**The NRAMP6 metal transporter contributes to cadmium toxicity**

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NRAMP (natural resistance-associated macrophage protein) homologues are evolutionarily conserved bivalent metal transporters. In *Arabidopsis*, AtNRAMP3 and AtNRAMP4 play a key role in iron nutrition of the germinating plantlet by remobilizing vacuolar iron stores. In the present paper 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 hypocotyl, as well as in the vascular tissue region of leaf and flower. We show that the *AtNRAMP6* transcript coexists with a partially spliced isoform in all shoot cell types tested. When expressed in yeast, *AtNRAMP6*, but not its misspliced derivative, increased sensitivity to cadmium without affecting cadmium content in the cell. Likewise, *Arabidopsis* transgenic plants overexpressing *AtNRAMP6* were hypersensitive to cadmium, although plant cadmium content remained unchanged. Consistently, a null allele of *AtNRAMP6*, named *nramp6-1*, was more tolerant to cadmium toxicity, a phenotype that was reverted by expressing *AtNRAMP6* in the mutant background. We used an *AtNRAMP6::HA* (where HA is haemagglutinin) 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 cadmium within the cell.

Key words: cadmium, endomembrane, heavy metal, natural resistance-associated macrophage protein (NRAMP), toxicity, transport.

**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], cadmium is commonly considered to be a non-essential transition metal. Cadmium is classified as a human carcinogen that affects many cellular processes including the cell cycle, cell proliferation and DNA repair [2]. Cadmium also disrupts homeoestasis of essential metals, which it displaces in metal-binding proteins such as transcription factors or enzymes. Although Cd²⁺ 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 cadmium 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 cadmium is taken up by cells or detoxified could therefore provide potential targets to reduce its toxicity.

Cadmium is supposed to enter the plant as an opportunistic hitchhiker using transporters of essential metals. IRT1 (iron-regulated transporter 1), a metal transporter essential for root iron uptake in response to iron deficiency, is also known to accept cadmium as a substrate [5,6]. As a result, the *Arabidopsis* *irt1* loss-of-function mutant fails to accumulate heavy metals, including cadmium, when grown in iron-limited conditions [7]. Furthermore a mammalian homologue of IRT1, ZIP8, was shown in mice 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 PC (phytochelatin) [9]. Genetic studies have confirmed that PC-deficient mutants are hypersensitive to cadmium [10]. Another strategy consists of 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 ABC (ATP-binding cassette) transporters, ScYCF1 (Sc is Saccharomyces cerevisiae) and SpHMT1 (Sp is Schizosaccharomyces pombe), sequester heavy metals by pumping GSH-conjugated cadmium and PC-conjugated cadmium respectively into the vacuole [11,12]. No similar function has yet been found among plant ABC transporter homologues, but the ABC transporter AtPDR8 was recently shown to mediate the extrusion of cadmium out of the plasma membrane of root epidermal cells [13]. Detoxification of cadmium is also achieved by members of the HMA (heavy metal-transporting P-type ATPase) subfamily [14]. HMA4 is a zinc/cadmium transporter that confers plant tolerance to cadmium 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 NRAMP (natural resistance-associated macrophage protein) 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\textsuperscript{2+}, Zn\textsuperscript{2+}, Mn\textsuperscript{2+}, Co\textsuperscript{2+}, Cd\textsuperscript{2+}, Cu\textsuperscript{2+}, Ni\textsuperscript{2+} and Pb\textsuperscript{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\textsuperscript{2+} and Mn\textsuperscript{2+} recycling from the transferrin [20].

Members of the NRAMP family have been characterized in many plant species including rice, soy bean and Thlaspi as well as in the green alga Chlamydomonas [21–27]. Through complementation of yeast metal transport mutants, they have been shown to function as metal ion transporters; however, very few of them have been assigned a physiological function. The Glycine max GmDMT1 homologue, 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 nickel hyperaccumulator Thlaspi japonicum, TjNRAMP4 specifically transports nickel when expressed in yeast, and might therefore contribute to nickel hyperaccumulation in the plant [24]. In Arabidopsis, six genes AtNRAMP1–6 encode NRAMP proteins. The most closely related members, AtNRAMP3 and AtNRAMP4, are metal transporters with a broad range of substrate specificities including iron, manganese, cadmium and zinc, as determined by functional complementation of yeast mutants [26,28–30]. AtNRAMP3 and AtNRAMP4 are tonoplastic proteins with redundant functions. Under iron-limiting growth conditions, germination of the double nrramp3 nrramp4 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 elucidated. However, AtNRAMP1 expression in yeast has been 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 the present study, we have 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 results of the present study suggest that AtNRAMP6 is a cadmium transporter that functions inside the cell either by mobilizing cadmium from its storage compartment or by taking up cadmium into a cellular compartment where it is toxic.

EXPERIMENTAL

Plasmids

For the reconstruction of AtNRAMP6 cDNA, an AtNRAMP6 cDNA retaining I6 (where I is intron) of the gene was amplified from reverse-transcribed total Arabidopsis RNA and cloned into the pGEM\textsuperscript{®}-T Easy vector (a gift of Dr Sébastien Thomine, Gif-sur-Yvette, France). To eliminate the I6 77 bp sequence, the 194 bp Scal-Xhol 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 Scal site in E5 (where E is exon) and a reverse primer encompassing the E6–E7 junction up to the Xhol site located close to the 5’ end of E7. The resulting clone was sequenced (see the sequence surrounding the E6-E7 junction in Figure 1). The 35S-AtNRAMP6 construct for overexpression in plants was obtained by subcloning the AtNRAMP6 cDNA KpnI-BamHI fragment into the pCHF3 vector [31] in translational fusion with the CaMV (carnation mosaic virus) 35S promoter. For expression in yeast, AtNRAMP6 cDNA sequences, containing I6 or without it, were subcloned as a SacI-BamHI fragment into the Xhol and BamHI sites of the pDR195 [32] yeast expression vector. The HA (haemagglutinin) epitope-tagged AtNRAMP6 construct was obtained by cloning an amplified 2 × HA fragment (primers: TagHA F, 5'-GCCAGATCTCATGCATGGCCATCTAGATCCATACTAC-3' and TagHA R, 5'-GGTGGATCCCGATCGTATGCTGCCCAGGAC-3') into the BamHI site located at the 3’ end of AtNRAMP6 cDNA into the pCHF3-AtNRAMP6 plasmid, and subsequently subcloning the Xhol-BamHI [AtNRAMP6:HA] cassette thus obtained into the Xhol and BamHI sites of the pDR195 vector. To generate a fusion between AtNRAMP6 promoter and GUS (β-glucuronidase), a 1.45 kb fragment of the AtNRAMP6 promoter sequence located upstream of the translation initiation codon was amplified by PCR on BAC T24D18 using the forward primer OCC51 (5’-CCCAGCTTGTGTTGTGCTTCTGAGA-3’) and the reverse primer OCC52 (5’-CCGCTATGCGCTGCCTCTCTGTGTTACG-3’) which contain a HindIII and a NcoI site respectively. The resulting promoter fragment was cloned in translational fusion with the uidA gene in the pBK/SUS vector [33] digested with HindIII and NcoI. The [AtNRAMP6 promoter–GUS] cassette thus obtained was then subcloned into the binary vector pBIN19 [34], and 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 0.5 × Murashige and Skoog medium [35]. When indicated, CdSO\textsubscript{4} was added into the medium from a 10 mM stock solution. The Arabidopsis GABI-Kat line ID 550D06 contains a T-DNA (transferred DNA) inserted 1940 bp downstream of the translation initiation codon of AtNRAMP6 in I9. Homozygous plants were screened by PCR using primers NR6S (5’-GCTGAGTACTCCCTGAGTTCCTGGCTAATCCCATATTGTTGAAGTGCTAG-3’) and either NR6AS (5’-GGGACTCGAGCTTTCTAATCCCATATTGTTGAAGTGCTAG-3’) or GKL (T-DNA left border). GKL amplified a fragment with both NR6S and NR6AS primers, indicating that at least two T-DNAs are inserted at the left border. GKLB amplified a fragment containing a 117 bp fragment corresponding to the spliced RNA. The 117 bp intron-less fragment was generated by PCR using primers: Forward primer encompassing the Scal site in E5 (where E is exon) and a reverse primer encompassing the E6–E7 junction up to the Xhol site located close to the 5’ end of E7. The resulting clone was sequenced (see the sequence surrounding the E6-E7 junction in Figure 1). The 35S-AtNRAMP6 construct for overexpression in plants was obtained by subcloning the AtNRAMP6 cDNA KpnI-BamHI fragment into the pCHF3 vector [31] in translational fusion with the CaMV (carnation mosaic virus) 35S promoter. For expression in yeast, AtNRAMP6 cDNA sequences, containing I6 or without it, were subcloned as a SacI-BamHI fragment into the Xhol and BamHI sites of the pDR195 [32] yeast expression vector. The HA (haemagglutinin) epitope-tagged AtNRAMP6 construct was obtained by cloning an amplified 2 × HA fragment (primers: TagHA F, 5’-GCCAGATCTCATGCATGGCCATCTAGATCCATACTAC-3’ and TagHA R, 5’-GGTGGATCCCGATCGTATGCTGCCCAGGAC-3’) into the BamHI site located at the 3’ end of AtNRAMP6 cDNA into the pCHF3-AtNRAMP6 plasmid, and subsequently subcloning the Xhol-BamHI [AtNRAMP6:HA] cassette thus obtained into the Xhol and BamHI sites of the pDR195 vector. To generate a fusion between AtNRAMP6 promoter and GUS (β-glucuronidase), a 1.45 kb fragment of the AtNRAMP6 promoter sequence located upstream of the translation initiation codon was amplified by PCR on BAC T24D18 using the forward primer OCC51 (5’-CCCAGCTTGTGTTGTGCTTCTGAGA-3’) and the reverse primer OCC52 (5’-CCGCTATGCGCTGCCTCTCTGTGTTACG-3’) which contain a HindIII and a NcoI site respectively. The resulting promoter fragment was cloned in translational fusion with the uidA gene in the pBK/SUS vector [33] digested with HindIII and NcoI. The [AtNRAMP6 promoter–GUS] cassette thus obtained was then subcloned into the binary vector pBIN19 [34], and digested with the same enzymes to generate the plasmid pBIN19/AtNRAMP6-GUS.

Histochemical analysis of GUS activity

Eight independent transgenic Arabidopsis lines were analysed for GUS activity. Plants were grown on plates containing iron-replete medium for 12 days. Plantlets were harvested in phosphate buffer [50 mM NaPO\textsubscript{4} and 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 three times in phosphate buffer prior to vacuum infiltration for 15 min, and then were soaked overnight at 37°C in a solution containing 1 mM 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 and 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 TRIzol® reagent (Invitrogen), according to the manufacturer’s protocol. A DNase (Promega) treatment was performed to avoid genomic DNA contamination. The integrity of DNA-free RNA was verified on agarose gel electrophoresis and equal amounts (3 µg) were used for RT (reverse transcription) [MMLV-RT (Moloney-murine-leukaemia virus-reverse transcriptase); Promega] with anchored oligo(dT)₁₂₋₁₅. Real-time PCR was performed using the LightCycler® FastStart DNA MasterPLUS SYBR green I (Roche) using NRAMP6 specific primers (forward, 5'-GAGTCTCTCGTCCACACATTTCC-3' reverse, 5'-TTTAATGTCCTCTATAACCCGCTAATCTG-3'). Relative transcript levels were calculated relative to the transcript amount of constitutively expressed actin gene (At3g18780) (forward primer, 5'-GGTAACATTGTGCTCAGTGGTGG-3' reverse, 5'-GAGTCCTCGTCCACACATTTCC-3').

Yeast strains and growth conditions

W303 (ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3) and Δsmf1 (MATa/MATα ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 smf1::URA3ura3::TRP1) cultures were grown in YNB (yeast nitrogen base) medium +2% glucose in liquid YNB medium supplemented with 2 µM CdSO₄ to a D₆₀₀ of 1.5. Cells were harvested and washed three times with water. For mineralization, pelleted yeast cells or harvested plant parts were desiccated for 2 days at 80°C. Samples were boiled for 1–3 h at 200°C in 1 ml of 69% HNO₃ in a Pyrex tube. The cadmium concentration in the samples was measured by AAS (atomic absorption spectrometry; SpectraAA220 Fast sequential; Varian).

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 located in the cytoplasmic loop between transmembrane domains 8 and 9, as well as one putative N-linked glycosylation site (Figure 1B). Attempts to isolate AtNRAMP6 cDNA led to clones retaining part or all of I6 of the gene. Figure 2(A) shows examples of misspliced AtNRAMP6 clones around I6, 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 programme (http://www.brc.riken.jp/ lab/epd/Eng/catalog/RAFL.shtml). Retention of the full I6, which is most frequently seen, introduces a stop codon 36 nucleotides downstream from the E6–I6 junction (labelled with an asterisk in Figure 1A), resulting in the addition of 12 amino acids

Immuno blot detection

The following antibodies were used: mouse anti-HA (Zymed Laboratories) diluted 1:4000, mouse anti-yeast V-ATPase diluted 1:2000 (as a yeast vacular marker), mouse anti-yeast cox2p diluted 1:500 (as a mitochondrial marker; Molecular Probes), mouse anti-yeast Pma1p diluted 1:10000 (as a plasma membrane marker; Abcam) and an anti-mouse conjugated to alkaline phosphatase (Invitrogen) diluted 1:10000. Membranes were washed three times, first with blocking buffer [PBS-T (1 × PBS with 0.1% Tween 20) containing 0.2% blocking reagent (Aurora™ Western blot chemiluminescence detection system, ICN Biochemicals)], secondly with 1 × PBS, and finally with 20 mM Tris/HCl and 1 mM MgCl₂ for 5 min. Membranes were then incubated for 5 min in the presence of Aurora Chemiluminescence Substrate Solution (Millipore). Chemiluminescence was revealed on a LAS 3000 Imager (Fujifilm).

Immuno fluorescence detection in yeast

Cells were treated following methods described in [39], using a mouse monoclonal anti-HA (Sigma) antibody as a primary antibody and a goat anti-mouse antibody coupled to Alexa Fluor® 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 (synthetic defined) medium supplemented with 2 µM CdSO₄ to a D₆₀₀ of 1.5. Cells were harvested and washed three times with water. For mineralization, pelleted yeast cells or harvested plant parts were desiccated for 2 days at 80°C. Samples were boiled for 1–3 h at 200°C in 1 ml of 69% HNO₃ in a Pyrex tube. The cadmium concentration in the samples was measured by AAS (atomic absorption spectrometry; SpectraAA220 Fast sequential; Varian).
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 (TMs). The amino acid residues that are circled in the cytoplasmic loop between TM8 and TM9 constitute the CTM. 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 v2.0 program (http://www.cbs.dtu.dk/services/TMHMM-2.0) coupled to the TMD pred® software.

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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 reconstructed a fully spliced AtNRAMP6 cDNA (see the Experimental section).

To check whether plants produce the two mRNA species, the I6-containing and the intron-less forms, we performed RT–PCR using primers encompassing I6 (Figure 2B). The Riken cDNA (RAFL05-14-K06 clone) and the fully spliced, reconstructed 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 I6. Indeed, semi-quantitative RT–PCR analysis showed a variation of the ratio of spliced to missspliced forms between the organs (Figure 2C, lanes c–i). Two organs, open flowers and siliques, showed the highest concentration of spliced AtNRAMP6 cDNA. Nevertheless, all tissues contained a significant amount of the missspliced form.

**A** Nucleotide sequence alignment between the intron-retaining ATNRAMP6 clone (ATNRAMP6-I6; RAFL05-14-K06 clone, Riken), the fully spliced deduced ATNRAMP6 cDNA (NRAMP6) and the ATNRAMP1 cDNA, in the region encompassing E5–E7. Nucleotides are numbered from the initiation codon of the ATG initiation codon. The in-frame stop codon in I6 is boxed. **B** Structure of the missspliced ATNRAMP6 transcript and position of the primers used in the RT–PCR shown in (C). **C** Differential expression of the two isoforms of ATNRAMP6 mRNA in planta; PCR control on plasmid DNA containing either the missspliced cDNA showing a DNA fragment at 284 bp (a) or the spliced cDNA showing a DNA fragment at 216 bp (b); the size markers (lane M) show a DNA fragment at 0.2 kb and 0.3 kb. 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) and siliques (i).

**Figure 2** Two ATNRAMP6 transcript isoforms coexist in the plant

**A** Nucleotide sequence alignment between the intron-retaining ATNRAMP6 clone (ATNRAMP6-I6; RAFL05-14-K06 clone, Riken), the fully spliced deduced ATNRAMP6 cDNA (NRAMP6) and the ATNRAMP1 cDNA, in the region encompassing E5–E7. Nucleotides are numbered from the initiation codon of the ATG initiation codon. The in-frame stop codon in I6 is boxed. **B** Structure of the missspliced ATNRAMP6 transcript and position of the primers used in the RT–PCR shown in (C). **C** Differential expression of the two isoforms of ATNRAMP6 mRNA in planta; PCR control on plasmid DNA containing either the missspliced cDNA showing a DNA fragment at 284 bp (a) or the spliced cDNA showing a DNA fragment at 216 bp (b); the size markers (lane M) show a DNA fragment at 0.2 kb and 0.3 kb. 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) and siliques (i).

**A**INRAMP6 mediates cadmium toxicity in yeast

All of the NRAMP homologues described have been shown to rescue the phenotype of *S. cerevisiae* metal transport mutants. Among the Arabidopsis members, AtNRAMP1, AtNRAMP3 and AtNRAMP4 were shown to complement Δfet3Δfet4 and Δsmf1 strains, defective in iron and manganese uptake respectively [22,26]. AtNRAMP4 was additionally shown to complement the phenotype of Δznr1Δznr2, a zinc uptake-defective mutant [28]. In the case of AtNRAMP6, however, its expression as a fully spliced cDNA or as a cDNA retaining I6 failed to restore growth of any of the yeast metal transport mutants (results 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, WT yeast cells expressing the AtNRAMP6 reconstructed cDNA were grown in standard YNB medium (Figure 3A) or in the presence of 25 μM CdSO₄ in the culture medium (Figure 3B). Although AtNRAMP6 did not impair yeast growth in standard medium, its presence increased cadmium-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 cadmium. The concentration–response curves indicated an IC₅₀ (the cadmium 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 μM cadmium (Figure 3C). Therefore, when expressed in yeast, AtNRAMP6 confers higher sensitivity to the external cadmium concentration.

**Expression pattern of ATNRAMP6**

Consistent with reported expression levels in public microarray 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 (Figures 4B and 4C). Accumulation of AtNRAMP6 transcripts was detected in leaves, senescing leaves, floral buds, opened flowers, siliques and silique peduncles (Figure 4C).

To investigate the tissue specificity of AtNRAMP6, transgenic plants expressing the *uidA* gene, encoding 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 was active in dry seeds where it is diffusely distributed in cotyledons and hypocotyl of the embryo (Figure 4D, a). Staining was also detected in the vascular bundles of shoot tissues including cotyledons (Figure 4D, b), young leaves (Figure 4D, d), sepals and petals (Figure 4D, c), and at the top of the flower stem and in the style (Figure 4D, c). Developing siliques showed GUS activity in the peduncle (Figure 4D, e), as well as in the septum and the funiculi (Figure 4D, f).

Neither transcript accumulation nor AtNRAMP6 promoter-driven GUS activity was modified upon treatments by deficiency or excess of iron, manganese or zinc, or by cadmium supply (results not shown).
containing two inverted repeated copies of T-DNA inserted in I9 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 nramp6-1 line, indicating that
this mutant was a null allele of AtNRAMP6 (Figure 5B).

nramp6-1 plants had no apparent phenotype; however, in order
to investigate whether the lack of AtNRAMP6 disturbed plant
metal homeostasis, nramp6-1 seeds were germinated in vitro
on medium either lacking iron, manganese, zinc or copper,
or containing excess amounts of these metals. In response to
these treatments, growth of Arabidopsis WT and nramp6-1
mutant plants was not differentially affected, indicating that
knocking out AtNRAMP6 does not impair the ability of the
plant to maintain iron, manganese, zinc or copper homeo-
ostasis (Supplementary Figure S1 at http://www.BiochemJ.org/
bj/422/bj4220217add.htm). Elemental analysis of whole seed,
root or shoot tissues, performed using AAS, did not reveal any
change in total metal content in the nramp6-1 mutant.

Because we observed that the expression of AtNRAMP6
in yeast mediates hypersensitivity to cadmium, we tested the
possibility that nramp6-1 might be resistant to cadmium
treatment. Four-week-old WT and nramp6-1 plants were supplied
with 50 μM CdSO₄ over 10 days. While WT plants developed
toxicity symptoms such as yellow leaves, nramp6-1 leaves
remained mostly green during the treatment (Figure 5C),
indicating a better tolerance of the nramp6-1 mutant to cadmium
toxicity. Transformation of nramp6-1 with the AtNRAMP6
cDNA expressed under the control of the strong ubiquitous
CaMV 35S promoter restored cadmium sensitivity of the mutant
(Supplementary Figure S2 at http://www.BiochemJ.org/bj/422/
bj4220217add.htm). This result suggested that AtNRAMP6 might
correlate to the uptake of cadmium by the plant. To test this
hypothesis, the concentration of cadmium was measured in leaves
of WT and nramp6-1 plants grown as described for Figure 5C
using AAS. Figure 5(D) shows that the cadmium concentration
did not significantly decrease in nramp6-1 compared with WT
plants, either in isolated petioles and limbs of rosette leaves
or in caulinar leaves. Similar results were obtained in 2-week-
old seedlings (Table 1). Therefore, rather than being involved in
cadmium entry into the cell, AtNRAMP6 may modify cadmium
distribution between subcellular compartments so as to promote
cadmium-induced damage.

**AtNRAMP6-overexpressing lines are hypersensitive to cadmium
toxicity**

WT Arabidopsis plants were transformed with the 35S-
AtNRAMP6 construct previously used to complement the nramp6-
1 phenotype. A total of ten 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
(results not shown). We investigated the capacity of germination
in the presence of cadmium of lines #2, #3 and #6, which har-
boured the highest level of AtNRAMP6 overexpression relative to
WT. Compared with WT plants, growth of 35S-AtNRAMP6 plants
was more strongly inhibited by 2 μM CdSO₄ in the medium, as
shown for line #2 (Figure 6B). Quantification of this phenotype
was estimated by measuring FW (fresh weight) production in lines
#2, #3 and #6 (Figure 6C). Compared with WT, FW dropped to
approx. 50% at 2 μM cadmium and 70% at 10 μM cadmium.
Consistent with this observation, nramp6-1 plants grew better
than WT plants during the treatment (Figure 6C). Similar to the
situation with the nramp6-1 mutant, however, the concentration
of

**The nramp6-1 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)
NRAMP6 and cadmium toxicity

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 seedlings germinated for the amount of time indicated on the Figure (A), 14-day-old plantlets grown in vitro (B) or mature aerial organs harvested from soil-grown plants (C). (D) Tissue-specific activity of the AtNRAMP6 promoter. Histochemical staining of GUS activity in Arabidopsis transgenic plants expressing the uidA gene under the control of 1.45 kb of the 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.

Membrane localization of AtNRAMP6 protein

One hypothesis that could explain these results is that AtNRAMP6 is a cadmium 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 GFP (green fluorescent protein) or the 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 analysis or by imaging of the GFP fluorescence (results not shown). We also failed to detect HA or GFP when fused to AtNRAMP6 truncated at the in-frame stop codon present in I6 (results not shown). When the 35S-AtNRAMP6::GFP construct was introduced into the nramp6-1 mutant, the fusion protein did accumulate in the plant, as indicated by Western blot analysis; however, the fluorescence signal of the GFP was very weak and mostly cytoplasmic (results 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 WT yeast strain under the control of the strong PMA1 promoter. The increase of cadmium sensitivity mediated by this construct, although slightly weaker, was in the same range as that observed with the non-tagged AtNRAMP6 protein (Figures 3B and 3C), indicating that the AtNRAMP6::HA fusion used was 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 2 × HA, and a less abundant polypeptide of approx. 30 kDa which is likely to result from a proteolytic cleavage of the full-size polypeptide (Figure 7A). Despite AtNRAMP6 mediating cadmium hypersensitivity, yeast cells contained the same amount of cadmium 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 by SDS/PAGE, Western 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 was relatively pure. Therefore AtNRAMP6::HA is not targeted to the plasma membrane of yeast.

We next addressed the intracellular distribution of AtNRAMP6 by immunofluorescence microscopy (Figure 7D). Whereas no cadmium measured in the overexpressing lines was not different from that in WT plants (Table 1).
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 I9 of the 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 an nramp6-1 plant grown in soil and irrigated with 10 μM CdSO₄ for 10 days. Compared with the yellow rosette leaves of the WT plant, nramp6-1 leaves remain green upon cadmium treatment. (D) Cadmium concentration measured by AAS on tissues harvested from plants cultivated as described in (C). Values represent the means ± S.D. from five individually grown adult plants. DW, dry weight.

Table 1  AtNRAMP6 does not affect total cadmium concentration in plants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean value</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>131.892</td>
<td>10.018</td>
</tr>
<tr>
<td>nramp6-1</td>
<td>116.630</td>
<td>7.110</td>
</tr>
<tr>
<td>35S-NR6 #2</td>
<td>107.200</td>
<td>20.244</td>
</tr>
<tr>
<td>35S-NR6 #3</td>
<td>118.759</td>
<td>15.645</td>
</tr>
</tbody>
</table>

Table 1 Measurement of cadmium in plants grown in vitro for 10 days in the presence of 5 μM CdSO₄. The cadmium concentration (mg · g⁻¹) was measured by AAS in Arabidopsis seedlings.

DISCUSSION

Among the six NRAMP-encoding genes of Arabidopsis, metal transport activity has been reported for AtNRAMP1, 3 and 4. In the present paper, 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 I6, resulting in shortening of the open reading frame by approximately 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 of the tissues tested and were unable to find conditions where the fully spliced isoform is produced exclusively, thus arguing against the idea...
Plants were grown for 14 days on plates containing the indicated amount of CdSO$_4$ prior to cellular cadmium content when expressed in yeast, whereas were previously shown to mediate cadmium toxicity and increased other NRAMP proteins. First, in plants, AtNRAMP1, 3 and 4 be capable of transporting cadmium is reminiscent of several other Arabidopsis lines overexpressing AtNRAMP3 were found to be hypersensitive to cadmium [26]. Similar results were obtained for the Thlaspi caerulescens member TcNRAMP3 when expressed in yeast or in tobacco [27]. MntH, the prokaryotic NRAMP orthologue, is an iron/manganese transporter that confers cadmium hypersensitivity when overexpressed in bacteria [40]. Very close to MntH, Chlamydomonas reinhardtii DMT1 restores growth of the yeast Δsmf1 mutant and mediates hypersensitivity to cadmium [25]. NRAMP2 in mammals was also shown in vitro to work as a cadmium transporter [17], but although it represents a candidate for cadmium uptake at the brush border of the duodenum [41], intestinal cadmium absorption was recently shown to be unaffected in the DMT1-dysfunctional mk1/mk mice [42].

Using immunological approaches, we have established that AtNRAMP6 is not located at the cell surface, but is located in a punctate-shaped endomembrane compartment, distinct from mitochondria and from the vacuole. This intracellular localization is consistent with AtNRAMP6 conferring cadmium sensitivity without affecting cadmium content in yeast cells and plant tissues, as one would expect if localized on the plasma membrane. Consequently, the cadmium sensitivity induced by AtNRAMP6 is not due to net influx of cadmium, but rather results from the redistribution of internal cadmium pools. AtNRAMP6 could induce cadmium 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 cadmium-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 catalyse the flux of cadmium towards the cytosol. In spite of the fact that the vacuole in yeast is responsible for cadmium detoxification through the transport of Cd–GS$_2$ complexes [11], we have clearly shown by immunolocalization that AtNRAMP6 is not located in the yeast vacuolar membrane. In addition to the vacuole, ER (endoplasmic reticulum)-derived vesicles represent putative sites of cadmium accumulation in plants. Indeed, in onion epidermal cells exposed to cadmium and lead, accumulation of these metals is observed in vesicles derived from the fragmentation of the ER [43]. But whether the ER is a detoxifying compartment for cadmium or a location where it exerts its toxicity is not clear. In mammalian cells, the well-known phenomenon of cadmium-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 the ER could be a target of cadmium toxicity. Immunolabelling 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, which requires a proton gradient across the membrane to function. In contrast, 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 playa centro role in the delivery of manganese to various cellular sites including mitochondria [47]. Whether AtNRAMP6 and Smf2p have similar cellular functions should be investigated in future studies.

Although cadmium seems to be a substrate for AtNRAMP6, which enabled us to reveal its role as a metal transporter, cadmium transport is likely not to be the primary function of AtNRAMP6 in the plant. What then could be the physiological substrate of AtNRAMP6? Elemental analysis by AAS of nramp6-1

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

(A) Accumulation of AtNRAMP6 mRNA in three overexpressing lines as measured by semi-quantitative RT–PCR on leaf total RNA extracts. Actin mRNA was used to control for RNA loading. (B) Phenotype of a representative overexpressing line after 7 days of growth in vitro in the presence of 2 μM CdSO$_4$. (C) Growth rate in the presence of cadmium of three AtNRAMP6-overexpressing lines compared with WT plants (Col-0) and the nramp6-1 mutant. Plants were grown for 14 days on plates containing the indicated amount of CdSO$_4$ prior to harvesting shoots for FW determination. Each value was obtained from 14 plants and represents the mean ± S.D. of three independent measurements.

that AtNRAMP6 expression is regulated at the level of mRNA splicing. Furthermore, plants overexpressing the truncated form of AtNRAMP6 did not harbour any growth defect whatsoever (results not shown).

Our results suggest that AtNRAMP6 is a cadmium transporter since, when expressed in S. cerevisiae or when overexpressed in Arabidopsis, AtNRAMP6 confers increased sensitivity to cadmium. Consistently, growth of the nramp6-1 loss-of-function mutant is unaffected by otherwise toxic concentrations of cadmium and this tolerance is reverted by complementation with a 35S-AtNRAMP6 construct. The finding that AtNRAMP6 may be capable of transporting cadmium is reminiscent of several other NRAMP proteins. First, in plants, AtNRAMP1, 3 and 4 were previously shown to mediate cadmium toxicity and increased cellular cadmium content when expressed in yeast, whereas...
Figure 7  AtNRAMP6 is localized in an endomembrane compartment in yeast

(A) Immunoblot detection of AtNRAMP6::HA in total protein 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 (inductively coupled plasma MS) in WT yeast expressing AtNRAMP6 (white bar), HA-tagged AtNRAMP6 (grey bar) or the empty vector pDR195 (black bar) and grown in the presence of 2 μM CdSO4. (C) Immunoblot detection of AtNRAMP6::HA in protein fractions. Total, total protein extract; M, microsomal fraction; PM, plasma membrane-enriched fraction. The purity of the protein fractions was controlled by hybridization with antibodies directed against the yeast plasma membrane ATPase Pma1p, the yeast mitochondrial protein Cox2p or the yeast vacuolar membrane V-ATPase. Total and M lanes on the left-hand side of the Figure, and PM lanes on the right-hand side of the Figure, are part of the same gel, exposed at the same time, that were seamed together to compose the Figure. (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 Fluor® 546; (b) DAPI staining reveals structures containing DNA, in this panel nuclei and mitochondria; and (c) overlay. Insert: enlargement of one cell revealing that HA staining is distinct from the mitochondrial DNA staining.

plant tissues did not reveal any change in metal content, which might provide a clue as to the physiological function of AtNRAMP6 (results not shown). In addition, we were unable to see growth restoration of yeast iron, zinc or manganese uptake-defective mutants by AtNRAMP6. However, we cannot exclude the possibility that AtNRAMP6 accepts physiological metals such as iron or manganese 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 iron, manganese or copper (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; however, this nramp1 nramp6 double mutant also failed to develop any metal-related phenotype (results not shown). Although phylogenetically 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 (the present study). Combination of the nramp6 mutation with a mutation in another AtNRAMP gene expressed in overlapping territories may provide information on the function of AtNRAMP6 in the plant. We found that the AtNRAMP6 promoter was mostly active in the cotyledons at the embryonic stage, which is consistent with the increased tolerance to cadmium of nramp6-1 embryos at germination. However, we did not measure any variation of the total amount of metals (iron, manganese, zinc, copper, cadmium) in nramp6-1 seeds (results 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 cadmium ([26] and the present study), AtNRAMP3 and AtNRAMP4 are tonoplastic 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 cadmium [45], which suggests that AtNRAMP3, AtNRAMP4 and AtNRAMP6 may contribute, through parallel transport pathways, to the availability of cadmium in cellular compartments sensitive to cadmium 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, the present study describes a new member of the NRAMP family of metal transporters. The AtNRAMP6 gene encodes a protein shown in the present study to be functional and potentially involved in cadmium tolerance. The protein, shown in yeast to be targeted to intracellular vesicles, may mediate the transport of cadmium from internal pools. Taken together,
our results and the already known mechanisms of cadmium 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.

AUTHOR CONTRIBUTION
Catherine Curie, Stéphane Mari and Jean-François Briat planned the experiments and analysed the data. Remy Calilatte undertook most of the experimentation, except for the immunofluorescence analyses in yeast which were performed by Bruno Lapryre. The manuscript was organized and written by Catherine Curie.

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SUPPLEMENTARY ONLINE DATA
The NRAMP6 metal transporter contributes to cadmium toxicity

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Supplementary Figures S1 and S2 are found on the subsequent pages.
Figure S1  *nramp6-1* is not affected by metal treatments

WT and *nramp6-1* plants were grown vertically for 8 days on either 0.5 × Murashige and Skoog medium (C) or 0.5 × Murashige and Skoog medium containing various metal concentrations: no Fe (−Fe), 600 μM FeEDTA (+++Fe), no Cu (−Cu), 50 μM CuSO₄ (+++Cu), no Mn (−Mn), 600 μM MnSO₄ (+++Mn), no Zn (−Zn) and 1 mM ZnSO₄ (+++Zn).
**Figure S2  Complementation of the nramp6-1 Cd-tolerance phenotype**

The nramp6-1 mutant was transformed with the pCHF3/35S-AtNRAMP6 construct and ten transformant lines were selected on kanamycin. (A) Accumulation of AtNRAMP6 mRNA was analysed in four complemented mutant lines by RT–PCR on total RNA extracted from rosette leaves of 10-day-old plants. Actin was used as an RNA loading control. (B) Shoot biomass production of 2-week-old WT, nramp6-1 and nramp6-1 complemented lines #1, #2 and #4, grown in the presence of 5 μM CdSO₄, showing restoration of cadmium-induced growth defect in the complemented lines. Each value represents the mean for 9–12 plants.