated induction of iron uptake responses. At sufficient iron supply, nicotianamine may repress iron uptake responses via FER. We suggest that nicotianamine acts in the fer gene signaling pathway as the sensor for iron availability. Modulation of nicotianamine action could be achieved by differential binding of metals to nicotianamine. For example, upon sufficient iron nutrition, iron could be bound to nicotianamine, resulting in suppression of these responses, whereas upon iron deficiency other metals may be bound, causing induction of iron deficiency responses. Future studies will show whether nicotianamine may bind directly to a regulator protein or whether nicotianamine acts indirectly by delivering metals to final targets, which then regulate the fer gene pathway.

8 P. Bauer, personal observation.

3 Z. Bereczky, T. Bnumbarova, and P. Bauer, unpublished results.
Differential Regulation of Metal Transporter Genes

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Differential Regulation of \textit{nramp} and \textit{irt} Metal Transporter Genes in Wild Type and Iron Uptake Mutants of Tomato

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