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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 homeostasis 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 heterologous expression in *Xenopus* oocytes of the rat *Nramp2* isologue DCT1 [4]. DCT1 was shown to be a proton-coupled iron transporter whose activity depends on cell-membrane potential. In addition to Fe<sup>2+</sup>, DCT1 also transports Zn<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, Ni<sup>2+</sup> and Pb<sup>2+</sup>. It is expressed ubiquitously, most notably in the proximal duodenum, where it is upregulated in response to iron deficiency. Based on the transport function of NRAMP2, NRAMP1 was also proposed to act as an iron transporter [5,6]. However, it is not clear whether it depletes the phagosome of the iron necessary to support bacterial growth, or induces an oxidative damage in the bacteria by overloading the phagosome with this metal.

*Saccharomyces cerevisiae* also expresses two *Nramp* homologues, *SMF1* and *SMF2* [7], whose function in transport of Mn<sup>2+</sup> [7], Cu<sup>2+</sup>, Co<sup>2+</sup> and Cd<sup>2+</sup> [8], and more recently transport of Fe<sup>2+</sup> [9], has been reported. The similarity between mammalian NRAMP2 and yeast SMF proteins was confirmed by complementation of the metal-transport defect of the yeast *smf* mutant by the mouse *Nramp2* gene [10]. *Drosophila* provides another example of the role of NRAMP in metal transport. The *malvolio* (*mlv*) 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<sub>4</sub>, 1 mM NH<sub>4</sub>NO<sub>3</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 50 μM NaFe-EDTA, 50 μM H<sub>3</sub>BO<sub>3</sub>, 12 μM MnCl<sub>2</sub>, 1 μM CuCl<sub>2</sub>, 1 μM ZnCl<sub>2</sub>, 30 nM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 10 g·l<sup>-1</sup> sucrose and 0.5 g·l<sup>-1</sup> 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

Homozygous 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 4 (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 λZAPII 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 (*IRT1* and *EF-1α*).

Expression level of the transgene in *A. thaliana* lines overexpressing *AtNramp*

Total RNA from T2 transgenic plants was prepared as described in [23]. Northern blots were performed with 20 μg of total RNA per lane and probed sequentially with *AtNramp1* and rDNA. Hybridization and washing conditions were as described above.

### Plasmid constructs

Plasmids for expression in yeast

The entire open reading frames of *AtNramp1* and *AtNramp2* cDNAs were subcloned from pBluescript into the yeast expression vector pYPGE15 [24]. The 1.8-kbp *Bam*HI–*Kpn*I fragment of *AtNramp1* cDNA was cloned into the pYPGE15 vector cut by the same enzymes, whereas the 1.8-kbp *Eco*RI fragment of *AtNramp2* cDNA was cloned into the pYPGE15 vector cut by *Eco*RI and dephosphorylated.

Plasmids for overexpression of *AtNramp1* in *A. thaliana*

The entire coding region of the *AtNramp1* cDNA was subcloned from the pBluescript vector into the plant expression vector pRok2 [25]. The *Bam*HI–*Kpn*I *AtNramp1* cDNA fragment was introduced into the corresponding sites of the pRok2 vector to generate the pRok-Nramp1 plasmid.

### Plant transformation

The C58 strain of *Agrobacterium tumefaciens* containing the pRok-Nramp1 construct was used to transform *A. thaliana* ecotype Col-0 by vacuum infiltration [26]. Transformed T1 plants were selected on kanamycin-containing M–S plates and transferred to soil for propagation.

### Yeast experiments

Yeast cells were grown essentially as in [27] except that the medium was supplemented with 50 μM Fe(III)-EDTA. The pH of the SD minimal medium (0.67 % Bacto-Yeast Nitrogen Base without amino acids/2 % D-glucose) was adjusted to 4.2 for optimal growth of the *fet3fet4* strain. In growth-test experiments, cultures were spotted at an absorbance of 0.2 and 0.02 on a synthetic minimal medium supplemented with iron(III) citrate (20:1, sodium citrate/FeSO<sub>4</sub>) at the concentrations indicated in the legend to Figure 2 (see below). The yeast double mutant *fet3fet4*, strain DEY1453 (*MATa/MATα ade2/+ can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2::HIS3/fet3-2::HIS3 fet4-1::LEU2/fet4-1::LEU2*) was transformed using standard procedures [28]. Iron-uptake experiments were performed essentially as described in [16] except that MG buffer (10 mM Mes, pH 6.1/2 % glucose) without nitrilotriacetic acid was used for the assay buffer. Iron was maintained under its ferrous form by adding 1 mM sodium ascorbate in the assays.

## 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 hydrophobic 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 ATAC004401), *AtNramp4* (TAC genomic sequence, GenBank® accession number AB007645) and *AtNramp5* (genomic sequence, GenBank® accession number ATF28A21). 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 over 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  $\mu$ M (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  $\mu$ M 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  $\mu$ M 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  $\mu$ M 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  $^{55}\text{Fe(II)}$  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  $\mu$ M (Figure 2A). It showed that *OsNramp1*, but not *OsNramp2*, is able to improve *fet3fet4* growth. However complementation occurred solely above 20  $\mu$ M iron citrate, whereas *IRT1* and *AtNramp1* complemented at 0 and 5  $\mu$ M 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

**A**

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AtNramp1      1 .....MAATGSGRSQFISSSGGNRFSFNSPLIENSDSNQIIVSEKRSWKNFFAYLGP
OsNramp3      1 .....IPGP
OsNramp1      1 .....MGVTRAAEAATGKVVDDIEALADLRKEPAWKKFLSHIGP
AtNramp2      1 MENVVKENLEEEEDRLLFPFPPSQ...SLPSTDS.EEAAAFETNEILVDFESPDDPTTGD...PFFFSWRKRLWLF...
AtNramp3      1 MTGSTVSRQENSPKRPNSNGEFKRLLVPEFSQPEDELDHESPPENQILNVEEDRD...KYVDS...VPPFSWKKLWLF...
AtNramp4      1 .....MQLENNEPLTNEE...E.ETAYDETHVHIVRNEEEDLEHGVCGGAPF...FSWKKLWLF...
AtNramp5      1 .....MSETDRRERPLASEER...AYET...V...V...G...D...E...D...Y...D...D...P...G...S...P...K...F...S...W...K...L...W...L...F...T...G...P...
OsNramp2      1 .....MRA...F...S...W...R...K...L...W...L...F...T...G...P...

AtNramp1      53 GFLVSIAYIDDPGNFETDLQAGAHYKYVLLWVILIASCAALVLOSIAANLGVVVTGRHLA...QCRAEYSKVPNFHMLWVVAEY
OsNramp3      5 GFLVSIAYIDDPGNFETDLQAGAHYKYVLLWVILIASCAALVLOSIAANLGVVVTGRHLA...QCRAEYSKVPNFHMLWVVAEY
AtNramp1      41 GFLVSIAYIDDPGNFETDLQAGAHYKYVLLWVILIASCAALVLOSIAANLGVVVTGRHLA...QCRAEYSKVPNFHMLWVVAEY
AtNramp2      75 GFLVSIAYIDDPGNFETDLQAGAHYKYVLLWVILIASCAALVLOSIAANLGVVVTGRHLA...QCRAEYSKVPNFHMLWVVAEY
AtNramp5      77 GFLVSIAYIDDPGNFETDLQAGAHYKYVLLWVILIASCAALVLOSIAANLGVVVTGRHLA...QCRAEYSKVPNFHMLWVVAEY
AtNramp3      63 GFLVSIAYIDDPGNFETDLQAGAHYKYVLLWVILIASCAALVLOSIAANLGVVVTGRHLA...QCRAEYSKVPNFHMLWVVAEY
AtNramp4      59 GFLVSIAYIDDPGNFETDLQAGAHYKYVLLWVILIASCAALVLOSIAANLGVVVTGRHLA...QCRAEYSKVPNFHMLWVVAEY
OsNramp2      17 GFLVSIAYIDDPGNFETDLQAGAHYKYVLLWVILIASCAALVLOSIAANLGVVVTGRHLA...QCRAEYSKVPNFHMLWVVAEY

AtNramp1      132 AVVACDIPFVIGTAFALNMLFS...IFVWVIGYVLLGPTSTLILITAJONKYGVRKLEFLIAFVVFH...ICFFVFLHYKFD...
OsNramp3      85 AVVACDIPFVIGTAFALNMLFK...SLCGVLLITGISTLMLLLIQYGVRRKLEFLIAFVVFH...ICFFVFLHYKFD...
OsNramp1      120 AVVACDIPFVIGTAFALNMLFH...IFVWVIGYVLLGPTSTLILITAJONKYGVRKLEFLIAFVVFH...ICFFVFLHYKFD...
AtNramp2      154 ALIGADIQEVIGSAIAT...LLSRGFLPLWAGVVI...TASDCFFLFLS...Y...K...C...G...M...R...K...L...E...G...L...F...A...V...L...I...A...T...M...L...S...F...A...M...F...G...Q...K...P...S...G...E...
AtNramp5      156 ALIGADIQEVIGSAIAT...LLSRGFLPLWAGVVI...TASDCFFLFLS...Y...K...C...G...M...R...K...L...E...G...L...F...A...V...L...I...A...T...M...L...S...F...A...M...F...G...Q...K...P...S...G...E...
AtNramp3      142 ALIGADIQEVIGSAIAT...LLSRGFLPLWAGVVI...TASDCFFLFLS...Y...K...C...G...M...R...K...L...E...G...L...F...A...V...L...I...A...T...M...L...S...F...A...M...F...G...Q...K...P...S...G...E...
AtNramp4      138 ALIGADIQEVIGSAIAT...LLSRGFLPLWAGVVI...TASDCFFLFLS...Y...K...C...G...M...R...K...L...E...G...L...F...A...V...L...I...A...T...M...L...S...F...A...M...F...G...Q...K...P...S...G...E...
OsNramp2      96 ALIGADIQEVIGSAIAT...LLSRGFLPLWAGVVI...TASDCFFLFLS...Y...K...C...G...M...R...K...L...E...G...L...F...A...V...L...I...A...T...M...L...S...F...A...M...F...G...Q...K...P...S...G...E...

AtNramp1      210 VLEHGLFVPCQKNGATGLAISLLGAVVPHN...FLHSALV...SRKI...ERSASGIER...CRFV...L...E...S...G...L...A...L...M...V...A...F...L...N...I...S...V...I...S...
OsNramp3      163 VLEHGLFVPCQKNGATGLAISLLGAVVPHN...FLHSALV...SRKI...ERSASGIER...CRFV...L...E...S...G...L...A...L...M...V...A...F...L...N...I...S...V...I...S...
OsNramp1      198 VLEHGLFVPCQKNGATGLAISLLGAVVPHN...FLHSALV...SRKI...ERSASGIER...CRFV...L...E...S...G...L...A...L...M...V...A...F...L...N...I...S...V...I...S...
AtNramp2      234 LMLGILLPRLSSK...TIRQAVGVV...C...V...I...M...P...H...N...V...F...L...H...S...A...L...V...O...S...R...E...V...D...E...K...K...R...R...V...O...A...L...N...Y...T...I...E...S...T...A...L...E...S...F...L...N...L...F...V...T...
AtNramp5      236 LMLGILLPRLSSK...TIRQAVGVV...C...V...I...M...P...H...N...V...F...L...H...S...A...L...V...O...S...R...E...V...D...E...K...K...R...R...V...O...A...L...N...Y...T...I...E...S...T...A...L...E...S...F...L...N...L...F...V...T...
AtNramp3      222 LMLGILLPRLSSK...TIRQAVGVV...C...V...I...M...P...H...N...V...F...L...H...S...A...L...V...O...S...R...E...V...D...E...K...K...R...R...V...O...A...L...N...Y...T...I...E...S...T...A...L...E...S...F...L...N...L...F...V...T...
AtNramp4      218 LMLGILLPRLSSK...TIRQAVGVV...C...V...I...M...P...H...N...V...F...L...H...S...A...L...V...O...S...R...E...V...D...E...K...K...R...R...V...O...A...L...N...Y...T...I...E...S...T...A...L...E...S...F...L...N...L...F...V...T...
OsNramp2      176 LMLGILLPRLSSK...TIRQAVGVV...C...V...I...M...P...H...N...V...F...L...H...S...A...L...V...O...S...R...E...V...D...E...K...K...R...R...V...O...A...L...N...Y...T...I...E...S...T...A...L...E...S...F...L...N...L...F...V...T...

AtNramp1      288 VSGAVCNAPNLSPEDRAN...CED...LDINR...K...A...S...F...L...L...R...N...V...V...K...W...S...S...K...L...F...A...I...A...L...L...A...S...G...Q...S...T...I...G...T...Y...A...G...Q...Y...V...M...G...C...F...L...D...R...L...E...P...W...L...
OsNramp3      242 VNNVTCGSDNLSPE...D...Q...N...C...S...D...LDINR...K...A...S...F...L...L...R...N...V...V...K...W...S...S...K...L...F...A...I...A...L...L...A...S...G...Q...S...T...I...G...T...Y...A...G...Q...Y...V...M...G...C...F...L...D...R...L...E...P...W...L...
OsNramp1      276 VSGTVCNAPNLSPE...D...A...V...R...C...S...D...LDINR...K...A...S...F...L...L...R...N...V...V...K...W...S...S...K...L...F...A...I...A...L...L...A...S...G...Q...S...T...I...G...T...Y...A...G...Q...Y...V...M...G...C...F...L...D...R...L...E...P...W...L...
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AtNramp4      370 RNLVTRCLAIIP...S...I...V...A...L...I...G...G...S...A...G...A...G...L...I...I...A...S...M...I...L...S...F...E...L...P...F...A...L...V...P...L...L...K...F...T...C...K...T...R...G...S...H...V...N...P...M...A...I...T...A...L...T...V...I...G...G...L...M...
OsNramp2      328 RNLVTRCLAIIP...S...I...V...A...L...I...G...G...S...A...G...A...G...L...I...I...A...S...M...I...L...S...F...E...L...P...F...A...L...V...P...L...L...K...F...T...C...K...T...R...G...S...H...V...N...P...M...A...I...T...A...L...T...V...I...G...G...L...M...

AtNramp1      444 GNLNHYVYVSS...F...I...K...L...L...I...H...S...H...M...K...L...L...V...V...F...C...G...I...G...F...A...G...A...L...V...L...A...A...Y...L...V...F...R...K...N...R...V...A...T...S...L...L...S...R...D...S...Q...N...V...E...T...I...P...R...Q...D...I...V...N...M...Q...L...P...
OsNramp3      432 GNLNHYVYVSS...F...I...K...L...L...I...H...S...H...M...K...L...L...V...V...F...C...G...I...G...F...A...G...A...L...V...L...A...A...Y...L...V...F...R...K...N...R...V...A...T...S...L...L...S...R...D...S...Q...N...V...E...T...I...P...R...Q...D...I...V...N...M...Q...L...P...
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AtNramp5      468 IINGYLLDFF...F...M...E...V...D...G...L...V...G...V...V...C...V...W...T...A...Y...A...F...I...V...L...I...S...H...S...F...F...S...P...W...S...S...S...I...E...L...P...K...R...V...S...T...S...
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AtNramp4      450 IINGYLLDFF...F...M...E...V...D...G...L...V...G...V...V...C...V...W...T...A...Y...A...F...I...V...L...I...S...H...S...F...F...S...P...W...S...S...S...I...E...L...P...K...R...V...S...T...S...
OsNramp2      408 IINGYLLDFF...F...M...E...V...D...G...L...V...G...V...V...C...V...W...T...A...Y...A...F...I...V...L...I...S...H...S...F...F...S...P...W...S...S...S...I...E...L...P...K...R...V...S...T...S...

AtNramp1      524 CRVSTSDVD
OsNramp3
OsNramp1      510 EDSKEPPV
AtNramp2
AtNramp5
AtNramp3
AtNramp4
OsNramp2

```

**B**

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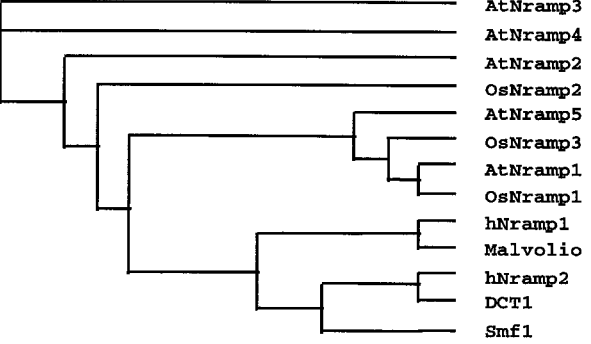
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Malvolio     GQSSTMTGTYAGQFVME...GFLN

AtNramp1      GQSSTITGTYAGQYV...M...G...C...F...L...D
OsNramp1      GQSSTITGTYAGQYV...M...G...C...F...L...D
OsNramp3      GQSSTITGTYAGQYV...M...G...C...F...L...D
AtNramp5      GQSSTITGTYAGQF...I...M...G...F...L...N
AtNramp3      GQSSTITGTYAGQF...I...M...G...F...L...N
AtNramp4      GQSSTITGTYAGQF...I...M...G...F...L...N
AtNramp2      GQSSTITGTYAGQF...I...M...G...F...L...N
OsNramp2      GQSSTITGTYAGQF...I...M...G...F...L...N

smf1         GQSAGVVC...T...M...S...G...Q...I...V...S...E...G...H...I...N

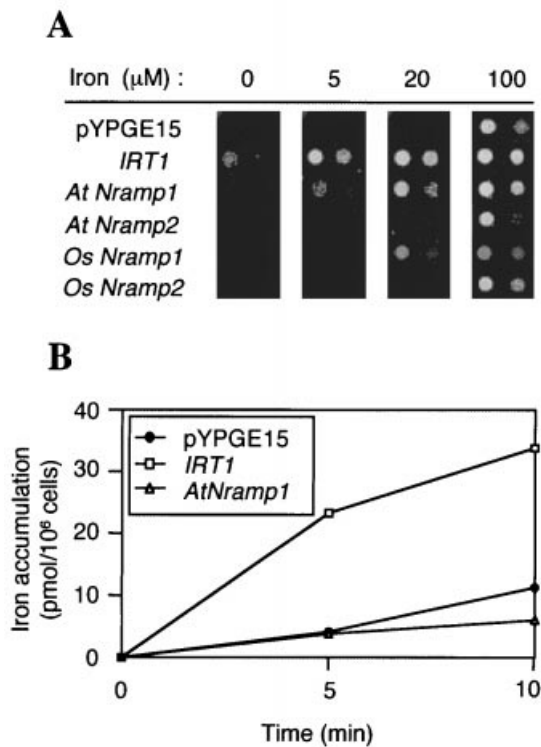
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**C**



**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*, AF165125; *AtNramp2*, AF141204; *AtNramp3*, ATAC004401; *AtNramp4*, AB007645; *AtNramp5*, ATF28A21.



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

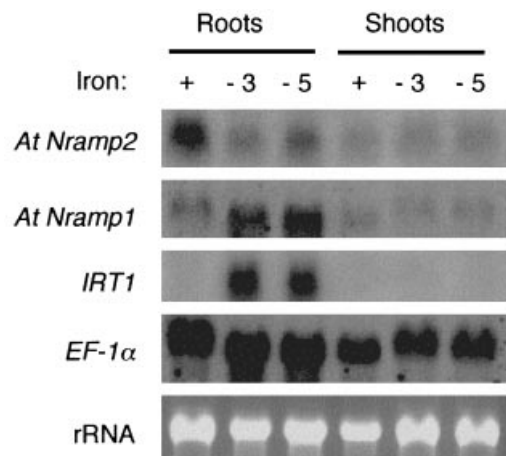
(A) The cDNAs expressed in *fet3fet4* (strain DEY1453) are indicated to the left. Overnight yeast cultures (0.2 and 0.02 absorbance units) in SD minimal medium were deposited on synthetic minimal medium supplemented with 0, 5, 20 or 100  $\mu\text{M}$  iron(III) citrate and grown for 5 days at 30 °C. (B) Iron accumulation in strain DEY1453 transformed with the same plasmids as in (A). Assays were performed with 5  $\mu\text{M}$   $^{55}\text{Fe(II)}$  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.

probes (Figure 3). *IRT1* mRNA had previously been shown to accumulate specifically in roots of iron-deficient plants [16] and was therefore used as a positive control, whereas the constitutively expressed translation elongation factor EF-1 $\alpha$  served as a loading control (Figure 3). *AtNramp1* mRNA is expressed weakly in shoots and roots and is upregulated by iron starvation. Likewise, *IRT1* expression is induced under iron deficiency specifically in roots, but unlike *AtNramp1*, *IRT1* transcript is neither detectable in roots of iron-sufficient plants nor in shoots of both iron-deficient and iron-sufficient plants. *IRT1* and *AtNramp1* seem to respond with a different time course to the iron status; indeed, while the *IRT1* hybridization signal was stronger at day 3 of iron starvation, *AtNramp1* mRNA accumulation increased between 3 and 5 days of iron starvation. Hence, it is likely that the *IRT1* response to iron starvation is faster than that of *AtNramp1*.

In contrast to *AtNramp1* and *IRT1*, *AtNramp2* transcript is highly expressed in roots of iron-sufficient plants and is slightly down-regulated by iron starvation (Figure 3). Like *AtNramp1*, *AtNramp2* is faintly expressed in shoots where it does not respond to iron status.

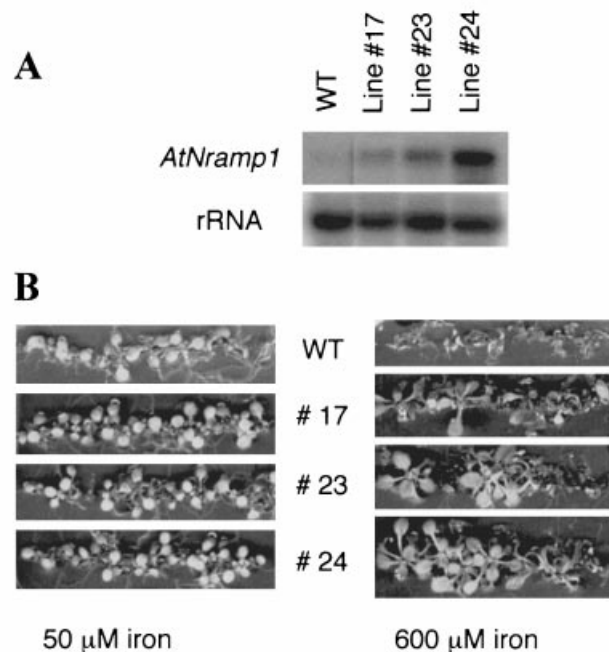
#### Overexpressing *AtNramp1* in *A. thaliana* decreases sensitivity to toxic iron levels

To study the effect of altered levels of AtNRAMP1 in *A. thaliana*, we expressed *AtNramp1* mRNA under the control of



**Figure 3** Regulation of *AtNramp1* and *AtNramp2* mRNA levels by iron availability

Northern blot of *A. thaliana* transcripts from roots and shoots of plants grown in the presence of 50  $\mu\text{M}$  Fe-EDTA (+) or in absence of iron for 3 (–3) or 5 (–5) days. Ethidium bromide staining of rRNA as well as hybridization with the *A. thaliana* EF-1 $\alpha$  gene encoding a translation elongation factor are shown and serve as loading controls.



**Figure 4** Resistance to iron toxicity of transgenic *A. thaliana* plants overexpressing *AtNramp1* cDNA

Three such independent transgenic lines, #17, #23 and #24, were analysed and compared with the wild-type Columbia (WT). (A) Northern blot probed with *AtNramp1* to show the level of expression of the transgene. rRNA hybridization signal is indicated as a loading control. (B) Phenotype of the same *AtNramp1*-overexpressing lines on medium containing normal (50  $\mu\text{M}$ ; grown for 8 days) or toxic (600  $\mu\text{M}$ ; grown for 25 days) levels of Fe-EDTA.

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  $\mu\text{M}$  Fe-EDTA or a

range of toxic iron levels, from 300 to 800  $\mu\text{M}$ . In the presence of 50  $\mu\text{M}$  iron, a concentration non-toxic for *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  $\mu\text{M}$  and higher inhibited germination and growth of all the plants. At 600  $\mu\text{M}$  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 AtNRAMP1 remained green and continued to grow (Figure 4B). Therefore, increasing *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 AtNRAMP1 in iron distribution in the cell, rather than in iron uptake from the soil. This phenotype was also confirmed by the observation that *A. thaliana* plants disrupted in the *AtNramp1* gene showed a higher sensitivity to toxic iron levels than the wild-type plants (results not shown).

## DISCUSSION

### AtNRAMP1 is involved in iron homeostasis in yeast and plants

In *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 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 *A. thaliana* multigene family, *AtNramp1* constitutes a distinct subclass, based on sequence comparisons. Choosing the *AtNramp1* and *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 *A. thaliana* plantlets, *AtNramp1* expression is upregulated whereas *AtNramp2* seems to be downregulated (Figure 3). In addition, complementation of the yeast *fet3fet4* strain on low iron content occurred with *AtNramp1* but not with *AtNramp2* (Figure 2). This result was confirmed with the two rice isologues, *OsNramp1* and *OsNramp2*, that respectively behaved like *AtNramp1* and *AtNramp2* in the yeast complementation test (Figure 2). Since both *AtNramp1* and *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 *AtNramp1* in *A. thaliana* leads to an increase in the plant's resistance to toxic levels of extracellular iron (Figure 4). Therefore, the 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 *Drosophila* [3–6,12,30], but was only recently reported for Smf1p, the major yeast NRAMP protein [9]. We found that *AtNramp1* expression efficiently complements the growth defect of the *smf1* yeast mutant (results not shown) as was previously reported with the mouse *Nramp2* gene [10]. This suggests that *A. thaliana* NRAMP, like its yeast, mammalian and fruitfly counterparts, may exhibit a broader substrate specificity than just iron. The recent complementation of *Drosophila* mutant *mv1* by a mammalian *Nramp* gene [31] underscores the level of conservation in the function of this family of proteins.

However, regulation of the expression of the *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 *DCT1/Nramp2* and plant *Nramp1* expression are, at least in part, regulated at the level of their transcription and/or mRNA stability. The *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 *DCT1* mRNA levels by RNA degradation. However, we did not find any IREs in the plant isologues.

### Is AtNRAMP1 localized in an intracellular compartment?

We could not prove that iron uptake in yeast is enhanced by the expression of *AtNramp1* (Figure 2). It is possible that AtNRAMP1 protein is mis-addressed in a subcellular compartment in yeast upon expression from a multicopy plasmid, instead of being targeted to the plasmalemma. Many proteins, when overexpressed in yeast, follow a default pathway towards the vacuole. Alternatively, 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 Smf1p that exhibit wild-type complementation activity of an *smf1* $\Delta$  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 *A. thaliana* plants overexpressing *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 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 overexpressors, 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 *A. thaliana* line disrupted in the *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 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 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 *AtNramp1* under the control of the ubiquitous strong 35 S promoter leads to an increase of 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 ( $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cd}^{2+}$ ) 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.

We thank Professor Philippe Gros (Department of Biochemistry, McGill University, Montreal, Canada) for the gift of the *OsNramp1* and *OsNramp2* cDNA clones and Dr Emmanuel Lesuisse for the gift of the yeast *fet3fet4* strain, as well as for his advice in iron-uptake assays. We are grateful to Dr Frederic Gaymard, who isolated an *A. thaliana* T-DNA insertion mutant in the *AtNramp1* gene, to Benoit Lacombe who performed electrophysiological tests in *Xenopus* oocytes and to Dr Laurence Marques for assaying metal concentrations in transgenic plants. We are indebted to Anna Stepanova for her comments on the manuscript.

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