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The NRAMP6 Metal Transporter Contributes to Cadmium Toxicity

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Short title: NRAMP6 and cadmium toxicity

SYNOPSIS

Natural Resistance Associated Macrophage Protein (NRAMP) homologues are evolutionarily conserved divalent metal transporters. In *Arabidopsis*, AtNRAMP3 and AtNRAMP4 play a key role in iron nutrition of the germinating plantlet by remobilizing vacuolar Fe stores. Here we describe the molecular and physiological characterization of AtNRAMP6. *AtNRAMP6* is predominantly expressed in the dry seed embryo and to a lesser extent in aerial parts. Its promoter activity is found diffusely distributed in cotyledons and hypocotyls as well as in the vascular tissue region of leaf and flower. We show that *AtNRAMP6* transcript coexists with a partially spliced isoform in all tested shoot cell types. When expressed in yeast, *AtNRAMP6*, but not its misspliced derivative, increased sensitivity to cadmium without affecting Cd content in the cell. Likewise, *Arabidopsis* transgenic plants overexpressing *AtNRAMP6* were hypersensitive to Cd although plant Cd content remained unchanged. Consistently, a null allele of *AtNRAMP6*, named *nramp6-1*, was more tolerant to Cd toxicity, a phenotype that was reverted by expressing *AtNRAMP6* in the mutant background. We used an AtNRAMP6::HA fusion, shown to be functional in yeast, to demonstrate through immunoblot analysis of membrane fractions and immunofluorescence localization that, in yeast cells, AtNRAMP6 is targeted to a vesicular-shaped endomembrane compartment distinct from the vacuole or mitochondria. We therefore propose that AtNRAMP6 functions as an intracellular metal transporter, whose presence, when modified, is likely to affect distribution/availability of Cd within the cell.

Keywords: NRAMP, Transport, Cadmium, Toxicity, Heavy metal, Endomembrane

Abbreviations used: NRAMP: Natural Resistance Associated Macrophage Protein. DCT1: Divalent Cation Transporter 1. DMT1: Divalent Metal Transporter 1. MntH: Mn Transporter H. DAPI: 4',6' Di Amidino-2-Phenyl Indole.

INTRODUCTION

Cadmium is an environmental pollutant with high toxicity to plants and animals. Despite the existence of a cadmium-containing carbonic anhydrase in a marine diatom [1], Cd is commonly considered as a nonessential transition metal. Cd is classified as a human carcinogen that affects many cellular processes including cell cycle, cell proliferation, and DNA repair [2]. Cd also disrupts homeostasis of essential metals, which it displaces in metal-binding proteins such as transcription factors or enzymes. Although Cd^{2+} is not a redox-active metal, it indirectly induces oxidative stress. In plants, cadmium affects root growth and biomass production by inhibiting photosynthesis, respiration and mineral uptake and by disturbing the plant water status [3, 4]. The presence of Cd in plants and more particularly its translocation to edible parts represents its main entry route into the food chain. Knowledge of the molecular events by which Cd is taken up by cells or detoxified therefore, could provide potential targets to reduce its toxicity.

Cd is supposed to enter the plant as an opportunistic hitchhiker using transporters of essential metals. IRT1, a metal transporter essential for root iron uptake in response to iron deficiency, is also known to accept Cd as a substrate [5, 6]. As a result, the *Arabidopsis irt1* loss-of-function mutant fails to accumulate heavy metals, including Cd, when grown in iron-limited conditions [7]. Furthermore a mammalian homolog of IRT1, ZIP8, was shown in mouse to be implicated in cadmium-induced toxicity in the testis [8].

Once cadmium has entered the cells, plants use various strategies to cope with its toxicity. One such strategy involves the chelation of cadmium by a family of thiol-containing ligands such as glutathione and its derivative phytochelatins (PC) [9]. Genetic studies have confirmed that PC-deficient mutants are hypersensitive to Cd [10]. Another strategy consists in transporting cadmium out of the cell or sequestering it into organelles, thereby removing it from the cytosol. A number of transporter families contribute to cadmium resistance. The yeast ATP-binding cassette (ABC) transporters, ScYCF1 and SpHMT1, sequester heavy metals by pumping GSH-conjugated cadmium and PC-conjugated cadmium, respectively into the vacuole [11, 12]. No similar function has been found yet among plant ABC transporter homologues but the ABC transporter AtPDR8 was recently shown to mediate the extrusion of Cd out of the plasma membrane of root epidermal cells [13]. Detoxification of cadmium is also achieved by members of the heavy metal-transporting P-type ATPases (HMA) subfamily [14]. HMA4 is a Zn/Cd transporter that confers plant tolerance to Cd by loading it into the xylem, thus increasing translocation to the shoot where it might have less damaging effects [15, 16].

Another family of metal transporters potentially involved in the mobilization of cadmium is the natural resistance-associated macrophage protein (NRAMP) family. First identified in mammals, the NRAMP/DCT1/DMT1 proteins are evolutionarily conserved, with homologues in bacteria, algae, plants and animals. Measurements of transport activity conferred by heterologous expression in *Xenopus laevis* oocytes have revealed that NRAMP transporters accept a broad range of substrates including Fe^{2+} , Zn^{2+} , Mn^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Ni^{2+} and Pb^{2+} and that this active transport is coupled with a proton [17]. However, the physiological role of NRAMP is mainly recognized for iron and to a lesser extent for manganese. Nramp1 is specific to macrophages where it has been shown to confer resistance to pathogen invasion probably by modulating metal ion content in the phagosome [18]. Nramp2 is thought to be the main transporter for the absorption of dietary iron at the brush-border membrane of the mammalian intestine [19] and to contribute to Fe^{2+} and Mn^{2+} recycling from the transferrin [20].

Members of the NRAMP family have been characterized in many plants species including rice, soybean and *Thlaspi* as well as in the green algae *Chlamydomonas* [21-27]. Through complementation of yeast metal transport mutants, they have been shown to function as metal ions transporters, however very few of them have been assigned a physiological function. The soybean GmDmt1, located on the peribacteroid membrane of root nodules and shown to mediate ferrous iron uptake in yeast, was proposed to provide iron to developing rhizobia [23]. In the Ni hyperaccumulator *Thlaspi japonicum*, TjNRAMP4 specifically transports Ni when expressed in yeast and might therefore contribute to Ni hyperaccumulation in the plant [24]. In *Arabidopsis*, 6 genes *AtNRAMP1-6* encode NRAMP proteins. The most closely related members, AtNRAMP3 and AtNRAMP4, are metal transporters with a broad range of substrate specificity including Fe, Mn, Cd and Zn, as determined by functional complementation of yeast mutants [26, 28-30]. AtNRAMP3 and AtNRAMP4 are tonoplasmic proteins with redundant functions. In iron-limiting growth conditions, germination of the double *nramp3 nramp4* mutant is arrested. Mutant seeds were shown to be unable to retrieve iron from vacuolar globoids upon germination, indicating that AtNRAMP3 and AtNRAMP4 function in the mobilization of iron from vacuolar stores to feed the developing plant [29]. The role of AtNRAMP1 *in planta* has not yet been deciphered. However, *AtNRAMP1* expression in yeast was shown to rescue the growth defect of iron and manganese transport defective mutants [22, 26]. In addition, overexpressing AtNRAMP1 in *Arabidopsis* yielded plants with increased tolerance to excess iron, suggesting a contribution of AtNRAMP1 to plant iron homeostasis [22].

In this paper, we identified AtNRAMP6 as an intracellular cadmium transporter. We show that AtNRAMP6 confers cadmium hypersensitivity when expressed in yeast or when overexpressed in *Arabidopsis*, without affecting yeast or plant cadmium content. Conversely, knocking out *AtNRAMP6* resulted in *Arabidopsis* plants with increased tolerance to cadmium. AtNRAMP6 protein accumulation was observed in a yeast intracellular membrane. The data presented here suggest that AtNRAMP6 is a cadmium transporter that functions inside the cell either by mobilizing Cd from its storage compartment or by taking up Cd into a cellular compartment where it is toxic.

EXPERIMENTAL

Plasmids

For the reconstruction of *AtNRAMP6* cDNA, an *AtNRAMP6* cDNA retaining the intron 6 (I6) of the gene was amplified from reverse transcribed total *Arabidopsis* RNA and cloned into the pGEM®-T Easy vector (gift of Sébastien Thomine, Gif-sur-Yvette, France). To eliminate the I6 77 bp sequence, the 194 bp ScaI-XhoI fragment surrounding I6 was replaced by a 117 bp fragment corresponding to the spliced RNA. The 117 bp intron-less fragment was generated by PCR using I6 containing-*AtNRAMP6* cDNA as a template, a forward primer encompassing the ScaI site in exon 5 and a reverse primer encompassing the E6-E7 junction up to the XhoI site located close to the 5' end of exon 7. The resulting clone was sequenced (see the sequence surrounding the E6-E7 junction in Fig1). The 35S-*AtNRAMP6* construct for overexpression in plants was obtained by subcloning the *AtNRAMP6* cDNA KpnI - BamHI into the pCHF3 vector [31] in translational fusion with the CaMV 35S promoter. For expression in yeast, *AtNRAMP6* cDNA sequences, containing the intron 6 or without it, were subcloned as a Sall- BamHI

fragment into the XhoI and BamHI sites of the pDR195 [32] yeast expression vector. The HA epitope-tagged *AtNRAMP6* construct was obtained by cloning an amplified 2 X HA fragment (primers TagHA F and TagHA R) in the BamHI site located in 3' of *AtNRAMP6* cDNA in the pCHF3-*AtNRAMP6* plasmid, and subsequently subcloning the XhoI-BamHI [*AtNRAMP6*::HA] cassette thus obtained in the XhoI and BamHI sites of the pDR195 vector. To generate a fusion between *AtNRAMP6* promoter and the β -Glucuronidase (GUS), a 1.45 kb fragment of *AtNRAMP6* promoter sequence located upstream the translation initiation codon was amplified by PCR on BAC T24D18 using the forward primer OCC51 and the reverse primer OCC52 which contain a HindIII and a NcoI site respectively. The resulting promoter fragment was cloned in translational fusion with the uidA gene in the pBKS-GUS vector [33] digested with HindIII and NcoI. The [*AtNRAMP6* promoter-GUS] cassette thus obtained was then subcloned into the binary vector pBIN19 [34] digested with the same enzymes to generate the plasmid pBIN19/*AtNRAMP6*-GUS.

Plant growth conditions, genotypes and transformation

Arabidopsis seedlings were grown on plates containing $\frac{1}{2}$ MS [35]. When indicated, CdSO₄ was added into the medium from a 10mM stock solution. The *Arabidopsis* GABI-Kat line ID 550D06 contains a T-DNA inserted 1940 bp downstream the translation initiation codon of *AtNRAMP6* in the intron 9. Homozygous plants were screened by PCR using primers NR6S and either NR6AS or GKLb (T-DNA left border). GKLb amplified a fragment with both NR6S and NR6AS primers, indicating that at least two T-DNAs are inserted in inverted orientation, with their left border towards *AtNRAMP6* gene sequences (see Figure 5). Sequencing of the T-DNA/gene junctions reveals a deletion of 19 bp of *AtNRAMP6* sequence at the insertion site. Transformation of *A. thaliana* ecotype Col-0, either wild-type or *nramp6-1* mutant, was performed using the GV301 strain of *Agrobacterium tumefaciens* following the floral deep method [36]. Transformed T1 plants were selected on kanamycin.

Histochemical analysis of GUS activity

Eight independent transgenic *Arabidopsis* lines were analyzed for GUS activity. Plants were grown on plates containing iron-replete medium for 12 days. Plantlets were harvested in phosphate buffer (50 mM NaPO₄, 0.05% Triton X-100, pH 7.2) and pre-fixed in 4% v/v formaldehyde in phosphate buffer by vacuum infiltration for 45 min. Samples were washed 3 times in phosphate buffer prior to be vacuum infiltrated for 15 min then soaked overnight at 37°C in a solution containing 1mM X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) in GUS buffer (50 mM NaPO₄, 0.5 mM ferrocyanide, 0.5 mM ferricyanide, 0.05% Triton X-100, pH 7.2). Samples were cleared by successive steps of increasing ethanol concentration before observation.

Expression analyses

Total RNA was extracted using the TRIZOL reagent (Invitrogen, San Diego) as described by the manufacturer. A DNase (Promega, Madison, WI, USA) treatment was performed to avoid genomic DNA contamination. The integrity of DNA-free RNA was verified on agarose gel electrophoresis and equal amounts (3 μ g) were used for reverse transcription (MMLV-RT,

Promega) with anchored oligo(dT₂₃). Real-time PCR was performed using the LightCycler® FastStart DNA Master^{PLUS} SYBER GREEN I (Roche, <http://www.roche.com>) using the *NRAMP6* specific (forward GAGTCCTCGTCCACACATTTC, reverse TTTAAGTCTCCTATAACCGCTACTC) primers. Relative transcripts levels were calculated relative to the transcript amount of the constitutively expressed *Clathrin* gene (At4g24550) (forward AGCATACACTGCGTGCAAAG, reverse TCGCCTGTGTCACATATCTC). *NRAMP6* cDNA polymorphism: cDNA isoforms were amplified from total RNA extracts using NR6 I6F forward primer and E7 GCGATCAAGAACTCGAGC reverse primer. Relative transcripts levels were calculated relative to the transcript amount of the constitutively expressed *actin* gene (At3g18780) (forward GGTAACATTGTGCTCAGTGGTGG, reverse AACGACCTTAATCTTCATGCTGC).

Yeast strains and growth conditions

W303 (ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3) and Δ smf1 (MATa/MATa ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 smf1::URA3ura3::TRP1). Cultures were performed in YNB + 2% D-glucose - Uracyl. For complementation of Δ smf1, the medium was supplemented or not with 5mM EGTA. Cadmium-induced growth inhibition was tested in the W303 wild-type strain as follows: 4 independent clones containing either pDR195-NRAMP6 or the empty pDR195 vector were grown overnight in liquid YNB. These precultures were used to inoculate 2 ml cultures containing increasing concentrations of CdSO₄ (0; 2; 5; 10; 20; 40μM) at a D₆₀₀ = 0.02 and grown overnight to reach the stationary phase. Growth inhibition was estimated by measuring the final D₆₀₀ of the cultures.

Yeast protein extract

Total protein extracts were prepared from yeast cultures grown until a D₆₀₀ = 1.5 in YNB. Cells were harvested, washed with water, resuspended in extraction buffer (50mM KH₂PO₄ pH 7.8, 1mM EDTA, 0.1% Triton X-100). Protein lysates were obtained using glass beads in the presence of protease inhibitors (100μg/ml phenylmethylsulfonyl fluoride, 1μg/ml pepstatin A, 1μg/ml leupeptin) and vortexing 15 X 20 s, with 30 s on ice between pulses. The cell debris were pelleted by centrifuging 5 min at 2500 x g at 4°C. The resulting supernatant represented the total protein extract. Plasma membrane proteins were prepared from W303 yeast strain transformed either with the empty pDR195 vector or pDR195-*AtNRAMP6-HA* and grown for 2 days in 2 liters YNB -Ura containing 2% glucose at 30°C according to [37]. Protein content was determined according to Bradford [38]. Proteins were denatured for 10 min at 65°C and loaded onto SDS-PAGE for immunoblotting according to standard procedures.

Immunoblot detection

The following antibodies were used: mouse Anti-HA (ZYMED Laboratories, Carlsbad, CA) diluted 1:4000, mouse Anti-yeast V-ATPase diluted 1:2000 as a yeast vacuolar marker, mouse Anti-yeast cox2p diluted 1:500 as a mitochondrial marker (Molecular probes Inc., PO Box 22010, Eugene, OR 97402-0469), mouse Anti-yeast Pma1p diluted 1:10000 as a plasma membrane marker (Abcam plc Cambridge, CB4 0WN, UK) and an Anti-mouse conjugated to

alkaline phosphatase (Invitrogen) diluted 1:10000. Membranes were washed three times, first with blocking buffer, secondly with PBS 1X and finally with Tris HCl 20mM, MgCl₂ 1mM for 5 min. Membranes were then incubated 5 min in the presence of “Aurora Chemiluminescence Substrate Solution” (Millipore Corporation, Bellerica, MA 01821, USA). Chemiluminescence was revealed on a LAS 3000 Imager (Fujifilm).

Immunofluorescence detection in yeast

Cells were treated according to [39] using a mouse monoclonal anti-HA (Sigma) as a primary antibody and a goat anti-mouse antibody coupled to Alexa 546 as a secondary antibody (Molecular Probes). Cells were examined using a Zeiss Axioplan2 confocal microscope equipped with a LSM 510 META head.

Elemental analyses

Yeast cultures were grown in SD medium supplemented with 2μM CdSO₄ to a D₆₀₀ = 1.5. Cells were harvested, washed 3 times with water. For mineralization, pelleted yeast cells or harvested plant parts were desiccated for two days at 80°C. Samples were boiled for 1 to 3 h at 200 °C in 1 ml of 69% HNO₃ in a pyrex tube. Cadmium concentration in the samples was measured by atomic absorption spectrometry (SpectrAA220 Fast sequential, Varian, Australia).

RESULTS

Cloning of a functional AtNRAMP6 cDNA

The *Arabidopsis* annotated genome indicates the existence of six members of the NRAMP protein family, among which AtNRAMP1 and AtNRAMP6 proteins form a separate cluster and share 81.5% identity (Figure 1A). AtNRAMP6 polypeptide contains 12 putative membrane-spanning domains, a consensus transport motif common to NRAMP homologues and located in the cytoplasmic loop between transmembrane domains 8 and 9, as well as 1 putative *N*-linked glycosylation site (Figure 1B).

Attempts to isolate *AtNRAMP6* cDNA led clones retaining part or all of the sixth intron of the gene. Figure 2A shows examples of mis-spliced *AtNRAMP6* clones around the intron 6, obtained independently either by RT-PCR on total RNA extracted from whole plantlets or by the Riken Bioresource Center in its *Arabidopsis* full-length cDNA cloning program (<http://www.brc.riken.jp/lab/epd/Eng/catalog/RAFL.shtml>). Retention of the full intron 6, which is most frequently seen, introduces a stop codon 36 nucleotides downstream the E6-I6 junction (labeled with an asterisk in Figure 1A), resulting in the addition of 12 amino acids and in premature translation termination. Based on the amino acid sequence homology between AtNRAMP6 and AtNRAMP1 as well as on the known consensus sequences of splice junctions, we have re-constructed a fully spliced *AtNRAMP6* cDNA (see Material and Methods).

To check whether plants produce the two mRNA species, the intron 6-containing and the intron-less forms, we performed RT-PCR using primers encompassing intron 6 (Figure 2B). The Riken cDNA (RAFL05-14-K06 clone) and the fully spliced, re-constructed cDNA were used as control templates. Both root- and shoot-extracted RNA produced two amplified fragments, whose

sizes corresponded to the fully spliced species and to the intron-retaining species, respectively (Figure 2C, lanes a and b). Moreover, the identity of the two amplified fragments was verified by sequencing. Both the misspliced and the spliced forms of *AtNRAMP6* mRNA therefore, coexist *in vivo*. Since it is well known that regulation of gene expression can occur at the level of intron retention, we investigated the possibility that *AtNRAMP6* is differentially expressed in the various organs through the retention of its intron 6. Indeed, semi-quantitative RT-PCR analysis showed a variation of the ratio of spliced to misspliced forms between the organs (Figure 2C, lanes c to i). Two organs, open flowers and siliques, showed the highest concentration of spliced *AtNRAMP6* cDNA. Nevertheless, all tissues contained a significant amount of the misspliced form.

AtNRAMP6 mediates cadmium toxicity in yeast

All of the NRAMP homologues described have been shown to rescue the phenotype of *Saccharomyces cerevisiae* metal transport mutants. Among the *Arabidopsis* members, *AtNRAMP1*, *AtNRAMP3* and *AtNRAMP4* were shown to complement $\Delta fet3\Delta fet4$ and $\Delta smf1$ strains, defective in Fe and Mn uptake respectively [22, 26]. *AtNRAMP4* was additionally shown to complement the phenotype of $\Delta zrt1\Delta zrt2$, a Zn uptake-defective mutant [28]. In the case of *AtNRAMP6* however, its expression as a fully spliced cDNA or as a cDNA retaining the intron 6 failed to restore growth of any of the yeast metal transport mutants (data not shown).

In addition to essential metals, NRAMP homologues have been shown to be involved in the transport of toxic metals such as cadmium or nickel [24, 26]. To reveal such a function for *AtNRAMP6*, wild-type yeast cells expressing the *AtNRAMP6* reconstructed cDNA were grown in standard YNB medium (Figure 3A) or in the presence of 25 μ M CdSO₄ in the culture medium (Fig 3B). Although *AtNRAMP6* did not impair yeast growth in standard medium, its presence increased Cd-induced growth inhibition (Figure 3B). When yeast was transformed with the intron-containing version of *AtNRAMP6* cDNA, growth inhibition was not observed (Figure 3B). We concluded that, at least in yeast, only the fully spliced form of *AtNRAMP6* cDNA is functional.

To quantify *AtNRAMP6*-induced toxicity, we performed a growth inhibition experiment in liquid medium containing increasing concentrations of Cd. The concentration-response curves indicated an IC₅₀ (Cd concentration necessary to reach 50% growth inhibition) of 1.0 and 1.7 for pDR195-*AtNRAMP6* and pDR195 respectively, with a maximum difference of growth inhibition reaching 4-fold at 5 μ M Cd (Figure 3C). Therefore, when expressed in yeast, *AtNRAMP6* confers higher sensitivity to external cadmium concentration.

Expression pattern of AtNRAMP6

Consistent with reported expression levels in public microarrays databases (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>, At1g15960), *AtNRAMP6* transcript, as measured by real time RT-PCR, was found to accumulate predominantly in seeds and levels decreased rapidly upon germination (Figure 4A). *AtNRAMP6* expression was also detected in shoot tissues, although to a lesser extent, but not in roots (Figure 4B,C). Accumulation of *AtNRAMP6* transcripts was detected in leaves, senescing leaves, floral buds, opened flowers, siliques and silique peduncles (Fig 4C).

To investigate the tissue specificity of *AtNRAMP6*, transgenic plants expressing the *uidA* gene, encoding the β -Glucuronidase (GUS), under the control of a 1.45 kb-long fragment of *AtNRAMP6* promoter were produced. Histochemical staining of GUS activity (Figure 4D) confirmed that the *AtNRAMP6* promoter is active in dry seeds where it is diffusely distributed in cotyledons and hypocotyls of the embryo (a). Staining was also detected in the vascular bundles of shoot tissues including cotyledons (b), young leaves (d), sepals and petals (c), at the top of the flower stem and in the style (c). Developing siliques showed GUS activity in the peduncle (e), as well as in the septum and the funiculi (f).

Neither transcripts accumulation nor *AtNRAMP6* promoter-driven GUS activity was modified upon treatments by deficiency or excess of Fe, Mn, or Zn or by Cd supply (data not shown).

The *namp6-1* mutant is tolerant to cadmium toxicity

We next used reverse genetics to study the physiological function of *AtNRAMP6*. An *Arabidopsis* line (GK Line ID: 550D06) containing 2 inverted repeated copies of T-DNA inserted in the intron 9 of the *AtNRAMP6* gene (Figure 5A) was identified in the GABI-Kat library of mutants (<http://www.mpiz-koeln.mpg.de>). *AtNRAMP6* transcripts were undetectable in the *nrap6-1* line, indicating that this mutant is a null allele of *AtNRAMP6* (Figure 5B).

nrap6-1 plants had no apparent phenotype; however, in order to investigate whether the lack of *AtNRAMP6* disturbed plant metal homeostasis, *nrap6-1* seeds were germinated *in vitro* on medium either lacking Fe, Mn, Zn or Cu, or containing excess amounts of these metals. In response to these treatments, growth of *Arabidopsis* wild-type and *nrap6-1* mutant plants was not differentially affected, indicating that knocking out *AtNRAMP6* does not impair the ability of the plant to maintain Fe, Mn, Zn or Cu homeostasis (Supplementary Figure S1). Elemental analysis of whole seed, root or shoot tissues, performed using Atomic Absorption Spectrometry (AAS), did not reveal any change in total metal content in the *nrap6-1* mutant.

Because we observed that the expression of *AtNRAMP6* in yeast mediates hypersensitivity to Cd, we tested the possibility that *nrap6-1* might be resistant to a Cd treatment. Four week-old WT and *nrap6-1* plants were supplied with 50 μ M CdSO₄ during 10 days. While WT plants developed toxicity symptoms such as yellow leaves, *nrap6-1* leaves remained mostly green during the treatment (Figure 5C), indicating a better tolerance of the *nrap6-1* mutant to Cd toxicity. Transformation of *nrap6-1* with the *AtNRAMP6* cDNA expressed under the control of the strong ubiquitous Cauliflower Mosaic Virus (CaMV) 35S promoter restored Cd sensitivity of the mutant (Supplementary Figure S2). This result suggested that *AtNRAMP6* might contribute to the uptake of Cd by the plant. To test this hypothesis, the concentration of Cd was measured in leaves of WT and *nrap6-1* plants grown as described for Figure 5C using AAS. Figure 5D shows that the Cd concentration did not significantly decrease in *nrap6-1* compared to WT plants, neither in isolated petioles and limbs of rosette leaves nor in caulinar leaves. Similar results were obtained in 2 week-old seedlings (Table 1). Therefore, rather than being involved in Cd entry into the cell, *AtNRAMP6* may modify Cd distribution between subcellular compartments so as to promote Cd-induced damage.

AtNRAMP6 overexpressing lines are hypersensitive to cadmium toxicity

Wild-type *Arabidopsis* plants were transformed with the 35S-*AtNRAMP6* construct previously used to complement the *nramp6-1* phenotype. 10 independent transformants were selected on kanamycin-containing medium and the level of *AtNRAMP6* expression was measured by semi-quantitative RT-PCR (Figure 6A). The macroscopic phenotype of these overexpressor lines was indistinguishable from the parental line (data not shown). We investigated the capacity of germination in the presence of Cd of lines #2, #3 and #6, which harbored the highest level of *AtNRAMP6* overexpression relative to WT. Compared to WT plants, growth of 35S-*AtNRAMP6* plants was more strongly inhibited by 2 μ M CdSO₄ in the medium, as shown for line #2 (Figure 6B). Quantification of this phenotype was estimated by measuring fresh weight (FW) production in lines #2, 3 and 6 (Figure 6C). Compared to WT, FW dropped to about of 50 % at 2 μ M Cd and 70% at 10 μ M Cd. Consistent with this observation, *nramp6-1* plants grew better than wild-type plants during the treatment (Figure 6C). Similar to the situation with the *nramp6-1* mutant however, the concentration of Cd measured in the overexpressing lines was not different from that in wild-type plants (Table 1).

Membrane localization of AtNRAMP6 protein

One hypothesis that could explain these data is that AtNRAMP6 is a Cd transporter responsible for releasing the metal into or from an intracellular organelle. To identify the subcellular location of AtNRAMP6, we have generated transgenic plants producing C-terminal fusions of AtNRAMP6 with either the green fluorescent protein (GFP) or the hemagglutinin (HA) epitope under the control of the CaMV 35S promoter. Although expression of the transgene was strongly detected at the level of transcript accumulation, we failed to detect the fusion proteins either by Western blot or by imaging of the GFP fluorescence (data not shown). We also failed to detect HA or GFP when fused to AtNRAMP6 truncated at the in frame stop codon present in the intron 6 (data not shown). When the 35S-*AtNRAMP6::GFP* construct was introduced in the *nramp6-1* mutant, the fusion protein did accumulate in the plant, as indicated by Western blot, however the fluorescence signal of the GFP was very weak and mostly cytoplasmic (data not shown).

In the absence of functional tools *in planta*, we addressed the question of the membrane localization of AtNRAMP6 in *S. cerevisiae*. To identify the target membrane of AtNRAMP6, the protein was epitope-tagged with two repeats of HA and expressed in a wild-type yeast strain under the control of the strong PMA1 promoter. The increase of Cd sensitivity mediated by this construct, though slightly weaker, was in the same range as that observed with the non-tagged AtNRAMP6 protein (Figure 3B, C), indicating that the AtNRAMP6::HA fusion used is functional. Immunoblot analysis of total protein extracted from yeast hybridized with a monoclonal anti-HA antibody enabled the detection of two polypeptides: a major polypeptide at 60 kDa corresponding to the calculated size of AtNRAMP6 fused to 2HA, and a less abundant polypeptide around 30 kDa which is likely to result from a proteolytic cleavage of the full size polypeptide (Figure 7A). Despite AtNRAMP6 mediating Cd hypersensitivity, yeast cells contained the same amount of Cd whether it was transformed with the empty vector, AtNRAMP6 or AtNRAMP6::HA constructs (Figure 7B). This result suggested that in yeast too, AtNRAMP6 may be targeted to an intracellular membrane.

We next fractionated yeast total proteins by differential centrifugation to obtain total membrane proteins (M: microsomal fraction) and a plasmalemma-enriched fraction (PM). Proteins were separated on a SDS-PAGE acrylamide gel, blotted and probed with the anti-HA antibody. The HA signal observed in total protein extracts was amplified in the microsomal

extract but was totally undetectable in the PM fraction (Figure 7C). Hybridization of the Western blot with antibodies recognizing proteins specific for the plasma membrane (Pma1p), the mitochondria (Cox2p) and the vacuolar membrane (V-ATPase) confirmed that the PM fraction is relatively pure. Therefore AtNRAMP6::HA is not addressed to the plasma membrane of yeast.

We next addressed the intracellular distribution of AtNRAMP6 by immunofluorescence microscopy (Figure 7D). While no fluorescence was observed in cells expressing the untagged allele (data not shown), AtNRAMP6::HA fluorescence was seen as a punctuate signal in the cytoplasm, thereby eliminating the vacuole as a target compartment for AtNRAMP6. In addition, staining with DAPI, a dye that reveals nuclear as well as mitochondrial DNA, indicated that AtNRAMP6 does not co-localize with mitochondria. These observations confirm that AtNRAMP6 is located in an endomembrane compartment different from vacuole or mitochondria.

DISCUSSION

Among the 6 NRAMP-encoding genes of *Arabidopsis*, metal transport activity has been reported for AtNRAMP1, 3 and 4. In this study, we describe the molecular and physiological characterization of AtNRAMP6 and show that it alters cellular cadmium sensitivity probably by perturbing intracellular distribution of this metal.

During the process of cloning *AtNRAMP6* cDNA, we found that *AtNRAMP6* transcripts are often stabilized as partially spliced isoforms, the most common of these retaining the entire intron 6, resulting in shortening of the open reading frame by about half the length of that encoding the predicted AtNRAMP6 protein. Although alternative splicing is a well-known way to increase protein diversity, a cDNA corresponding to the misspliced isoform turned out to be non functional in our yeast assay. An *AtNRAMP6* cDNA reconstructed according to the prediction of splicing however, was functional in yeast. The biological significance of this missplicing is unclear. We found that both *AtNRAMP6* species coexist in all the tissues tested and were unable to find conditions where the fully spliced isoform exclusively is produced, thus arguing against the idea that *AtNRAMP6* expression is regulated at the level of mRNA splicing. Furthermore, plants overexpressing the truncated form of *AtNRAMP6* did not harbor any growth defect whatsoever (data not shown).

Our data suggest that AtNRAMP6 is a cadmium transporter since when expressed in *Saccharomyces cerevisiae* or when overexpressed in *Arabidopsis*, *AtNRAMP6* confers increased sensitivity to Cd. Consistently, growth of the *nramp6-1* loss-of-function mutant is unaffected by otherwise toxic concentrations of Cd and this tolerance is reverted by complementation with a 35S-*AtNRAMP6* construct. The finding that AtNRAMP6 may be capable of transporting Cd is reminiscent of several other NRAMP proteins. First in plants, AtNRAMP1, 3 and 4 were previously shown to mediate Cd toxicity and increased cellular Cd content when expressed in yeast, whereas *Arabidopsis* lines overexpressing *AtNRAMP3* were found hypersensitive to Cd [26]. Similar results were obtained for the *Thlaspi caerulescens* member TcNRAMP3 when expressed in yeast or in tobacco [27]. MntH, the prokaryotic NRAMP ortholog, is a Fe/Mn transporter that confers Cd hypersensitivity when overexpressed in bacteria [40]. Very close to MntH, *Chlamydomonas reinhardtii* DMT1 restores growth of the yeast $\Delta smf1$ mutant and mediates hypersensitivity to Cd [25]. Nramp2 in mammals was also shown *in vitro* to work as a

cadmium transporter [17] but although it represents a candidate for Cd uptake at the brush border of the duodenum [41], intestinal cadmium absorption was recently shown to be unaffected in the DMT1-dysfunctional *mk/mk* mice [42].

Using immunological approaches, we have established that AtNRAMP6 is not located at the cell surface, but is located in a punctuate-shaped endomembrane compartment, distinct from mitochondria and from the vacuole. This intracellular localization is consistent with AtNRAMP6 conferring Cd sensitivity without affecting Cd content in yeast cells and plant tissues, as one would expect if localized on the plasma membrane. Consequently, the Cd sensitivity induced by AtNRAMP6 is not due to net influx of Cd but rather results from the redistribution of internal Cd pools. AtNRAMP6 could induce Cd toxicity in two ways: either by remobilizing the metal from a detoxifying compartment into the cytosol, thus increasing its availability/toxicity, or by promoting its allocation into an organelle sensitive to Cd-induced damages. However the fact that NRAMP family members use the proton motive force to transport metals [17] supports the view that AtNRAMP6 is targeted to an acidic compartment in order to catalyze the flux of Cd toward the cytosol. In spite of the fact that the vacuole in yeast is responsible for Cd detoxification through the transport of Cd-GS₂ complexes [11], we have clearly shown by immunolocalization that AtNRAMP6 is not located in the yeast vacuolar membrane. In addition to the vacuole, ER-derived vesicles represent putative sites of Cd accumulation in plants. Indeed, in onion epidermal cells exposed to Cd and lead, accumulation of these metals is observed in vesicles derived from the fragmentation of the endoplasmic reticulum [43]. But whether the ER is a detoxifying compartment for Cd or a location where it exerts its toxicity is not clear. In mammalian cells, the well known phenomenon of Cd-triggered apoptosis, which is in part due to dysfunction of mitochondria, was recently also attributed to ER stress in some cell types [44]. This suggests that ER could be a target of Cd toxicity. Immunolabeling of AtNRAMP6 in yeast is compatible with an ER localization. However, the pH of the ER, which was reported to be neutral [45], is not optimal for NRAMP activity that requires a proton gradient across the membrane to function. On the contrary, the lumen of the peroxisomes is alkaline [46] and could provide the proton motive force for AtNRAMP6, which would thus transport metals from the cytosol towards the peroxisome. Finally, the vesicular localization of AtNRAMP6 is reminiscent of that of its yeast homologue, Smf2p, which resides in intracellular vesicles and seems to play a central role in the delivery of Mn to various cellular sites including mitochondria [47]. Whether AtNRAMP6 and Smf2p have similar cellular functions should be investigated in future work.

Although Cd seems to be a substrate for AtNRAMP6, which enabled us to reveal its role as a metal transporter, Cd transport is likely not the primary function of AtNRAMP6 in the plant. What then could be the physiological substrate of AtNRAMP6? Elemental analysis by AAS of *nramp6-1* plant tissues did not reveal any change in metal content, which might provide a clue as to AtNRAMP6's physiological function (data not shown). In addition, we were unable to see growth restoration of yeast Fe, Zn or Mn uptake defective mutants by AtNRAMP6. However, we cannot exclude the possibility that AtNRAMP6 accepts physiological metals such as Fe or Mn as a substrate because, based on immunofluorescence microscopy and immunoblot analyses, AtNRAMP6 is exclusively targeted to intracellular membranes of yeast. In plants, we failed to detect any alteration of growth of the *nramp6-1* mutant in response to deficiency or excess of Fe, Mn or Cu (Supplementary Figure S1), suggesting that AtNRAMP6 is not essential to maintain the homeostasis of these metals.

One explanation for the lack of metal-related phenotype of the *nramp6-1* mutant could be the existence of a functional redundancy between AtNRAMP members, as previously shown for AtNRAMP3 and AtNRAMP4 [29]. Given that AtNRAMP1 and AtNRAMP6 are highly

homologous proteins, we generated an *Arabidopsis* line knocked out in both *AtNRAMP1* and *AtNRAMP6* genes; this *nramp1 nramp6* double mutant also failed to develop any metal-related phenotype however (data not shown). Though phylogenically very close, *AtNRAMP1* and *AtNRAMP6* do not share the same territories of expression, the former being mostly expressed in roots [22] and the latter in seeds and shoots (this work). Combination of the *nramp6* mutation with a mutation in another *AtNRAMP* gene expressed in overlapping territories may provide information on AtNRAMP6 function in the plant. We found AtNRAMP6 promoter to be mostly active in the cotyledons at the embryonic stage, which is consistent with the increased tolerance to Cd of *nramp6-1* embryos at germination. However we did not measure any variation of the total amount of metals (Fe, Mn, Zn, Cu, Cd) in *nramp6-1* seeds (data not shown). Both the expression pattern in the cotyledons and the lack of metal content variation in seeds are reminiscent of *AtNRAMP3* and *AtNRAMP4* [29]. In addition overexpression of *AtNRAMP3*, like *AtNRAMP6*, generates *Arabidopsis* plants hypersensitive to Cd [26] (this study). AtNRAMP3 and AtNRAMP4 are tonoplasmic proteins, an intracellular localization that is clearly different from the vesicular location observed for AtNRAMP6. Like AtNRAMP6, the overexpression of AtNRAMP3 increases the plant sensitivity to Cd [45], which suggests that AtNRAMP3, AtNRAMP4 and AtNRAMP6 may contribute, through parallel transport pathways, to the availability of Cd in cellular compartments sensitive to Cd toxicity. In this scenario, analysis of the double *nramp3 nramp6* or *nramp4 nramp6* mutants may reveal a role for AtNRAMP6 in metal mobilization/sequestration during germination.

In conclusion, this report describes a new member of the NRAMP family of metal transporters. The *AtNRAMP6* gene encodes a protein shown in this study to be functional and potentially involved in Cd tolerance. The protein, shown in yeast to be targeted to intracellular vesicles, may mediate the transport of Cd from internal pools. Taken together, our results and the already known mechanisms of Cd toxicity and detoxification open new perspectives to integrate the role of NRAMP proteins in the homeostasis of metals and the detoxification of non-essential pollutants.

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FIGURE LEGENDS

Table 1 AtNRAMP6 does not affect total cadmium concentration in plants

AAS measurement of Cd in plants grown *in vitro* for 10 days in the presence of 5 μ M CdSO₄. S.E. stands for standard error.

Figure 1 AtNRAMP6 protein sequence and topology

(A) Alignment of the *Arabidopsis* NRAMP protein family generated using ClustalW (<http://align.genome.jp/>) and Boxshade (http://www.ch.embnet.org/software/BOX_doc.html). The Consensus transport motif (CTM) is indicated. (B) AtNRAMP6 is an integral membrane protein predicted to have 12 transmembrane domains TM. The amino acid residues that are circled in the cytoplasmic loop between TM8 and TM9 constitute the consensus transport motif. The position of the putative N-glycosylation site is indicated as a circle in the third extracellular loop. The topology was predicted using the TMHMM v. 2.0 program (<http://www.cbs.dtu.dk/services/TMHMM-2.0>) coupled to the TMD pred^R software.

Figure 2 Two AtNRAMP6 transcript isoforms coexist in the plant

(A) Nucleotide sequence alignment between the intron-retaining *AtNRAMP6* clone (*NRAMP6-I6*: RAFL05-14-K06 clone, Riken), the fully spliced deduced *AtNRAMP6* cDNA (*NRAMP6*) and the *AtNRAMP1* cDNA, in the region encompassing exon 5 to exon 7. Nucleotides are numbered from the ATG initiation codon. The in-frame stop codon in the intron 6 is boxed. (B) Structure of the misspliced *AtNRAMP6* transcript and position of the primers used in the RT-PCR reaction presented in panel C. (C) Differential expression of the two isoforms of *AtNRAMP6* mRNA *in planta*; PCR control on plasmid DNA containing either the misspliced cDNA showing a DNA fragment at 284 pb (a) or the spliced cDNA showing a DNA fragment at 216 pb (b); The size markers lane (M) shows a DNA fragment at 0.2 kbp and 0.3 kbp. Semi-quantitative RT-PCR performed on total RNA extracted from various plant tissues: roots (c), shoots (d), young leaves (e), floral buds (f), open flowers (g), peduncles of siliques (h), siliques (i).

Figure 3 Expression of AtNRAMP6 in yeast results in increased cadmium sensitivity

(A) (B) Assay of cadmium tolerance. Wild-type W303 cells transformed with the pDR195 empty vector, or the pDR195 vector containing either the misspliced *AtNRAMP6* cDNA (NR6-I6), or the reconstructed *AtNRAMP6* cDNA untagged (NR6) or tagged with HA (NR6::HA) were grown for 3 days in YNB medium supplemented (B) or not (A) with 25 μ M CdSO₄. 5X serial dilutions of the cultures, starting at D₆₀₀ 1, were plated. (C) Cadmium induced-growth inhibition test. Clones described in panel A and B were grown for 12 h in YNB liquid medium containing increasing concentrations of CdSO₄ as indicated in the Figure and D₆₀₀ was determined. For each construct, the values represent the mean of values obtained with 3 independent clones. The graph shown is representative of two independent experiments.

Figure 4 Expression analysis of AtNRAMP6

(A-C) Real time RT-PCR measurement of *AtNRAMP6* transcript accumulation, calculated relative to the actin transcript level, on total RNA extracted from either seedlings germinated for the amount of time indicated on the figure (A), or from 14 day-old plantlets grown in vitro (B), or mature aerial organs harvested from soil-grown plants (C). (D) Tissue-specific activity of *AtNRAMP6* promoter. Histochemical staining of β -Glucuronidase (GUS) activity in *Arabidopsis* transgenic plants expressing the *uidA* gene under the control of 1.45 kb of *AtNRAMP6* promoter sequence. (a) Embryo dissected from imbibed seed; (b) Plantlet 3 days post germination; (c) flowers; (d) Mature rosette leaf; (e) silique peduncle; (f) open silique showing staining of the funiculi. L: leaves, SL: senescent leaves, FB: flower buds, OF: open flowers, SP: silique peduncles, S: siliques.

Figure 5 The *nramp6-1* mutant is tolerant to cadmium

(A) Position of insertion of the two inverted repeated T-DNAs, represented by an arrow that points to the left border, in the intron 9 of *AtNRAMP6* genomic sequence. (B) RT-PCR experiment on total RNA extracted from WT and *nramp6-1* *in vitro*-grown plants confirming the absence of *AtNRAMP6* mRNA in the mutant. Amplification of actin is shown to control for RNA loading. (C) Typical phenotype of hypertolerance to cadmium of a *nramp6-1* plant grown in soil and irrigated with 10 μ M CdSO₄ during 10 days. Compared to the yellow rosette leaves of the wild-type plant, *nramp6-1* leaves remain green upon Cd treatment. (D) Cadmium concentration measured by atomic absorption spectrometry (AAS) on tissues harvested from plants cultivated as in (C). Values represent the mean from 5 individually grown adult plants. Error bars represent the standard deviations.

Figure 6 *AtNRAMP6* overexpressing plants are hypersensitive to cadmium toxicity

(A) Accumulation of *AtNRAMP6* mRNA in 3 overexpressing lines as measured by semi-quantitative RT-PCR on leaf total RNA extracts. Actin mRNA was used to control RNA loading. (B) Phenotype of a representative overexpressing line after 7 days of growth *in vitro* in the presence of 2 μ M CdSO₄. (C) Growth rate in the presence of cadmium of 3 *AtNRAMP6* overexpressing lines compared to wild-type plants (Col-0) and the *nramp6-1* mutant. Plants were grown 14 days on plates containing the indicated amount of CdSO₄ prior to harvest shoots for fresh weight determination. Each value was obtained from 14 plants and represents the mean of 3 independent measurements. Error bars represent the standard deviations.

Figure 7 *AtNRAMP6* is localized in an endomembrane compartment in yeast

(A) Immunoblot detection of AtNRAMP6::HA in total proteins extracts obtained from yeast transformed with either the pDR195/NRAMP6::HA construct or the empty pDR195 vector and probed with an anti-HA antibody. (B) Cadmium content measured by ICP-MS in wild-type yeast expressing AtNRAMP6 (white bar), or HA-tagged AtNRAMP6 (grey bar), or the empty vector pDR195 (black bar) and grown in the presence of 2 μ M CdSO₄. (C) Immunoblot detection of AtNRAMP6::HA in protein fractions. Total: total proteins extract; M: microsomal fraction; PM: plasma membrane-enriched fraction. The purity of the plasma membrane fraction is controlled by

hybridization with antibodies directed against the yeast plasma membrane ATPase Pma1p, the yeast mitochondrial protein Cox2p, and the yeast vacuolar membrane V-ATPase. **(D)** Indirect immunofluorescence detection of AtNRAMP6::HA in fixed yeast cells. a) cells were stained with mouse monoclonal anti-HA antibodies, which were then revealed with an anti-mouse coupled to Alexa 546; b) DAPI staining reveals structures containing DNA, here nuclei and mitochondria; c) overlay. Insert: enlargement of one cell revealing that HA-staining is distinct from the mitochondrial DNA staining.

Table 1

**Cadmium concentration (mg.g⁻¹) measured
by AAS in *Arabidopsis* seedlings**

Genotype	Mean value	S.E.
WT	131.892	10.018
<i>nramp6-1</i>	116.630	7.110
35S-NR6 # 2	107.200	20,244
35S-NR6 # 3	118.759	15.645

A

AtNRAMP1 1 -----MAATGSGRQFTSSSGNRSFNSPLIENSDSNQIIVS---EKKSWKNFFAYLGGPGLVSIAYID
AtNRAMP6 1 -----MAAE-----TASGNSRISNSPLIENSDSNQIIVP---EKKSWKNFFSYLGGPGLVSIAYID
AtNRAMP2 1 MENDVKENLEEEEDRLPPPPPSQSLPS---TDSSEAAAFETNEKILIVDFESPDDPTTGDTTP---PPFSWKLKLETPGPGFLMSIAFID
1 MTGSTVSRQENS PKRPNDNSNGEFKRLVPESSQPEEDLHESPPENQILNDEEDRDKTYDSVP---PFSTAKLKKETGPGFLMSIAFID
AtNRAMP3 1 ----MPQLENNE-----PLLIN-----EEEEEEAYDETEKVHIVRNEEDDLEHGCGCGAPPFSWKLKLETPGPGFLMSIAFID
AtNRAMP4 1 ----MSETDRER-----PLLAS-----EER-----AYEETEKVLIVGDEEDADYDDDPGNSPFSWKLKLETPGPGFLMSIAFID

AtNRAMP1 63 PGNFETDLQAGAHYKYELLWIIIVASCAALVIQSLAANLGVVTGKHLAEQCRAEYSKVPNFMLWVVAELAVVACDEPEVIGTAFALNMLF
AtNRAMP6 55 PGNFETDLQSGAQYKYELLWIIIVASCAALVIQSLAANLGVVTGKHLAEHCRAEYSKVPNFMLWVVAELAVVA-----IGTAFALNMLF
AtNRAMP2 85 PGNTEGDLQAGAIAGYSLWLLMWATAMGLIQMLSARVGVATGRLHAEICRDEYPTWARYVLWSMAELALICADIQEVIQSAIALQILS
AtNRAMP5 87 PGNTEGDLQAGAVAGYSLWLLMWATMGLMLQSLSARVGVATGRLHAEICRSEYPSWARILTLAEVALICADIQEVIQSAIALQILT
AtNRAMP3 73 PGNTEGDLQAGAVAGYSLWLLMWATAMGLLVCLLSARVGVATGRLHAEICRDEYPTWARMVLVMAELALIGSDIQEVIQSAIALKILS
AtNRAMP4 69 PGNTEGDLQAGAIAGYSLWLLMWATAGLLIQMLSARVGVATGRLHAEICRDEYPTWARMVLVMAELALICADIQEVIQSAIALKILS

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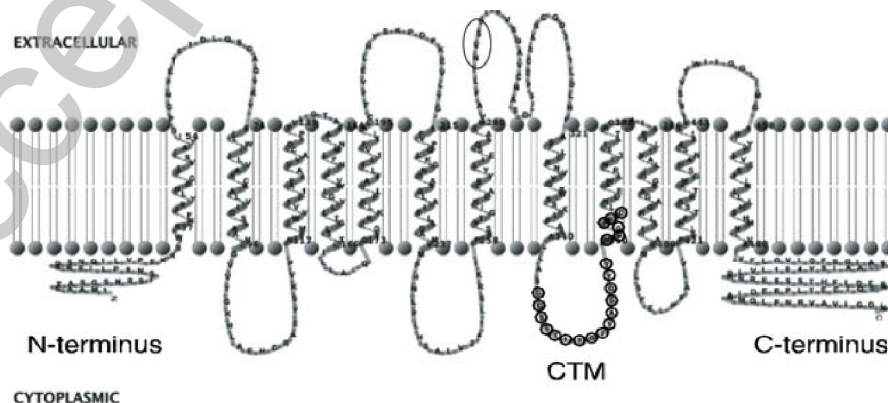
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A

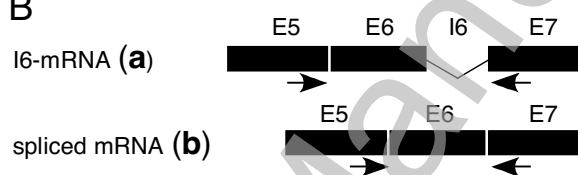
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B



C



