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Divalent metal transport in the green microalga *Chlamydomonas reinhardtii* is mediated by a protein similar to prokaryotic Nramp homologues

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Abstract

Information about the molecular mechanisms of metal transport in algae is scarce, despite the significant status these organisms have in aquatic ecosystems. In the present study, we describe the cloning and functional characterization of a divalent metal transporter (named DMT1) in the green microalga *Chlamydomonas reinhardtii* Dangeard. The longest open reading frame of the cloned DMT1 cDNA encodes a protein of 513 amino acids with 11 putative transmembrane domains. The protein belongs to the Nramp family of divalent metal transporters and shows surprisingly higher similarity to some prokaryotic than to eukaryotic polypeptides. Especially the N-terminus, which is longer than of every other homologue considered in this study, displays – uniquely among selected eukaryotic Nramps – exclusively prokaryotic characteristics. Functional complementation experiments in yeast strains with impaired metal transport systems, revealed that *C. reinhardtii* DMT1 has a broad specificity, acting in the transport of several divalent metals (manganese, iron, cadmium, copper), but excluding zinc.

Introduction

Metal ions are indispensable for cell survival since they fulfill important functions at several sites in the cell, including stabilization of cellular structures, enabling of redox reactions and activation of enzymes (Fraústo da Silva & Williams 1991). Different metal elements do not occur at the same concentration in the environment, are not equally bioavailable, and have to be maintained at different concentrations in the cell. Iron for instance, is needed in greater amounts than copper, but occurs mainly in the form of insoluble and thus not readily bioavailable ferric complexes. This reality forces the cell to express a variety of transport systems in order to acquire a broad range of these essential micronutrients in the required concentrations. A number of metal transport proteins have been characterized in bacteria, yeast, mammals and plants, some of them displaying similar features and belonging to the same family (for an overall classification see, Saier 2000). Less is known regarding divalent metal transporters in green algae at a molecular level. Only recently, the molecular identification and characterization of genes involved in high affinity iron transport in the green microalga *Chlamydomonas reinhardtii* (Herbik *et al.* 2002; La Fontaine *et al.* 2002) was published.

Nramps (*n*atural *r*esistance *a*ssociated *m*acrophage *p*roteins) form a group of divalent metal cation transporters which contribute to cell metal homeostasis and have been highly conserved throughout evolution. They are expressed in many different organisms belonging to the

pro- and eukaryotic domains, and share high similarity at the amino acid level. In the yeast *Saccharomyces cerevisiae* three Nramp homologues have been identified and characterized, *SMF1*, *SMF2* and *SMF3* (Supek *et al.* 1996; Portnoy *et al.* 2000). *SMF1* is expressed at the cell surface and is specific for Mn^{2+} ions, but it was also shown to contribute to copper and cadmium accumulation (Liu *et al.* 1997). *SMF2* is also specific for Mn^{2+}, it is expressed in intracellular vesicles and was suggested to supply the manganese superoxide dismutase (MnSOD) in the mitochondria with the essential Mn^{2+} ions (Luk & Culotta 2001). *SMF2* was also shown to play a role in cobalt transport (Liu *et al.* 1997). *SMF1* and *SMF2* are regulated at the post-translational level by the protein BSD2 (Liu *et al.* 1997; Liu & Culotta 1999). *SMF3*, expressed in the vacuole, is believed to mobilize vacuolar iron stores and is down-regulated by iron (Portnoy *et al.* 2000). In the thale cress *Arabidopsis thaliana* several Nramp homologues were found. *AtNramp1*, *3* and *4* contribute to manganese, iron and cadmium transport as was shown by yeast complementation tests (Curie *et al.* 2000; Thomine *et al.* 2000). Mammalian *Nramp2* (DMT1, DCT1) is described as the major transferrin-independend iron transport system in the intestine. It functions as a pH-dependent divalent cation transporter (Gunshin *et al.* 1997) with broad specificity. The mammalian *Nramp1* is expressed in the endosomal/lysosomal vesicles of macrophages and is recruited to the phagosomal membrane upon phagocytosis of pathogens (Gruenheid *et al.* 1997). It was shown to transport manganese (Mn^{2+}) ions out of the phagosome, suggesting that its role lies in mediating metal depletion of divalent metals from the phagosomal space (Jabado *et al.* 2000). Since Mn^{2+} is a cofactor for MnSOD, its lack would deprive the pathogen from an important defense mechanism, resulting to decreased pathogenicity. Some pathogens have also been described to express Nramp homologues – MntH in the case of the Gram-negative bacterium *Salmonella typhimurium* – which might compete with Nramp1 for metal ions in the phagosome. The MntH proteins from *Salmonella typhimurium* and *Escherichia coli* were shown to be Mn^{2+} transporters involved in the response to reactive oxygen (Kehres *et al.* 2000). Several other organisms were described to express Nramp homologues (Rodrigues *et al.* 1995; Feng *et al.* 1996; Chen *et al.* 2002; Donovan *et al.* 2002) and putative genes encoding members of the Nramp family have been identified in many other organisms based on sequence homology.

The present study describes the cloning and functional characterization of a divalent metal transporter from the green microalga *Chlamydomonas reinhardtii*. The protein designated DMT1 shows highest amino acid sequence similarity to prokaryotic members of the Nramp family. Functional complementation in yeast strains with impaired manganese, zinc and iron transport systems revealed that the DMT1 protein displays broad specificity, transporting manganese, cadmium, copper and iron but no zinc.

Materials and methods

Strains and plasmids

Saccharomyces cerevisiae strain Y16272 (BY4742; MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; smf1::kanMX4) is a yeast mutant in which the *SMF1* gene has been disrupted and was obtained from EUROSCARF, Frankfurt (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). The following two strains were kindly provided by D. Eide, University of Missouri-Columbia and have disruptions in genes for zinc and iron transport, respectively: *S. cerevisiae* strain ZHY3 (MATα; ade6; can1-100oc; his3; leu2; trp1; ura3; zrt1::LEU2; zrt2::HIS3) (Zhao & Eide 1996) and strain DDY4 (MAT α; ura3; trp1; leu2; his3; can1; fet3::HIS3; fet4::LEU2) (Dix *et al.* 1997). *C. reinhardtii* strain cw15arg7 (kindly provided by E.H. Harris, Harris 1989) was used for RNA isolation for RACE and RT-PCR.

To express the *C. reinhardtii DMT1* in yeast the corresponding full-length cDNA was cloned into the yeast expression vector pUG35 (kindly provided by J.H. Hegemann, University of Duesseldorf, Germany, (Guldener and Hegemann, in preparation). pUG35 has a CEN/ARS origin of replication (1–2 copies per cell) and uses the MET-25 promoter for moderate expression of heterologous genes.

Culture conditions

S. cerevisiae: For selection of transformants cells were grown on solid synthetic minimal medium containing 0.17% yeast nitrogen base (without amino acids and ammonium sulfate, DIFCO), 0.5% ammonium sulfate, 2% glucose, a mixture of amino acids and 1.8% agar. For complementation experiments cells were grown in liquid medium with the same composition as above supplemented with various concentrations (see Results) of metals or metal chelators EGTA (ethylene glycol-bis(2-aminoethyl)-tetra acetic acid) and EDTA (ethylene diamine tetra acetic acid). For growth tests 5 ml medium were inoculated with a single colony and incubated with shaking (200 rpm) at 30 °C for a few hours. Forty five ml of growth medium in a 250-ml Erlenmeyer flask were inoculated with this yeast preculture and incubated with shaking at 30 °C overnight. The late-exponential overnight culture was diluted to an optical density (OD_{600}) of 0.1–0.3 at 600 nm and divided into several flasks containing medium with and without stress (metal chelators or metals). These were then incubated with shaking at 30 °C for the course of the growth experiment. Growth was monitored by measuring OD_{600} at 1–2 h intervals for 7–10 h. The maximal growth rate of each strain in the medium without any stress conditions was defined as 100% growth of the strain and growth of the same cells in different concentrations of stress was expressed in relation to it. All growth tests were performed at least in triplicate and either a representative experiment or the mean of several experiments is shown in the corresponding figures.

C. reinhardtii: For RNA isolation *C. reinhardtii* strain cw15arg7 was grown in liquid Tris–acetate–phosphate medium TAP (Harris 1989) supplemented with 50 mg/l arginine under constant illumination and agitation.

Cloning

A search in the *C. reinhardtii* EST (expressed sequence tag) library of Kazusa DNA Research Institute, Japan identified a partial EST sequence (GenBank accession no. AV389837) with high homology to known Nramps. The 5′ end of the corresponding cDNA was identified by 5′ RACE (*r*apid *a*mplification of *c*DNA *e*nds). Full-length cDNA was then amplified by nested PCR following reverse transcription and cloned into the yeast vector pUG35. Total RNA of *C. reinhardtii* was extracted with Trizol Reagent (GibcoBRL) according to supplier's instructions. RACE-primers were designed specific to the partial EST-sequence and RACE was performed as described elsewhere (Haring & Beck 1997). Briefly, one reverse transcription step was followed by a tailing reaction and by two PCR steps. For cloning of the full-length cDNA by RT-PCR, two restriction sites were inserted at the 5′ (XbaI) and 3′ (EcoRI) end of the cDNA in the second PCR step by use of appropriate primers. A hemagglutinin tag (HA-tag) was fused to the 5′end immediately after the start codon and the resulting plasmid was named pAR1. The insert of pAR1 contains the start codon, followed by the HA-tag and ends 129 bp downstream of the native stop codon. Yeast mutants were transformed with pAR1 and pUG35 with the YEASTMAKER yeast transformation system (Clontech).

DMT1 expression

Heterologous expression of *DMT1* in *S. cerevisiae* was tested by western blotting according to standard protocols (Sambrook *et al.* 1989). Total yeast protein content was extracted by the method described in (Ooi *et al.* 1996). SDS gel electrophoresis was performed according to standard protocols (Sambrook *et al.* 1989) and proteins were blotted onto nitrocellulose membrane (Schleicher & Schuell). Anti-HA High Affinity (Roche) was used as the primary antibody and Anti-Rat-IgG (peroxidase conjugate, Sigma) as the secondary. Subsequent detection was performed with the ECL kit (Amersham Biotech).

Similarity tree (Figure 3).

A similarity tree with selected Nramp homologues was generated by the software DNAMAN. Parameters for optimal, full, dynamic alignment:

Gap open penalty: 10
Gap extension penalty: 5
DNA transition weight: 0.5
Protein weight matrix: GONNET
Alignment optimization
Gap open penalty: 10
Gap extension penalty: 1
Delay divergent Seqs %: 30

Protein weight matrix: GONNET
Hydrophilic penalties: On
Hydrophilic residues: GPSNDQEKR
Gap separation distance: 4
Residue specific penalties: On

GenBank accession numbers
Cloned *C. reinhardtii* DMT1: AF515631; EST clone from Kazusa institute: AV389837.

Nramp homologues in Figure 3: *Escherichia coli* MntH P77145; *Salmonella enterica* MntH CAD07646; *Deinococcus radiodurans* Nramph AAF11265; *C. reinhardtii* DMT1 AF515631; *Nostoc* sp. PCC 7120 MntH BAB77244; *Xylella fastidiosa* 9a5c Nramph AAF83825; *Pseudomonas aeruginosa* MntH2 Q9RPF2; *Staphylococcus aureus* Nramph NP_374223; *Oryza sativa* Nramp1 S62667; *Arabidopsis thaliana* Nramp1 AAF36535; *Saccharomyces cerevisiae* Smf1 CAA64547, Smf2 AAB68900, Smf3 NP_013134; *A. thaliana* Nramp2 AF141204, Nramp4 AAF13279, *O. sativa* Nramp2 AAB61961; *A. thaliana* Nramp5 CAC27822; *Homo sapiens* Nramp1 AAG15405, Nramp2 P49281.

Nramp homologues in Figure 4: *Salmonella enterica* MntH CAD07646; *Escherichia coli* MntH P77145; *Yersinia pestis* MntH CAC92226; *Chlorobium tepidum* Nramph NP_661903; *Wigglesworthia brevipalpis* Nramph NP_714977; *Listeria monocytogenes* Nramph CAC99502; *Nostoc sp.* PCC 7120 MntH BAB77244; *Leuconostoc mesenteroides* Nramph ZP_00063650; *Enterococcus faecalis* Nramph NP_815583; *Streptococcus agalactiae* Nramph NP_689011; *Lactococcus lactis* Nramph NP_267238; *Oenococcus oeni* Nramph ZP_00069508; *Lactobacillus brevis* Nramph BAB47552; *Xylella fastidiosa* 9a5c Nramph AAF83825; *Xanthomonas axonopodis* Nramph NP_642362; *Pseudomonas aeruginosa* MntH2 Q9RPF2; *Mesorhizobium loti* Nramph BAB49617; *Brucella melitensis* MntH NP_539486; *Agrobacterium tumefaciens* Nramph NP_354720; *Ralstonia solanacearum* Nramph NP_522081; *Staphylococcus aureus* Nramph NP_374223; *Deinococcus radiodurans* Nramph AAF11265; *Chlamydomonas reinhardtii* DMT1 AF515631; *Bos taurus* Nramp NP_777077; *Ovis aries* Nramp1 P49280; *Homo sapiens* Nramp1 AAG15405, *Mus musculus* Nramp1 NP_038640; *Homo sapiens* Nramp2 P49281; *Macaca fascicularis* Nramp2 AF153279; *Rattus norvegicus* Nramp2 O54902; *Takifugu rubripes* Nramph CAD43053; *Ictalurus punctatus* Nramph AAM73759; *Gallus gallus* Nramp1 P51027; *Drosophila melanogaster* Mvl S56140; *Caenorhabditis elegans* Nramph NP_509132; *Arabidopsis thaliana* Nramp1 AAF36535, Nramp3 Q9SNV9, Nramp4 AAF13279, Nramp2 AF141204, Nramp5 CAC27822; *Oryza sativa* Nramp1 S62667; *Schizosaccharomyces pombe* Nramph T38466; *S. cerevisiae* Smf1 CAA64547, Smf2 AAB68900, Smf3 NP_013134.

Results

Identification and cloning of the Chlamydomonas reinhardtii DMT1 gene. A search for Nramp homologous sequences in the *C. reinhardtii* EST database of the Kazusa DNA Research Institute led to the identification of an EST sequence displaying high similarity to genes of the *Nramp* family. Sequencing of the clone (CM051d09, GenBank accession no. AV389837) obtained from the Kazusa Institute revealed that the cDNA was complete only at the 3′ end. We identified the 5′ end with the help of 5′ RACE and cloned the full-length cDNA via RT-PCR (GenBank accession no. AF515631). The cloned gene is named *DMT1* (*d*ivalent *m*etal *t*ransporter 1) in this study, in analogy to *DMT1* (*DCT1*, *Nramp2*) in mammals. The longest cDNA product obtained by RT-PCR reached 1936 nucleotides and the longest open reading frame, which showed similarity to Nramps, consisted of 1542 base pairs (bp) with the translation start (ATG) at position 64. The 3′ untranslated region was 330 nucleotides long and contained a putative polyadenylation signal (TGTAA) 13 bp upstream the polyadenylation site, as has already been described for other *C. reinhardtii* genes (Rochaix *et al.* 1998). The *DMT1* GC content of 62.9% reflects the typical codon usage of *C. reinhardtii*, which favors codons containing a G or C at the third position.

Recently, sequencing of the *C. reinhardtii* genome was accomplished at the Doe Joint Genome Institute, California (Grossman *et al.* 2003) and the sequence is available in http://genome.jgi-psf.org/chlre1/chlre1.home.html since February 2003. Homology search in this database (version 1.0) revealed that the coding sequence of the cloned *DMT1* corresponds to genomic sequence scaffold_489, contig 3, base pairs 30332:34793.

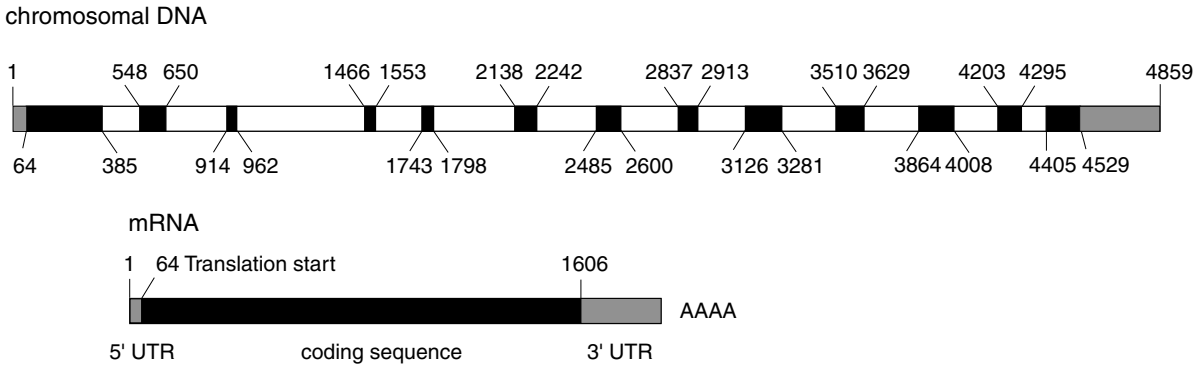

Figure 1. Organization of the DMT1 gene. Total length of the genomic DMT1 DNA region is 4859 base pairs, of mRNA without the poly-A tail (cDNA) 1936 nucleotides and of the coding region 1542. Exons and the coding region are represented by black, introns by white and the 3′ and 5′ untranslated regions by gray boxes. Number 1 indicates the first nucleotide of the sequence which was obtained by 5′-RACE and the other numbers mark the position of start and end of exons (genomic DNA) or of the coding sequence (mRNA). Boxes are drawn to scale in order to give an impression of the organization of exons and introns in the genomic sequence.

Comparison of the genomic sequence to the cDNA sequence determined by us showed that the *DMT1* gene consists of 13 exons and 12 introns (Figure 1). The introns are rather small with the largest one reaching 504 bp and the smallest 110 bp. However, total length of intronic sequence is 2923 bp, while the coding sequence reaches only 1542 bp. This is rather unusual for *C. reinhardtii* genes where total intronic sequence is described to equal total coding sequence (Rochaix *et al.* 1998). Analysis of the boundaries between exons and introns showed that they correspond well to the consensus sequence for *C. reinhardtii* splice sites (data not shown) (Rochaix *et al.* 1998). At least three further Nramp homologues were identified by comparative sequence analysis (Rosakis & Köster 2004) in the recently released genome of *C. reinhardtii* (Grossman *et al.* 2003).

Deduced amino acid sequence analysis. Translation of the *DMT1* cDNA gives rise to a 513 amino acids protein sequence which contains key structural features of the Nramp family (Figure 2). DMT1 is predicted to comprise 11 transmembrane domains (by the programme TMHMM 2.0, (Krogh *et al.* 2001), with the N-terminus in the cytoplasm and the C-terminus extracellularly. The CTM (consensus transport motif) sequence motif described for other Nramp homologues (Cellier *et al.* 1996) is also present in DMT1 (Figure 2). Putative N-glycosylation sites as described for other Nramps (Vidal *et al.* 1996; Gunshin *et al.* 1997) were predicted only with low probability by the programme NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/, Gupta *et al.* in preparation) (not shown).

Interestingly, comparison of Nramp homologues from various species to the *C. reinhardtii* Nramp showed that the predicted protein sequence of DMT1 shares more similarity to some prokaryotic than to the eukaryotic Nramps (Figure 3). Pairwise alignment of the hydrophobic core (as defined in Cellier *et al.* 1996) of the proteins in Figure 3 reveals the best similarity of DMT1 to the Nramp homologue of the cyanobacterium *Nostoc sp. PCC 7120* (58.06% identities) and lowest similarity to *Oryza sativa* Nramp2 (32.51% identities) (data not shown). The identified sequence of further Nramp homologues in *C. reinhardtii* (Rosakis and Köster 2004) allowed sequence comparisons in only one case (due to short length), which also showed higher similarity to the prokaryotic Nramps used in the present study (data not shown).

Another interesting feature of DMT1 involves the N-terminus of the protein. We compared the N-termini of selected Nramp homologues and found that the length of the *C. reinhardtii* DMT1 N-terminus exceeds that of the other analyzed homologues (Figure 4). Presuming that the N-terminus faces the inside of the cell as predicted, it might form an extra domain with a function distinct from the actual transport function of the protein and for example play a role in the regulation of protein stability. However, analysis of the first 80 amino acids with ScanProsite (Gattiker *et al.* 2002) produced only patterns with a high

Figure 2. Deduced amino acid sequence of DMT1. Putative transmembrane domains (predicted by TMHMM2.0) are indicated by black arrows under the corresponding sequences and numbered 1 through 11. The arrowheads point to the outside of the cell. Predicted extracytoplasmic loops are shaded in grey, predicted intracellular loops are not shaded. The consensus transport motif (CTM) appears in bold types.

probability of occurrence (*e.g.*, phosphorylation sites) but no apparent protein motif known to play a role in regulatory posttranslational modification (*e.g.*, ubiquitination, which was shown for yeast ZRT1, Gitan & Eide 2000).

In a further investigation of the DMT1 N-terminus we compared selected Nramp homologues in respect of the N-terminal sequence, which is not as conserved as the core. For this alignment we used the N-terminal sequence overlapping the first 9 amino acids of the core up to the first completely conserved residue (glycine). Despite the overall variability of the sequence there are distinct conserved residues shared only by eukaryotic or prokaryotic homologues (Figure 4). Some prokaryotic Nramps (*e.g.*, *Salmonella*, *E. coli*, *Yersinia*, *Chlorobium*, *Ralstonia*) do not include these conserved residues mainly because they are shorter. On the other hand, there are also eukaryotic homologues which break the ranks, namely the plants, yeasts, *Drosophila* and *C. elegans*, which do not possess the typical eukaryotic conserved amino acids or possess them to a lesser extend. Nevertheless, the vertebrates display a matching pattern. Seemingly, the two major groups formed regarding the N-terminal sequence correspond to the pro- and eukaryotic domains. Clearly, the *C. reinhardtii* DMT1 takes its place among the selected prokaryotic Nramp homologues since it only displays prokaryotic and no eukaryotic features on the N-terminus. This is in agreement with the placement of DMT1 in the prokaryotic cluster when the sequences of the core are compared (Figure 3). Once again, DMT1 takes a unique position among the analyzed Nramps as a eukaryotic homologue displaying prokaryotic characteristics.

Heterologous expression of C. reinhardtii DMT1 *in* S. cerevisiae. For functional analysis of the *C. reinhardtii* DMT1 protein an approach utilizing yeast *S. cerevisiae* transport mutants was chosen, since targeted nuclear gene disruption by homologous recombination in *C. reinhardtii* is not established (Nelson & Lefebvre 1995). The complete cDNA of *DMT1* was cloned into the yeast vector pUG35 resulting in plasmid pAR1, which was transformed into various yeast mutants (see Materials and methods). Expression of the *DMT1* gene in yeast was confirmed by western blot analysis (Figure 5). A specific immunoreactive band was detected in cells transformed with pAR1, but not in cells transformed with the control vector

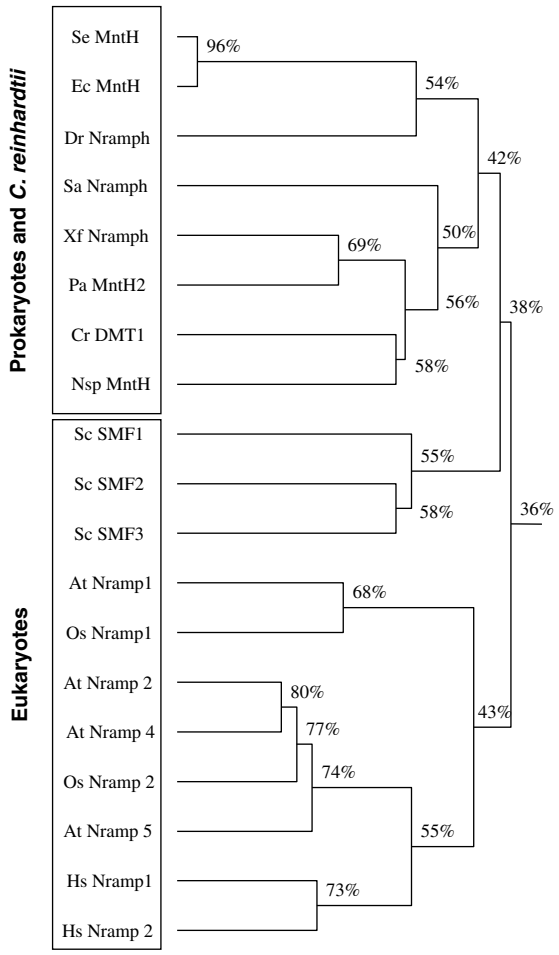

Figure 3. Similarity tree of selected members of the Nramp family. The similarity tree for the hydrophobic core (as defined by (Cellier *et al*. 1996)) of 19 Nramp homologues was constructed by the program DNAMAN. Prokaryotic and eukaryotic groups of Nramp homologues are boxed. Accession numbers for the used protein sequences in order of appearance in the tree can be found in the Materials and methods. Parameters for generation of the tree can be found in the Materials and methods. Ec: *Escherichia coli*; Se: *Salmonella enterica*; Dr: *Deinococcus radiodurans*; Cr: *Chlamydomonas reinhardtii*; Nsp: *Nostoc* sp. PCC 7120; Xf: *Xylella fastidiosa* 9a5c; Pa: *Pseudomonas aeruginosa*; Sa: *Staphylococcus aureus*; Os: *Oryza sativa*; At: *Arabidopsis thaliana*; Sc: *Saccharomyces cerevisiae*; Hs: *Homo sapiens*. Nramph stands for Nramp homologue.

pUG35 or in untransformed cells. As was already observed with highly hydrophobic membrane proteins (Beyreuther *et al*. 1980; Köster & Braun 1986) heating of the cell extract in SDS sample buffer at 95 °C before loading on the gel led to aggregation of the DMT1 protein which barely entered the separation gel (Figure 5, lane 4). When the cell extract was loaded on the gel without previous heating, the DMT1 protein was identified as a rather diffuse band in the gel corresponding to the predicted 57 kDa (Figure 5, lane 1). Expression of the *DMT1* gene was confirmed in all studied mutants (data not shown).

DMT1 restores growth of a S. cerevisiae *smf1 mutant under manganese limiting conditions*. The Smf1p protein of *S. cerevisiae* has been identified as a manganese transporter belonging to the Nramp family (Supek *et al*. 1996) and shows 38% similarity to *C. reinhardtii* DMT1. Yeast strains with disrupted *SMF1* gene fail to grow on synthetic medium containing the divalent cation chelator EGTA. As a first step to elucidate the function of DMT1, its ability to complement the yeast strain Y16272 (*smf1*) with respect to growth restoration in inhibiting concentrations of EGTA was tested. For this, yeast strain Y16272 (*smf1*) was transformed with plasmid pAR1 containing *C. reinhardtii* Nramp homologue *DMT1*, or with the empty yeast overexpression vector pUG35 as a control. Growth tests were performed in liquid minimal medium containing 0–10 mM EGTA and the maximal growth rate of each strain in medium with no EGTA was defined as 100% growth of the strain. Growth rates of the two strains were different and the strain with the control vector showed higher sensitivity to EGTA than the strain expressing DMT1 (Figure 6). At an EGTA concentration of 5 mM, relative growth of strain *smf1*/pUG35 was reduced to 78%, whereas relative growth of strain *smf1*/pAR1 remained at 100%. Higher EGTA concentrations eventually impaired growth of both strains. These results indicate that the strain *smf1*/pAR1 is able to compete with EGTA for manganese ions, in contrast to strain *smf1*/pUG35. Thus, DMT1 most likely functions as a metal transporter which imports metal ions into the yeast cell. At EGTA concentrations below 5 mM, growth of strain *smf1*/pAR1 exceeded 100%. This might suggest that the overexpressed DMT1 protein imported too many metal ions into the cell in medium without the metal chelator and thus lead to a slight growth decline.

Overexpression of DMT1 in S. cerevisiae mutant smf1 increases sensitivity to manganese, cadmium, and copper, but not to zinc. The fact that metals can become toxic when their concentration exceeds physiological levels in the cell was utilized to study

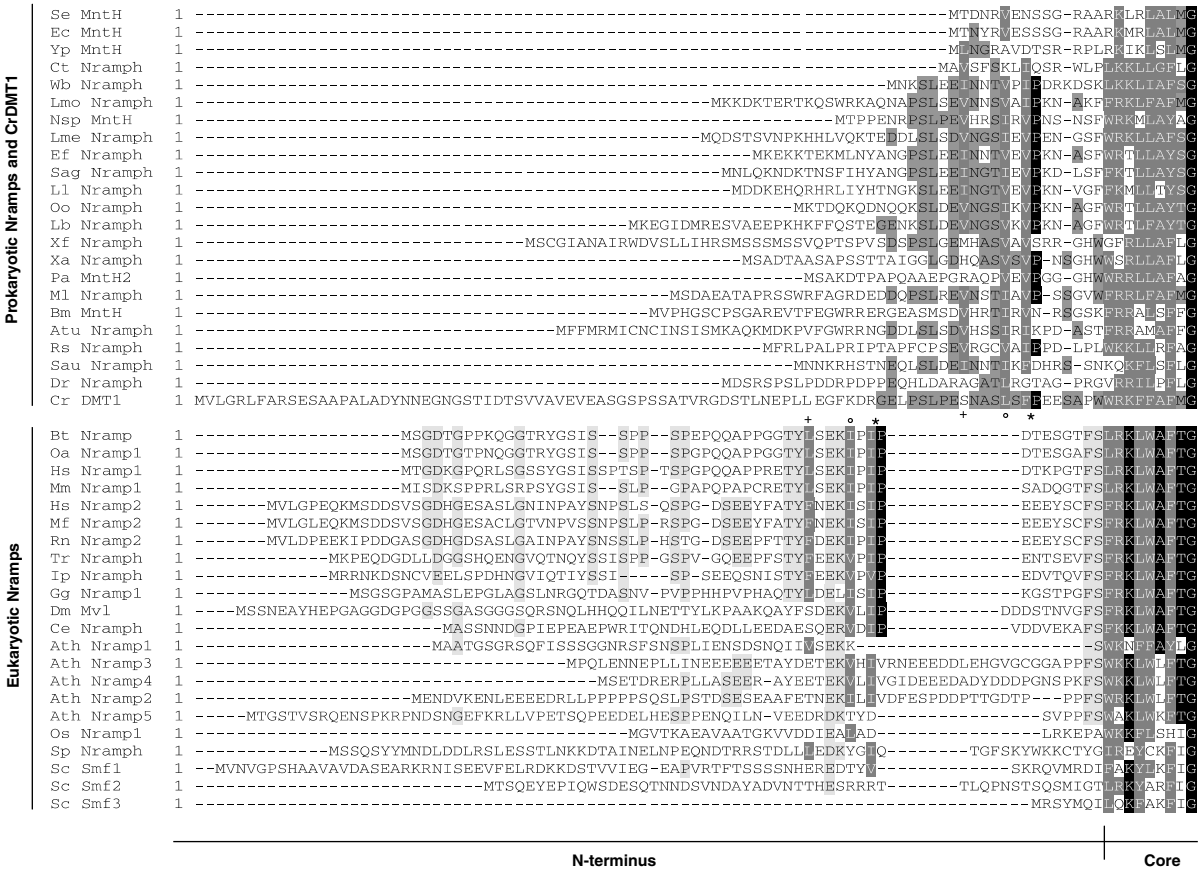

Figure 4. Alignment of the N-terminal sequence of selected Nramp homologues and of *C. reinhardtii* DMT1. The alignment was performed with the N-terminal sequence of selected Nramp homologues including the first 9 amino acids of the core region, which display high similarity among the organisms (rightmost in the alignment). The alignment is split in two blocks. The upper block is composed of prokaryotic Nramps and CrDMT1, the lower block of eukaryotic Nramps. White letters shaded in black or dark grey represent residues conserved in both blocks. Black letters shaded in dark grey represent residues conserved only in the prokaryotic block and CrDMT1, and black letters shaded in light grey represent residues conserved only in the eukaryotic block. The cross, the circle and the star indicate residues which are conserved in both blocks, but are somewhat shifted (because of additional amino acid stretches in the eukaryotic block). The first two letters correspond to the initials of the organism (*e.g.*, Se for *Salmonella enterica*) with the designation of the protein following. Nramph stands for Nramp homologue. Accession numbers for the used sequences (in order of appearance in the alignment) can be found in the Materials and methods.

the specificity of the DMT1 protein. If uptake of a particular metal ion is mediated by DMT1, the strain expressing the DMT1 protein should be less tolerant to that metal than the strain with the control vector. In the following experiments the two strains used above (*smf1*/pUG35 and *smf1*/pAR1) were tested for differences in growth under increasing metal concentrations.

The first element tested was manganese, in order to confirm the result of the previous experiment, which pointed to a role of DMT1 in manganese (Mn^{2+}) uptake. For this, cells were grown in liquid minimal medium containing 0, 10, 100 or 1000 μM $MnCl_2$. Addition of $MnCl_2$ showed the expected effect. That is, the yeast mutant expressing *DMT1* was more sensitive to increasing concentrations of $MnCl_2$ than the control strain with the empty vector (Figure 7a). The maximal growth rate of each strain in medium with no $MnCl_2$ was defined as 100% growth of the strain and growth of the same cells in different concentrations of $MnCl_2$ was expressed in relation to it. Relative growth of strain *smf1*/pUG35 was still 100% at a $MnCl_2$ concentration of 100 μM and 99% at 1000 μM $MnCl_2$. Growth in strain *smf1*/pAR1 was reduced to 89 and 83%,

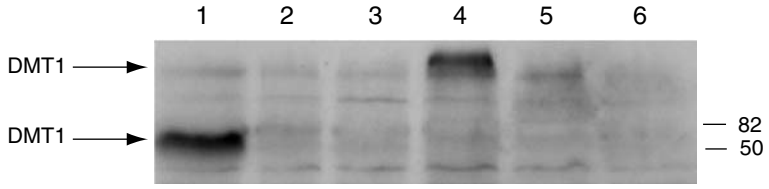

Figure 5. Heterologous expression of DMT1 in *S. cerevisiae* strain Y16272 (smf1). Yeast strain Y16272 was transformed with pAR1 or with the control vector pUG35 and whole cell extract was analyzed for *DMT1* expression by western blotting. The molecular weight in kDa is indicated on the right and arrows point to the DMT1 protein band. Samples 1–3 were loaded on an SDS PAGE gel without previous boiling, samples 4–6 were boiled. Lanes 1 and 4: mutant *smf1* with DMT1, lanes 2 and 5: mutant *smf1* with control plasmid, lanes 3 and 6: mutant *smf1*.

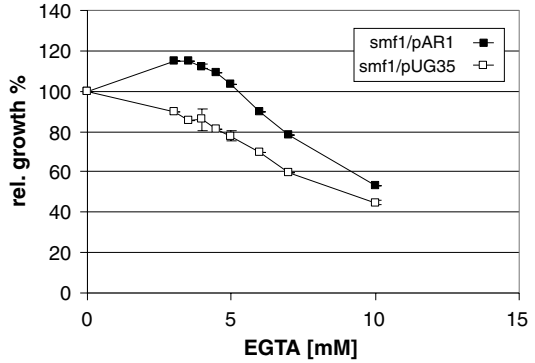

Figure 6. Yeast strain smf1/pAR1 was tested for altered EGTA tolerance. The yeast manganese transport mutant strain Y16272 (*smf1*) was transformed with the *DMT1* containing plasmid pAR1 (filled squares) or with the control expression vector pUG35 (open squares) and growth of the two strains was compared in medium containing increasing concentrations of the metal chelator EGTA. The maximal growth rate of each strain in the medium without EGTA was defined as 100% and growth of the same cells in different concentrations of EGTA was expressed in relation to it. The graph is derived from two independent experiments.

respectively. These results indicate that Mn^{2+} ions must have entered the *smf1*/pAR1 cells via the DMT1 transporter where they reached toxic concentrations leading to growth impairment.

In order to investigate the spectrum of metal cations transported by DMT1 in addition to Mn^{2+}, cadmium (Cd^{2+}), copper (Cu^{2+}) and zinc (Zn^{2+}) were tested for potential growth inhibition of the yeast mutant strain *smf1* expressing *DMT1* on the plasmid pAR1. Addition of 1 μM $CdCl_2$ into the medium led to an average growth reduction of 18% in the strain expressing *DMT1* but only 2% in the strain with the control vector (Figure 7b). At 10 μM $CdCl_2$ strain *smf1*/pAR1 showed a growth reduction of 52% whereas relative growth of *smf1*/pUG35 decreased for only 9%. These results led to the conclusion that DMT1 is able to transport cadmium ions into the yeast cells. Addition of $CuSO_4$ to the medium had a similar effect and a clear growth difference between the two strains was observed at a concentration of 1 mM $CuSO_4$ (Figure 7c). Strain *smf1*/pAR1 showed a growth reduction of 73%, while growth of strain *smf1*/pUG35 was reduced by 48%. The fourth metal tested was zinc. In this case there was no difference in the response of the two strains after addition of up to 10 mM $ZnCl_2$ into the growth medium. Both strains showed growth inhibition in the same degree at concentrations exceeding 1 mM $ZnCl_2$ (Figure 7d), showing that the yeast strain expressing *DMT1* did not have any growth advantage over the strain with the empty vector in toxic zinc concentrations.

DMT1 does not complement EDTA sensitivity of S. cerevisiae zrt1/zrt2 double mutant. ZHY3 is a yeast mutant in which the high and low affinity zinc transporter genes *ZRT1* and *ZRT2* have been disrupted and which, consequently, shows reduced growth in medium with the metal chelator EDTA (Zhao & Eide 1996). The mutant was transformed with the *DMT1* containing plasmid pAR1 or the control vector pUG35 in order to test whether *DMT1* expression would alter the growth behavior of the strain. Growth tests were performed in liquid minimal medium containing 1 mM EDTA and 0–1 mM $ZnCl_2$. Maximal growth rate of each strain under zinc replete conditions (EDTA 1 mM, $ZnCl_2$ 1 mM) was defined as 100% growth of the strain and growth of the same cells in lower concentrations of $ZnCl_2$ was expressed in relation to it (Figure 8). At lower zinc concentrations in the medium growth of both strains was reduced to the same degree and no difference was observed between them. Since the strain expressing the *C. reinhardtii DMT1* did not show any advantage in growth, it can be assumed that the overexpressed

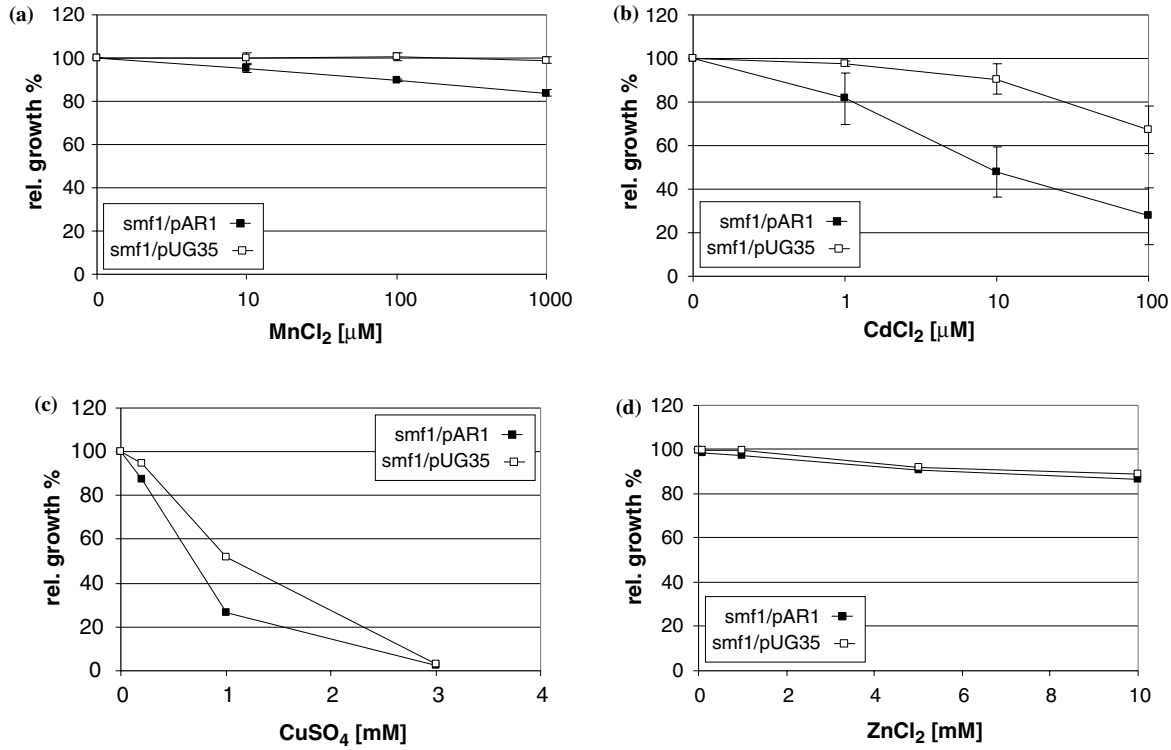

Figure 7. Altered sensitivity to divalent metals in yeast strain smf1/pAR1. The yeast manganese transport mutant strain Y16272 (*smf1*) was transformed with the *DMT1* containing plasmid pAR1 (filled squares) or with the control expression vector pUG35 (open squares) and growth of the two strains was compared in minimal medium containing increasing concentrations of a metal solution. The maximal growth rate of each strain in medium without additional metal was defined as 100% and growth of the same cells in different concentrations of the metal solution was expressed in relation to it. a: $MnCl_2$, b: $CdCl_2$, c: $CuSO_4$, d: $ZnCl_2$. The graphs for $MnCl_2$ and $CdCl_2$ are derived from three independent experiments and for $CuSO_4$ and $ZnCl_2$ a representative experiment is shown.

protein was not able to transport zinc. This is in agreement with the results obtained for the *smf1* mutant grown in medium with $ZnCl_2$, which showed no enhanced sensitivity to zinc when expressing *DMT1*.

DMT1 function in iron transport. Next, we investigated whether CrDMT1 acts in iron transport. Yeast mutant DDY4 (*fet3*/*fet4*), in which high and low affinity iron transport is impaired (Dix *et al*. 1997), was tested for growth improvement after transformation with the *DMT1* containing plasmid pAR1, and compared to the same strain transformed with the control vector pUG35. Growth tests were performed in liquid minimal medium containing 1 mM EDTA and 0–1.25 mM $FeCl_3$. Maximal growth rate of each strain in iron excess conditions (EDTA 1 mM, $FeCl_3$ 1.25 mM) was defined as 100% growth of the strain and growth of the same cells in lower concentrations of $FeCl_3$ was expressed in relation to it. At lower $FeCl_3$ concentrations, growth of all strains was impaired (Figure 9). However, the strain expressing *DMT1* had clearly a growth advantage over the strain with the control vector (growth reduction of approximately 40% as opposed to 70% at a $FeCl_3$ concentration of 0.5 mM). This result suggests a role of DMT1 in iron transport.

Discussion

We have cloned a member of the Nramp gene family in the green microalga *C. reinhardtii*, named *DMT1* for *d*ivalent *m*etal *t*ransporter 1. The cloned cDNA displays characteristics typical of *C. reinhardtii*, that is a GC content of over 60% and a polyadenylation signal 13 base pairs upstream the polyadenylation site. Analysis of the genomic sequence which was recently released (Doe Joint Genome Institute, California) (Grossman *et al*. 2003), showed that the

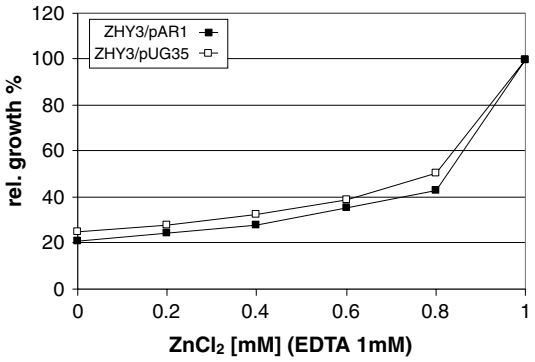

Figure 8. Growth of yeast strain ZHY3/pAR1 under zinc limiting conditions. The yeast zinc transport mutant strain ZHY3 (Zhao and Eide 1996) was transformed with the *DMT1* containing plasmid pAR1 (filled squares) or with the control expression vector pUG35 (open squares) and growth of the two strains was compared in minimal medium containing 1 mM EDTA and increasing concentrations of $ZnCl_2$. The maximal growth rate of each strain under zinc replete conditions (EDTA 1 mM, $ZnCl_2$ 1 mM) was defined as 100% and growth of the same cells in limiting concentrations of $ZnCl_2$ was expressed in relation to it. Shown is a representative experiment.

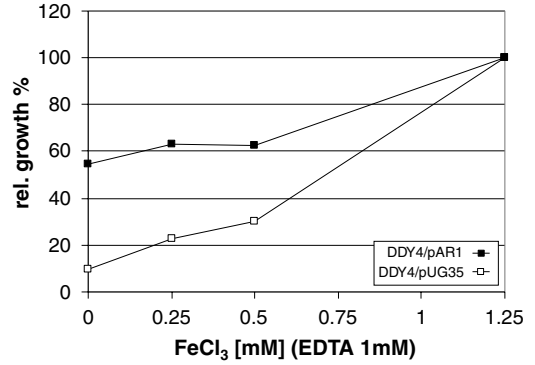

Figure 9. Growth of yeast strain DDY4/pAR1 under iron limiting conditions. The yeast iron transport mutant strain DDY4 (Dix *et al.* 1997) was transformed with the *DMT1* containing plasmid pAR1 (filled squares) or with the control expression vector pUG35 (open squares) and growth of the two strains was compared in minimal medium containing 1 mM EDTA and increasing concentrations of $FeCl_3$. The maximal growth rate of each strain under iron excess conditions (EDTA 1 mM, $FeCl_3$ 1.25 mM) was defined as 100% and growth of the same cells in limiting concentrations of $FeCl_3$ was expressed in relation to it. Shown is a representative experiment.

DMT1 gene consists of 13 exons and 12 introns, with the introns reaching more than double the length of the coding sequence. This is rather uncommon for *C. reinhardtii*, where intronic sequence equals the coding sequence length (Rochaix *et al.* 1998).

The deduced amino acid sequence reached 513 amino acids and contained structural features common for the Nramp family. Firstly, the DMT1 protein was predicted to comprise 11 transmembrane domains (TMs). In agreement to that it was shown by topological analysis that the *E. coli* MntH forms 11 TMs, with a cytoplasmic N-terminus and a periplasmic C-terminus (Courville *et al.* 2003). For other Nramp homologues a number of 10–12 TMs was predicted (Cellier *et al.* 1995; Gunshin *et al.* 1997; Reeve *et al.* 2002). Secondly, the highly conserved 'consensus transport motif' (CTM) found in every Nramp homologue is also present in DMT1 within the same region (between TM8 and TM9). This motif was suggested to be involved in the transport mechanism of Nramps, since it is similar to the prokaryotic binding-protein-dependent transport signature (EAA region of prokaryotic ABC transporters) and to the pore region of K^+ channels (Cellier *et al.* 1996). The EAA region is found in the hydrophobic subunits of the ABC transporters and most likely interacts with the ATPase subunit (Böhm *et al.* 1996; Mourez *et al.* 1997; Locher *et al.* 2002). For the Nramp proteins an interaction with an ATPase subunit in order to facilitate transport by ATP hydrolysis seems unlikely, since uptake of cations was shown to be coupled to H^+ import (Gunshin *et al.* 1997). Nevertheless, the consensus sequence described for the CTM motif (Vidal *et al.* 1993) might indicate an important function in the Nramp protein but distinct to the function of the EAA region in bacterial ABC transporters. Regarding voltage gated K^+channels, the similarity to Nramps is restricted to the pore region sequence (Wood *et al.* 1995; Cellier *et al.* 1996), which is thought to be responsible for ion selectivity (Heginbotham *et al.* 1994). Since the CTM of Nramps is predicted in a cytoplasmic loop, it seems unlikely that the motif should have a similar function as in K^+channels.

Strikingly, the algal DMT1 protein sequence showed higher similarity to some prokaryotic than to eukaryotic members of the Nramp family. A scenario for the evolution of Nramps which includes horizontal gene transfer events between eukaryotes and prokaryotes was proposed (Cellier *et al.* 2001). In spite of such events the investigated eukaryotic Nramps in that study clustered exclusively in one group and they all showed

higher homology to each other than to prokaryotic homologues. The DMT1 protein seems to be an exception as it clusters within a prokaryotic group of Nramps (Figure 3). Likewise, it was shown for the globin protein in *Chlamydomonas eugametos* that it is more similar to globins from ciliates and *Nostoc* than to eukaryotic homologues, and gene transfer events between the *Nostoc* ancestor and a common ancestor to *Paramecium*, *Tetrahymena* and *Chlamydomonas* were considered as an explanation (Moens *et al.* 1996). In addition, regarding only the N-terminus of DMT1, it stands out that it possesses only features of some prokaryotic Nramps, in contrast to other selected eukaryotic homologues (Figure 4). Thus *C. reinhardtii* DMT1 might prove useful for further studies on the phylogenetic evolution of Nramps.

Since the N-terminus of DMT1 is longer than that of any other homologue considered in this study, it seems possible that it plays some important role in the function or regulation of the protein, although no familiar protein motif seems to be present. Tabuchi *et al.* (1999) studied the function of *H. sapiens* Nramp1 and Nramp2 by exchanging the N-termini of the two proteins. Complementation experiments showed that the native N-terminus is essential for a fully functional Nramp1 or Nramp2. This is an interesting result, considering that the N-terminus of some Nramps (*e.g.*, *E. coli*) is extremely short.

Heterologous expression in a manganese transport mutant of *S. cerevisiae* was utilized to investigate the function of the DMT1 protein. A deletion mutant for *SMF1* – the yeast Nramp homologue – was transformed with a moderate level expression plasmid containing *DMT1* and tested for growth under metal depleted conditions. Growth impairment in medium with the divalent metal chelator EGTA was partially restored in cultures expressing *C. reinhardtii DMT1*, suggesting a role for DMT1 as a divalent metal transporter. This finding was affirmed by the fact that the strain expressing *DMT1* was less tolerant to increasing $MnCl_2$ concentrations than the strain with the control plasmid. These results demonstrate the ability of DMT1 to transport manganese ions into the yeast cell.

In order to check the ability of DMT1 to transport other divalent metal ions, we performed growth impairment experiments with the same yeast strains as above (*smf1*/pAR1, *smf1*/pUG35). Addition of $CdCl_2$ to the growth medium resulted in growth impairment for both yeast strains, but the strain expressing DMT1 responded to lower concentrations, that way displaying greater sensitivity against the metal. Cadmium was also described to pass the cell membrane via the Nramp transporter in *Arabidopsis thaliana* (Thomine *et al.* 2000) and it was shown that Mn^{2+} uptake by the Nramp homologue MntH of *E. coli* and *S. typhimurium* was inhibited strongly by Cd^{2+} (Kehres *et al.* 2000). In contrast, MntH was inhibited only weakly by Cu^{2+}, an element which was transported by DMT1 as was shown by the increased sensitivity of the *smf1* yeast mutant strain expressing the algal Nramp, compared to the strain with the control vector. Apparently, this was not true for $ZnCl_2$ were both strains showed exactly the same growth behavior after addition of increasing $ZnCl_2$ concentrations. In order to confirm whether zinc was indeed not transported by the DMT1 protein, *DMT1* was expressed in the yeast strain ZHY3 (*zrt1*/*zrt2*) which shows defects in zinc transport. The phenotype of ZHY3, namely reduced growth in medium with the metal chelator EDTA, was not complemented by *DMT1* expression. Thus, both experiments – the zinc sensitivity test with the *smf1* mutant and the complementation test with the *zrt1*/*zrt2* mutant – lead to the conclusion that DMT1 was not able to transport zinc ions. Concerning zinc, different results were obtained for its transport by Nramp homologues. MntH of *E. coli* and *S. typhimurium* were not inhibited considerably by zinc in manganese uptake (Kehres *et al.* 2000), whereas Supek *et al.* (Supek *et al.* 1996) mention inhibition of Smf1p manganese import by zinc. In rat, it was shown that zinc inhibited iron and manganese uptake via the Nramp homologue DCT1 (DMT1, Nramp2) (Gunshin *et al.* 1997), but Sacher *et al.* (2001) could not show transport of $^{65}Zn^{2+}$ via DCT1 or Smf1p. Thus, Zn^{2+} might bind at the same site on the transporter protein as the other metal ions but without entering the cell.

For a further characterization, *DMT1* was expressed in the yeast strain DDY4 (*fet3*/*fet4*) in which the high and low affinity iron transport systems are impaired. The phenotype of growth impairment under iron limiting conditions was partially complemented by expression of *DMT1*, indicating a role for the DMT1 protein in iron transport. Recently, components of a high affinity

iron transport system similar to the *S. cerevisiae* frt1/fet3 iron assimilation pathway were described in *C. reinhardtii* (Herbik *et al*. 2002; La Fontaine *et al*. 2002). This does not disagree with our results, considering that generally there are several uptake systems present in the cell, mediating high and low affinity transport of the same substrate. An efficient transport system for iron is mostly important for the cell as this element is essential for many vital processes. However, under iron excess conditions, the high affinity system can be shut down and a low affinity system can take on the uptake of iron. Accordingly, DMT1 might play a role in low affinity iron assimilation when the high affinity system is down regulated.

In conclusion, we have cloned and characterized a divalent metal transporter in an alga – the DMT1 of *C. reinhardtii* – which shows interestingly higher sequence similarity to some prokaryotic than to eukaryotic Nramp homologues. Furthermore, we elucidated the function of the protein by complementation experiments in several yeast mutants and showed that the specificity of the transporter was quite broad, including manganese, cadmium, copper and iron but no zinc transport.

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