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The macrophage-specific membrane protein Nramp controlling natural resistance to infections in mice has homologues expressed in the root system of plants

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Abstract

In mice, natural resistance or susceptibility to infection with *Mycobacteria*, *Salmonella*, and *Leishmania* is controlled by a gene named *Bcg*. *Bcg* regulates the capacity of macrophages to limit intracellular replication of the ingested parasites, and is believed to regulate a key bactericidal mechanism of this cell. Recently, we have cloned the *Bcg* gene and shown that it encodes a novel macrophage-specific membrane protein designated Nramp. A routine search of the public databases for sequences homologous to Nramp identified 3 expressed sequence tags (EST) that show strong similarities to the mammalian protein.

We report the identification and cloning of a full-length cDNA clone corresponding to a plant homologue (*OsNramp1*) of mammalian *Nramp*. Predicted amino acid sequence analysis of the plant protein indicates a remarkable degree of similarity (60% homology) with its mammalian counterpart, including identical number, position, and composition of transmembrane domains, glycosylation signals, and consensus transport motif, suggesting an identical overall secondary structure and membrane organization for the two proteins. This high degree of structural similarity indicates that the two proteins may be functionally related, possibly through a common mechanism of transport. RNA hybridization studies and RT-PCR analyses indicate that *OsNramp1* mRNA is expressed primarily in roots and only at very low levels in leaves/stem. DNA hybridization studies indicate that *OsNramp1* is not a single gene, but rather forms part of a novel gene family which has several members in all plants tested including cereals such as rice, wheat, and corn, and also in common weed species. The striking degree of conservation between the macrophage-specific mammalian *Nramp* and its *OsNramp1* plant homologue is discussed with respect to possible implications in the metabolism of nitrate in both organisms.

Introduction

In mice, natural resistance to infection with intracellular parasites such as *Salmonella*, *Leishmania*, and *Mycobacteria* is controlled by the expression of a single dominant gene on chromosome 1, alternatively designated *Bcg*, *Ity*, or *Lsh* [42]. This gene controls the replication of these antigenically

and taxonomically unrelated microbes in the reticuloendothelial organs of the mouse (spleen, liver, lungs), early during the non-immune phase of infection [18, 19]. Detailed *in vivo* and *in vitro* analyses have demonstrated that the mature tissue macrophage is the host cell type phenotypically expressing the genetic difference at the *Bcg* locus [16, 19, 26, 43]. The mechanism of macrophage defense controlled by *Bcg* is intriguing since it appears to be unrelated to the immune system, is active against antigenically unrelated microbes, and seems to act very early during the course of infection.

In the absence of a biochemical assay or immunological reagent against the *Bcg* gene product, we have used a positional cloning approach to isolate *Bcg*. This approach is based on the construction of genetic [27, 28] and physical maps [29] of the mouse chromosome 1 region carrying *Bcg*, its cloning in yeast artificial chromosomes (YACs), and the search for candidate transcription units by exon trapping [47]. Using this approach, a total of seven candidate transcription units were identified, and one of them corresponded to an mRNA expressed exclusively in reticuloendothelial organs, and which was greatly enriched in the macrophage populations from these organs [47]. This gene was given the appellation *Nramp* for Natural Resistance Associated Macrophage Protein. The mRNA encodes a protein of 550 amino acids in length which is extremely hydrophobic and most likely an integral membrane protein; indeed the hydropathy profile suggests a protein encoding 12 putative transmembrane domains, with several domains containing a charged residue [47]. Cloning and sequencing of the human, rat, and chicken homologues of this gene reveals that the sequences of the membrane domains, including the charged residues are the most conserved domains, suggesting an important structural or functional role [30]. Sequencing the *Nramp* gene from 27 different resistant and susceptible inbred strains of mice revealed that susceptibility to infection was exclusively associated with a single non-conservative glycine to aspartic acid substitution in one of the predicted TM domains of the protein [30]. Recently, we have isolated a second member of the *Nramp* family that we have called *Nramp2*. Therefore, there are at least two *Nramp* genes (and probably more) in mammals. The characterization of a mouse mutant bearing a null mutation at *Nramp1* has confirmed that *Nramp1* and *Bcg* are indeed allelic [48].

The role of the Nramp1 protein in macrophage physiology and how mutations in *Nramp* could affect macrophage function and host response to infection remain unclear. Sequence analysis of the Nramp1 protein revealed that it contains a consensus sequence motif known as the 'binding protein-dependent transport system inner membrane component signature' which was originally discovered in the membrane subunits of bacterial permeases such as the histidine, maltose, or oligopeptide transporters of *Escherichia coli* [23]. This motif is predicted to be located on the intracellular phase of the membrane subunits of these transporters and has been postulated to mediate the interaction of peripheral ATP binding subunits with the membrane components to energize transport [23]. This transport motif has been found in very few eukaryotic membrane proteins, but one of them is the CrnA protein of the fungus *Aspergillus nidulans* [46]. CrnA has been demonstrated to function as a nitrate/nitrite concentrator in this microorganism. Interestingly, reactive nitrogen intermediates (RNI), in particular nitric oxide (NO) and its nitrite (NO_2^-) and nitrate (NO_3^-) derivatives, play a crucial role in the cytostatic and cytolytic activities of macrophages against a variety of microbial and tumor targets [33, 44]. *In vitro* experiments show that RNI are cytocidal for a variety of intracellular parasites, including several species of *Leishmania*, *Mycobacteria*, and many others [17, 33, 44]. We have proposed a model in which Nramp1 could function as a nitrate/nitrite concentrator in the membrane of phagolysosomes [47]; alterations in this putative transport system (insertion of a charged residue in one of the transmembrane domain) would affect the capacity of Bcg^S macrophages to control intracellular replication of antigenically unrelated ingested microbial targets. This model remains highly speculative and awaits the bio-

chemical characterization of the Nramp1 protein.

We now report the cloning of a plant(rice) homolog of *Nramp1* (*OsNramp1*) which shares 60% sequence homology with its mammalian counterpart, is highly conserved throughout the plant kingdom, and whose expression is mostly limited to roots. The possibility that *OsNramp1* codes for a novel nitrate transport system in plants is discussed.

Materials and methods

Plant materials

Certified seeds of *Oryza sativa* (cv. Lacassine) were obtained from Stratton Seed Co. (Stuttgart, Arkansas). They were germinated in Petri dishes kept in darkness and filled with distilled water for 24 h at 25 °C. The water was drained off and eight germinated seeds were planted in 15 cm diameter pots filled with steam pasteurized potting soil consisting of sand, peat-moss, vermiculite and perlite (2:2:1:1 v/v/v/v). Pots were kept in plastic trays filled with tap water in order to sub-irrigate the rice plants. Plants were grown in a green house (25 °C day and 18 °C night) with supplemental fluorescent lighting (400 μmol m^{-2} s^{-1} for 16 h/day). Plants were fertilized weekly with a solution containing 200 mg/l each of N, P and K.

Plants were harvested at two growth stages (3 and 5 leaves). The above-ground portion for each plant was harvested and immediately stored at −80 °C in sealed plastic bags. Roots were excavated from the soil and washed off under a stream of water, leaving no debris, and then put at −80 °C. Sample size of stored tissues averaged 2 g fresh weight. Seeds of four annual weeds, redwood pigweed (*Amaranthus retroflexus*), Lamb's quarters (*Chenopodium album*), Barnyard grass (*Echinochloa crus-galli*), and Proso millet (*Panicum miliaceum*) were obtained from weeds present in fields crops in rural localities near Québec city. They were germinated and grown as above. Potato tubers (*Solanum tuberosum*) and seeds of tomato (*Lycopersicon esculentum*), cabbage (*Brassica oleracea*), field bean (*Vicia faba*), wheat (*Triticum aestivum*) and corn (*Zea mays*) were obtained commercially, grown in a soil/vermiculite mixture (1:1 v/v) at 21–27 °C, and shoots were harvested 7 days later. All plant tissues were washed and frozen immediately at −80 °C until analysis.

Isolation and characterization of Oryza sativa Nramp1 (OsNramp1) *cDNA clones*

A cDNA library (5×10^5 PFUs) constructed in bacteriophage vector λgt10 with RNA extracted from etiolated shoots of *Oryza sativa* (indica, cv. IR 36) (FL1041a; Clontech Laboratories, Palo Alto, CA) germinated and grown in vermiculite for 5 days in complete darkness, was plated on *E. coli* host strain C600. The library was screened for the presence of clones corresponding to an *O. sativa* expressed sequence tag (EST, accession number D15268) showing sequence homology to mammalian *Nramp1* and detected during a computer-assisted search [1] of the NCBI non-redundant databases. For this, an oligonucleotide primer (OSS3) of sequence 5′-ATCAGGTTC-CGAAGCCACTGT-3′ corresponding to pst 252 to 272 of the EST was labelled with γ-[^{32}P]ATP (ICN Biochemicals, specific activity 7000 Ci/mmol) using T4 polynucleotide kinase under conditions recommended by the supplier of enzyme (Pharmacia-LKB, Montréal, Canada). Briefly, duplicate sets of filters (Hybond-N, Amersham) were pre-hybridized in a buffer containing 5 × SSC (1 × SSC is 0.15 M NaCl, 0.15 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.1% bovine serum albumin, 0.1% Ficoll and 0.1% polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate (SDS), 0.2 mg/ml denatured salmon sperm DNA for 16 h at 55 °C. Hybridization was carried out in the same solution containing the [^{32}P]-labelled oligonucleotide (1×10^6 cpm/mL hybridization buffer) at 55 °C for 24 h. Filters were washed under conditions of increasing stringency up to 55 °C in 2 × SSC, 0.1% SDS for 1 h, and exposed to Kodak XAR film for 4 days at −80 °C. A total of twenty six positive clones were obtained, plaque purified and DNA was prepared from them [37], followed by

partial restriction mapping of their inserts. The inserts of five overlapping clones were subcloned in the corresponding *Eco* RI site of plasmid vector pBluescript KS II (Stratagene, La Jolla, CA) and their nucleotide sequences were established by the dideoxy chain termination method of Sanger [38], using modified T7 polymerase (Sequenase, USB), and double-stranded DNA templates. Subcloning of cDNA inserts into the filamentous bacteriophage vector M13mp18 was carried out to prepare additional single-stranded DNA templates for nucleotide sequencing. The sequence of the *OsNrampl* cDNA was determined on both strands of two independent clones, using either oligonucleotide primers derived from the cDNA sequence or primers derived from the plasmid vectors and flanking the cloning site.

Genomic DNA isolation and hybridization studies

Total genomic DNA was isolated from either fresh or frozen plant tissues kept at −80 °C. Briefly, 2–3 g of tissue was ground to a fine powder in sterile mortar and pestle in the presence of liquid nitrogen. This powder was first dissolved in ice-cold extraction buffer (0.5 M sorbitol, 0.1 M Tris-HCl pH 8.0, 70 mM EDTA, and 20 mM sodium metabisulfite) by vigourous vortexing, followed by the addition of an equal volume of ice-cold lysis buffer (0.9 M NaCl, 90 mM Tris-HCl pH 8.0, 30 mM EDTA, 55 mM hexadecitrimethylammonium bromide), and one third volume of a 5% *N*-lauroyl sarcosine solution. The mixture was then incubated at 65 °C for 15 min, cleared by centrifugation (3000 × ***g***, 15 min) and genomic DNA extracted once with chloroform isoamyl alcohol (24:1) followed by precipitation with isopropyl alcohol. The precipitate was then dissolved in a buffer containing 10 mM Tris pH. 8, 0.1 M NaCl, and 1 mM EDTA and treated with Proteinase K (100 μg/ml, Bethesda Research Laboratories) at 50 °C for 16 h. Nucleic acids were further purified by sequential phenol and chloroform extractions followed by ethanol precipitation. The final DNA pellet was dissolved in 10 mM Tris pH. 8, 1 mM EDTA and stored at 4 °C. A 10 μg portion of total genomic DNA from all plants, except *Arabidopsis thaliana* (1.2 μg), was digested to completion with restriction endonucleases and the fragmentation products were analyzed by electrophoresis in a 0.8% agarose gel containing TBE buffer (89 mM Tris pH 8, 89 mM boric acid) and blotted onto a nylon hybridization membrane (GeneScreen Plus, NEN-Dupont de Numours) by capillary transfer in 10 × SSC. Blots were prehybridized for 4 h at 55 °C in a solution composed of 5 × SSC, 5 × Denhardt's solution, 20 mM Tris-HCl pH 7.5, 1% SDS, and denatured salmon sperm DNA (0.2 mg/ml). Hybridization was carried out for 16 h at 55 °C in the same solution containing a *OsNrampl* cDNA hybridization probe (1×10^6 cpm/ml hybridization solution). The cDNA probe (204 bp *Rsa* I/*Cla* I fragment, pst 1013 to 1217) was labelled with α[^{32}P]-dATP (NEN-Dupont de Numours, specific activity 3000 CI/mmol) to high specific activity ($> 1 \times 10^8$ cpm/μg DNA) by random priming [14]. Blots were washed first at moderate (2 × SSC, 0.1% SDS) and then high stringency (0.1 × SSC, 0.1% SDS), followed by autoradiography for 20 h to 4 days at −80 °C to Kodak XAR-5 film with an intensifying screen.

RNA isolation and hybridization studies

For the isolation of total cellular RNA, frozen plant tissues (roots and leaves) were ground to a fine powder using a mortar and pestle pre-cooled with liquid nitrogen. Briefly, ca. 1–2 g of frozen tissue was dissolved by vigourous vortexing on ice in a mixture of extraction buffer (10 mM Tris pH. 8, 0.1 M NaCl, 1 mM EDTA pH 8, 0.1% SDS), phenol, and chloroform at a 5:3:3 ratio. The suspension was cleared by centrifugation (3000 × ***g***, 15 min) then, the aqueous phase was recovered and extracted three times with an equal volume of chloroform. Nucleic acids were recovered by ethanol precipitation, and total cellular RNA was further purified using a method based on guanidinium hydrochloride extraction and differential ethanol precipitations [7]. The polyade-

nylated fraction of the RNA [poly(A)$^+$ RNA] was isolated using oligo-dT coupled to magnetic microspheres (Dynabead, Dynal) according to the protocol of Jakobsen *et al.* [22]. A 500 ng portion of poly(A)$^+$ RNA from each tissue was separated by electrophoresis in a denaturing gel containing 0.66 M formaldehyde [37], and transferred to a nylon hybridization membrane (GeneScreen Plus; NEN-DuPont de Numours) by capillarity in the presence of 10 × SSC. The northern blot was pre-hybridized (4 h, 65 °C) in a solution consisting of 1 M NaCl, 10% dextran sulfate, 1% SDS, and 10 mM Tris pH 8, and hybridized in the same solution (16 h, 65 °C) containing an *OsNramp1* cDNA (*Apa* I to *Eco* RI, 687–1723) probe (2×10^6 cpm/ml hybridization solution) labelled with $\alpha[^{32}P]$-dATP to high specific activity by random priming. The blots were washed under conditions of increasing stringency up to 65 °C, 0.1 × SSC, 0.5% SDS for 30 min at 65 °C, and exposed to Kodak X-ray films with an intensifying screen at −80 °C. The filter was then stripped of the probe (90 °C, 0.1 × SSC, 0.1% SDS) and re-hybridized with a control orchid (*Phalaenopsis orchid*) actin cDNA probe (generous gift of Dr S. O'Neil, UC Irvine), to verify equal RNA loading on the gel and equal transfer to the membrane.

RNA expression analysis by RT-PCR

The level of *OsNramp1* mRNA expression was also analyzed by reverse transcriptase cDNA synthesis followed by amplification by the polymerase chain reaction (RT-PCR), according to a protocol that we have described in detail elsewhere [13]. Briefly, poly(A)$^+$ RNA (100 ng) from rice leaves or roots was reverse transcribed into cDNA using random hexadeoxyribonucleotides (Pharmacia-LKB) primers followed by PCR amplification using either *OsNramp1*-specific oligonucleotide primers RN5 (5′-GACCCGGGAAATATGGAGACTGATCT-3′, 216–235) and RNapa (5′-TGGCACCGGGCCCACTGAGC-3′, 680–699) or orchid actin-specific oligonucleotide forward (5′GGCTGCAGGATATGGAAAAAATTTGG-3′) and reverse (5′-GGGAATTCATCAACATCACATTTCAT-3′) primers [34]. Conditions for PCR amplification were: 92 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min for either 20 or 25 cycles. PCR amplification products were separated by electrophoresis in agarose gels (1.6%) containing TBE buffer, and visualized under UV after ethidium bromide staining of the gel. In some experiments, the gel was transferred to a hybridization membrane that was analyzed by Southern blotting with either *OsNramp1* or actin hybridization probes (1×10^5/ml hybridization solution), as described for northern blotting.

Computer-assisted sequence analyses

Computer-assisted nucleotide sequence analysis was done using either the DNA Strider software package [31] or using the GCG software package (University of Wisconsin, Madison, WI) [10] with the VAX/VMS server of Université de Montréal. The NCBI databases were searched using the TBLASTN program [1], and the SwissProt database was searched with the FASTA program of the GCG package.

Results

Identification of plant sequences bearing homology to mammalian Nramp1

The predicted amino acid sequences of the mouse [47] and human [6] Nramp1 proteins were used as query sequences to screen the NCBI sequence databases for the presence of homologous protein sequences. This search led to the identification of several short anonymous 'expressed sequence tags' (EST) whose translation products showed significant homology to discrete regions of mammalian Nramp1. Three ESTs identified originated from plant genes, with two sequences from *Oryza sativa* (GenBank accession numbers D15268 and D25033) and one sequence from *Arabidopsis thaliana* (GenBank, Z30530). D15268 originates from a rice (*O. sativa*) callus cDNA library and

encodes a polypeptide sequence which shows a high degree of homology (Poisson probability of random association, 4.3 e^{-18}) over a 67 amino acid residue segment of human Nramp1 from 351 to 417 (see Figure 1), which includes but is not limited to the 'binding protein-dependent transport system inner membrane component signature'; within this region, the rice and mammalian sequences share 31 identical residues and an additional 22 highly conservative substitutions for a remarkable total homology of 79%. The second rice EST, D25033, was identified in a roots cDNA library and can encode a polypeptide showing high homology (probability 1.1 e^{-13}) to a 49-residue segment of human Nramp1 between psts 86 to 135 (Fig. 1) which includes the first two predicted TM domains of the mammalian protein; in this 49 residue alignment, the two polypeptides share 28 identical residues, with 17 additional conserved substitutions for a total homology of 71% (37/49) (Fig. 1). Finally, a third EST (*A. thaliana*, Z30530) encodes a protein showing a somewhat lower but still highly significant degree of homology to Nramp1 (probability 1.5 e^{-10}) over a 52 residues segment. As was the case for D15268, the segment of homology between Nramp1 and Z30530 overlaps but is not restricted to the 'transport motif' with 27 identical residues and 11 additional conserved substitutions for a total homology of 73%.

Although the region of homology between D15268 and mammalian Nramp1 contains the transport motif which is also found in many other prokaryotic and eukaryotic proteins, it also overlaps sequences outside this motif in a proposed intracellular loop, and a putative adjacent transmembrane domain, prompting us to conclude that the corresponding plant protein(s) may be structural and/or functional homologs of Nramp1.

*Cloning of an Nramp homologue (OsNramp1) from rice (*Oryza sativa*)*

A cDNA library (5×10^5 PFUs) constructed using RNA from etiolated shoots of *Oryza sativa* (indica, cv. IR 36) was screened for the presence of clones corresponding to the *O. sativa* gene using an oligonucleotide (OSS3) corresponding to p 252–272 of the EST D15268. A total of 26 positive clones (frequency 1:20000) were identified, plaque-purified, and their preliminary restriction established. The cDNA inserts of 5 overlapping clones were subcloned and their nucleotide

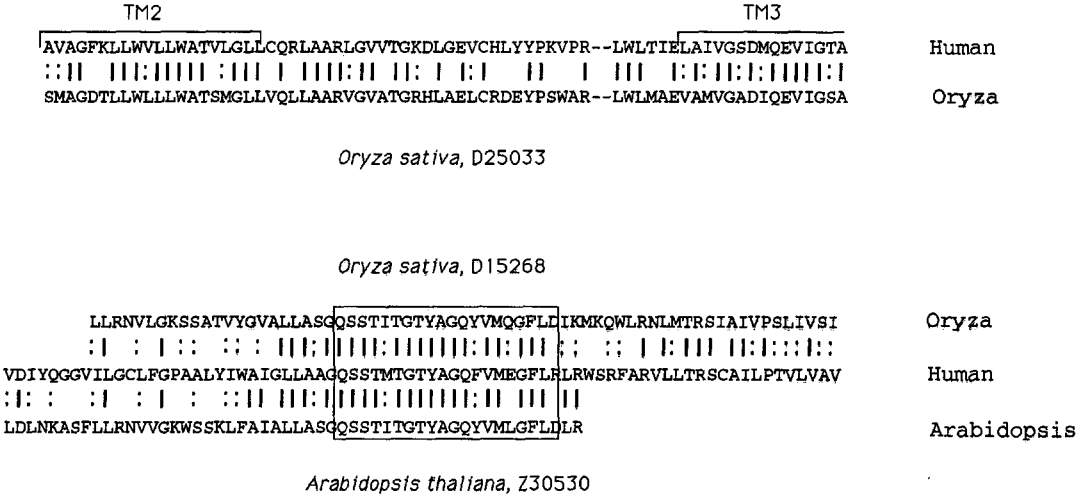

Fig. 1. Amino acid sequence comparison between the *Oryza sativa* and the *Arabidopsis thaliana* expressed sequence tags (EST) and the human Nramp1 protein. Identical residues between human Nramp1 and the 3 ESTs are indicated by the vertical line (|), and highly conservative substitutions by two dots (:), according to the standard of the GCG package. The binding protein dependent inner membrane component signature is boxed and the putative transmembrane domain 2 and 3 are identified (TM2, TM3).

sequences were determined (Fig. 2). This analysis showed that the 5 clones were overlapping and corresponded to the same mRNA transcript; the 1967 nucleotide sequence of the longest clone is shown in Fig. 2. At the 5′ end, the first ATG codon was found at nucleotide pst 63 of the sequence and was followed by an open reading frame consisting of 1551 nucleotides capable of encoding a 517 amino acids polypeptide, terminating with a TAG codon located immediately downstream of Val-517. The presence of a TAA termination codon at nucleotide position 45 indicates that the sequence upstream of the proposed initiator ATG codon (position 63) is indeed untranslated. The termination TGA codon at 1613 is followed by 351 nucleotides of 3′ untranslated region. The 5 clones analyzed showed poly(A) segments varying between 10 and 100 nucleotides in length, and the position of these poly(A) tails indicated that at least 4 possible polyadenylation

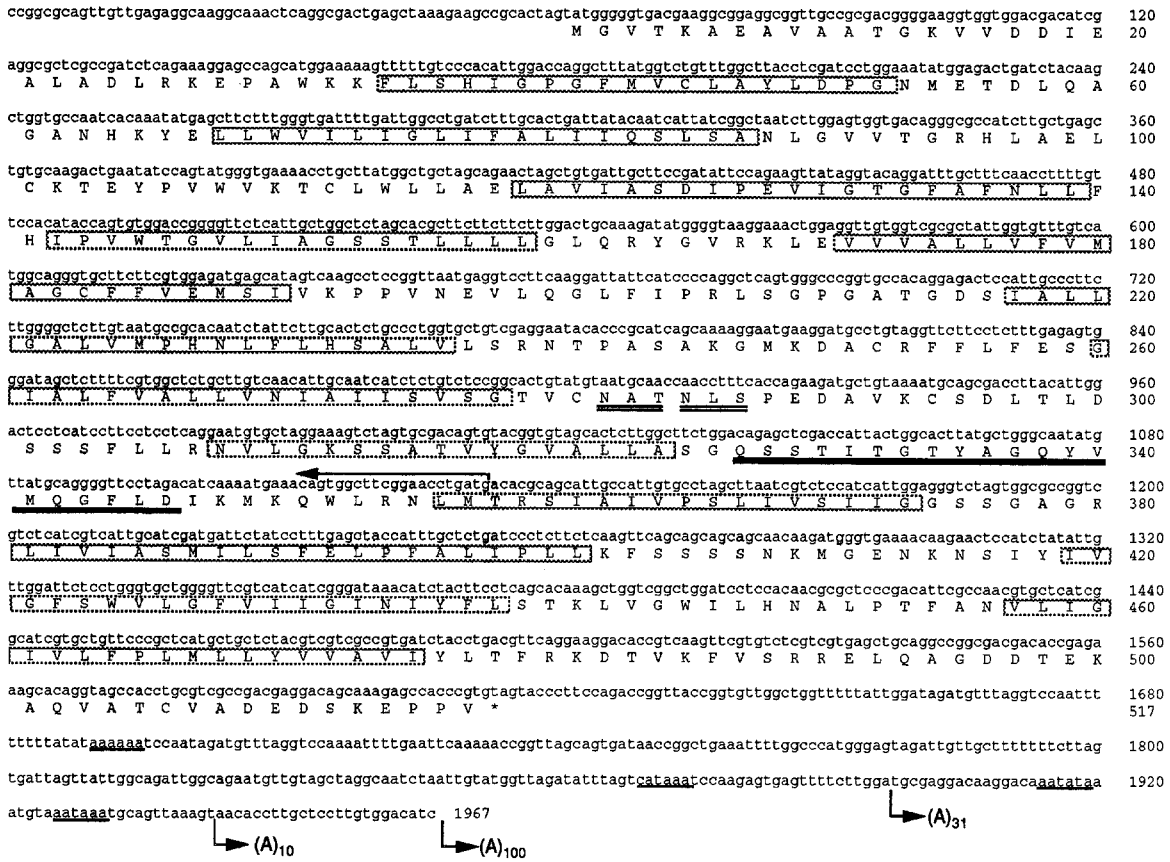

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the cDNA clone encoding OsNramp1. Nucleotides are numbered positively in the 5′ to 3′ orientation to the right and left of each lane (top row), starting with the first nucleotide of the 5′-untranslated region and ending with the last nucleotide, immediately upstream of the observed poly(dA) tails within the 3′-untranslated (3′ UT) region which size and positions (arrows) are indicated. Putative polyadenylation signals are underlined within the 3′ UT. The deduced amino acid sequence is shown below the nucleotide sequence. Amino acids are numbered to the right and left of each lane (bottom row), starting with the first in-frame methionine, and ending with the TAG termination codon (*), located immediately downstream of Val-517. Predicted N-linked glycosylation sites corresponding to the sequence N-X-S/T are underlined by two lines, and highly hydrophobic segments corresponding to putative membrane-spanning (TM) domains are underlined and boxed. The 'binding-protein-dependent transport system inner membrane component signature' [9, 23] located between predicted TM 8 and TM 9 is underlined by a thick line. The position of the oligonucleotide OSS3 derived from the EST D15268 and used to identify *OsNramp1* is identified by an arrow immediately above the corresponding nucleotide sequence.

sites could be used *in vivo* (Fig. 2). The mRNA and protein defined by this gene were given the temporary appellation *OsNramp1*.

The predicted amino acid sequence of the OsNramp1 protein shows a high degree of sequence similarity and overall secondary structure with its mammalian counterpart. OsNramp1 is predicted to encode a highly hydrophobic protein of minimum molecular mass 55.6 kDa, with all the characteristics of an integral membrane protein. Indeed, highly hydrophobic residues account for 46% of the polypeptide (leucine, 14%; valine, 10%; alanine, 9%; isoleucine, 8%; phenylalanine, 5%), while the apolar residue glycine accounts for 8%. The hydropathy profile of the OsNramp1 protein using the Kyte and Doolittle algorithm (Fig. 3B) or the hydrophobic moment analysis [12] identifies 12 highly hydrophobic putative membrane associated (TM) domains (Fig. 2). One of the interesting and unusual characteristics of the protein is that several of these predicted TM domains (TM1, 5, 8, 9, and 10) contain one charged residue, while predicted TM 3 contains two such charges. The OsNramp1 protein contains an almost equal number of 38 positively charged (24 lysines, 14 arginines) and 37 negatively charged amino acid residues (17 aspartates, 20 glutamates), and therefore has an apparent net charge of +1 at pH 7.0; a large number of these charged residues are clustered within two short segments of 33 (1–32) and 60 residues (458–517) located at the amino- and carboxy-terminal ends of the protein, respectively. These charged domains are incompatible with membrane insertion and are most likely associated with the aqueous phase of the membrane compartment. Additional predicted structural features of OsNramp1 include the presence of two putative N-linked glycosylation sites (N-X-S/T) at 282 and 285 in the intervening segment separating predicted TM 7 and 8, suggesting that this domain is an extracellular glycosylated loop. Finally, the sequence motif known as the 'binding protein-dependent transport system inner membrane component signature' [(E, Q)(S, T, A)$_2$ (X)$_3$ G (X)$_6$ (L,I,V,M,Y,F,A) (X)$_4$ (F, L, I, V) (P, K)] [36] was found located at 327 to 346, between predicted TM domains 8 and 9 (Fig. 2). Taken together, these results indicate that the OsNramp1 protein is a novel plant integral membrane protein with putative transport function. The proposed structural model for membrane association of OsNramp1 presented in Fig. 3 is based on an even number of membrane domains, and on the positioning of the cluster of N-linked glycosylation signals to a putative extracellular loop, and the 'transport motif' to an intracellular loop.

Homology between the human and plant Nramp polypeptides

An alignment of the OsNramp1 and mammalian Nramp1 protein sequences is presented in Fig. 3. The two proteins are of different length (517 residues, OsNramp1; 550 residues, Nramp1), this being caused by differences in their amino (25 additional residues in Nramp1), and carboxy terminus (10 additional residues in OsNramp1), and in the segment separating TM7 and TM8 (16 additional residues in Nramp1). The amino acid composition of the two proteins are very similar, both with respect to the high proportion of hydrophobic residues (OsNramp1, 240/517; Nramp1, 241/550) but also for the absolute number of positively (OsNramp1, 36; Nramp1, 35) and negatively charged residues (OsNramp1, 37; Nramp1, 34). Over their region of overlap the two proteins are very homologous, sharing 179 identical residues (35% identity) with an additional 126 highly conservative substitutions for an overall homology of 60%. Unconserved substitutions were not randomly distributed, but were clustered within three discrete segments, the extreme amino terminus (1–30, 23% similarity), the predicted loop between TM7 and TM8 (279–304, 38%), and the segment from TM10 to the carboxy terminus (402–507, 39%). In addition, only very few small gaps needed to be inserted to optimize sequence alignment, and the position of these gaps was only within regions of low sequence conservation between the two proteins. The rest of the two sequences are very homologous, and both proteins show very similar hydropathy profiles

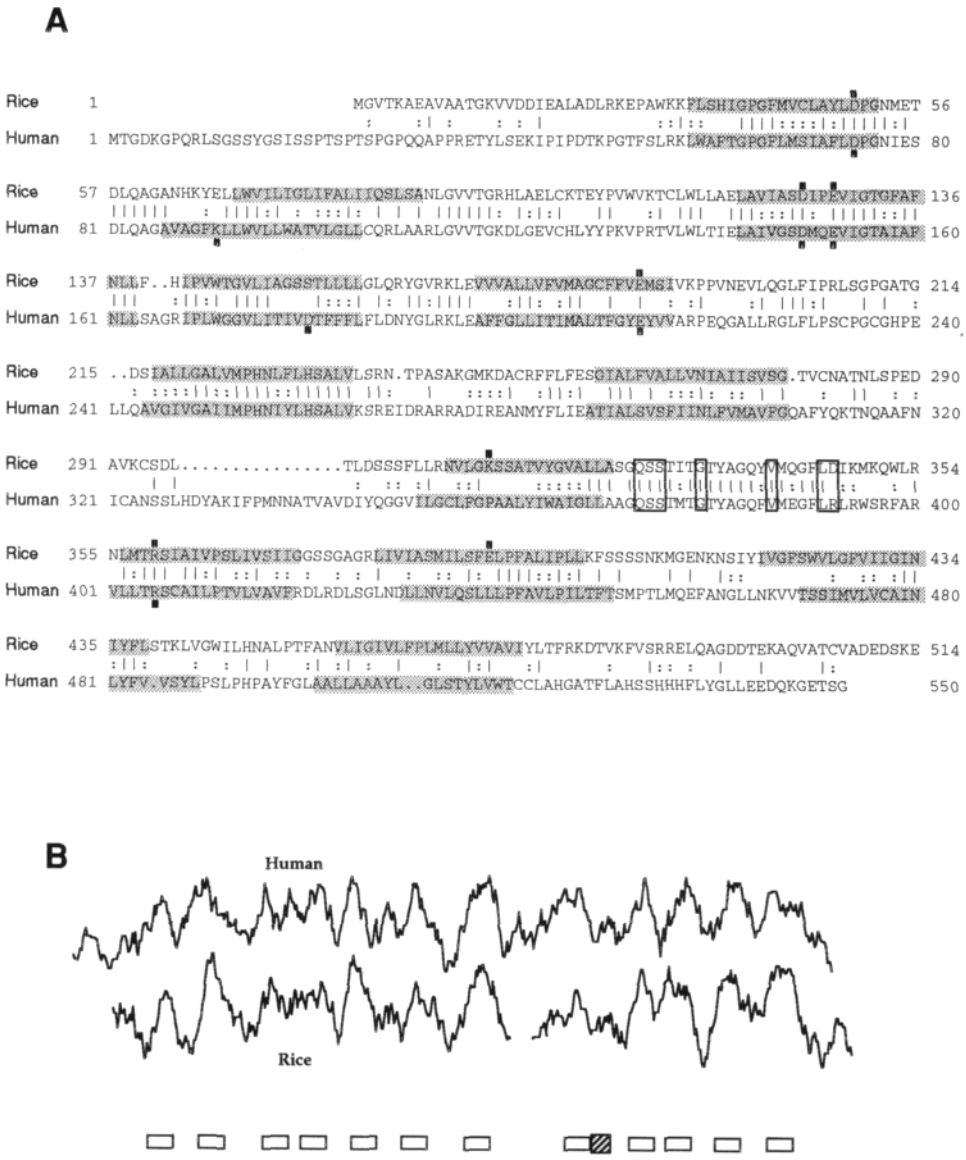

Fig. 3. Amino acid sequence (A) and hydropathy profile (B) alignments of the rice OsNramp1 and the human Nramp1 proteins. A. Amino acids are numbered starting with the initiator methionines in each sequence. The binding protein dependent inner membrane component signature (boxed) and putative transmembrane domains (shaded) are shown. The sequences of OsNramp 1 and human Nramp1 [6] were aligned using the GAP program of the GCG package and gaps were introduced to optimize homology. Identical residues between OsNramp 1 and human Nramp1 are indicated by a vertical line (|), and highly conservative substitutions by two dots (:), according to the standard of the GCG package. B. The hydropathy profiles of the predicted amino acid sequences of human Nramp1 (human, top) and rice OsNramp 1 (rice, bottom) were aligned. The profiles were derived with the DNA Strider program, using the algorithm and hydropathy values of Kyte and Doolittle [25] for a window of 11 amino acids, and a small gap was introduced in the predicted glycosylated loop of OsNramp (defined by TM7-8 interval) to optimize alignment. The position of the predicted TM domains (empty boxes) and transport signatures (hatched box) is indicated immediately under the two hydropathy profiles.

(Fig. 3). The predicted amino acid sequences of the TM domains show a remarkable degree of conservation between the two proteins (42% identity, 75% homology). Finally, both OsN-

ramp1 and Nramp1 display 7 charged residues at neutral pH within their proposed TM domains; five of these charged residues are precisely conserved in both proteins with respect to position, charge and nature of the residue (Fig. 3). The high degree of conservation of these domains and residues suggest that they play a key role in common structural and functional aspects of the two proteins. Although mapping within an otherwise non-conserved segment, the clusters of two N-linked glycosylation sites (Fig. 3) were preserved within the predicted extracellular loop defined by TM7 and TM8, suggesting an important role of these sites for proper targeting and/or processing of the two proteins. Finally, the sequence of the 'transport motif' and its position within the predicted intracellular loop delineated by TM8 and TM9 were precisely conserved between the two protein sequences, also suggesting an important role for this sequence in the structure or mechanism of action common to both proteins.

Taken together, these results indicate that OsNramp1 and its mammalian counterpart are highly similar, and share almost identical predicted structural features and overall secondary structure.

OsNramp1 mRNA expression in plant tissues

The pattern of *OsNramp1* mRNA expression in normal plant tissues was investigated. For this, polyadenylated RNA was isolated from roots and leaves of *O. sativa* plantules and analyzed by northern blotting, using as a hybridization probe an *OsNramp1* cDNA fragment corresponding to the 5′ end of the mRNA (Fig. 4A). This probe detected a single mRNA species of approximate size 2.3 kb which appeared to be expressed exclusively in roots and could not be detected in leaves under experimental conditions used. The level of expression of *OsNramp1* mRNA in roots was very low, and could not be detected when total RNA was used for the blot. Prolonged exposure (5 days) of poly(A)$^+$ containing blots was required to observe a clear hybridization signal. Re-hybridization of the Northern blot with a control Orchid actin probe showed that the leaves RNA lane was underloaded compared to the roots lane (factor of two-fold), but clearly indicated that the absence of *OsNramp1* mRNA in

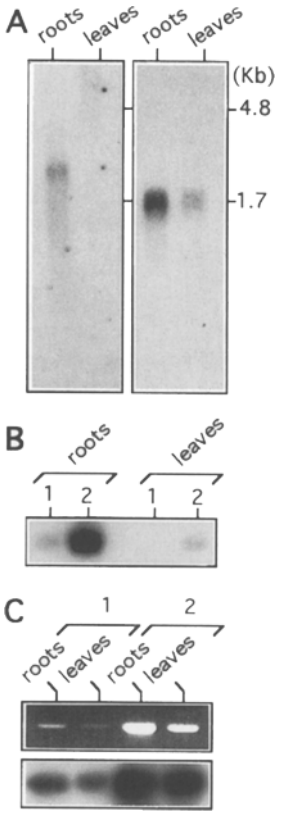

Fig. 4. Tissue-specific expression of the OsNramp1 mRNA in plant tissues. A. The polyadenylated fraction (0.5 μg) of RNA isolated from roots and leaves of *O. sativa* plantules was separated on denaturing formaldehyde agarose gel, and analyzed by Northern blotting using a specific *OsNramp1* probe (left panel). The blot was stripped of the probe and re-hybridized to a control actin cDNA probe (right panel). The position of ribosomal RNA markers is indicated to the right of the panel. B. Polyadenylated RNA from roots and leaves was used as a template for the amplification of *OsNramp1* cDNA fragments by RT-PCR, and amplification products after 20 (lanes 1) and 25 cycles (lanes 2) were separated by agarose gel electrophoresis and analyzed by Southern blotting with a *OsNramp1* cDNA probe. C. Polyadenylated RNA from roots and leaves was used as a template for the amplification of control actin cDNA fragments by RT-PCR, and amplification products after 20 (lanes 1) and 25 cycles (lanes 2) were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under UV (top panel) and analyzed by Southern blotting with an *actin* cDNA probe (bottom panel).

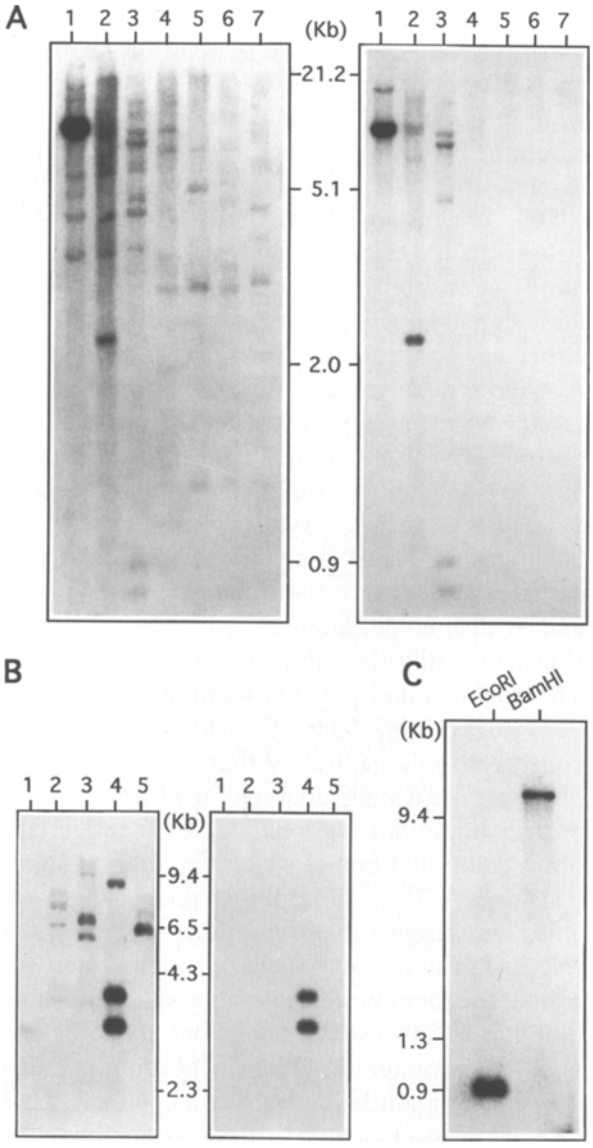

Fig. 5. Detection of OsNramp1 homologues in different plant species. A. Genomic DNA from rice (1, *O. sativa*), wheat (2, *Triticum aestivum*), corn (3, *Zea mays*), potato (4, *Solanum tuberosum*), tomato (5, *Lycopersicon esculentum*), cabbage (6, *Brassica oleracea*) and field bean (7, *Vicia faba*) were digested to completion with *Bam* HI, separated by agarose gel electrophoresis, and analyzed by Southern blotting using a *OsNramp1* cDNA probe (Fig. 1, 1237–1747), under conditions of reduced (left panel) or full stringency (right panel). B. Genomic DNA from common weed species redwood pigweed (1, *Amaranthus retroflexus*), Lamb's quarters (2, *Chenopodium album*), Barnyard grass (3, *Echinochloa crus-galli*), rice (4, *O. sativa*), and Proso millet (5, *Panicum miliaceum*) were digested to completion with *Hin*d III, separated by agarose gel electrophoresis, and analyzed by Southern blotting using a *OsNramp1* cDNA probe (Fig. 1, 1013–1217), under conditions of reduced (left panel) or full stringency (right panel). C. genomic DNA from *Arabidopsis thaliana* was digested to completion with *Hin*d III and analyzed by Southern blotting with the same probe under conditions of low stringency. The position of the different molecular size markers used is indicated for each Southern blot.

leaves RNA was not due to absence of RNA in the lane. The level of *OsNramp1* RNA expression was also examined by PCR amplification of *OsNramp1* cDNA from total cDNA produced by reverse transcription of poly(A)$^+$ RNA (RT-PCR) from roots and leaves using random hexamers. Reaction products obtained after 20 (Fig. 4B, lanes 1) or 25 cycles (Fig. 4B, lanes 2) of amplification were separated by agarose gel electrophoresis and analyzed by Southern blotting with a *OsNramp1* cDNA probe. The same experiment was carried out using control actin-specific oligonucleotides, and reaction products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, followed by hybridization to an actin probe (Fig. 4C). Results from these analyses confirmed that *OsNramp1* mRNA expression was controlled in a tissue specific fashion: mostly restricted to roots, with only very low expression detected in leaves.

OsNramp1 is a member of a family of homologous genes conserved in the plant kingdom

Comparison of the nucleotide and predicted amino acid sequence encoded by the *OsNramp1* cDNA clone isolated here with the nucleotide and translated sequences of the second rice EST (D25033) showing homology to mammalian *Nramp1*, indicated that *OsNramp1* and D25033 were homologous but were encoded by distinct genes. In order to determine if additional sequence homologous *OsNramp* genes exist in the rice genome, we carried out Southern blotting studies under hybridization conditions of reduced stringency. For this, a short *OsNramp1* cDNA fragment (1013–1217) corresponding to a protein segment highly conserved in the plant and mam-

malian polypeptides (transport motif) was used to probe Southern blots of genomic DNA from different plant species digested with either *Bam* HI (Fig. 5A) or *Hin*d III (Fig. 5B, 5C). These include various crops (rice, corn, wheat; Fig. 5a), vegetables (tomato, potato, cabbage, field beans; Fig. 5A), common weed species (redwood pigweed, lamb's quarters, barnyard grass, proso millet; Fig. 5B), and *A. thaliana* (Fig. 5C). Results shown in Fig. 5A indicate that the *OsNramp1* probe detects under conditions of high stringency several hybridizing fragments only in rice, corn and wheat genomic DNA (right panel); however, under reduced stringency the probe detects additional fragments in these species but also detects homologous but more weakly hybridizing fragments in potato, tomato, cabbage and field beans genomic DNA (left panel). Likewise, the *OsNramp1* probe can detect under reduced stringency homologous genomic DNA fragments in all common weed species tested (Fig. 5B) as well as in *A. thaliana* (Fig. 5C).

Considering the very small size of the cDNA probe used and the complex pattern of hybridizing fragments detected at low stringency, these results indicate that *OsNramp1* forms part of a small family of closely related genes in all plant species tested to date.

Discussion

The macrophage-specific Nramp1 protein has a plant homologue expressed in the root system

During the course of routine screenings of sequence data bases, we detected sequence homology between Nramp1 and three partial cDNA fragments emanating from plant genome projects, and deposited as expressed sequence tags (EST). Therefore, we have used an oligonucleotide primer derived from one of these ESTs, D15268, to isolate a full length cDNA clone for the corresponding plant gene that we have named *OsNramp1*. The predicted amino acid sequence of OsNramp1 and mammalian Nramp1 share a high degree of homology which is remarkable for proteins separated by such a large evolutionary distance. Over their region of overlap the two proteins share 179 identical residues (35% identity) with an additional 126 highly conservative substitutions for an overall homology of 60%. They display extremely similar hydropathy profiles which, together with the conservation of key structural (TM domains) and functional sequence motifs (transport signature, N-linked glycosylation signals), suggest similar two-dimensional structures and transmembrane organization.

A more detailed analysis of the discrete areas of conservation along the sequences of the two polypeptides also identified critical segments that may contribute to common structural and/or functional aspects of both proteins. One of the most striking areas of conservation are the predicted TM domains: hydropathy profile (Fig. 3) and hydrophobic moment analyses (data not shown) identify the same number of 12 putative TM domains and position them at similar locations in both proteins. Considering that nonconservative substitutions that maintain hydrophobicity are usually tolerated in TM domains of protein homologs without loss of function, the observation that homology between the two proteins rises to 75% within these regions is remarkable. This suggest that these domains play a key role in intramolecular (helix packing within the membrane) or intermolecular interactions (formation of multimer) which are essential for the creation of a structure, such as a transport site, that may be common to both proteins. Finally, both OsNramp1 and Nramp1 display 7 charged residues within their proposed TM domains, and five of these charged residues are precisely conserved with respect to positions in the TM domains, charge (+ or −) and nature (R vs. K; D vs. E) of the residue (Figure 3). Likewise, this conservation may underlie either an important structural aspect of both proteins, such as the formation of a water filled pore or stabilizing ionic bridges within the membranes [36], or these may be important for the transport of similar substrates such as small charged molecules or ions across the membrane [2]. Indeed, the conservation of thermodynamically disfavored charged residues

within TM domains is a hallmark of several families of ions channels and transporters [24]. A second striking region of conservation is the segment overlapping the consensus transport motif, located in both proteins in the predicted intracellular loop defined by TM8-9. This motif was originally described as the 'EAA box' in integral membrane proteins that form membrane anchors of osmotically sensitive high affinity transport systems of Gram-negative bacteria, known as periplasmic permeases [9, 23]. The motif is located in one of the C-terminal intracytoplasmic loops of these membrane proteins and is believed to mediate the functional coupling of a peripheral ATP-binding subunit to energize transport [9, 23]. Recently, the sequence of this motif has been expended to include the signature '[(L,I,V,M,F,Y) $(X)_9$ (E,Q,R) (S,T,A) (S,T,A,G) $(X)_3$G (L,I,V,M-,F,Y,S,T,A,C) $(X)_5$ (L,I,V,M,Y,F,A,S,T) $(X)_4$ (F,L,I,V,M,F) (P,K,R)] [4], which is found in a total of 51 membrane transport proteins, 44 of which are prokaryotic origins and 7 of eukaryotic origin [4, 39]. The sequence of this motif is precisely conserved in both proteins, suggesting an important but still undetermined role. Also, despite the fact that they are located in a segment where amino acid sequence is otherwise poorly conserved, the cluster of N-linked glycosylation sites is preserved in the same extracellular loop. This suggests an important role of this glycosylated loop in processing or targeting of the two proteins. Finally, a proposed SH3 binding domain has been identified in the murine Nramp protein and proposed to be important for coupling of this protein to membrane signal transduction [5]. This domain is absent in the rice protein, reflecting either a non-essential role or different aspects of regulation.

The high degree of structural similarity between the mammalian Nramp1 and the plant OsNramp1 is highly suggestive of functional similarity between the two proteins. The exact biochemical function of Nramp1 is currently unknown and our understanding of its function at the cellular level is so far limited to the observation that an alteration in TM4 (introduction of a negative charge) or a null mutation results in the incapacity of the tissue macrophage to restrict intracellular replication of ingested microbes. However, one of the proteins which displays similarities with Nramp1 is the nitrate transporter CrnA of the eukaryote *Aspergillus nidulans* [46]. These include similar size of the two proteins, the presence and position within the molecule of the conserved transport signature, and the presence of several charged residues within TM domains. Although limited, this similarity is nevertheless interesting since nitrate and nitrite are oxidation products of nitric oxide, a key bactericidal species produced by macrophages to kill intracellular targets such as ingested microbes [33]. The induced macrophage specific enzyme, the nitric oxide synthase (iNOS) catalyzes in these cells the oxidation of the guanidino nitrogen of L-arginine to yield NO and L-citrulline, and can generate considerable amounts of NO [32]. NO is a highly reactive but short-lived intermediate which becomes oxidized to the more stable NO_2^- and NO_3^- ions in the presence of O_2 and water. However, NO_2^- can be converted back to the biologically active NO by dismutation at low pH [44]. In macrophages, NOS can be induced by specific cytokines or by non-specific bacterial products such as lipopolysaccharides of Gram-negative bacteria, muramyl dipeptide from mycobacterial cell wall, and live BCG [17, 32]. Since NOS is a cytosolic enzyme, and since intracellular parasites are usually present within phagosomes or fused phagolysosomes, RNI have to cross at least one membrane to reach their target. While NO is believed to readily diffuse across lipid bilayers, transmembrane diffusion of charged species such as N O_2^- and NO_3^- may be facilitated by a specific transporter. In particular, concentrating NO_2^- into phagolysosomes would increase cytocidal activity against ingested parasites after conversion to biologically active but short-lived NO in the acidic environment of this intracellular compartment, while reducing deleterious attacks against endogenous macrophage proteins. Therefore, one possible function for Nramp1 may be to transport a small anion such as nitrate/nitrite at that site.

The remarkable degree of homology between

mammalian Nramp1 and the rice OsNramp1 proteins raises the interesting possibility that the latter may also participate in the transport of small ions such as nitrate. Ammonium and nitrate are the two nutrient sources of nitrogen present in soil that are available to plants; although ammonium can directly enter the metabolic pathways through the synthesis of glutamine (from glutamic acid) or asparagine (from aspartic acid), nitrate has to be reduced first no nitrite by nitrate reductase, and ultimately to ammonium by nitrite reductase, using the reducing power of NADPH + . The import of a small negatively charged ion such as nitrate against a strong membrane electrical potential estimated at 150–200 mV (inside negative) requires the presence of specific transporters. Kinetic studies using nitrogen tracers [3, 8, 11, 21, 35] and electrophysiological measurements [8, 15, 40, 41] have indicated that several classes of nitrate transporters may be present in roots cells. The only nitrate transporter cloned so far from plants is encoded by the *CHL1* gene of *Arabidopsis thaliana* [45]. Chl1 is a low-affinity nitrate transporter (K_m 1–5 mM), and *chl1* mutants can grow normally on nitrate (in absence of the inhibitor chlorate), clearly indicating that additional nitrate transporters exist in *Arabidopsis* [45]. OsNramp1 does not show any sequence similarity of the Chl1 protein (data not shown). It is therefore tempting to speculate that *OsNramp1* may encode one of the missing nitrate transporters in plants. The restricted expression of *OsNramp1* to the root system together with its homology to Nramp1 would be in agreement with such a function. However, this proposition remains speculative and the possibility that OsNramp and Nramp proteins transport other types of substrates cannot be excluded in the absence of additional biochemical data.

OsNramp1 defines a new gene family in plants

The *OsNramp1* gene is not unique in rice but defines a novel gene family with several highly homologous members in this species. This conclusion is based partly on the presence of several cross-hybridizing genomic fragments detected under low stringency conditions by an *OsNramp1* probe (Fig. 5). In addition, the *OsNramp1* cDNA clone isolated here shares partial homology but is clearly distinct from the sequence of the second rice EST (D25033) scoring positive in our initial search for homologues of mammalian Nramp1. Therefore, the gene corresponding to D25033 most likely represents a second member of the rice *OsNramp* family; It is interesting to note that D25033 was isolated from a cDNA library constructed from roots RNA, suggesting that this second *OsNramp* gene is also expressed in the roots system. Indeed, we have recently isolated from a rice cDNA library DNA clones corresponding to two highly similar but distinct members of the Osnramp family (*OsNramp2*, *OsNramp3*; A. Belouchi and P. Gros, personal communication). Preliminary nucleotide sequencing indicate that *OsNramp2* corresponds to the second *Oryza sativa* EST (D25033) initially identified. Finally, the rice *OsNramp* family is also highly conserved across the plant kingdom, and this degree of conservation seems to follow established phylogenic relationships amongst plant species. Several homologs could be detected in these species by hybridization under conditions of reduced stringency, including cereals, vegetables and common weeds (Fig. 5). The high degree of conservation of this gene family in the plant kingdom, and the high level of sequence homology between the mammalian and plant genes together indicate that the Nramp protein plays a fundamental role which has been preserved across very large evolutionary distances. Elucidating the substrate(s) and mechanism of action of this new protein family is a difficult task that is presently being tackled.

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