AtIREG2 Encodes a Tonoplast Transport Protein Involved in Iron-dependent Nickel Detoxification in Arabidopsis thaliana Roots

Iron acquisition in Arabidopsis depends mainly on AtIRT1, a Fe$^{2+}$ transporter in the plasma membrane of root cells. However, substrate specificity of AtIRT1 is low, leading to an excess accumulation of other transition metals in iron-deficient plants. In the present study we describe AtIREG2 as a nickel transporter at the vacuolar membrane that counterbalances the low substrate specificity of AtIRT1 and possibly other iron transport systems in iron-deficient root cells. AtIREG2 is co-regulated with AtIRT1 by the transcription factor FRU/FIT1, encodes a membrane protein, which has 10 putative transmembrane domains and shares homology with vertebrate Fe$^{2+}$ exporters. Heterologous expression of AtIREG2 in various yeast mutants, however, did not demonstrate an iron transport function. Instead, expression in wild-type and nickel-sensitive cot1 yeast cells conferred enhanced tolerance to elevated concentrations of nickel at acidic pH. A role in vacuolar substrate transport was further supported by localization of AtIREG2-GFP fusion proteins to the tonoplast in Arabidopsis suspension cells and root cells of intact plants. Transgenic plants overexpressing AtIREG2 showed an increased tolerance to elevated concentrations of nickel, whereas T-DNA insertion lines lacking AtIREG2 expression were more sensitive to nickel, particularly under iron deficiency, and accumulated less nickel in roots. We therefore propose a role of AtIREG2 in vacuolar loading of nickel under iron deficiency and thus identify it as a novel component in the iron deficiency stress response.

Iron deficiency in plants is visually expressed as chlorosis, first appearing in the youngest developing leaves and accompanied by a reduction in growth rate, dry matter production, and in most cases by a decrease of iron concentration (1). At the same time, root uptake capacities and leaf concentrations of other divalent metal cations increase (2, 3). In soils or nutrient solutions with unbalanced microelement supply, iron deficiency can then promote the toxicity of other transition metals to plants (4, 5). Such toxicity seems also to be the case with nickel, supported by the observation that phytoxicity of nickel decreased with increasing iron:nickel ratios in the leaf tissue (6, 7). Thus, an increased sensitivity to heavy metals under iron deficiency might represent a secondary, growth-limiting factor besides the lack of iron itself.

Molecular studies in yeast showed that an iron deficiency-induced accumulation of transition metals other than iron was explained by a higher activity of non-selective low affinity iron transport. Deletion of FET3, an essential component for high affinity iron uptake in yeast, leads to a constitutive iron-deficient phenotype and a concomitant up-regulation of FET4, which encodes a low affinity Fe(II) transporter with poor substrate selectivity (8). As a consequence, sensitivity to elevated concentrations of the transition metals cobalt, copper, zinc, and manganese was higher in fet3 mutants than in the corresponding wild type, consistent with increased metal accumulation (8).

In Arabidopsis, iron-dependent overaccumulation of divalent metal cations was found to be mediated by the Fe(II) transporter AtIRT1, which in fact transports a broad range of transition metals (9). Atirt1 T-DNA insertion lines no longer accumulated manganese, zinc, and cobalt under iron deficiency and even showed an increased tolerance to toxic levels of cadmium (10). Thus, accumulation of certain transition metals in iron-deficient Arabidopsis plants directly depends on AtIRT1 and appears as an unavoidable side effect of iron deficiency-induced iron accumulation.

In the search for genes that might be involved in metal transport in Arabidopsis roots, homology to iron export proteins from animals pointed to the Arabidopsis gene At5g03570, named AtIREG2. The derived amino acid sequence of AtIREG2 shares 34.8% similarity to IREG1 (iron-regulated protein) from mouse, which encodes an iron exporter located in the basolateral membrane of duodenal enterocytes. IREG1 mediates iron transport across the basolateral membrane into the extracellular lumen of the portal circulation, where it is bound as Fe$^{3+}$ to transferrin (11). IREG1 is not only expressed in duodenal enterocytes but is present in all cells in which iron export appears as a major function, such as placental cells, macrophages, and the yolk sac of nonmammalian vertebrates like...
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AtIREG2 in iron export. To unravel the function of AtIREG2 in the cellular homeostasis of transition metals, we tested functionality in yeast, investigated membrane localization of GFP

protein fusions and analyzed transgenic plants either lacking the expression or overexpressing AtIREG2. Our results identify AtIREG2 as an as yet unrecognized component in metal homeostasis required to circumvent toxicity of nickel in iron-deficient Arabidopsis plants.

EXPERIMENTAL PROCEDURES

Plasmids, Transgenic Plants, and Yeast Growth—DNA manipulations were carried out using standard protocols (13). The open reading frame (ORF) of AtIREG2 was amplified by PCR from an Arabidopsis thaliana Col-0 CDNA library (kindly provided by Karin Schumacher, ZMBP, Tübingen, Germany) using the primers 5'-CGGGATCTCGAGGGAGAACA-GAAAAC-3' and 5'--GGCGGAGCTCTCATGAAGCAGAAAAAAGTTG-TTCAAGG-3'. PCR products were A-tailed, cloned into the pGEM®-T Easy Vector (Promega, Madison, WI), and subcloned into the yeast expression vector pDR195 (14) at the NotI site. The cDNA of AtIRT1 was subcloned from pFL61-AtIRT1 (kindly provided by C. Curie) into pDR195 at the NotI sites. For transient expression of an AtIREG2-GFP fusion protein in protoplasts, the AtIREG2 ORF without a stop codon was amplified using the primers 5'-GGATCCATGGAGGAGGAAACAAGA-AACTAGG-3' and 5'-GGATCTTGCAGTTCTGACTACTTTGA-3'. PCR products were inserted into the pGEM®-T Easy Vector and subcloned at the BamHI sites into the pSoup plasmid (15) to allow replication of the plasmid in strains GV3101:pMP90 and containing the respective metal as indicated.

For Northern analysis, 20 μg of total root or shoot RNA were used as gene-specific primers. The AtIREG2 forward primer 5'-TCTCTGCAGTTCTGACTACTTTGA-3' and reverse primer 5'-TTTCCTCGACTTCGATTTG-3' were hybridized and cloned into pTkan at the XbaI/PstI restriction sites resulting in pTkan+. The AtIREG2-GFP fusion was excised from the vector CF203 with Acc65I and PstI. A blunt end was created at the Acc65I cutting site and subcloned into the PstI/EcoRV restriction sites of pTkan+. A 1794-bp AtIREG2 promoter fragment was amplified from genomic DNA with the primers 5'-TTCTCGAGTTCTTCTGACTACTTTGA-TTCTTTC-3' and 5'-CCGCTCGAGGGGCGGAAGTCGAG-GAGAG-3'. The resulting PCR product was A-tailed, cloned into pGEM-T Easy, digested with NotI and subcloned into the pTkan+-AtIREG2-GFP construct at the Bsp120I restriction site resulting in the plasmid pTkan+-AtIREG2 promoter-AtIREG2-GFP.

The resulting binary plasmids were introduced into the Agrobacterium tumefaciens strain GV3101:pMP90 and selected on 100 μg ml⁻¹ rifampicin, 40 μg ml⁻¹ gentamycin, and 100 μg ml⁻¹ spectinomycin. Agrobacterium cells were pretransformed with pSoup to allow replication of the pGreen construct. A. thaliana Col-0 plants or tir2g-2 T-DNA insertion lines were then transformed via agrobacteria using the floral-dip method (16) and transformants were selected on BASTA (pGreen) or 75 μg ml⁻¹ kanamycin (pTkan+-AtIREG2 promoter-AtIREG2-GFP). The pTkan+-AtIREG2 promoter-AtIREG2-GFP construct was used for complementation of the ireg2-1 T-DNA insertion line (see below). Homozygous plants were identified in the T2 generation based on segregation analysis on 50 μg ml⁻¹ kanamycin. Arabidopsis T-DNA insertion lines SALK_074442 (ireg2-1) and SALK_127071 (ireg2-2) were obtained from the stock of T-DNA insertion lines provided by SALK (17). The lines were screened by PCR using AtIREG2 specific and left border T-DNA primers. To verify homozygosity in line ireg2-1, AtIREG2 forward primer 5'-TTTCTCTGACCTCTGATTGTG-3' and AtIREG2 reverse primer 5'-CCATCGAGCAAGA-AAATAGCC-3' were used to amplify the wild-type AtIREG2 gene. To screen for the T-DNA insertion, PCR was performed with the reverse primer (see above) and the T-DNA left border primer Lbb1 (5'-CGCTGTGAGCTTGTGACTCACT-3'). For line ireg2-2 the forward primer 5'-CGAAAAATTTAGAATC-GAATCTAAA-3' and the reverse primer 5'-TGATCGACCTTGTGACCCCAT-3' were used as gene-specific primers. The presence of the T-DNA insertion in ireg2-1 was verified by PCR with the reverse primer and Lbb1 (see above). The location of the T-DNA insertion in both lines was determined by sequencing of the PCR product.

Yeast cells were transformed by the LiAc method (18) and transformants were selected on uracil-deficient medium containing 1% arginine as nitrogen source and the appropriate supplements. To support growth of the fet3fett mutant, solid YNB medium contained additionally 30 μM FeCl₃, while the liquid YPD medium was acidified to pH 5.0 with HCl. For growth tests, saturated cultures of yeast transformants were spotted in YPD medium was acidified to pH 5.0 with HCl. For growth tests, saturated cultures of yeast transformants were spotted in 1 spectinomycin. Agrobacterium cells were pretransformed with pSoup to allow replication of the pGreen construct. A. thaliana Col-0 plants or tir2g-2 T-DNA insertion lines were then transformed via agrobacteria using the floral-dip method (16) and transformants were selected on BASTA (pGreen) or 75 μg ml⁻¹ kanamycin (pTkan+-AtIREG2 promoter-AtIREG2-GFP). The pTkan+-AtIREG2 promoter-AtIREG2-GFP construct was used for complementation of the ireg2-1 T-DNA insertion line (see below). Homozygous plants were identified in the T2 generation based on segregation analysis on 50 μg ml⁻¹ kanamycin. Arabidopsis T-DNA insertion lines SALK_074442 (ireg2-1) and SALK_127071 (ireg2-2) were obtained from the stock of T-DNA insertion lines provided by SALK (17). The lines were screened by PCR using AtIREG2 specific and left border T-DNA primers. To verify homozygosity in line ireg2-1, AtIREG2 forward primer 5'-TTTCTCTGACCTCTGATTGTG-3' and AtIREG2 reverse primer 5'-CCATCGAGCAAGA-AAATAGCC-3' were used to amplify the wild-type AtIREG2 gene. To screen for the T-DNA insertion, PCR was performed with the reverse primer (see above) and the T-DNA left border primer Lbb1 (5'-CGCTGTGAGCTTGTGACTCACT-3'). For line ireg2-2 the forward primer 5'-CGAAAAATTTAGAATC-GAATCTAAA-3' and the reverse primer 5'-TGATCGACCTTGTGACCCCAT-3' were used as gene-specific primers. The presence of the T-DNA insertion in ireg2-1 was verified by PCR with the reverse primer and Lbb1 (see above). The location of the T-DNA insertion in both lines was determined by sequencing of the PCR product.

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Plant Cultivation, Growth Conditions, and Gene Expression Analysis—A. thaliana Col-0 was grown in hydroponic culture under short day conditions as described in Schaaf et al. (19) for the iron deficiency experiment. Germination, preculture, growth conditions for the transition metal accumulation assay and Northern blot analysis of wild-type plants under heavy metal stress were conducted according to Loqué et al. (20) using NH₄NO₃ as a nitrogen source in the nutrient solution. For Northern analysis, 20 μg of total root or shoot RNA were separated by gel electrophoresis and transferred to a Hybond-N membrane (Amersham Biosciences). cDNA frag-

4 The abbreviations used are: GFP, green fluorescent protein; ORF, open reading frame; MES, 4-morpholineethanesulfonic acid.
**RESULTS**

**AtIREG2 Encodes an Iron-regulated Membrane Protein Related to Vertebrate Fe^{2+} Exporters**—A phylogenetic analysis of eukaryote and prokaryote sequences with the highest similarity to *Arabidopsis* IREG2 clearly pointed to a separation of a plant cluster from an animal cluster, with particular high sequence conservation among mammalian sequences. AtIREG1 (At2g38460) and AtIREG2, which share 84.6% sequence similarity, fell into a first clade, which share 84.6% sequence similarity, fell into a first clade, both of these AtIREG2 and AtIREG3 (At5g26820) into a second clade, both of these clades harboring an additional sequence from rice (Fig. 1A).

To verify an iron-dependent regulation of *AtIREG2*, a Northern analysis was conducted with hydroponically grown *Arabidopsis* plants cultured under different iron regimes. Transcript levels of *AtIREG2* in roots were up-regulated under iron deficiency and down-regulated after resupply of iron (Fig. 1B), similar to the Fe^{2+} transporter gene *AtIRT1* (22).

**AtIREG2 Increases Nickel Tolerance in Yeast**—Because of its homology with vertebrate iron exporters, we assumed a iron export function and tested in heterologous systems whether *AtIREG2* might alleviate iron toxicity in iron-sensitive yeast strains. Neither the AFT1up strain, which exhibits a constitutive expression of genes involved in iron acquisition (23), nor *ccc1*, a mutant suffering from a lower capacity to transport iron ments obtained by NotI restriction digests from pDR195-AtIREG2 and pFL61-AtIRT1 were used as probes.

In plate growth tests on different micronutrient or heavy metal concentrations, seeds of wild-type and transgenic plants were germinated in long day conditions in a growth chamber on 0.5× Murashige and Skoog (MS) medium (Duchefa, Haarlem, NL), 1% sucrose, solidified with 0.7% Difco agar (Becton Dickinson) and supplemented with metals at indicated concentrations. Plants were continued to grow on vertical plates for determination of root length. A^{63}Ni accumulation assay was conducted with 6-week-old wild-type plants, precultured under iron deficiency for 2–5 days. Plants were transferred to 20 μM ^{63}NiSO_{4} for 30 min, and then roots were washed for 10 min with EDTA, pH 5.7. For ^{63}Ni analysis plants were washed twice at 500 °C before and after wet digestion with 65% HNO_{3}, resuspended in 2 ml of 1% HCl and analyzed by liquid scintillation counting.

For subcellular localization, *Arabidopsis* protoplasts were transformed as described previously (21). Transformed protoplasts were analyzed by confocal laser scanning microscopy (TCP-SP Leica, Bensheim, Germany). Localization experiments with ieg2-1 recomplemented lines (see above) were conducted with homozygous T2 plants using an inverted fluorescence microscope equipped with an ApoTome (Zeiss Axiovert 200 M, Jena, Germany). Plant roots were stained with 25 μM FM4-64 (Molecular Probes) for 5 min and shortly rinsed in ultra pure water before observation under the microscope.

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![AtIREG2 is related to iron exporters from vertebrates and up-regulated under iron deficiency.](image)

**FIGURE 1.** AtIREG2 is related to iron exporters from vertebrates and up-regulated under iron deficiency. A, phylogenetic tree of AtIREG1, -2, and -3 from *A. thaliana* and related sequences from rice, fungi, bacteria, and animals. Maximum parsimony analyses were performed using PAUP 4.0b10 (43), with all DNA characters unweighted and gaps scored as missing characters. Heuristic tree searches were executed using 1000 random sequence additions and the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. The complete alignment was based on 798 sites; 600 were phylogenetically informative. Bootstrap values (%) are indicated at branch nodes. An, *Aspergillus nidulans*; At, *A. thaliana*; Bb, *Bdellovibrio bacteriovorus*; Ce, *Caenorhabditis elegans*; Cb, *Caenorhabditis briggsae*; Cf, *Canis familiaris*; Cn, *Cryptococcus neoformans*; Dd, *Dictyostelium discoideum*; Dr, *Danio rerio*; Gg, *Gallus gallus*; Gz, *Gibberella zeae*; Hs, *Homo sapiens*; Mg, *Magnaporthe grisea*; Mm, *Mus musculus*; Nc, *Neurospora crassa*; Os, *Oryza sativa*; Pt, *Pan troglodytes*; Rn, *Rattus norvegicus*; Tn, *Tetraodon nigroviridis*; Um, *Ustilago maydis*; Xl, *Xenopus laevis*. B, RNA gel blot analysis was performed to determine AtIREG2 and AtIRT1 expression in roots from hydroponically grown plants that were precultured for 5 weeks in presence of 50 μM Fe(III)-EDTA and starved for 10 days for iron, before resupply (RS) with 50 μM Fe(III)-EDTA for 24 h. Total RNA from roots (left) or shoots (right) were used for hybridization to the complete ORF of AtIREG2 or AtIRT1. EtBr-stained gel blots are shown as loading control.

Rattus norvegicus; Tn, Tetraodon nigroviridis; Um, Ustilago maydis; Xl, Xenopus laevis. 8, RNA gel blot analysis was performed to determine AtIREG2 and AtIRT1 expression in roots from hydroponically grown plants that were precultured for 5 weeks in presence of 50 μM Fe(III)-EDTA and starved for 10 days for iron, before resupply (RS) with 50 μM Fe(III)-EDTA for 24 h. Total RNA from roots (left) or shoots (right) were used for hybridization to the complete ORF of AtIREG2 or AtIRT1. EtBr-stained gel blots are shown as loading control.
into the vacuole, showed different growth on medium supplemented with increasing concentrations of iron when expressing AtIREG2 (data not shown). To test an iron import function

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AtIREG2 was expressed in the iron uptake-defective yeast mutant fet3fet4 (24) for a growth complementation test on 4–10 μM Fe(III). Growth of AtIREG2 transformants, however, did not differ significantly from that of control transformants (data not shown).

In general, plant iron transporters such as AtIRT1 or AtNRAMPs discriminate poorly between iron, manganese, zinc, cobalt, and cadmium (10, 25). We therefore decided to screen for a heavy metal transport function of AtIREG2 employing yeast mutants with increased heavy metal sensitivities, like sfn1, vma1, and cot1 at increasing concentrations of different transition metals (26–28). Expression of AtIREG2 in a cot1 deletion strain reversed its nickel sensitivity (Fig. 2). Remarkably, AtIREG2-mediated tolerance to nickel was only observed at pH 5, but could not be observed at pH 6 or higher. This pH-dependent contribution of AtIREG2 to nickel tolerance pointed to a putative function of AtIREG2 in proton-dependent heavy metal export from the cytoplasm into the vacuole.

**Intracellular Localization of AtIREG2 in Planta**—To examine the intracellular localization of AtIREG2 in planta, a GFP cDNA was fused to the 3'-end of AtIREG2 and the fusion construct was placed under control of a 35S promoter for transient expression in Arabidopsis protoplasts derived from a suspension cell culture. As observed by confocal laser scanning microscopy, AtIREG2-dependent green fluorescence appeared as ring-shaped structures (Fig. 3A). A comparison to the transmission view and merging both images allowed identification of these globular compartments as vacuoles. Even in fully differentiated cells with large vacuoles, a small cytoplasmic region including organellar structures separated the AtIREG2-dependent fluorescence from the plasma membrane, clearly indicating tonoplast localization (Fig. 3A). In contrast, fluorescence derived from GFP alone localized to the cytoplasm and to the nucleus. In an independent approach, transgenic Arabidopsis lines expressing an AtIREG2-GFP construct under control of a 1.8-kb fragment of the endogenous AtIREG2 promoter were analyzed (Fig. 3B). In root cortex cells, green fluorescence derived from AtIREG2-GFP was localized internal of red fluorescence derived from the lipophilic dye FM4-64, which labels the plasma membrane after short term incubation (29). These observations

**FIGURE 2. AtIREG2 mediates elevated tolerance to nickel in yeast.** Yeast cot1 cells were transformed with the empty vector pDR195 or with pDR195-AtIREG2. Single colonies were cultured in selective media for 48 h and adjusted to O.D500 = 1.0 before spotting 5-fold dilutions on uracil-free YNB medium or medium supplemented with NiCl₂. The pH was adjusted to pH 5 or 6 by 50 mM MES/TRIS.

**FIGURE 3. Tonoplast localization of GFP-fused AtIREG2.** A, upper panel, GFP-derived fluorescence from protoplasts transformed with pCF203-GFP alone (left) or pCF203-AtIREG2-GFP (panels a–c). Middle panel, phase contrast views. Lower panel, overlay of GFP-derived fluorescence and phase contrast; panel d, magnified insert from panel c. Protoplasts derived from a dark-adapted Arabidopsis cell suspension culture and were assayed by confocal laser scanning microscopy. B, root cell of a atireg2-1 plant retransformed with an AtIREG2-promoter-AtIREG2-GFP fusion construct (line 12) grown on half-strength Murashige and Skoog medium supplemented with 1% Suc for 2 weeks before image analysis using an ApoTome imaging system in an inverted fluorescence microscope. Bar, 10 μm.
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A

\textbf{CaMV 35S:AtIREG2}

\begin{tabular}{c|c|c|c|c|c}
 & \textbf{wt} & \textbf{1} & \textbf{4} & \textbf{7} & \textbf{10} \\
\hline
\textbf{AtIREG2} & & & & & \\
EtBr & & & & & \\
\end{tabular}

B

\textbf{FIGURE 4. Overexpression of AtIREG2 increases nickel tolerance in Arabidopsis.} A, RNA gel blot analysis of AtIREG2 expression in roots of wild type and 35S-AtIREG2 plants (lines 1, 4, 7, 10), which were cultured for 45 days under iron-sufficient conditions. The corresponding EtBr-stained RNA is shown as a loading control. B, fresh weight of 12-day-old wild type and 35S-AtIREG2 plants grown under elevated supply of nickel. Significant differences at \( p < 0.01 \) are indicated by different letters, \( n = 5 \). C, accumulation of nickel in roots of wild type and 35S-AtIREG2 plants, which were cultured for 5 days on nutrient solution supplied with 30 \( \mu \text{M} \) nickel. Significant differences at \( p < 0.05 \) are indicated by different letters, \( n = 6 \) (3 plants per replica).

![Graphs](image-url)

indicated that AtIREG2 is targeted to vacuolar membranes and suggested a role of AtIREG2 in substrate transport across the tonoplast.

Overexpression of AtIREG2 Results in Enhanced Nickel Tolerance—Based on the enhanced tolerance to nickel of yeast cells transformed with AtIREG2, we investigated whether overexpression of AtIREG2 also increases tolerance to elevated concentrations of this metal \textit{in planta}. Transgenic plants expressing AtIREG2 under control of the constitutive 35S promoter were generated and four independent homozygous lines were examined in the T2 generation. RNA gel blot analysis using plants grown under iron-sufficient conditions showed that AtIREG2 was highly expressed in roots of the lines 1, 4, and 10, while mRNA levels in line 7 were only slightly increased relative to the wild-type expression level (Fig. 4A). When seeds were germinated and grown for 12 days on agar plates supplemented with 100 or 200 \( \mu \text{M} \) nickel, wild-type seedlings experienced a severe loss of biomass (Fig. 4B). In contrast, the gain of fresh weight by the AtIREG2 overexpressor lines 1, 4, and 10 was unaffected even at 200 \( \mu \text{M} \) NiCl\(_2\). In agreement with its lower expression level of AtIREG2, line 7 showed an intermediate response with higher fresh weight than the wild type at 100 \( \mu \text{M} \) nickel but a similar reduction in fresh weight as wild-type plants at 200 \( \mu \text{M} \) nickel. This differential growth response was also reflected by visible signs of plant health. Wild-type plants developed chlorosis at 50 \( \mu \text{M} \) nickel and germinated poorly above 100 \( \mu \text{M} \) nickel, whereas lines 1, 4, and 10 developed cotyledons even up to 500 \( \mu \text{M} \) NiCl\(_2\) (supplemental Fig. S1A). To assess whether increased nickel tolerance was restricted to germination, non-stressed seedlings were subjected to different concentrations of nickel for 7 days. At 200 \( \mu \text{M} \) NiCl\(_2\), root growth was completely arrested in wild-type plants and in line 7, whereas root growth of the overexpressor lines 1, 4, and 10 was not impaired (supplemental Fig. S1B). A phenotypic analysis of the same lines on agar with elevated concentrations of cadmium, cobalt, manganese, zinc, or iron did not yield any significant differences related to the level of AtIREG2 gene expression (data not shown). Moreover, AtIREG2 gene expression levels in roots of wild-type plants were not increased after supply of 20 \( \mu \text{M} \) cobalt, cadmium, or nickel (supplemental Fig. S2).

To assess the nickel accumulation, \textit{Arabidopsis} lines were precultured on nutrient solution for 5.5 weeks and then continued to grow on nutrient solution supplemented with 30 \( \mu \text{M} \) nickel. After 5 days of nickel treatment, line 7 and wild-type plants started to show a slight growth depression, but nickel accumulation in roots of lines 1, 4, and 10 were approx. 40% higher (Fig. 4C), indicating that a higher nickel tolerance in transgenic lines was related to an enhanced capacity for nickel accumulation in roots.

AtIREG2 Plants Accumulate Less Nickel in Roots and Show an Increased Nickel Sensitivity under Iron Deficiency—Two lines were obtained from the SALK collection (SALK_074442 and SALK_127071) (17) that carry T-DNA insertions in the AtIREG2 gene (Fig. 5A). In homozygous progeny from the T3 generation, verified by segregation and PCR analyses, iron deficiency induced expression of AtIREG2 was not detected (Fig. 5B), but plants did not exhibit any visible phenotypes in soil culture. We then grew plants hydroponically for 6 weeks before supplementing the nutrient solution with 10 \( \mu \text{M} \) nickel for 10 days. A subsequent analysis of transition metals in shoots yielded no significant differences in accumulation of any measured metal (data not shown), but in roots of both \textit{ireg2} insertion lines, nickel concentrations were only half of those of wild-type plants (Fig. 5C). All other metal concentrations in \textit{ireg2} roots were similar to wild-type plants. In contrast, overexpression of AtIREG2 led to a significantly higher accumulation in roots of nickel but also of manganese, copper, and zinc (line 10 in Fig. 5C).

To confirm that the observed phenotype is indeed caused by loss-of-function of AtIREG2, we retransformed \textit{ireg2}-1 plants with an AtIREG2-promoter-AtIREG2-GFP construct resulting in high levels of AtIREG2-GFP mRNA (Fig. 6A) and grew homozygous plants from the T2 generation on nickel-supplied agar under different iron regimes. The \textit{ireg2}-1 insertion lines
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A \( \text{ireg2-1} \)

\[ \text{T-DNA} \quad \text{ATG} \quad \text{AtIREG2} \quad \text{TGA} \]

B \( \text{Col-0 ireg2-1 ireg2-2} \)

EtBr

C

| Metal conc. [ug g\(^{-1}\) DW] | Mn | Ni | Cu | Zn |
|----------------|----------------|----------------|----------------|----------------|
| a | b | c | d |

FIGURE 5. Loss of AtIREG2 leads to reduced nickel accumulation in roots. A, scheme of the T-DNA integration sites in \( \text{ireg2-1} \) (SALK_074442) and \( \text{ireg2-2} \) (SALK_127071). The location of the T-DNA insertions is indicated by triangles. Both insertions are located 200-bp upstream of the transcription start. B, RNA gel blot analysis of AtIREG2 expression in roots of iron-deficient wild type, \( \text{ireg2-1} \) and \( \text{ireg2-2} \). The corresponding EtBr-stained rRNA is shown as a loading control. C, accumulation of nickel, copper, zinc, and manganese in roots of wild type, \( \text{ireg2-1} \) and \( \text{ireg2-2} \) plants and the AtIREG2-overexpressing line 10, which were cultured for 10 days on nutrient solution supplied with 10 \( \mu M \) nickel. Significant differences at \( p < 0.05 \) are indicated by different letters, \( n = 4 \) (3 plants per replica).

Three independent approaches demonstrated that AtIREG2 acts as a transporter for nickel. First, transformation of wild-type or a nickel-sensitive yeast strain (cot1) with AtIREG2-conferred yeast growth on elevated nickel concentrations (Fig. 2 and supplemental Fig. S3B); second, overexpression of AtIREG2 in Arabidopsis increased nickel tolerance and nickel accumulation in roots (Fig. 4); and third, \( \text{iireg2} \) insertion lines were more sensitive to external nickel and accumulated less nickel (Fig. 5). The identification of nickel as a substrate for AtIREG2-mediated transport was surprising in view of the transport studies performed with vertebrate IREG homologs, which so far describe iron as the exclusive transport substrate (32). In fact, we cannot exclude that iron is transported by AtIREG2. However, our observations do not support such a function, because expression of AtIREG2 in yeast could neither alter iron sensitivity nor influence iron accumulation in yeast cells. Moreover, iron export rates from iron-preloaded oocytes were increased upon expression of mouse IREG1 but not of AtIREG2. Despite a substantial variation of the assay conditions, AtIREG2 never showed a significant effect on iron export or import in oocytes. Third, overexpression or deletion of AtIREG2 in Arabidopsis did not alter sensitivity to toxic iron concentrations in planta (data not shown). From these results we conclude that either the proof of AtIREG2 as an Fe(II) transporter in vivo is hampered by the low availability of free Fe\(^{2+} \) within living cells, or Fe\(^{2+} \) is not a suitable substrate for AtIREG2. In view of these observations and its membrane localization, we hypothesize that AtIREG2 encodes a nickel transporter.

DISCUSSION

\( \text{AtIREG2 Mediates V} \)

Iron acquisition by non-graminaceous plant roots, as in yeast cells and the vertebrate intestine, is based on the reduction of ferric iron via a membrane-bound reductase and on the subsequent uptake of ferrous iron across the plasma membrane via a transporter for Fe\(^{2+} \). In most organisms analyzed so far the relevant iron transporters discriminate poorly among transition metals (8, 10, 30) with the exception of yeast FTR1, which appears to be more specific because of the coupling with an iron oxidase (31). Analysis of the substrate selectivity of mutated plant IRT1 proteins supports the view that it might be difficult to evolve highly specific Fe\(^{3+} \) transporter proteins (9). An excess accumulation of other transition metals, however, provokes a secondary stress that increases with the extent of iron deficiency (6, 8).

The present study describes a novel process that operates in plant roots downstream of cellular iron acquisition and mediates detoxification of nickel, which is one of the potentially hazardous transition metals that accumulate under iron deficiency (6, 7).

Three independent approaches demonstrated that AtIREG2 acts as a transporter for nickel. First, transformation of wild-type or a nickel-sensitive yeast strain (cot1) with AtIREG2-conferred yeast growth on elevated nickel concentrations (Fig. 2 and supplemental Fig. S3B); second, overexpression of AtIREG2 in Arabidopsis increased nickel tolerance and nickel accumulation in roots (Fig. 4); and third, \( \text{iireg2} \) insertion lines were more sensitive to external nickel and accumulated less nickel (Fig. 5). The identification of nickel as a substrate for AtIREG2-mediated transport was surprising in view of the transport studies performed with vertebrate IREG homologs, which so far describe iron as the exclusive transport substrate (32). In fact, we cannot exclude that iron is transported by AtIREG2. However, our observations do not support such a function, because expression of AtIREG2 in yeast could neither alter iron sensitivity nor influence iron accumulation in yeast cells. Moreover, iron export rates from iron-preloaded oocytes were increased upon expression of mouse IREG1 but not of AtIREG2. Despite a substantial variation of the assay conditions, AtIREG2 never showed a significant effect on iron export or import in oocytes. Third, overexpression or deletion of AtIREG2 in Arabidopsis did not alter sensitivity to toxic iron concentrations in planta (data not shown). From these results we conclude that either the proof of AtIREG2 as an Fe(II) transporter in vivo is hampered by the low availability of free Fe\(^{2+} \) within living cells, or Fe\(^{2+} \) is not a suitable substrate for AtIREG2. In view of these observations and its membrane localization, we hypothesize...
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A

AtIREG2

Col-0 ireg2-1 12 19

AtIREG2-GFP

wild type ireg2-1 12 19

wild type ireg2-1 12 19

B

+Fe + 30 μM Ni

-Fe + 30 μM Ni

C

n.s.

12

-Fe

0 2 4 6 8 10 12

Root length [cm plant⁻¹]

control

20 μM Ni

30 μM Ni

50 μM Ni

70 μM Ni

0 2 4 6 8 10

Root length [cm plant⁻¹]

FIGURE 6. AtIREG2 mediates nickel tolerance in an iron-dependent manner. A, RNA gel blot analysis of AtIREG2 expression in roots of wild type, ireg2-1, and ireg2-1 plants retransformed with a AtIREG2-promoter-AtIREG2-GFP fusion construct (lines 12 and 19). The corresponding EtBr-stained rRNA is shown as a loading control. B, iron-dependent phenotype of the same lines precultured for 8 days on iron adequate (+ 75 μM Fe-EDTA) half-strength MS agar plates and then for 2 weeks on iron-deficient (+ 75 μM Fe-EDTA) or iron-deficient half-strength MS agar supplemented with 30 μM nickel. Bar, 1 cm. C, quantitative analysis of primary root length after plant cultivation like that described in B with increasing supply of nickel. Significant differences at p < 0.05 are indicated by different letters, n = 4 (3 plants per replica).

that iron transport is not a primary function of AtIREG2. To specify the range of metals being transported by AtIREG2, we determined the accumulation of radiolabeled ¹⁰⁹Cd, ⁵⁴Mn, ⁶⁵Zn, ⁶₃Ni, or ⁵⁹Fe supplied at different concentrations to AtIREG2-expressing yeast cells and found that only the accumulation of ⁶₃Ni was drastically altered (data not shown). Moreover, we also screened AtIREG2-expressing yeast cells on toxic concentrations of manganese, cadmium, and zinc, but no obvious growth differences relative to transformants expressing the empty vector were observed (data not shown). Although overexpression of AtIREG2 in Arabidopsis significantly increased manganese, copper, and zinc accumulation in roots (Fig. 5C), growth tests of AtIREG2-overexpressing Arabidopsis lines and wild-type plants on agar medium supplied with elevated concentrations of manganese, cadmium, iron, and zinc revealed no growth differences (data not shown). Taken together, these observations indicate that nickel is a preferential substrate of AtIREG2, even though other transition metals might be transported, in particular when AtIREG2 is overexpressed.

AtIREG2 as a pH-dependent Tonoplast Transporter—Transient expression of an AtIREG2-GFP fusion construct in Arabidopsis protoplasts and transgenic plants expressing an AtIREG2-promoter-AtIREG2-GFP construct clearly showed AtIREG2 protein localization at the tonoplast (Fig. 3). AtIREG2 is preferentially expressed in roots where cellular iron acquisition mechanisms are expressed at the highest levels, unlike transition metal transporters of the CDF family, which mediate vacuolar loading of metals such as zinc, cobalt, cadmium, and manganese, and are mainly expressed in shoots. The results presented in this study suggest that AtIREG2 mediates nickel transport out of the cytoplasm and into the vacuole, a less metal-sensitive compartment. This is supported by the tonoplast localization of AtIREG2 and the enhanced nickel tolerance and nickel accumulation in roots of iron-deficient wild-type plants relative to the insertion lines. In general, the dependence of metal tolerance in yeast on acidic pH is most likely reflected by pH-dependent metal detoxification mechanisms as represented by the CDF family members COT1 and ZRC1 (28, 33). Likewise, AtIREG2-mediated nickel tolerance was strongly pH-dependent and increased with decreasing pH. Studies on the V-ATPase-defective yeast mutant vma have shown that heavy metal transport into the vacuole is severely affected at increasing pH as a consequence of a smaller electrochemical gradient across the vacuolar membrane (27). This pH dependence most likely indicates that AtIREG2 in planta depends directly on the electrochemical gradient across the tonoplast and might function as a metal proton antiporter as is the case for substrate/proton antiporters of the CAX family (34).

A Role of AtIREG2 in Iron Deficiency-induced Metal Detoxification—Considering its function in nickel detoxification, AtIREG2 showed an unexpected transcriptional regulation. An increasing supply of nickel did not induce gene expression in roots (supplemental Fig. S2), whereas iron deficiency did (Fig. 1B). Most interestingly, iron deficiency-induced up-regulation of AtIREG2 was controlled by the transcription factor FRU/FIT1. Recent studies identified FRU/FIT1 as a major regulator coordinating the expression of genes involved in iron deficiency-induced iron acquisition in Arabidopsis, such as AtIRT1, AtFRO2, and others (35, 36). A comparative transcriptome analysis in Arabidopsis wild type and atfit1 T-DNA insertion lines identified AtIREG2 as another downstream target gene of FRU/FIT1 (35). In yeast, the nickel/cobalt transporter gene COT1 was also co-regulated with other iron deficiency-induced genes and appeared to be under control of the iron-regulated transcription factor AFT1 (37, 38). Thus, induction of gene expression by cobalt appeared as a major
difference in the regulation between COT1 in yeast and AtIREG2 in roots (supplemental Fig. S2). These observations indicated that the function of AtIREG2 is related to the iron deficiency stress response rather than to substrate-induced metal transport. A physiological requirement for metal detoxification under iron deficiency is further indicated by up-regulation of nicotianamine synthase 1 (NAS1), the metal-transporting ATPase HMA3 and a putative phytochelatine synthase (At1g07970) in atf1t mutant lines (35). In particular, a function for NAS1 in nickel detoxification has been suggested in which translocation of nicotianamine-chelated nickel to the shoots is enhanced (39–41). In contrast, AtIREG2-mediated nickel compartmentalization into the vacuole, a transport process that appears to be confined to the roots (Fig. 1B) where it strongly determines nickel accumulation (Fig. 5), represents an alternative pathway of nickel detoxification that prevents a further heavy metal load to the shoots.

We finally addressed the physiological requirement for a iron deficiency-induced vacuolar iron loader by investigating whether nickel accumulates in roots under iron-deficient growth conditions. Indeed, even short term 63Ni uptake rates in roots of wild-type plants increased with iron starvation (supplemental Fig. S3A), demonstrating that nickel is similar to other transition metals that become enriched in iron-deficient plants (2, 6, 7, 10). In strong agreement with the broad substrate specificity of AtIRT1 (9, 42), AtIRT1 was identified as a major pathway for the excess uptake of zinc, cobalt, and manganese under iron-deficient growth conditions (10). An enhanced nickel sensitivity in AtIRT1-expressing yeast cells supports the notion that induction of AtIRT1 under iron deficiency increases iron acquisition but is also coupled with the drawback of an enhanced accumulation of the undesired transition metal nickel (supplemental Fig. S3B). Because both AtIREG2 and AtIRT1 expression is enhanced in root cells, our results indicate that AtIREG2 can prevent toxicity of these transition metals in the same tissue that also expresses AtIRT1. We therefore conclude that the physiological function of AtIREG2 is the deposition of excess nickel into the vacuole to counterbalance the low substrate specificity of AtIRT1 and other iron transport systems (Fig. 7). Hence, AtIREG2 can be regarded as a so far unrecognized component in the iron deficiency stress response of plants.

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FIGURE 7. Model for the cellular function of AtIREG2 in nickel homeosta-
sis in roots. Under iron deficiency, the iron-regulated transcription factor FRU/FIT induces transcription of AtFRO2 and AtIRT1 in hizodermis and cortex cells (35) resulting in an enhanced Fe(II) uptake capacity (10). Alternatively, FRU/FIT may also regulate AtIRT1 at the post-transcriptional level (Ref. 35, not depicted). Because of the low specificity of AtIRT1 (9), the uptake of other transition metals is also increased. In same cells or neighboring cells, iron deficiency also up-regulates AtIREG2 via FRU/FIT (35). AtIREG2 then allows transport of excess nickel into the vacuole thereby increasing cellular toler-
ance to nickel under iron deficiency. N, nucleus.
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