AtIRT1, the Primary Iron Uptake Transporter in the Root, Mediates Excess Nickel Accumulation in Arabidopsis thaliana

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Nickel (Ni) is an essential nutrient for plants, but excessive amounts can be toxic. Ni competes with iron (Fe) in vivo, raising the possibility that Ni is competitively taken up via the Fe uptake system in plants. Here, we show evidence that AtIRT1, the primary Fe2+ uptake transporter in the root, mediates Ni accumulation in Arabidopsis thaliana. In hydroponic cultures, excess Ni exposure increased Fe accumulation and the relative transcription level of AtIRT1 in roots, indicating that excess Ni induces AtIRT1 expression in roots. An Fe-deficient treatment increased Ni accumulation in plants, suggesting that excess Ni was absorbed via the Fe uptake system, which was induced by Fe starvation. Moreover, Ni accumulation under Fe-deficient conditions was markedly lower in AtIRT1-defective mutants than in the wild-type, Col-0. Furthermore, AtIRT1 showed Ni2+ uptake activity in a yeast expression system. These data demonstrate that AtIRT1 transports Ni2+ in roots, and strongly suggest that Ni accumulation is further accelerated by AtIRT1 that is expressed in response to excess Ni.

Keywords: Arabidopsis thaliana • Iron • IRT1 • Metal transporter • Nickel • ZIP family.

Abbreviations: DMG, dimethylglyoxime; Fe, iron; IRT1, Iron-Regulated Transporter 1; Ni, nickel; RT–PCR, reverse transcription–PCR; YNB, yeast nitrogen base; YPG, yeast extract–peptone–galactose; ZIP, Zrt/Irt-like protein

Introduction

Nickel (Ni) is an essential nutrient for plants (Marschner 1995), but can be toxic at high concentrations in the environment. Ni contamination of soils occurs through the weathering of ultramafic rock in the natural environment (Mizuno and Nosaka 1992), and from human activities (Kukier and Chaney 2004). In contaminated areas, agricultural crops show various Ni-induced impairments, such as reduction in biomass, leaf deformities, chlorosis and necrosis. Contamination of soil by toxic metals, including Ni, is a worldwide problem that limits food production. Therefore, it is crucial to understand the mechanisms underlying Ni phytotoxicity in order to develop techniques to avoid crop damage in Ni-rich areas.

Ni(II) is the most common oxidation state of Ni in the environment, and the form most available to plants. Since Ni does not have redox activity in biological conditions, Ni phytotoxicity is attributed to the disruption of various physiological processes, including the activities of various enzymes, photosynthesis and mineral nutrition (Samarakoon and Rauser 1979, Veeranjaneyulu and Das 1982, Van Assche and Glijsters 1990, Pandey and Sharma 2002). In particular, iron (Fe) deficiency is a well-known symptom of Ni phytotoxicity. Ni belongs to the Fe group of transient elements and its chemical properties are similar to those of Fe. Thus, Ni competes with Fe in biochemical and physiological processes in plants (Seregin and Kozhevnikova 2006). Indeed, a high Fe to Ni ratio in the soil is significantly correlated with high Ni resistance in various plants (Crooke and Knight 1955, Mizuno 1968). Research has shown that excess Ni treatment decreases the activities of antioxidant Fe enzymes (e.g. Fe superoxide dismutase and catalase). This suggests that Ni-induced oxidative stress is associated with the competition of Ni with Fe (Pandey and Sharma 2002, Gajewska and Sklodowska 2007, Ghasemi et al. 2009).

Excess Ni also interrupts the Fe transport system in plants. It is thought that excess Ni2+ competitively inhibits the configuration of Fe2+/–ligand complexes, decreasing their mobility. For example, nicotianamine and citrate, which are the primary ligands responsible for the mobility of Fe in plants, can effectively chelate Ni2+ in plants (Krämer et al. 2000, Kim et al. 2005, Callahan et al. 2007, Saito et al. 2010). It has been demonstrated, in several plant species, that excess Ni inhibits the root to shoot translocation of Fe, causing overaccumulation of Fe in the root (Piccini and Malavolta 1992, Yang et al. 1996, Ghasemi et al. 2009). Furthermore, several Fe2+/–nicotianamine transporters in the Yellow Stripe-Like (YSL) family show Ni2+/–nicotianamine transport activity (Schaaf et al. 2004, Gendre et al. 2007), indicating that competition between Fe2+ and Ni2+ occurs within the membrane transport system.

According to several physiological studies in various plant species, Ni can be competitively taken up by the Fe transport...
system in roots (Cataldo et al. 1978, Yang et al. 1996, Pandey and Sharma 2002). Schaaf et al. (2006) showed that Ni accumulation in roots was increased by Fe-deficient treatments in Arabidopsis thaliana. In A. thaliana, Fe uptake in the root system mainly depends on an Fe2+ transporter protein, Iron-Regulated Transporter 1 (AtIRT1), a member of the Zrt/Irt-like protein (ZIP) family of transporters (Vert et al. 2002). AtIRT1 has broad specificity for divalent heavy metals, mediating the accumulation of zinc, manganese, cobalt and cadmium under Fe-deficient conditions (Vert et al. 2009). Schaaf et al. (2006) also showed that the Ni sensitivity of a yeast strain expressing AtIRT1 was higher than that of a control strain, suggesting that AtIRT1 takes up excess Ni2+ into cells. Therefore, it has been postulated that AtIRT1 also mediates Ni2+ uptake in A. thaliana (Schaaf et al. 2006, Morrissey et al. 2009). In this study, we examined the influence of a loss-of-function AtIRT1 mutation on Ni accumulation, and investigated the ability of AtIRT1 to transport Ni2+ using a yeast heterologous expression system. Our data demonstrate that AtIRT1 functions as an Ni2+ uptake transporter in A. thaliana. Furthermore, our data suggest that excess Ni2+ uptake of AtIRT1 is accelerated by Ni exposure. We propose that AtIRT1 is responsible for the excess Ni accumulation in plants growing in Ni-rich conditions.

Results

Evaluation of Ni tolerance of Arabidopsis thaliana in a hydroponic system

We evaluated the Ni tolerance of the A. thaliana ecotype Col-0. Four-week-old plants were exposed to various concentrations of NiCl2 (0–50 μM) for 7 d in hydroponic culture, and growth and mineral concentrations in roots and shoots were determined. Growth inhibition was observed at concentrations exceeding 25 μM NiCl2. Young leaves of plants affected by Ni toxicity were chlorotic between the veins, and those in the 50 μM NiCl2 treatment developed necrotic lesions (Fig. 1A). After 7 d in the 50 μM NiCl2 treatment, the FWs of shoots and roots were 50% of those of the unexposed controls (Fig. 1B). The Ni concentration in roots and shoots increased with increasing Ni concentrations in the hydroponic solution. Ni accumulation was significantly higher in roots than in shoots across all Ni treatments (Fig. 1C, D). Fe accumulation in roots increased with increasing concentrations of Ni. In the 50 μM NiCl2 treatment, the concentration of Fe in the roots was 6.2-fold higher compared with roots in the control plants (Fig. 1E). In contrast, the Fe concentration in shoots significantly decreased in response to Ni exposure (Fig. 1F). These results suggest that the accumulated Ni inhibited Fe translocation from roots to shoots, resulting in an overaccumulation of Fe in roots. However, the Fe accumulation in whole plants, grown in 50 μM NiCl2, significantly increased 2.2-fold (t-test, P < 0.001) compared with the unexposed controls. This result indicates that Ni exposure induced Fe uptake in roots.

Expression of AtIRT1 is induced under excess Ni conditions

To examine if the increase in Fe accumulation under excess Ni conditions was related to the Fe2+ uptake transporter AtIRT1, we compared the expression level of AtIRT1 in roots of unexposed controls and those exposed to 25 μM NiCl2 for 7 d. In Fe-sufficient conditions, the transcription level of AtIRT1 was approximately 6-fold higher in roots exposed to Ni than in the unexposed control (Fig. 2). When exposed to Ni under Fe-deficient conditions, the AtIRT1 expression in roots was further increased approximately 6-fold compared with roots exposed to Ni in Fe-sufficient conditions. These data indicate that excess Ni exposure induced AtIRT1 expression.

Mutation of AtIRT1 suppresses Ni accumulation in roots

To determine whether AtIRT1 mediates Ni2+ uptake in roots, we examined the phenotype of AtIRT1 mutants with respect to their Ni2+ uptake and accumulation patterns. We obtained two AtIRT1 mutants, iirt1-1 (SALK_024525) and iirt1-2 (SALK_054554), which carry T-DNA insertions in the promoter region (iirt1-1) and in the third exon (iirt1-2) (Fig. 3A). Fukao et al. (2011) characterized these lines as AtIRT1-defective mutants. Semi-quantitative reverse transcription–PCR (RT–PCR) analyses showed that AtIRT1 expression was not induced by Fe starvation in iirt1-1 (Fig. 3B), whereas AtIRT1 expression was detected in iirt1-2. It is predicted that iirt1-2 produces a mutated AtIRT1, lacking the two amino acid residues (tryptophan and alanine) at the C-terminal end (Fig. 3A). We constructed plasmids expressing AtIRT1 cloned from either the wild type or iirt1-2. These plasmids were transformed into the yeast fet3fet4 mutant, which lacks the Fe uptake system of Fet3 and Fet4 and cannot survive in low-iron medium (Eide et al. 1992). The plasmid carrying wild-type AtIRT1 rescued yeast fet3fet4 mutant, but the strain expressing the mutated AtIRT1 was not rescued (Fig. 3C). This result suggests that AtIRT1 in iirt1-2 codes for a non-functional form. Similar to previously identified mutants (Varotto et al. 2002, Vert et al. 2002), we found that iirt1-1 and iirt1-2 showed hypersensitivity to Fe deficiency, although on Fe-sufficient (50 μM Fe-EDTA) agar plates there was no significant difference in growth between the wild type (Col-0) and mutants (Fig. 4A). This result indicates that the function of AtIRT1 was lost in these lines. In this study we used these lines, iirt1-1 and iirt1-2, as AtIRT1-defective mutants.

Seedlings of the wild type (Col-0) and AtIRT1 mutants were exposed to 25 μM NiCl2 on either Fe-sufficient or Fe-deficient MGRL plates. The growth of Col-0 and both AtIRT1 mutants was severely inhibited when exposed to Ni under Fe-deficient conditions (Fig. 4A). The difference in growth inhibition between the lines was non-significant. The growth of Col-0 seedlings under excess Ni conditions recovered when supplied with Fe, while the AtIRT1 mutants still exhibited chlorosis, indicating that Ni sensitivity was increased by the defect in AtIRT1. The accumulated Ni in seedlings was visualized by
dimethylglyoxime (DMG), which selectively binds to Ni$^{2+}$, producing reddish-brown crystals (Mizuno et al. 2003, Richau et al. 2009). In Col-0 seedlings exposed to Ni under Fe-deficient conditions, strong staining was observed in the roots, particularly the lateral roots (Fig. 4B). Ni staining in roots was significantly weaker in AtIRT1 mutants. We also observed strongly stained regions in Col-0 and mutants under a light microscope (Fig. 4C). The images of Col-0 root, which were in focus on the surface (left panel) or the center (right panel), suggested that the red crystals of the Ni–DMG complex mainly accumulated at the epidermis and in root hair cells in Col-0. Significantly fewer cells accumulated Ni–DMG crystals in the mutant lines than in Col-0. When exposed to Ni under Fe-sufficient conditions, there was no significant staining in Col-0 or the mutants (data not shown). Under those conditions, the Ni concentrations in tissues may be lower than the threshold of detection by DMG.

To obtain quantitative data on Ni$^{2+}$ uptake in the AtIRT1 mutant, we examined Ni accumulation in the roots and shoots of 4-week-old plants. Plants were grown in hydroponic solutions containing 25 μM NiCl$_2$ for 7 d in either Fe-sufficient (50 μM Fe-EDTA) or Fe-deficient (0 μM) conditions. The Col-0 and iirt1-1 plants exposed to Ni under Fe-deficient condition displayed severe chlorosis. However, this symptom was reduced by Fe supplementation in both lines (Fig. 5A). The FW of roots exposed to Ni in Fe-sufficient conditions was lower in iirt1-1 than in Col-0 (Fig. 5B). Shoot biomass showed no significant differences between iirt1-1 and Col-0 (Fig. 5C). In Col-0, the Ni accumulation in roots was 3.1-fold higher in Fe-deficient conditions compared with Fe-sufficient conditions, whereas
the overaccumulation of Ni in Fe-deficient conditions was absent in *irt1-1* (Fig. 5D). The Ni concentration in shoots was not significantly different between Col-0 and *irt1-1* in both Fe-deficient and Fe-sufficient conditions (Fig. 5E). To eliminate the possibility of secondary effects from long-term exposure, we examined short-term (1 h) Ni$^{2+}$ uptake in roots of Col-0 and *irt1-1* using $^{65}$Ni. In Col-0, after 7 d in Fe-deficient conditions, $^{65}$Ni accumulation in roots was 3.4-fold greater than in roots of plants grown in Fe-sufficient conditions. There was no significant increase in $^{65}$Ni accumulation in *irt1-1* under Fe deficiency (Fig. 5F). These quantitative data indicate that the overaccumulation of Ni in Fe-deficient conditions is dependent on AtIRT1.

**AtIRT1 mediates Ni$^{2+}$ uptake in yeast cells**

Using yeast assays, we investigated if AtIRT1 could transport Ni$^{2+}$. The strain expressing AtIRT1 showed increased sensitivity to Ni compared with the vector control strain (Fig. 6A), suggesting that AtIRT1 takes up excess Ni$^{2+}$ into cells. The yeast expressing mutated AtIRT1 cloned from *irt1-2* did not show increased Ni sensitivity. The Ni accumulation in cells expressing AtIRT1 doubled compared with the vector control (Fig. 6B). These data demonstrated that Col-0 AtIRT1 has Ni$^{2+}$ uptake activity.

**Discussion**

Our study verified Ni$^{2+}$ uptake via the Fe$^{2+}$ uptake system in *A. thaliana*. Ni accumulation in roots is increased by Fe-deficient treatments in *A. thaliana* (SchAAF et al. 2006), suggesting that the Fe$^{2+}$ uptake system is responsible for Ni$^{2+}$ absorption. Our results showed that Ni accumulation in roots under Fe-deficient conditions decreased in mutants that were defective in primary Fe$^{2+}$ uptake by AtIRT1. We also showed that AtIRT1 has Ni$^{2+}$ uptake activity in yeast cells. These data demonstrated that AtIRT1 is one of the pathways of Ni$^{2+}$ uptake in the root of *A. thaliana*.

AtIRT1 is expressed in the outer layer of the root, and absorbs Fe$^{2+}$ from the soil (Vert et al. 2002). When the root takes up Fe$^{2+}$ in Ni-rich conditions, excess Ni$^{2+}$ can be taken up by AtIRT1 and concentrated in epidermal cells. Our data showed a strong accumulation of the Ni–DMG compound in the epidermis and root hairs of roots in Col-0, and reduced Ni–DMG accumulation in *AtIRT1* mutants. Excess Ni$^{2+}$ is transported to the endodermis via the symplastic pathway, then translocated from the root to the shoot. However, *A. thaliana* does not have an efficient system to translocate Ni, resulting in excess accumulation of Ni in the root, similar to other plant species (Cataldo et al. 1978, Krämer et al. 1997, Pandey and Sharma 2002). Accumulated Ni can cause oxidative or other physiological damage to root cells, although some Ni$^{2+}$ can be sequestered in the vacuole by FPN2 (IREG2) (SCHAAF et al. 2006, Morrissey et al. 2009). Furthermore, our data indicated that accumulated Ni$^{2+}$ inhibited Fe$^{2+}$ translocation from roots to shoots (Fig. 1E, F). Decreased Fe content in the shoot may accelerate the development of Ni toxicity as Ni$^{2+}$ competes with Fe$^{2+}$. Additionally, overaccumulation of Fe may cause oxidative stress in roots (Ghasemi et al. 2009).

In this study, AtIRT1 mutants under Fe-deficient conditions showed reduced Ni accumulation in roots, but showed no decreased sensitivity to Ni in agar plate or hydroponic cultures. Rather, the mutant seedlings displayed severe chlorosis from excess Ni even in Fe-sufficient conditions, unlike wild-type seedlings (Fig. 4A). These results suggest that the functional loss of AtIRT1 enhances sensitivity to Ni toxicity. As described in the Introduction, it has been suggested that Ni$^{2+}$ competes with Fe$^{2+}$ in plants. Physiological and biological systems requiring Fe are greatly affected by the loss of AtIRT1 (Henriques et al. 2002, VAROTTO et al. 2002), which could result in the enhancement of Fe deficiency at the molecular level due to Ni accumulation via other uptake pathways in the mutant seedlings. In contrast to the result with seedlings, the mature plants of AtIRT1 mutants did not show any chlorosis when exposed to Ni under Fe-sufficient conditions (Fig. 5A). We predict that the sufficient Fe, which was pooled during growth, decreased...
Fe deficiency stress induced by Ni. We are currently investigating the increased Ni sensitivity in \textit{AtIRT1} mutants by examining the total and biologically available Fe content, along with the expression of related genes in plants exposed to excess Ni.

To what extent does \textit{AtIRT1} contribute to excess Ni$^{2+}$ uptake in plants exposed to long-term excess Ni in Ni-contaminated soil? As an essential micronutrient for higher plants, the requirement for Fe is comparatively high (100 mg kg$^{-1}$ DW) (Marschner 1995). Therefore, we speculate that the Ni$^{2+}$ uptake via \textit{AtIRT1} responsible for Fe$^{2+}$ uptake greatly contributes to excess Ni accumulation in plants grown in Ni-contaminated soils. We are now attempting to estimate the amount of Ni$^{2+}$ uptake via \textit{AtIRT1} in Ni-contaminated soils, by investigating the transport affinity of \textit{AtIRT1} for Fe$^{2+}$ and Ni$^{2+}$, and the Fe$^{2+}$/Ni$^{2+}$ uptake ratio. Our quantitative RT–PCR data, one of the most significant results in this study, showed that excess Ni exposure induced \textit{AtIRT1} expression. This suggests that excess Ni further accelerates Ni$^{2+}$ uptake by \textit{AtIRT1} in Ni-rich soils. Ni exposure significantly increased Fe accumulation in the roots (Fig. 1E), suggesting that the up-regulated expression of \textit{AtIRT1} increased Fe$^{2+}$ uptake by \textit{AtIRT1} in roots.

The increased expression of \textit{IRT1} by metal exposure has been observed in rice and tobacco plants exposed to excess cadmium (Yoshihara et al. 2006, Ogawa et al. 2009), and it is postulated that the induction of \textit{IRT1} expression was a response to Fe deficiency at the molecular level due to cadmium toxicity. Accumulated Ni$^{2+}$ could induce an Fe deficiency response in plants, causing further Ni accumulation by \textit{AtIRT1}. However, \textit{AtIRT1} is deregulated at the protein level in response to high external concentrations of metals (Kerkeb et al. 2008). Therefore, we need to verify not only the transcript accumulation, but also the protein accumulation of \textit{AtIRT1} under excess Ni conditions. Further studies are in progress in our laboratory to determine the relative contribution of \textit{AtIRT1} to excess Ni uptake in \textit{A. thaliana} grown in Ni-contaminated soils.

We demonstrated the involvement of the Fe uptake system in excess Ni accumulation in plants at the molecular level. Our findings are important for the development of techniques to prevent crop damage in Ni-rich soils. Our results also showed that another pathway of Ni$^{2+}$ uptake exists in \textit{A. thaliana}. We predict that Ni$^{2+}$ is erroneously absorbed via pathways for other nutrients, including Fe$^{2+}$. In soybean, Cataldo et al. (1978) suggested that Ni$^{2+}$ is absorbed by the Zn$^{2+}$ and Cu$^{2+}$...
uptake system, which is mediated by ZIP-family transporters (Grotz et al. 1998, Wintz et al. 2003, Ishimaru et al. 2005). Other members of the ZIP family may also have Ni\(^{2+}\) transport activity. Currently, we are focusing on the ZIP family to identify other genes that are responsible for excess Ni accumulation in A. thaliana.

**Materials and Methods**

**Plant material**

Arabidopsis thaliana ecotype Col-0 was obtained from Dr. Kagaya (Mie University). The T-DNA mutant lines irt1-1 (SALK_024525) and irt1-2 (SALK_054554) were acquired from the Salk Collection via the Arabidopsis Biological Resource Center (ABRC) and Dr. Fukao (NAIST). Homozygous insertion mutants were identified using PCR with primers flanking the insertion site, as follows: irt1-1fw (5'-TGA CTA ACA GAA CTA TAG AAG ACG CAT G-3') and irt1-1rev (5'-CAT GCC ATT TAT TGT CGA TGA CGA-3') for irt1-1; irt1-2fw (5'-TCT TGG CGG TTT TAT CCT CCA G-3') and irt1-2rev (5'- TGA TTT TAT CAA CAG AAC CCG GTT AGA GAA C-3') for irt1-2 (Fukao et al. 2011).

**Determination of Ni tolerance and accumulation in hydroponics**

Half-strength MGRL solution (Fujiwara et al. 1992) containing Fe (8.6 \(\mu\)M Fe-EDTA) and 5 mM MES at pH 5.5 was used as the hydroponic solution. Plants were pre-cultured in the hydroponic system as follows: seeds that had been stored at 4°C were sown on rockwool (Nittobo Co.) soaked with hydroponic solution. After 10 d, seedlings were transferred onto floats and hydroponically cultured for 1 week. Subsequently, plants were transferred to pot cultures and grown for the indicated periods of time. Plants were cultivated in a growth chamber at 22°C and 70% relative humidity with 10 h light periods. Light was supplied by white fluorescent lamps (120 \(\mu\)E m\(^{-2}\) s\(^{-1}\)). Hydroponic solutions were continuously aerated and changed weekly.

To test the Ni tolerance of A. thaliana Col-0, plants cultured in pots for 2 weeks were exposed to 0–50 \(\mu\)M NiCl\(_2\) in hydroponic solution for 1 week. To determine the effect of Fe conditions on Ni\(^{2+}\) uptake, plants cultured in pots for 8 d were irrigated with a hydroponic solution containing 25 \(\mu\)M NiCl\(_2\) and either 0 or 50 \(\mu\)M Fe-EDTA, and cultured for 1 week. After exposure, plants were harvested, separated into roots and shoots, rinsed with deionized water, blotted dry, and weighed.

![Fig. 5](https://example.com/fig5.png)
to determine FW. Plant parts then were dried at 70 °C for 3 d and reweighed to determine DW. Samples were digested in concentrated HNO₃ in a heat block and decolorized with H₂O₂. Digests were diluted with deionized water and mixed with scintillation cocktail (Ecoscint™ XR, National Diagnostics). ⁶³Ni was determined using a liquid scintillation counter (LSC-5100, Aloka Co., Ltd.).

Assay for Ni sensitivity on agar plates and Ni staining using dimethylglyoxime

Seeds were surface-sterilized and sown on MGRL-based agar plates. The medium consisted of half-strength MGRL supplemented with 1.5% sucrose, 1.2% agar, 5 mM MES (pH 5.5) and 50 μM Fe-EDTA. This medium was sufficient for the healthy growth of int1-2, a putative null mutant of AtIRT1. After 4 d, seedlings were transferred to MGRL-based agar plates supplemented with 0 or 25 μM NiCl₂ under Fe-deficient (0 μM Fe-EDTA) or Fe-sufficient (50 μM Fe-EDTA) conditions, and cultured for 10 d. To prepare the Fe-deficient medium, we used ultra-pure agar (Sigma–Aldrich Co.). Plants were grown under 16 h light periods at 70 μE m⁻² s⁻¹ at 22 °C.

The staining of Ni using DMG was carried out as described previously (Mizuno et al. 2003). Seedlings were immersed in Ni staining solution (0.17% DMG, 0.83 M C₂H₃O₂NH₄, pH 10) and incubated for 5 min at room temperature. Images were obtained under a stereomicroscope (SX51, Olympus).

RNA extractions and cDNA synthesis

Total RNA was extracted following the method of Suzuki et al. (2004). Genomic DNA was digested with DNase I (TAKARA BIO INC.). The concentration and quality of RNA were determined using a flame atomic absorption spectrophotometer (AA6500S) or an inductively coupled plasma-atomic emission spectrometer (ICPS-7500, Shimadzu Co.).

Semi-quantitative and quantitative RT–PCR assays

Semi-quantitative RT–PCR analyses were performed using 1 μl of reverse transcription reaction product with gene-specific primers for AtIRT1 (IRT1por.fw, 5'-TGG AAT CTC TCC AGC AAC TTC AAC TG-3'; and IRT1por.rev, 5'-TCG CAA GAG CTG 0-3') and ACT1 (ACT1por.fw, 5'-AAT TGG GAT GAC ATG GAG AAG ATT TGG-3'; and ACT1por.rev, 5'-TGG AGT TAT AGG TGG TTT CAT GGA TAC-3') and KOD Dash DNA polymerase (Toyobo Co., Ltd.) in a total volume of 25 μl. Various cycle numbers (25–35 cycles) were used for the PCRs. A 10 μl aliquot of the reaction mixture was used for agarose gel electrophoresis, and the gels were stained with ethidium bromide.
Real-time DNA quantification was performed in 96-well plates with the ABI 7300 Real-Time PCR system (Applied Biosystems). cDNA corresponding to 5 ng of RNA was used for the reaction with Go Taq® qPCR Master Mix and 0.2 μM forward and reverse primers in a total volume of 25 μl. The following standard thermal profile was used: 2 min at 95°C, 40 repeats of 15 s at 95°C and 60 s at 60°C, and a final stage of 15 s at 95°C, 30 s at 60°C and 15 s at 95°C to determine dissociation curves of amplified products. Data were analyzed with SDS v. 1.3.1 (Applied Biosystems). The specific primers for AtIRT1 were IRT1RTfw (5’-GGT TGT ATC CTC CAG GCT GAG T-3’) and IRT1RTrev (5’-TG TGT TAC TAC CCG ACA AA-3’), which were designed using Prime Express® software v. 3 (Applied Biosystems). The specificities of primers were confirmed by analysis of the dissociation curves and agarose gel electrophoresis of the PCR products. The expression level of AtIRT1 was normalized against that of EF1α. The specific primers for EF1α were 5’-CCT TGG TCA GGA GAT GA-3’ (forward) and 5’-TGA AGA CAT CTC CTT GAT TT-3’ (reverse), as designed by Takano et al. (2006). Quantitative RT–PCR was performed using material from two independent biological samples, and representative data are shown.

**Yeast strain, growth conditions and plasmids**

Saccharomyces cerevisiae strain BY4741 (MATa his3 leu2 met15 ura3) was obtained from the European S. cerevisiae Archive for Functional Analysis (Frankfurt, Germany). The fet3fet4 DEY1453 (MATa/MATα ade21 can1 can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2:HIS3 fet3-2:HIS3 fet4-1::LEU2 fet4-1::LEU2) was provided by Professor Nishizawa (Ishikawa Prefectural University). The strain was grown in yeast nitrogen base (YNB) medium (0.67% YNB without amino acids) supplemented with appropriate amino acids and 2% glucose at pH 5.5.

The full-length AtIRT1 was amplified from the cDNA, which was generated from roots under Fe-deficient conditions, with KOD-Plus DNA polymerase (Toyobo) using the following specific primer sets: IRT1fw (5’-GGA TTC ATG GCT TCA AAT TCA G-3’) and IRT1rev (5’-GGA TTC TTA AGC CCA TTG GGC GAT AAT C-3’) for Col-0 AtIRT1, and IRT1fw and LBb1 (5’-GCG ACC CCT GTC TGC AAC T-3’) (O’Malley et al. 2007) for irt1-2 AtIRT1. PCR products were cloned into the pTall vector (Toyobo) and sequenced. AtIRT1 and the mutated AtIRT1 were subcloned into the pKT10-Gal-HA-BS yeast expression vector at the EcoRI–SalI sites and the EcoRI–PvuII sites, respectively. Escherichia coli and yeast transformations were performed using standard methods.

**Metal uptake assays in yeast**

For the complementation test of the fet3fet4 mutant, yeast extract–peptone–galactose (YPG) medium supplemented with 0 or 50 μM FeSO₄ and 50 mM MES at pH 6.0 was used. For the Ni sensitivity test, YNB medium containing 0 or 500 μM NiCl₂ and 2% galactose (pH 6.0) was used. Yeast cultures at the exponential phase were diluted and then spotted onto each assay plate. Cells were observed during a 3–5 d incubation at 30°C. All solid plate media contained 2% agar.

The Ni uptake assay was performed as described previously (Nishida et al. 2008, Nishida et al. 2011), except that 50 μM CdCl₂ was substituted for 500 μM NiCl₂.

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