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Involvement of NRAMP1 from Arabidopsis thaliana in iron transport

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NRAMP genes code for a widely distributed class of proteins involved in a variety of processes, ranging from the control of susceptibility to bacterial infection in mammalian cells and taste behaviour in Drosophila to manganese uptake in yeast. Some of the NRAMP proteins in mammals and in yeast are capable of transporting metal ions, including iron. In plants, iron transport was shown to require a reduction/Fe(II) transport system. In Arabidopsis thaliana this process involves the IRT1 and Fro2 genes. Here we report the sequence of five NRAMP proteins from A. thaliana. Sequence comparison suggests that there are two classes of NRAMP proteins in plants: A. thaliana (At) NRAMP1 and Oriza sativa (Os) NRAMP1 and 3 (two rice isologues) represent one class, and AtNRAMP2–5 and OsNRAMP2 the other. AtNramp1 and OsNramp1 are able to complement the fet3fet4 yeast mutant defective both in low- and high-affinity iron transports, whereas AtNramp2 and OsNramp2 fail to do so. In addition, AtNramp1 transcript, but not AtNramp2 transcript, accumulates in response to iron deficiency in roots but not in leaves. Finally, overexpression of AtNramp1 in transgenic A. thaliana plants leads to an increase in plant resistance to toxic iron concentration. Taken together, these results demonstrate that AtNramp1 participates in the control of iron homoeostasis in plants.

Key words: iron deficiency, iron toxicity, Nramp, plant, transport.

INTRODUCTION

The first Nramp gene identified in mammals, Nramp1, was shown to encode an integral membrane protein recruited in phagosomes of infected macrophages [1,2]. Upon mutation of Nramp1, mice become susceptible to mycobacterial infection. More recently, NRAMP proteins have been shown to participate in metal-ion transport. Microcytic anaemia (mk) in mice is due to a missense mutation in the Nramp2 gene [3] that causes a defect in dietary iron uptake, suggesting that mouse NRAMP2 is an iron transporter. The formal proof of this function of NRAMP2 came with the demonstration of the metal-transport defect of the yeast smf1 mutant by complementation of the yeast smf mutation in the mouse Nramp2 gene [10]. Drosophila provides another example of the role of NRAMP in metal transport. The maltoolio (mvI) gene encodes a protein of the NRAMP family which, when mutated, leads to an abnormal taste behaviour in the flies [11]. However this mutant phenotype can be suppressed by an iron-supplemented diet [12].

In plants, biochemical and physiological results have suggested that iron uptake by dicotyledons is mediated by Fe(III) reduction coupled to Fe(II) uptake [13,14] in a way similar to the iron-uptake system occurring in yeast [15]. Arabidopsis thaliana has been used to start to decipher these mechanisms at a molecular level. A new type of metal transporter, IRT1, involved in ferrous iron transport and specifically upregulated in response to iron deficiency in A. thaliana roots, has been cloned by functional complementation of the fet3fet4 yeast mutant defective in both high- and low-affinity iron transport [16]. In addition, the Fro2 gene from A. thaliana, encoding an Fe(III)-chelate reductase, has been cloned by PCR using oligonucleotides derived from the yeast ferric reductase FRE1 and FRE2 gene sequences [17]. Fro2 is able to complement an A. thaliana mutant deficient in root ferric iron reduction and is also upregulated in response to iron deficiency. Although the IRT1/FRO2 system is likely to be a major component of the A. thaliana iron-uptake system, other proteins could be involved in this process. Indeed, three rice genes encoding proteins similar to the mammalian Nramp genes have been cloned, but their role in metal, and more specifically iron, transport has not yet been addressed [18,19].

In this study, we report the sequences of five A. thaliana NRAMP (AtNRAMP) proteins deduced from their corresponding cDNA or gene sequences. Sequence comparison reveals two phylogenetically related subgroups in this multigene family, AtNramp1 and AtNramp2 being representatives of these two classes. We show that AtNramp1, but not AtNramp2, is able to complement a yeast mutant defective in iron uptake. In addition AtNramp1, but not AtNramp2, is upregulated in response to iron

Abbreviations used: AtNRAMP, Arabidopsis thaliana NRAMP; Os, Oriza sativa; M−S medium, Murashige–Skoog medium; TM, transmembrane domain; CTM, consensus transport motif.

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deficiency. Furthermore, transgenic *A. thaliana* overexpressing *AtNramp1* have an increased resistance to toxic iron concentration. Possible implications for *AtNRAMP1* subcellular localization are discussed.

**EXPERIMENTAL**

**Plant material**

Culture of iron-starved plantlets

*A. thaliana* plants, ecotype Columbia, were grown hydroponically for 20–24 days in the presence of sucrose, as described in [20]. Each vessel (Magenta) harboured 10–20 seedlings on 60 ml of nutrient solution (pH 5.7) containing 1 mM CaSO$_4$, 1 mM NH$_4$NO$_3$, 1 mM KH$_2$PO$_4$, 0.5 mM MgSO$_4$, 50 µM NaFe-EDTA, 50 µM H$_2$BO$_3$, 12 µM MnCl$_2$, 1 µM CuCl$_2$, 1 µM ZnCl$_2$, 30 nM (NH$_4$)$_6$Mo$_7$O$_{24}$, 10 g l$^{-1}$ sucrose and 0.5 g l$^{-1}$ Mes. In iron-depletion experiments, NaFe-EDTA was provided initially at 50 µM and omitted for the last 3 or 5 days of culture, as indicated in the legend to Figure 3 (see below). The nutrient medium was renewed every 5 days. Roots and shoots were harvested separately and frozen at −80°C until RNA extraction.

Iron-resistance assays

Homoyzoic T3 seeds were surface-sterilized and sown on plates containing half-strength Murashige–Skoog (M–S) medium without iron. The medium was then supplemented with various concentrations of Fe-EDTA as indicated in the legend to Figure 3 (see below). After 48 h at 4°C, seeds were germinated at 22°C under a 16-h light/8-h dark regime.

**cDNA cloning and sequencing**

An *A. thaliana* cDNA AZAPII library [21] was screened for the presence of clones corresponding to the *A. thaliana* expressed sequence tags Z30530 (*Nramp1*) and N38436 (*Nramp2*). Full-length cDNAs were identified among the positive clones by PCR-based size selection and end-sequencing of the inserts. Full-length cDNA clones for each of the *AtNramp* genes were sequenced using the ABI Prism dye-terminator cycle-sequencing kit Ready Reaction (Perkin-Elmer) and synthetic oligonucleotides.

Computer database searches were performed using BLAST software (version 2.0, revised December 1998) and sequences were aligned using ClustalW (http://www2.ebi.ac.uk/clustalw/) and boxshade (http://www.ch.embnet.org/software/BOX-form.html) software. The dendrogram was generated using the Treeview program.

**Northern blots**

Expression of *AtNramp1* and *AtNramp2* genes in response to iron starvation

RNA preparation and analysis by Northern blotting were as described previously [22]. First, 10 µg of total RNA were loaded per lane. The same blot was hybridized sequentially to each probe and stripped between two hybridization steps. Full-length cDNAs were used to make probes. Washes were performed at 60°C in 0.1× SSC/0.1% SDS (where 1× SSC is 0.15 M NaCl/0.015 M sodium citrate). The filter was exposed to a Phosphor-Imager screen (Molecular Dynamics, Sunnyvale, CA, U.S.A.) for 48 h (*AtNramp1* and *AtNramp2*) or for 17 h (IRTI and EF-1α).

**RESULTS**

Cloning of the *AtNramp1* and *AtNramp2* cDNAs

*AtNramp1* and *AtNramp2* cDNAs were cloned by screening an *A. thaliana* cDNA library [21] for the presence of clones...
corresponding to the A. thaliana expressed sequence tags Z30530 (Nramp1) and N38436 (Nramp2). The AtNramp2 cDNA sequence was reported elsewhere (GenBank accession number AF141204) [29], and the AtNramp1 sequence was determined during the course of this work (GenBank accession number AF165125).

The predicted amino acid sequences of the AtNramp1 and AtNramp2 proteins are very similar to those of their rice, mammalian and fruitfly counterparts. AtNramp1 and AtNramp2 are predicted to encode hydrophilic proteins of 532 and 530 amino acids, respectively (Figure 1A), with twelve putative transmembrane domains (TMs). Both proteins present the highly conserved consensus transport motif (CTM), first identified in bacterial transport proteins [1] in the region located between TMs 8 and 9.

Database searches identified three additional members of the AtNramp gene family, named AtNramp3 (BAC genomic sequence, GenBank accession number AB007645) and AtNramp5 (genomic sequence, GenBank accession number AT258251). Based on sequence homologies, these five genes group into two classes: AtNramp1 on one hand, and AtNramp2–5 on the other, with AtNramp5 being the most divergent (Figure 1A). Proteins of the second class, AtNramp2–5, share 80% identity and 90% similarity with each other, whereas AtNramp1 protein is 47% identical and 66% similar to AtNramp2. Members of the same two classes are also present in rice [18,19], with Oriza sativa (Os) Nramp1 and 3 being most homologous with AtNramp1 and OsNramp2 showing a higher degree of conservation with the AtNramp2–5 class (Figure 1A).

Mammalian NRAMP proteins share 65% similarity with AtNramp1 and as much as 75% similarity with the AtNramp2 class, which is more than the conservation between the two classes of A. thaliana proteins.

When comparing animal and plant NRAMP proteins in the CTM region, we found a remarkable conservation among all organisms (Figures 1B and 1C). The CTM has been proposed, on the basis of its homology with bacterial transport proteins, to be involved in the interaction with ATP-coupling subunits. The evolutionary conservation of this motif reveals its fundamental role in the function of NRAMP proteins.

**AtNramp1 restores the growth of the iron-deficient yeast mutant under iron-limited conditions**

The mammalian Nramp2/DCT1 genes have been shown to encode the primary transport system involved in dietary iron uptake in the small intestine. Therefore, in order to investigate the role of NRAMP in iron assimilation in plants, we set out to test whether expression of the A. thaliana Nramp genes in the yeast double mutant fet3fet4 (strain DEY1453) could correct the phenotype associated with the inactivation of the yeast high- and low-affinity iron-uptake systems of this strain. The AtNramp1 cDNA and one representative of the second class, the AtNramp2 cDNA, were chosen to be expressed in yeast. Both cDNAs were subcloned in the yeast expression vector pYPGE15 under the control of the strong pGK promoter (see Materials and methods). The A. thaliana iron transporter IRT1 cDNA, cloned by functional complementation of the fet3fet4 mutant [16] was used as a positive control in this experiment. Plasmids were introduced into the fet3fet4 mutant and transformants were selected on selective medium. Since the fet3fet4 mutant cannot grow under iron-limited conditions, we tested the ability of the transformants to grow on minimal medium supplemented with iron in a range of concentrations, from 1 to 100 μM (Figure 2A) compared with the growth of the same strain transformed with the original pYPGE15 vector. Figure 2(A) indicates that transformants with the pYPGE15 control vector were detected at 100 μM iron(III) citrate, whereas IRT1 expression allowed cells to grow in the absence of added iron. Expression of AtNramp1 restored the growth of the mutant above 5 μM iron(III) citrate, at a level intermediate between that of the IRT1 transformant and that of the pYPGE15 control. In contrast to AtNramp1, the AtNramp2 expression plasmid did not restore cell growth since AtNramp2-expressing yeast only grew at 100 μM iron(III) citrate, like the pYPGE15 control. These results indicate that, like IRT1, A. thaliana Nramp1 can functionally complement the growth defect on iron-limited medium of a yeast lacking functional high- and low-affinity iron uptakes.

To test whether expression of AtNramp1 can restore iron uptake in the fet3fet4 mutant, we examined the uptake of radioactive 55Fe(II) in this strain. Although we found an enhanced rate of Fe uptake by the IRT1-transformed cells, we failed to show Fe uptake in yeast expressing AtNramp1 using the same experimental conditions (Figure 2B). Moreover, expression of AtNramp1 in Xenopus oocytes did not produce detectable currents (results not shown), as did DCT1 under similar conditions [4]. Therefore, we have been unable to show transport properties of AtNRAMP1.

**The function of the A. thaliana NRAMP1 and NRAMP2 proteins is conserved in rice**

The cloning of three rice cDNAs encoding NRAMP, OsNramp1, OsNramp2 and OsNramp3, has been reported previously [18,19]. The authors have shown a differential expression for the three genes, with OsNramp1 being mainly expressed in roots, OsNramp2 in shoots and OsNramp3 in both roots and shoots [19]. However, the function of the proteins encoded by these three genes has not been investigated yet. Figure 1(A) shows the high degree of homology between AtNRAMP1 and OsNramp1 and 3 on one hand, and between AtNRAMP2 and OsNramp2 on the other. Because the OsNramp3 cDNA was incomplete, we chose the OsNramp1 and OsNramp2 cDNAs as representatives for the two subclasses of plant Nramp genes to investigate their role in iron assimilation. Functional complementation of the yeast mutant fet3fet4 was used as previously for this analysis. The OsNramp1 and OsNramp2 cDNAs, provided kindly by Professor Gros’ laboratory (Department of Biochemistry, McGill University, Montreal, Canada), were subcloned into the pYPGE15 vector and the resulting plasmid was transformed into the fet3fet4 strain. Growth of the transformants was monitored on minimal medium supplemented with a range of iron concentrations, from 0 to 100 μM (Figure 2A). It showed that OsNramp1, but not OsNramp2, is able to improve fet3fet4 growth. However complementation occurred solely above 20 μM iron citrate, whereas IRT1 and AtNramp1 complemented at 0 and 5 μM iron citrate, respectively. We conclude from these data that, in rice as in A. thaliana, representatives of the first NRAMP subclass are able to complement the yeast iron-uptake function, whereas representatives of the second subclass are not.

**Expression of AtNramp1 and AtNramp2 in response to iron**

We wished to determine the pattern of expression of AtNramp1 and AtNramp2 in response to iron. Expression of the transcripts was assessed by high-stringency Northern hybridization using total RNA from roots and shoots of A. thaliana plants grown under iron-sufficient or iron-deficient conditions. The entire cDNAs of AtNramp1 and AtNramp2 were used as gene-specific
Figure 1 Sequence conservation between plant NRAMP proteins

(A) Multiple alignments of deduced amino acid sequences of five *A. thaliana* and three *O. sativa* Nramp genes. Lines above the sequences indicate the positions of the predicted transmembrane domains (TMs). The consensus transport motif (CTM) between TMs 8 and 9 is boxed. 

(B) Alignment of mammalian, fruitfly, plant and yeast NRAMP sequences in the CTM domain. 

(C) Phylogenetic tree of the NRAMP proteins from the species aligned in (B) and based on their CTM sequence. GenBank® accession numbers: OsNramp1, L41217; OsNramp2, L81152; OsNramp3, U60767; AtNramp1, AF141204; AtNramp2, AF141204; AtNramp3, ATAC004401; AtNramp4, AB007645; AtNramp5, ATF28A21.

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NRAMP from Arabidopsis thaliana

Figure 2 Concentration-dependent growth restoration of the yeast fet3fet4 mutant by plant NRAMP proteins

(A) The cDNAs expressed in fet3fet4 (strain DEY1453) are indicated to the left. Overnight yeast cultures (0.2 and 0.02 absorbance units) in SD minimal medium were deposited on synthetic minimal medium supplemented with 0, 5, 20 or 100 µM iron(III) citrate and grown for 5 days at 30 °C. (B) Iron accumulation in strain DEY1453 transformed with the same plasmids as in (A). Assays were performed with 5 µM 55Fe(II) ascorbate-containing MG medium. Values represent the difference between iron accumulation at 30 °C and that at 0 °C and are the means for at least three independent experiments.

Overexpressing AtNramp1 in A. thaliana decreases sensitivity to toxic iron levels

To study the effect of altered levels of AtNRAMP1 in A. thaliana, we expressed AtNramp1 mRNA under the control of the strong cauliflower mosaic virus 35 S promoter in transformed A. thaliana plant lines. The level of expression of the transgene is shown on a Northern blot for three different transgenic lines in Figure 4(A). Homozygous T3 seeds were germinated on half-strength M-S medium containing either 50 µM Fe-EDTA or a
range of toxic iron levels, from 300 to 800 \(\mu M\). In the presence of 50 \(\mu M\) iron, a concentration non-toxic for \textit{A. thaliana} germination and growth, both wild-type and overexpressing lines (+17, +23 and +24) grew equally well (Figure 4B). Concentrations of iron reaching 700 \(\mu M\) and higher inhibited germination and growth of all the plants. At 600 \(\mu M\) all the plants germinated and showed equal growth for the first 8–10 days. However, at this concentration growth of wild-type plants suddenly stopped and the plants turned brown, whereas plants overexpressing \textit{AtNRAMP1} remained green and continued to grow (Figure 4B). Therefore, increasing \textit{AtNramp1} expression in the plants allowed better growth in the presence of high iron concentrations, with a decrease in the sensitivity of the plants to iron toxicity. This finding suggests a role for \textit{AtNRAMP1} in iron distribution in the cell, rather than in iron uptake from the soil. This phenotype was also confirmed by the observation that \textit{A. thaliana} plants disrupted in the \textit{At Nramp} gene showed a higher sensitivity to toxic iron levels than the wild-type plants (results not shown).

DISCUSSION

\textbf{\textit{AtNRAMP1} is involved in iron homeostasis in yeast and plants}

In \textit{A. thaliana} there are at least five genes coding for members of the NRAMP family of metal-ion transporters, first described in mammals and yeast. These proteins are highly conserved throughout evolution. The \textit{AtNRAMP} proteins contain 12 predicted TMs as well as the characteristic CTM between TMs 8 and 9 (Figure 1). Such NRAMP proteins were previously described in rice [18,19] but no function in metal transport had been assigned to them.

Among the five members of the \textit{A. thaliana} multigene family, \textit{AtNramp1} constitutes a distinct subclass, based on sequence comparisons. Choosing the \textit{AtNramp1} and \textit{AtNramp2} genes as representatives of the two subclasses, we found that they are inversely regulated in response to the iron status. Indeed, in roots of iron-starved \textit{A. thaliana} plantlets, \textit{AtNramp1} expression is upregulated whereas \textit{AtNramp2} seems to be downregulated (Figure 3). In addition, complementation of the yeast \textit{fet3 fet4} strain on low iron content occurred with \textit{AtNramp1} but not with \textit{AtNramp2} (Figure 2). This result was confirmed with the two rice isolouges, \textit{OsNramp1} and \textit{OsNramp2}, that respectively behaved like \textit{AtNramp1} and \textit{AtNramp2} in the yeast complementation test (Figure 2). Since both \textit{AtNramp1} and \textit{OsNramp1} genes restore growth of the yeast iron-uptake mutant in iron-limiting conditions, we conclude that their products are indeed involved in iron assimilation. In addition, we show that overexpression of \textit{AtNramp1} in \textit{A. thaliana} leads to an increase in the plant’s resistance to toxic levels of extracellular iron (Figure 4). Therefore, the \textit{AtNRAMP1} protein must play a role in iron homeostasis in transformed yeast and in plants.

Involvement of NRAMP in the transport of iron, in addition to other divalent metal cations, has already been shown in mammals and \textit{Drosophila} [3–6,12,30], but was only recently reported for Smf1p, the major yeast NRAMP protein [9]. We found that \textit{AtNramp1} expression efficiently complements the growth defect of the \textit{smf1} yeast mutant (results not shown) as was previously reported with the mouse \textit{Nramp2} gene [10]. This suggests that \textit{A. thaliana} NRAMP, like its yeast, mammalian and fruitfly counterparts, may exhibit a broader substrate specificity than just iron. The recent complementation of \textit{Drosophila} mutant \textit{mvl} by a mammalian \textit{Nramp} gene [31] underscores the level of conservation in the function of this family of proteins.

However, regulation of the expression of the \textit{Nramp} genes in response to iron conditions differs in these species. Yeast Smf1p is exclusively controlled at the level of protein stability and protein sorting to the vacuole involving the BSD2 gene product [30,32]. On the other hand, both mammalian \textit{DCT1/Nramp2} and plant \textit{Nramp1} expression are, at least in part, regulated at the level of their transcription and/or mRNA stability. The \textit{DCT1} gene contains a putative iron-responsive element (IRE) in its 3’ untranslated region [4] which could, by analogy with the transferrin receptor mRNA, regulate \textit{DCT1} mRNA levels by RNA degradation. However, we did not find any IREs in the plant isologues.

Is \textit{AtNRAMP1} localized in an intracellular compartment?

We could not prove that iron uptake in yeast is enhanced by the expression of \textit{AtNramp1} (Figure 2). It is possible that \textit{AtNRAMP1} protein is mis-addressed in a subcellular compartment in yeast upon expression from a multicopy plasmid, instead of being targeted to the plasma membrane. Many proteins, when overexpressed in yeast, follow a default pathway towards the vacuole. Alternatively, \textit{AtNRAMP1} may be localized correctly, for instance in an internal membrane of the yeast cell where it could act in the re-distribution of iron in the cell, unloading it from a specific storage compartment. A study with a vacuolar mutant showed that vacuoles are involved in iron storage in yeast [33]. In addition, a dynamic flux of iron through yeast mitochondria was shown to be at least in part regulated by YFH1, a protein that mediates iron efflux from mitochondria [34]. Interestingly, mutated versions of yeast \textit{Smf1p} that exhibit wild-type complementation activity of an \textit{smf1A} strain show an intracellular localization [32], which suggests that Smf1p can act either at the cell surface or in an intracellular compartment to modulate the cytosolic metal content according to cellular needs.

The fact that \textit{A. thaliana} plants overexpressing \textit{AtNramp1} are less sensitive to toxic iron is also in contradiction with a localization of the transporter on the plasma membrane and rather suggests that \textit{AtNRAMP1} is targeted to an internal membrane of the cell. Co-suppression between the transgene and the endogenous gene, which could explain such resistance of the overexpressers, can be excluded since (i) all five independent transgenic lines tested (three of which are shown in Figure 4) had the same phenotype, even though the transgene mRNA accumulation differed greatly between these lines, and (ii) preliminary experiments performed with an \textit{A. thaliana} line disrupted in the \textit{AtNramp1} gene indicate that this mutant plant is more sensitive to toxic iron levels than the wild-type plant (results not shown). These data suggest that the \textit{AtNRAMP1} protein could be involved in intracellular iron transport to a sequestering compartment, thereby contributing to iron resistance.

A likely compartment to perform such iron storage in plants is the vacuole. Although there is evidence of zinc transport and storage in the plant vacuole [35,36] and of iron storage in yeast vacuoles [37], no data yet indicate that plant vacuoles can store iron. On the other hand, plastids represent a well-known storage compartment in plants for excess cellular iron where it is sequestered by ferritins. Computer prediction of the \textit{AtNRAMP1} protein sorting in the cell suggests a localization in plastids, based on the presence of a putative N-terminal transit peptide. It is possible that overexpressing \textit{AtNramp1} under the control of the ubiquitous strong 35 S promoter leads to an increase of \textit{AtNRAMP1} protein in the membrane of all the plant plastids, including chloroplasts in the shoots, thereby leading to iron sequestration in this compartment and to a higher resistance. However, no significant differences were detected in shoot iron content between wild-type and overexpressing lines (results not shown).
Hypothetical role of AtNRAMP1 in plants

How can we reconcile the upregulation of AtNramp1 expression by iron starvation with its detoxifying role and putative intracellular localization? Northern-blotting experiments do indicate that both IRT1 and AtNramp1 mRNAs accumulate when iron is limiting. However, the timing of expression of the two mRNAs is slightly different: IRT1 responds more rapidly than AtNramp1 since IRT1 mRNA signal decreases between 3 and 5 days of iron starvation while the AtNramp1 mRNA signal keeps increasing (Figure 3). A possible explanation could be that, once IRT1 expression is induced, cations (Fe\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\) and Cd\(^{2+}\)) are massively taken up by plant cells, which must then react quickly to prevent accumulation of free ions in the cytosol that can cause cell damage. In turn, AtNramp1 could be turned on to mediate sequestration of free cytosolic iron, and possibly other ions, into a cellular compartment, such as the plastids or the vacuole.

The precise role of AtNramp1 in iron homeostasis in normal plant cells remains elusive. However, based on the data presented here, we propose that it is involved in iron distribution in the cell, mainly in the roots, in response to iron starvation. We will need to address AtNramp1 specificity towards the various metal cations known to be substrates for the animal and yeast NRAMP proteins. At last, the establishment of its pattern of expression in the plant tissues, as well as its subcellular localization, will help clarify the function of the AtNRAMP1 protein in the plant.

Note added in proof (received 22 March 2000)

cDNAs corresponding to three of the five AtNramp genes have been cloned and characterized by J. Schroeder and co-workers [38]. They show that AtNRAMP proteins can transport cadmium as well as iron.

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