Metal Ion Homeostasis Mediated by NRAMP Transporters in Plant Cells – Focused on Increased Resistance to Iron and Cadmium Ion

Toshio Sano[1], Toshihiro Yoshihara[1], Koichi Handa[2],
Masa H. Sato[3], Toshiyuki Nagata[1] and Seiichiro Hasezawa[2,4]
[1]Faculty of Bioscience and Applied Chemistry, Hosei University
[2]Graduate School of Frontier Sciences, The University of Tokyo
[3]Graduate School of Life and Environmental Sciences,
Kyoto Prefectural University
[4]Advanced Measurement and Analysis,
Japan Science and Technology Agency (JST)
Japan

1. Introduction

Plants have developed several adaptive systems to control the cellular concentrations of essential metals in which the ion transporters play significant roles. At the cell surface, transporters localized on plasmamembrane controlled metal ion uptake and release whereas inside of the plant cells, those localized on endomembrane sequestered and remobilized metal ions in organella, such as vacuoles and plastids (Pilon et al. 2009, Puig and Peñarrubia, 2009).

Iron is one of several essential nutrients but a problematic one for living organisms (Conte and Walker 2011). At the cellular level, iron is used as a cofactor in enzymatic activities based on the reversible reaction between Fe^{2+} (ferrous) and Fe^{3+} (ferric) ions (Hell and Stephan 2003). In plants, it is essential for chlorophyll synthesis and hence iron deficiency results in chlorosis and pale-yellow or white leaves (Wiedenhoeft 2006). Usually, iron is chelated to organic matter in insoluble forms in soils that causes iron deficiency whereas in anaerobic and acidic conditions, iron toxicity occurs because of the increase of iron solubility (Ricachenevsky et al. 2010). The basis of iron toxicity was usually discussed to be oxidative stress by generation of reactive-hydroxyl radicals (Neyens and Baeyens 2003). Iron homeostasis in plant cells is partly achieved through the control of iron transport across membranous structures, and several families of putative iron transporters, including ZIP (ZRT, IRT-like proteins) and NRAMP (natural resistance associated macrophage protein), have been described (Guerinot 2000, Curie and Briat 2003, Kim and Guerinot 2007). Among the ZIP transporters, the *Arabidopsis AtIRT1* was the first iron transport molecule identified in plants (Eide et al. 1996) and was shown to be the major transporter mediating iron uptake into roots (Vert et al. 2002). Recently, IRT2, a close homolog of IRT1 in *Arabidopsis*, was suggested to compartmentalize iron into vesicles to prevent toxicity from excess free iron in the cytosol (Vert et al. 2009). On the other hand, among the ubiquitous NRAMP family of

metal transporters, it was the mouse *Nramp1* that was first cloned as the gene responsible for resistance to mycobacterial infection (Nevo and Nelson 2006). In *Arabidopsis*, six NRAMP transporters, AtNRAMP1-6, have been identified and categorized by phylogenic analysis into two subfamilies: AtNRAMP1 and 6 forming the first group and AtNRAMP2 through 5 comprising the second group (Mäser et al. 2001). Of these, *AtNRAMP1*, *3*, *4* and *6* have been shown to encode functional plant metal transporters (Krämer et al. 2007, Cailliatte et al. 2009). *AtNRAMP1* can complement the *fet3fet4* yeast mutant that is defective in both low- and high-affinity iron transporters, while overexpression of *AtNRAMP1* in *Arabidopsis* increases plant resistance to toxic iron concentrations (Curie et al. 2000). AtNRAMP3 and AtNRAMP4 mediate the remobilization of iron from the vacuolar store and are essential for seed germination under low iron conditions (Thomine et al. 2003, Lanquar et al. 2005).

In addition to iron transport activities, these transporters can mediate the transport of a wide range of metal cations because of their similar chemical characteristics (Hall and Williams 2003, Krämer et al. 2007). AtNRAMP1 can functionally complement a manganese-uptake defective mutant and confer cadmium sensitivity to yeast (Thomine et al. 2000). This transporter was recently demonstrated to act as a physiological manganese transporter in *Arabidopsis* (Cailliatte et al. 2010). Similarly, TcNRAMP3 and TcNRAMP4 from the metal hyperaccumulator, *Thlaspi caerulescens*, can transport various metal cations, including Fe^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+} when expressed in yeast, and MbNRAMP1 from apple trees of *Malus baccata* was found to mediate Mn^{2+} uptake in addition to Fe^{2+} (Oomen et al. 2008, Xiao et al. 2008, Wei et al. 2009). Recently, rice Nrat1 that belongs to the NRAMP family has been reported to transport trivalent aluminum ion, but not other divalent ions such as manganese, iron and cadmium (Xia et al. 2010).

In the present chapter, we demonstrate that tobacco NtNRAMP1 is a plasma membrane transporter, and that overexpression of this protein in tobacco BY-2 cells increases the resistance of the cells to both iron and cadmium ions. We propose that NtNRAMP1 moderates metal ion-uptake and prevents toxicity resulting from excess iron or cadmium application.

2. Results

2.1 Excess iron application induces cell death and arrests cell cycle progression

To examine the effect of excess iron application to plant cells, we monitored the growth of tobacco BY-2 cells in medium containing high amounts of iron. The cells took up about 50 to 90 μg iron g cells^{-1} 6 h after transfer to a medium containing 1.0, 2.0 or 5.0 mM $FeSO_4$ and lacking other divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+}), but only about 5 μg iron g cells^{-1} in standard medium with 0.1 mM $FeSO_4$ (Fig. 1A).

Under these conditions, about 50 % or more than 80 % of the cells died 24 h after transfer to medium containing 1.0 and 2.0 or 5.0 mM $FeSO_4$, respectively, whereas only a few percent of the cells died in the standard medium (Fig. 1B).

As cell death is known to relate to the arrest of cell cycle progression (Kadota et al. 2004, Sano et al. 2006), we monitored the latter by flow cytometric and mitotic index (MI) analyses upon excess iron application.

In the control condition containing 0.1 mM $FeSO_4$, the cell cycle progressed from the S to G_2 phase 2 h after aphidicolin release, and entered mitosis at 8 h and the G_1 phase at 10 h (Fig.
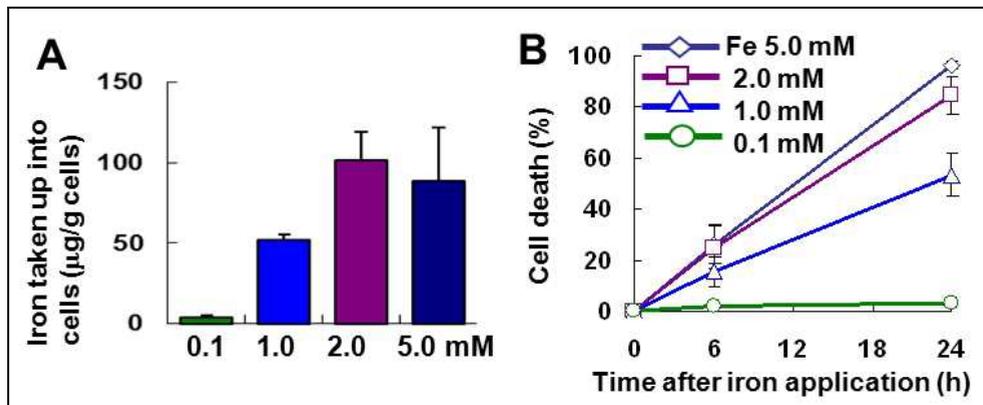
Fig. 1. Effect of excess iron application on tobacco BY-2 cell growth. (A) Amount of iron taken up into cells. After transfer of 7-day-old cells to medium containing 0.1, 1.0, 2.0 or 5.0 mM $FeSO_4$, the amount of iron taken up into the cells in 6 h was measured. Values shown are those after subtraction of measurements taken just after transfer to the medium as background. Data show the means ± SE of three independent experiments. (B) In the culture conditions in (A), the population undergoing cell death was measured. Data represent means ± SE of three independent experiments with more than 400 cells in each experiment.

2A, B). When the cells were additionally treated with 1.0 mM $FeSO_4$ after aphidicolin release, with the cell cycle restarting from S phase, cell cycle progression was delayed and the percentage of cells entering mitosis decreased (Fig. 2A). Further flow cytometric analysis demonstrated the cell cycle arrest of these cells in the S to G_2 phase (Fig. 2B).
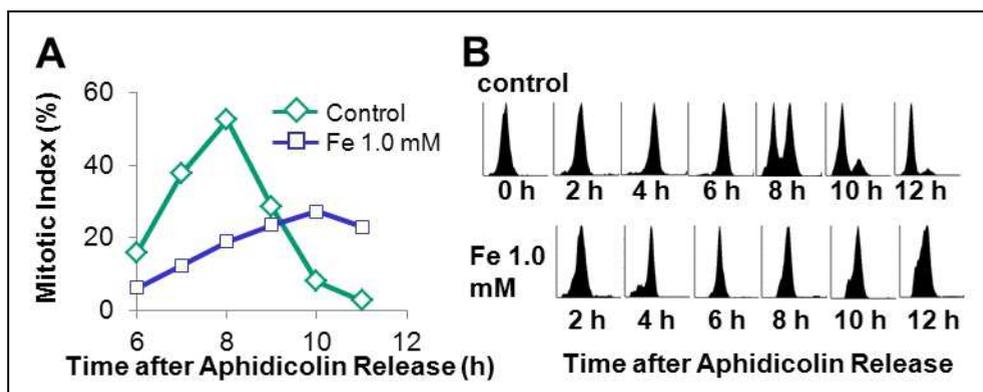

Fig. 2. Effects of excess iron application on cell cycle progression. Cell cycle progression of cells in control conditions (open diamonds) and those cultured with 1.0 mM $FeSO_4$ after aphidicolon treatment (open squares). Cell cycle progression was monitored by the mitotic index (A) and by flow cytometry (B). The data show representatives of three independent experiments.

2.2 Overexpression of *NtNRAMP1* decreases sensitivity to excess iron application

To investigate the molecular mechanisms of iron uptake and cell death of tobacco BY-2 cells, we identified and characterized several tobacco iron transporter genes. As the ZIP and NRAMP family proteins are known as iron/metal transporters in plants, we identified two tobacco cDNA clones that encoded proteins with high sequence similarity to ZIP or NRAMP and named them *NtZIP1* and *NtNRAMP1*, respectively. The amino acid sequence of NtZIP1 was 61 % identical to the MtZIP3 of *Medicago truncatula* (Lopez-Millan et al. 2004) and 53 % to AtZIP5 of *Arabidopsis thaliana*, whereas NtNRAMP1 was 71 % identical to *Arabidopsis* AtNRAMP1 and AtNRAMP6. Gene expression analysis revealed that 1.0 mM $FeSO_4$ application increased the relative transcript levels of *NtNRAMP1* but decreased those of *NtZIP1* (Fig. 3A, B).

As the increased level of *NtNRAMP1* gene expression upon iron application implied an involvement of this transporter under these culture conditions, we prepared transgenic tobacco BY-2 cell lines that overexpressed *NtNRAMP1* by placing the gene under control of the cauliflower mosaic virus 35S promoter. In one (NR1) of the four transgenic lines obtained, *NtNRAMP1* transcript levels were about 2-fold those of the non-transformed BY-2 cells whereas the *NtZIP1* transcript levels were reduced (Fig. 3A, B). Similar increases in *NtNRAMP1* transcript levels were also observed in the other three transgenic lines obtained (data not shown).
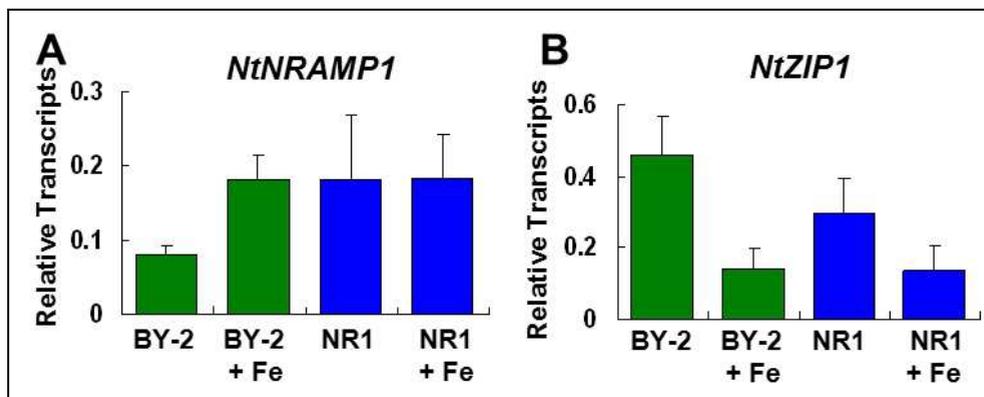

Fig. 3. Gene expression of the *NtNRAMP1* and *NtZIP1* iron transporters. *NtNRAMP1* (A) and *NtZIP1* (B) gene expression in non-transformed BY-2 cells cultured in control conditions for 24 h (BY-2) or with 1.0 mM $FeSO_4$ for 24 h (BY-2 + Fe), and in *NtNRAMP1* overexpressing cells cultured in control conditions (NR1) or with 1.0 mM $FeSO_4$ for 24 h (NR1 + Fe). Gene expression was monitored by real-time quantitative PCR and the data show relative transcripts normalized with GAPdH gene expression. The data show the means ± SE of three independent experiments.

When the iron uptake activities of NtNRAMP1 and NtZIP1 were measured in yeast cells, the amount of iron accumulated in the yeast cells expressing NtNRAMP1 or NtZIP1 was about 1.5 times high compared to control cells expressing LacZ. The amout was comparable to those expressing *Arabidopsis* AtNRAMP1 or AtNRAMP3 whereas that expressing an effulux pump AtHMA4 (Verret et al. 2004, Mills et al. 2005) was comparable to that

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expressing LacZ (Fig. 4). Therefore, both NtNRAMP1 and NtZIP1 could have the iron uptake activity comparable to *Arabidopsis* AtNRAMP1 and AtNRAMP3.
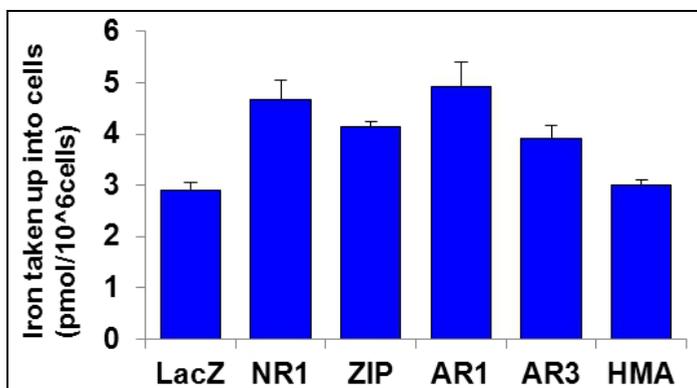

Fig. 4. Iron uptake activity of metal transporters in yeast cells. Iron accumulation in yeast cells transformed with the vector containing LacZ, NtNRAMP1 (NR1), NtZIP1 (ZIP), AtNRAMP1 (AR1), AtNRAMP3 (AR3) and AtHMA4 (HMA) was measured by atomic absorption spectrograph after incubation for 18 h in a medium with 0.2 mM $FeCl_3$. The data show the means ± SE of four independent experiments.

In the NtNRAMP1-overexpressing tobacco line (NR1), cell cycle progression was similar to that of the non-transformed BY-2 cells under control conditions in which 0.1 mM $FeSO_4$ was included. When 0.3 mM $FeSO_4$ was applied at the S phase, cell cycle progression of the non-transformed cells was delayed and the value of the peak MI reduced (Fig. 5A). In contrast, in the NR1 cells, the tendency of cell cycle progression was comparable to that in the control condition even though these cells took up as much as amounts of iron compared to the non-transformed cells (Fig. 5A, B). Furthermore, the proportion of NR1 cells undergoing cell death was reduced in comparison with the non-transformed cells following 1.0 mM $FeSO_4$ application (Fig. 5C), even though the amount of iron taken up by the NR1 cells was comparable to that in the non-transformed cells under these conditions (Fig. 5D).

To investigate the role of NtNRAMP1 on the suppression of cell cycle arrest and cell death upon excess iron application, we examined the subcellular localization of NtNRAMP1 by transient expression of NtNRAMP1-GFP fusion proteins. The GFP fluorescence was localized primarily on the plasma membrane, and confirmed by the plasma membrane marker, SYP132 (Enami et al. 2009, Fig. 6A-C). In contrast, cells transiently expressing GFP only showed cytoplasmic-localized fluorescence (Fig. 6D).

As the above results suggested that NtNRAMP1 is a plasma membrane transporter, we examined the effect of *NtNRAMP1* overexpression on iron uptake. The total amount of iron taken up into cells 24 h after 1.0 mM $FeSO_4$ application was comparable in the NR1 and non-transformed cells (Fig. 7A). However, when calculated on the basis of the rate of iron uptake, the non-transformed BY-2 cells had about 3-fold higher rates than the NR1 cells in the initial 1 hour after iron application (Fig. 7B). In subsequent periods, uptake rates were comparable in the two cell lines (Fig. 7B).
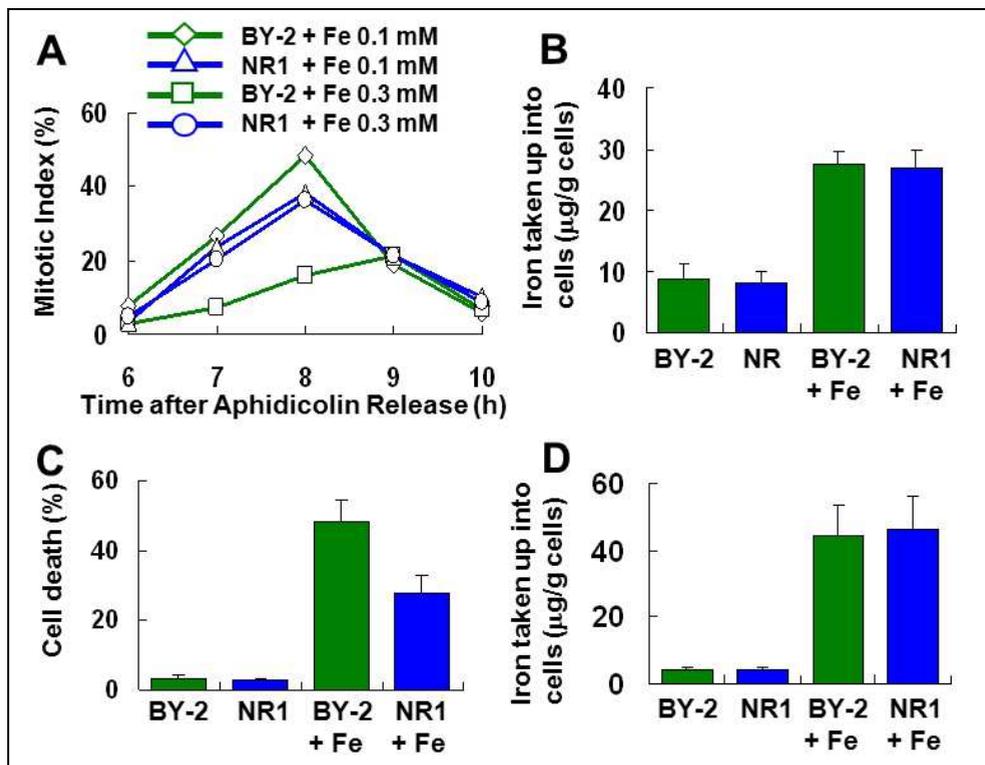
Fig. 5. Cell cycle progression and the population of NtNRAMP1 overexpressing cells undergoing cell death. (A) Cell cycle progression of non-transformed BY-2 cells (BY-2 + Fe 0.1 mM) and NtNRAMP1 overexpressing cells (NR1 + Fe 0.1 mM) cultured in control conditions or with 0.3 mM $FeSO_4$, respectively (BY-2 + Fe 0.3 mM, NR1 + Fe 0.3 mM). The data show a representative sample of three independent experiments. (B) Amount of iron taken up into cells cultured for 6 h in the culture conditions shown in (A). (C) Population of cells undergoing cell death in non-transformed BY-2 cells (BY-2) and NtNRAMP1 overexpressing cells cultured in control conditions (NR1) for 24 h, or those cultured with 1.0 mM $FeSO_4$ for 24 h, respectively (BY-2 + Fe, NR1 + Fe). (D) Amount of iron taken up into cells cultured for 24 h in culture conditions in (C). In (B), (C) and (D), the data show the means ± SE of three independent experiments.
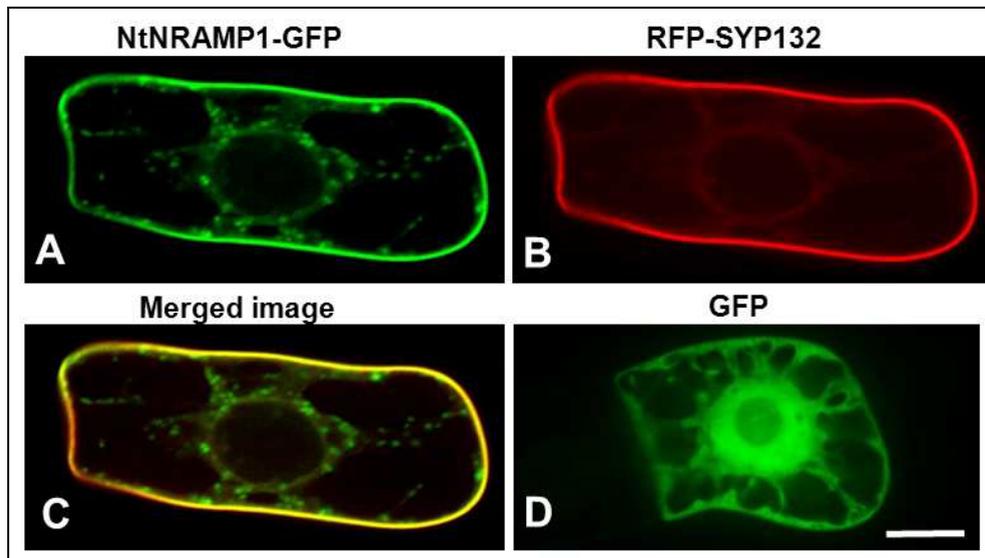
Fig. 6. Subcellular localization of NtNRAMP1. (A) NtNRAMP1 localization was monitored in a tobacco BY-2 cell transiently expressing NtNRAMP1-GFP. (B) Plasma membrane localization of the syntaxin, SYP132, monitored in cells transiently expressing tagRFP-SYP132. (C) Merged image of images (A) and (B). (D) GFP fluorescence in a tobacco BY-2 cell transiently expressing GFP. Scale bar represents 20 μm.
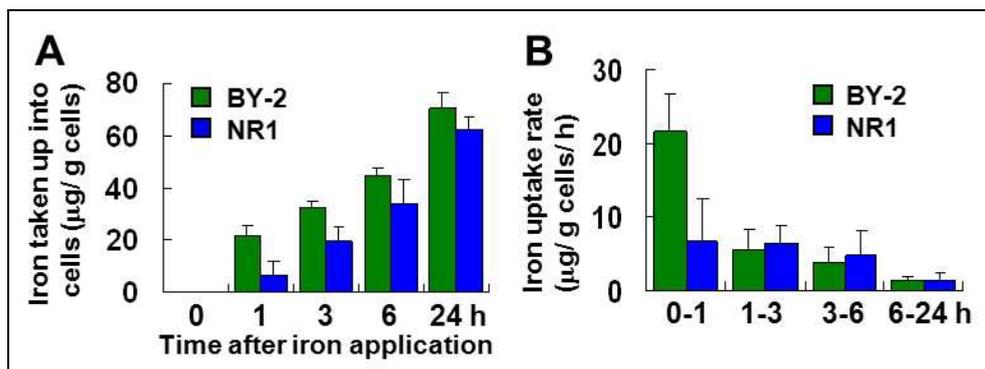

Fig. 7. Effect of NtNRAMP1 overexpression on iron uptake. (A) Changes in the amount of iron taken up into control BY-2 cells (BY-2) and NtNRAMP1 overexpressing cells (NR1). (B) Changes in iron uptake rate calculated from the data in (A). Data show the means ± SE of four independent experiments.

To further characterize NtNRAMP1, we examined the effects of cadmium on cell growth since NRAMP transporters are known to transport a variety of metal ions (Nevo and Nelson 2006, Krämer et al. 2007). In control medium without cadmium, both non-transformed and

NR1 cells proliferated about 70 times per week (Fig. 8A). When 1.0 or 10 μM $CdSO_4$ was added to the medium, the growth rate of controls cells decreased to 40 or 20 times per week, respectively (Fig. 8A). In contrast, the NR1 cell growth rates in 1.0 μM $CdSO_4$ were comparable to those without cadmium treatment, and were still about 55 times per week in 10 μM $CdSO_4$ (Fig 8A). After 10 μM $CdSO_4$ application, the amount of cadmium taken up into the control BY-2 cells increased in 24 h but decreased thereafter (Fig. 8B). In the NR1 cells, the amount of cadmium was smaller than that in the control BY-2 cells in 24 h (Fig. 8B).
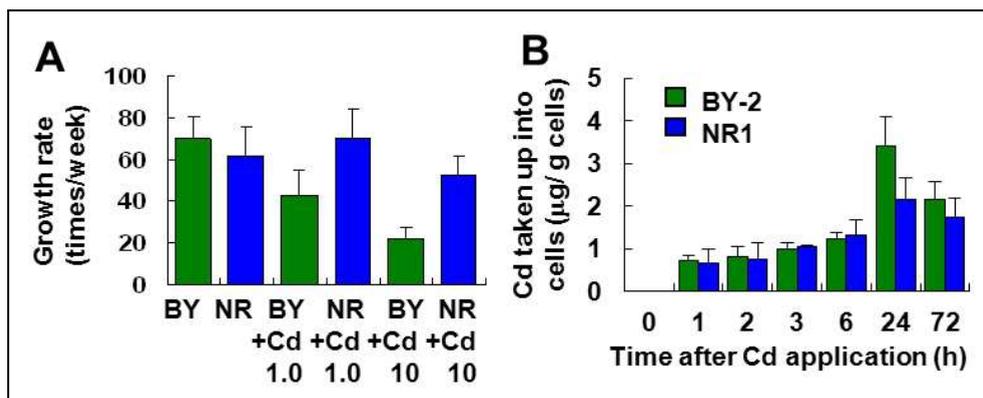

Fig. 8. Effect of NtNRAMP1 overexpression on cell growth following cadmium application. (A) Growth rate of control BY-2 cells and NtNRAMP1 overexpressing cells during culture for 7 days in control conditions (BY, NR), or with 1.0 μM (BY + Cd 1.0, NR + Cd 1.0) or 10 μM $CdSO_4$ (BY + Cd 10, NR + Cd 10). (B) Changes in the amount of cadmium taken up into cells cultured with 10 μM $CdSO_4$. Data show the means ± SE of four independent experiments.

3. Discussion

3.1 Role of the NtNRAMP1 transporter following excess iron application

The NRAMP family transporters function as general metal ion transporters (Nevo and Nelson 2006), and we have shown in this study that NtNRAMP1 overexpression in tobacco BY-2 cells suppressed cell cycle arrest and cell death upon excess iron application (Fig. 5). Plasma membrane localization of NtNRAMP1 (Fig. 6) and the decreased rate of iron uptake in the NtNRAMP1 overexpressing cells (Fig. 7B) implies the role of NtNRAMP1 as a modulator of iron uptake or an iron exporter.

Concerning the latter hypothes, the metal ion efflux activity of the NRAMP family members are somewhat uncommon. The TcNRAMP3 transporter was found to exclude Ni when expressed in yeast but transported iron and cadmium into cells in yeast and plants (Wei et al. 2009). Our iron uptake experiments in yeast implies the iron uptake of NtNRAMP1 rather than the export since the amout of iron uptake was higher than that in the cells expressing the efflux pump of AtHMA4 (Fig. 4).

In this context, the increaed iron resistance upon NtNRAMP1 overexpression might be explaned by the role of AtNRAMP1 as a physiological manganese (Mn) transporter

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(Cailliatte et al. 2010). Although the AtNRAMP1 was capable of transporting both iron and Mn in yeast cells (Curie et al. 2000, Thomine et al. 2000), Cailliatte et al. (2010) discussed the competence of iron uptake by Mn uptake increased the resistance to iron toxicity. Similar competence of iron uptake might be occured in the NtNRAMP1 overexpressing cells (Fig. 9). In this model, the supposed metal transporters other than NtNRAMP1 that actively mediate iron uptake are remained to be determined.
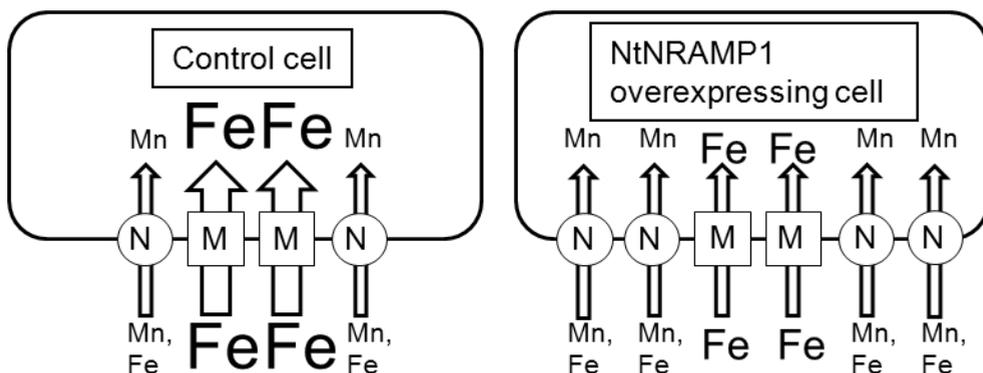

Fig. 9. A model for the increased resistance to iron in NtNRAMP1 overexpressing cells. In the control cells (left), excess iron application increases the rate of iron uptake by metal transporters (M) with high iron uptake activity other than NtNRAMP1 (N). In contrast in the NtNRAMP1 overexpressing cells (right), iron uptake is competed by manganese uptake through the increased number of the NtNRAMP1 proteins.

In graminaceous plants, the enhanced tolerance upon excess iron application was achieved by overproduction of a metal chelator, nicotianamine (Lee et al. 2009). The chelated iron was discussed to be an inactive form for reactive-hydroxyl radical generation as well as to be easily transported from roots to aerial organs (Curie et al. 2009). The increased translocation of iron to rice seeds was expected to provide iron-fortified plants and improve human health (Lee et al. 2009, Wirth et al. 2009, Zheng et al. 2010). Recently, transporters involved in iron translocation was identified in which iron-nocotianamine complex was transported to the rice shoots and phytosiderophore for iron acquisition was secreted to the soil (Ishimaru et al. 2010, Nozoye et al. 2011). In dicot plants, loss of nicotianamine synthase genes did not to fully supply iron to flowers and seeds (Klatte et al. 2009) whereas overaccumulation of nicotianamine did not affect iron translocation (Cassin et al. 2009). The role of metal chelator in dicot plants on iron translocation and resistance against iron application has still been controversial. The combination of the iron uptake moduration and the enhanced iron translocation could enhance the iron fortification and torelance in dicot plants.

3.2 Increased resistance of NtNRAMP1 overexpressing cells to cadmium

In plants, cadmium has various effects, such as the inhibition of photosynthesis, respiration and metabolism, and may finally lead to plant growth inhibition (Deckert 2005). NRAMP family members can potentially transport toxic heavy metals, including cadmium, and further characterization of the NtNRAMP1 overexpressing cells in this study revealed their

enhanced resistance to cadmium application (Fig. 8A). Changes in plant cadmium sensitivity as a consequence of NRAMP transporter activity have also been reported in which overexpression of AtNRAMP3 or AtNRAMP6 resulted in cadmium hypersensitivity of *Arabidopsis* growth (Thomine et al. 2000, Cailliatte et al. 2009). These proteins were considered to remobilize cadmium to cytoplasm from a detoxifying compartment such as a vacuole and an endomembrane compartment (Thomine et al. 2003, Cailliatte et al. 2009). The plasmamembrane localization of NtNRAMP1 may explain the increased resistance of NtNRAMP1 overexpressing cells to cadmium by the moderation of Cd^{2+} uptake similar to iron uptake.

The basis of cadmium toxicity is not completely understood, but it appears to affect cellular metabolism through its high affinity for sulfydryl compounds that therefore leads to the misfolding of enzymes, while its chemical similarity to other divalent cations reduces the activity of enzymes similar to the divalent trace metals described above (DalCorso et al. 2008, Verbruggen et al. 2009). In addition, cadmium is thought to be related to ROS generation and subsequent oxidative stress, although primarily through reduced antioxidative capacities rather than a direct effect on ROS generation (Schützendübel and Polle 2002, Deckert 2005, Heyno et al. 2008). In tobacco BY-2 cells, H_2O_2 production and subsequent cell death was reported upon application of 3 or 5 mM $CdCl_2$ (Olmos et al. 2003, Garnier et al. 2006). Upon application of 50 μM $CdSO_4$, cell cycle phase-specific death was also observed in these cells (Kuthanova et al. 2008). In our observation, as cell death was not clearly observed 24 h after application of 10 μM $CdSO_4$, effect of cadmium application was monitored by measurement of cell growth (Fig. 8A). Although we can not exclude the possible effects of cadmium on ROS generation, the reduced cellular growth rates shown in this study may have resulted from the reduced enzymatic activities of metabolic pathways since the amounts of cadmium accumulated in the NtNRAMP1 overexpressing cells decreased (Fig. 8B).

4. Conclusion

Upon excess metal ion application, plant cells modulated transporter activities. The activation of the plasma membrane transporters upon excess metal application might be inconsistent to keep the cellular metal homeostasis. Our findings suggest that activation of transporters with low affinity to metal ions could be involved in avoiding metal ion toxicity.

5. Material and methods

5.1 Plant materials and culture conditions

A tobacco BY-2 cell line (*Nicotiana tabacum* L. cv. Bright Yellow 2) was maintained by weekly subculture in a modified Linsmaier and Skoog medium supplemented with 2,4-D (LSD medium), in which KH_2PO_4 and thiamine HCl were increased to 370 and 1 mg l^{-1}, respectively. To this basal medium, sucrose and 2,4-D were supplemented to 3 % and 0.2 mg l^{-1}, respectively, and the pH was adjusted to 5.8 before autoclaving (Nagata et al. 1992). The cell suspension was cultured on a rotary shaker at 130 rpm and 27°C in the dark.

Cell synchrony was established by treatment with 5 μg l^{-1} aphidicolin (Sigma Chemical Co., St. Louis, MO, USA) essentially as described by Kumagai-Sano et al. (2006). After 24 h of

aphidicolin treatment, the cell culture was washed with 1 L of LSD medium on a glass filter and then incubated further in this medium. The cell culture was divided into two to four portions, and several different $FeSO_4$ concentrations were applied as described in Results before cell cycle and cell death analyses were conducted as described below.

5.2 Cell cycle and cell death analyses

The mitotic index (MI) was determined by fluorescence microscopy after the nuclei were stained with 1 µM of SYTOX (Molecular Probes Inc., Eugene, OR, USA). For flow cytometry, cells were fixed with 100 % ethanol, then rehydrated in Galbraith's buffer (45 mM $MgCl_2$, 30 mM Na-Citrate, 20 mM MOPS and 1 g $l^{-1}l$ Triton X-100, pH 7.0, Galbraith et al. 1983), and finally treated with 20 µg l^{-1} RNase A (Sigma) and 10 µg l^{-1} propidium iodide (Sigma) for 1 h at room temperature. Cytometric analysis was performed on 5×10^3 cells with a laser scanning cytometer (LSC101, Olympus, Tokyo, Japan) as described by Sano et al. (2006). Cell death was determined after staining the cells with 0.05 % Evans Blue (Sigma) as described in Kadota et al. (2004).

5.3 Quantification of iron and cadmium concentrations

Intracellular iron and cadmium concentrations were measured by atomic absorption spectrograph. Cells were sedimented by centrifugation to determine their packed cell volumes, and were then washed with 3 % sucrose on a glass filter before being resuspended in distilled water. For iron or cadmium extraction, cells were disrupted by a bead cell disrupter (MS-100, Tomy Seiko Co. Tokyo, Japan) and the iron or cadmium concentrations determined by atomic absorption spectrograph (AA-6800, Shimadzu Co., Kyoto, Japan).

5.4 Molecular cloning of tobacco iron transporter genes

Tobacco total RNA was isolated with the E.Z.N.A. Plant RNA Kit (Omega Bio-tek, Inc. Doraville, GA, USA), and cDNA synthesized using M-MLV reverse transcriptase (Promega, Heidelberg, Germany) with oligo-dT primers. Tobacco BY-2 *NRAMP* cDNA fragments were amplified with degenerate primers of 5'-CCNCAYAAYCTNTTYCTNCAYTSNGC-3' and 5'-TGNCCNGCRTANGTNCCNGTDATNGT-3' designed from homologous regions of known plant NRAMP proteins. *NtZIP1* cDNA was obtained based on the sequence information with high homology to *AtZIP* gene families deposited in the tobacco BY-2 EST database (TAB, Transcriptome Analysis of BY-2, http://mrg.psc.riken.jp/strc/). Amplification of the 5' and 3' cDNA ends was performed by RACE (SMART RACE cDNA Amplification kit, Clontech, Palo Alto, CA, USA), and the amplified fragments then subcloned into the pCR2.1 vector (Invitrogen Corp., Carlsbad, CA, USA).

5.5 Gene expression analysis by quantitative RT-PCR

Real-time quantitative PCR was performed in a Smart Cycler II System (Takara Bio Inc., Shiga, Japan) using the SYBR Green Real time PCR Master Mix (Toyobo Co., LTD., Osaka, Japan). *NtNRAMP1* and *NtZIP1* fragments from nucleotides 635 to 833 and 313 to 487 were amplified with primers 5'-TCTTCAAGGGATTCCCAGGA-3' (NRAMP1 FW) and 5'-TGTTATCCCACGGCATGCAAC-3' (NRAMP1 RV) or 5'- TCGCCATGTTTG AAAGAGAATCC-3' (ZIP1 FW) and 5'- CCAGACTGAGCCACCAATCCA-3' (ZIP1 RW),

respectively. As internal standards of the cDNA amounts, GAPdH fragments were amplified with primers 5′-CCGGACAAGGCTGCTGCTAC-3′ (GAP FW) and 5′-GACCCTCCACAATGCCAAACC-3′ (GAP RW), designed on the basis of the tobacco GAPdH (cytosolic glyceraldehyde-3-phosphate dehydrogenase) gene (Accession number: M14419, Dambrauskas et al., 2003) and the relative transcript values then calculated.

5.6 Transformation of tobacco BY-2 cells

The coding region of *NtNRAMP1* was amplified by PCR using gene specific primers of 5′-CACCATGGCGGCGAACTCGTCCCC-3′ and 5′-ATTAGTGGTCCTCTGCTGAGGCAA-3′, then cloned into the pENTR/D-TOPO vector (Invitrogen) and finally introduced into the pGWB502 binary vector (Nakagawa et al. 2007) by the Gateway cloning system using LR clonase (Invitrogen). The pGWB502 vector gave the cauliflower 35S promoter sequence to the PCR products. *Agrobacterium*-mediated transformation of the tobacco BY-2 cells was performed as described by Mayo et al. (2006). Transformants were selected with 50 mg l^{-1} hygromycin.

Transient gene expression was carried out by particle bombardment. A cell suspension of 2 d-old BY-2 cells was filtrated onto filter paper, and the cells bombarded with gold particles (1.0 μm) coated with the appropriate vector constructs using a particle delivery system (PDS-1000/He, Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. Filtrated BY-2 cells were placed at a distance of 6 cm under the stopping screen and were bombarded in a vacuum of 28 inches Hg at a helium pressure of 1100 psi. Following bombardment, the cells were diluted in LSD medium and kept in the dark at 27 ˚C for 6 to 12 h before observation. The GFP fluorescence was detected on the inverted platform of a fluorescence microscope equipped with a spinning disc confocal laser scanning system (CSU-X1, Yokogawa, Tokyo, Japan) and a cooled CCD camera (Cool-SNAP HQ, PhotoMetrics, Huntington Beach, Canada).

5.7 Yeast experiments

Yeast cells INVSc1 (Invtrogen) were transformed by pYES2.1/V5-His-TOPO vectors (Invitrogen) containing an entire ORF region of the respective metal transporter cDNAs according to standard procedures (Invitrogen). The transformants were selected on synthesic complete medium omitted uracil (SC-uracil) containing 2 % glucose, 0.67 % yeast nitrogen base (without amino acids, Difco), amino acids omitting uracil (-Ura DO Supplement, Clontech Laboratories Inc.), 0.5 % ammmoniumu sulfate and 2 % agar. The transporter proteins were induced by application of 2 % galactose instead of glucose in the SC-uracil medium. For iron uptake measurements, yeast cells precultured in the SC-uracil medium were diluted to OD_{600} of 0.3 and cultured in the medium supplied with 2 % galactose and 0.2 mM FeCl$_3$. After 18 h incubation, OD_{600} were measured and the yeast culture was washed with deionized water twice. For iron extraction, yeast cells were digested with 2N HCl and the iron concentrations were determined by atomic absorption spectrograph (AA-660, Shimadzu Co., Kyoto, Japan).

6. Acknowledgment

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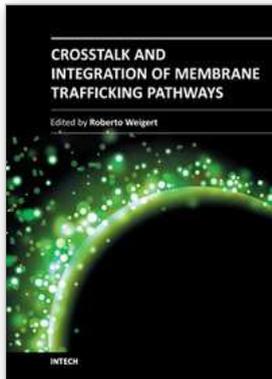
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Membrane traffic is a broad field that studies the complex exchange of membranes that occurs inside the cell. Protein, lipids and other molecules traffic among intracellular organelles, and are delivered to, or transported from the cell surface by virtue of membranous carriers generally referred as "transport intermediates". These carriers have different shapes and sizes, and their biogenesis, modality of transport, and delivery to the final destination are regulated by a multitude of very complex molecular machineries. A concept that has clearly emerged in the last decade is that each membrane pathway does not represent a close system, but is fully integrated with all the other trafficking pathways. The aim of this book is to provide a general overview of the extent of this crosstalk.

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