Manganese Efficiency in Barley: Identification and Characterization of the Metal Ion Transporter HvIRT1\(^1\)\^[OA]\^[1]


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Manganese (Mn) deficiency is an important plant nutritional disorder in many parts of the world. Barley (Hordeum vulgare) genotypes differ considerably in their ability to grow in soils with low Mn\(^{2+}\) availability. Differential genotypic Mn efficiency can be attributed to differences in Mn\(^{2+}\) uptake kinetics in the low nanomolar concentration range. However, the molecular basis for these differences has not yet been clarified. We present here the identification and characterization of the first barley gene encoding a plasma membrane-localized metal transport protein able to transport Mn\(^{2+}\). The gene is designated HvIRT1 (for IRON-REGULATED TRANSPORTER1) because it belongs to the ZIP gene family and has a high similarity to rice (Oryza sativa) OsIRT1. A novel yeast uptake assay based on inductively coupled plasma-mass spectrometry analysis of 31 different metal and metalloid ions showed that the HvIRT1 protein, in addition to Mn\(^{2+}\), also transported Fe\(^{2+}/Fe^{3+}\), Zn\(^{2+}\), and Cd\(^{2+}\).

Both Mn and iron deficiency induced an up-regulation of HvIRT1 (for sativa roots and contributes to genotypic differences in Mn\(^{2+}\) uptake. We conclude that HvIRT1 is an important component controlling Mn\(^{2+}\) uptake in barley roots and contributes to genotypic differences in Mn\(^{2+}\) uptake kinetics.

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The first identified plant member of the ZIP family, AtIRT1 (for IRON-REGULATED TRANSPORTER1) from Arabidopsis (Arabidopsis thaliana), transports a wide range of trace elements, including Fe\(^{2+}/Fe^{3+}\), Cd\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\) (Korshunova et al., 1999). Using a knockout mutant of AtIRT1, it was shown that this transporter is a key component in regulating iron (Fe) acquisition at low soil Fe availability (Vert et al., 2002). The chlorotic phenotype of the Atirt1 mutants could not be rescued by applying exogenous Mn\(^{2+}\), but the mutants showed a significant reduction in root Mn\(^{2+}\) concentrations relative to the wild type when grown under Fe limitation. This indicates that AtIRT1 functions as a Mn\(^{2+}\) transporter in planta and that it is the primary pathway for Mn\(^{2+}\) transport during Fe deficiency. Several members of the ZIP transporter family from other plant species than Arabidopsis also appear to have the ability to transport Mn\(^{2+}\), including MtZIP4 and MtZIP7 from Medicago truncatula (López-Millán et al., 2004), LeIIRT1 and LeIIRT2 from tomato (Solanum lycopersicum; Eckhardt et al., 2001), and PsRIT1 from pea (Pisum sativum; Cohen et al., 2004). Some of these transporters are most likely localized in the plasma membrane, but their metal ion specificity remains to be clarified. Several members of the ZIP transporter family from other plant species than Arabidopsis also appear to have the ability to transport Mn\(^{2+}\), including MtZIP4 and MtZIP7 from Medicago truncatula (López-Millán et al., 2004), LeIIRT1 and LeIIRT2 from tomato (Solanum lycopersicum; Eckhardt et al., 2001), and PsRIT1 from pea (Pisum sativum; Cohen et al., 2004). Some of these transporters are most likely localized in the plasma membrane, but their metal ion specificity remains to be clarified. Several members of the ZIP transporter family from other plant species than Arabidopsis also appear to have the ability to transport Mn\(^{2+}\), including MtZIP4 and MtZIP7 from Medicago truncatula (López-Millán et al., 2004), LeIIRT1 and LeIIRT2 from tomato (Solanum lycopersicum; Eckhardt et al., 2001), and PsRIT1 from pea (Pisum sativum; Cohen et al., 2004).

In this study, yeast was used as a tool to isolate and characterize the first Mn\(^{2+}\) transporter from barley. The gene had a high similarity with OsIRT1 and was accordingly named HvIIRT1. Heterologous expression indicated that HvIIRT1 also had specificity for Fe\(^{2+}/Fe^{3+}\), Co\(^{2+}\), and Cd\(^{2+}\). Transient expression localized HvIIRT1 to the plasma membrane, suggesting a role in metal ion uptake from the soil solution. Expression patterns of HvIIRT1 and corresponding Mn\(^{2+}\) uptake rates in intact barley roots were positively correlated. Furthermore, HvIIRT1 expression levels in all cases were considerably higher (up to 40%) in the Mn-efficient genotype Vanessa compared with the Mn-inefficient genotype Antonia. This suggests an important role of HvIIRT1 in Mn\(^{2+}\) acquisition and in controlling differential Mn efficiency among barley genotypes.

RESULTS

Isolation and Sequence Analysis of a Putative Root Mn\(^{2+}\) Transporter

To identify transport proteins with specificity for Mn\(^{2+}\), a yeast screen using the Δsmf1 mutant was employed, containing a null mutation in the gene for high-affinity Mn\(^{2+}\) uptake (Supek et al., 1996). We produced a cDNA library from roots of the barley genotypes Vanessa and Antonia grown at insufficient or ample Mn\(^{2+}\) supply. The library was used for transformation of the Δsmf1 mutant, and primary transformants were plated on medium containing Mn\(^{2+}\), resulting in more than 10\(^7\) colonies. Approximately 5% of the primary transformants were restreaked on Mn\(^{2+}\)-limited medium (controlled by 10 mM EGTA). On the order of 500 colonies were able to complement the Δsmf1 mutant. Among these positive transformants, about 200 were analyzed, and 40% of the clones were found to carry the same barley cDNA clone, whereas the remaining 60% were false positives identified by the absence of growth on Mn\(^{2+}\)-limited medium after retransformation, giving a fairly safe screen. The barley cDNA clone had high similarity to the rice IRT1 gene and was consequently named HvIIRT1, in accordance with the founding member of the IRT family isolated from Arabidopsis (Eide et al., 1996). After identification from the yeast screening, the HvIIRT1 gene was cloned from both the Mn-efficient genotype Vanessa and the Mn-inefficient genotype Antonia, and no differences in the coding gene sequences were seen. Thus, the differential Mn\(^{2+}\) uptake kinetics for the two genotypes observed previously (Pedas et al., 2005) were not caused by different isoforms. The open reading frame of the HvIIRT1 gene was 1,113 bp in length, corresponding to a polypeptide of 371 amino acids. The predicted HvIIRT1 protein exhibited 69% and 55% amino acid sequence identity to OsIRT1 and AtIRT1, respectively. In agreement with the known structure of other IRT1 family members, the HvIIRT1 protein was predicted to contain eight transmembrane domains, a very short C-terminal tail, and a hydrophilic region between transmembrane domains III and IV directed toward the cytosolic side of the membrane (Fig. 1). This region contains the conserved His residues that are anticipated to be involved in the formation of a cytoplasmic metal ion-binding site (Guerinot, 2000). However, the proposed ZIP signature sequence located in transmembrane domain IV (Eng et al., 1998) does not fully match with the three IRT1 protein sequences reported for rice, pea, and barley. Therefore, an extended version of the ZIP signature sequence is presented here and the additions are indicated in boldface: [LIVFAM] [GAS] [LIVMD] [LIVSGC] [LIVFAS] [H] [SAN] [LIVFA] [LIVFMAT] [LIVDE] [G] [LIVF] [SANG] [LIFVF]M [GS], where [] refer to one of the amino acids between brackets being possible (Fig. 1). Thus, the sequence analysis of the barley HvIIRT1 gene suggests that it encodes a metal ion transport protein belonging to the ZIP family. A phylogenetic tree obtained after comparing HvIIRT1 with the sequences of eight other known plant IRT1 members (Fig. 2) revealed that HvIIRT1 is most closely related to OsIIRT1. Furthermore, the two IRT1 proteins from rice and barley fall into a subgroup separated from the dicotyledonous IRT1 proteins, indicating their evolutionary divergence.

Yeast Functional Complementation

AtIRT1 has been shown previously to be involved in the transport of Fe\(^{2+}/Fe^{3+}\), Zn\(^{2+}\), Mn\(^{2+}\), and Cd\(^{2+}\) (Korshunova et al., 1999), why AtIRT1 was used as a positive control in this study. Transformation of the
yeast Δsmf1 mutant with HvIRT1 or AtIRT1 showed that both genes had the ability to restore growth on Mn\(^{2+}\) -limited medium controlled by the chelator EGTA (Fig. 3A). Over the range of EGTA concentrations tested, the transformants containing the empty vector grew poorly in medium containing 1 to 2 mM EGTA and did not grow at all in medium containing 5 mM and higher. The transformants containing HvIRT1 and AtIRT1 grew almost as well as wild-type yeast at all EGTA concentrations tested. Since AtIRT1 is known to transport Zn\(^{2+}\) and Fe\(^{2+}/Fe^{3+}\), we also examined HvIRT1 for those transport properties.
mutant (Fig. 3B). However, the complementation test showed that the cells expressing AtIRT1 grew faster than those expressing HvIRT1, which might suggest that HvIRT1 encodes a transporter with a lower uptake rate or affinity for Zn<sup>2+</sup> than AtIRT1. Likewise, HvIRT1 and AtIRT1 were able to restore the growth defect of the Fe<sup>2+</sup>/Fe<sup>3+</sup> transport-defective Δfet3Δfet4 double mutant with similar efficiency, demonstrating the importance of HvIRT1 for Fe<sup>2+</sup>/Fe<sup>3+</sup> transport (Fig. 3C). Thus, the complementation study suggests that HvIRT1 is involved in the transport of Mn<sup>2+</sup>, Zn<sup>2+</sup>, and Fe<sup>2+</sup>/Fe<sup>3+</sup>.

**Yeast Trace Element Uptake Studies**

ICP-MS was used to study the HvIRT1 specificity for 31 different trace elements and metalloids across the periodic table. Yeast cells transformed with either empty vector or HvIRT1 were harvested from log-phase cultures. The cells were washed and used for a 5.5-h uptake study. The following elements showed no differences between HvIRT1- and empty vector-transformed yeast cells in any of the three mutant strains (data not shown): aluminum, arsenic, silver, barium, beryllium, boron, calcium, cobalt, chromium, copper (Cu), europium, holmium, lanthanum, magnesium, molybdenum, sodium, nickel, lead, antimony, scandium, selenium, strontium, thorium, thallium, uranium, vanadium, and ytterbium. On the contrary, significant differences were observed for the uptake of Mn<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup>/Fe<sup>3+</sup>, and Cd<sup>2+</sup> (Figs. 4 and 5).

Background values resulting from unspecific adsorption were determined by performing the uptake experiment below 5°C and subtracting these values from those measured at 30°C (Fig. 4, B and C). In general, the values obtained below 5°C were less than 10% of those measured at 30°C. In order to ensure optimal cell viability and still avoid metal toxicity, the initial trace element concentrations were not equimolar. Widely different uptake rates were observed for the individual elements, with no clear relation to the differences in their initial concentration (Fig. 4). Taking Zn<sup>2+</sup> as an example, the uptake rate was significantly higher than that of Mn<sup>2+</sup>, even though the initial concentration at time zero was more than 50% lower (Fig. 4A).

Δsmf1 cells transformed with HvIRT1 showed approximately 50% higher uptake rates of Mn<sup>2+</sup>, Fe<sup>2+</sup>/Fe<sup>3+</sup>, and Cd<sup>2+</sup> when compared with yeast cells transformed with empty vector, whereas no difference was
seen in Zn\textsuperscript{2+} uptake (Fig. 5A). The same experiment carried out with the Zn\textsuperscript{2+}-deficient Δzrt1Δzrt2 yeast strain resulted in an approximately 100% higher uptake rate of Zn\textsuperscript{2+} and a 15% higher Cd\textsuperscript{2+} uptake rate in HvIRT1-transformed cells compared with empty vector transformants, while Mn\textsuperscript{2+} and Fe\textsuperscript{2+}/Fe\textsuperscript{3+} uptake rates were higher for yeast cells transformed with empty vector (Fig. 5B). The Fe\textsuperscript{2+}/Fe\textsuperscript{3+} uptake rate was 150% higher in the Fe\textsuperscript{2+}/Fe\textsuperscript{3+} uptake-defective Δfet3Δfet4 mutant when transformed with HvIRT1 compared with empty vector (Fig. 5C). In comparison, the Cd\textsuperscript{2+} and Mn\textsuperscript{2+} uptake rates were increased by 70% and 20%, respectively. Thus, the yeast uptake assays using ICP-MS support the results obtained from the complementation assays, confirming that HvIRT1 transports Mn\textsuperscript{2+}, Zn\textsuperscript{2+}, Fe\textsuperscript{2+}/Fe\textsuperscript{3+}, and Cd\textsuperscript{2+} when expressed in yeast.

**Cellular Localization of HvIRT1-GFP Fusion Protein**

A HvIRT1:GFP fusion construct was transiently expressed in epidermal cells of onion (*Allium cepa*) bulb scales under the control of two times the cauliflower mosaic virus (2×CaMV) 35S promoter. This resulted in a high expression level with substantial accumulation of the fusion protein in the endoplasmic reticulum (ER), as evidenced by a well-defined ER network (Fig. 6H), and by staining of the nuclear envelope when looking at an optical section (data not shown). In addition, GFP labeled the border of the cell, indicating plasma membrane localization of HvIRT1:GFP (Fig. 6H). In order to test if there indeed is HvIRT1:GFP protein at the plasma membrane, we incubated the cells with cycloheximide to block the protein synthesis and thereby reduce the amount of protein accumulating within the ER. Figure 6 (A–G) shows cells expressing HvIRT1:GFP after incubation with cycloheximide for 5 h. In these cells, there was a clear signal at the

**Figure 4.** Trace element uptake by Δsmf1 yeast cells. A, Box plot of the initial concentration of the trace elements analyzed before the start of the experiment (12 replicates). B and C, Uptake rates were measured at 30°C. Background values resulting from unspecific adsorption were determined in the cold below 5°C. Data represent triplicate means ± se of yeast mutant Δsmf1 transformed with HvIRT1 measured at 5°C (B) or 30°C (C).

**Figure 5.** The uptake rates of Mn\textsuperscript{2+}, Fe\textsuperscript{2+}/Fe\textsuperscript{3+}, Zn\textsuperscript{2+}, and Cd\textsuperscript{2+} were measured for 5.5 h in yeast mutants transformed with HvIRT1 and normalized on the basis of the uptake rates of similar yeast mutants transformed with empty vector (pFL61). Three yeast mutants were used: Δsmf1, defective in Mn\textsuperscript{2+} uptake (A), Δzrt1Δzrt2, defective in Zn\textsuperscript{2+} uptake (B), and Δfet3Δfet4, defective in Fe\textsuperscript{2+}/Fe\textsuperscript{3+} uptake (C). Unspecific adsorption of metal ions was adjusted by subtracting the uptake values from similar experiments performed at 5°C. The experiment was replicated three times with different transformation lines with similar results, and representative data are shown. Data are means ± se of three independent yeast samples. ns. refers to no significant difference in uptake rate between HvIRT1 and empty vector transformants (P<0.05).
HvIRT1 localizes to the plasma membrane. HvIRT1:GFP fusion protein was transiently expressed in onion epidermal cells (A–H). Eighteen hours of expression of the fusion protein resulted in massive protein buildup in the ER (H). This effect was reduced by shortening the expression time to 5 h followed by 5 h of treatment with cycloheximide to block protein synthesis (G). Optical sections of cells show localization of HvIRT1 to the plasma membrane (A and E). This localization was confirmed by plasmolyzing the cell from A in a mannitol solution for 30 min (B). Expression of free GFP resulted in an entirely different localization pattern, with staining of the cytosol and the entire nucleus (I). A to F, Optical sections through cells. A, B, and E, GFP staining. C, D, and F, Transmission and GFP channel overlay. G to I, Whole cell projections of the GFP staining show the differences in localization patterns between constructs and treatments. Bars = 100 µm.

Expression of HvIRT1 in roots was induced by Mn or Fe deficiency and responded to a much lesser extent to deprivation of Zn and Cu (Fig. 7). Under Mn or Fe deficiency, the expression level of HvIRT1 increased 40% to 50% in the Mn-efficient genotype Vanessa compared with 25% in the Mn-inefficient genotype Antonia. Significantly higher expression of HvIRT1 in Vanessa compared with Antonia was also evident under control conditions as well as under Zn deficiency (Fig. 7). The observed differences in the expression response of HvIRT1 were reflected by an up to 65% higher Mn$^{2+}$ uptake rate in roots of Vanessa compared with Antonia under control conditions (Fig. 8A). Under Mn deficiency, the Mn$^{2+}$ uptake rate in Vanessa was 95% higher than that in Antonia, whereas under Fe-deficient conditions, the difference was 60% in favor of Vanessa. Relative to the control conditions, the increase in Mn$^{2+}$ uptake rate was highest for Vanessa, as the Mn$^{2+}$ uptake rate increased 80% and 40% in Mn- and Fe-deficient plants, respectively. For Antonia, the corresponding increases were 55% and 40%. The differences in uptake rate were not related to differences in biomass production, as no significant differences between the two genotypes were observed (Fig. 8, B and C). Resupply of Mn$^{2+}$ to Mn-deficient plants caused Mn$^{2+}$ uptake rates to drop in both genotypes, most distinctly in Vanessa (Fig. 9). Although ample Mn$^{2+}$ supply resulted in a decreased difference in Mn$^{2+}$ uptake rates between the two genotypes, Vanessa maintained a significantly higher Mn$^{2+}$ uptake rate than Antonia, consistent with the results obtained for plants with steady-state Mn status (Fig. 8). Elemental tissue analysis showed a clear effect of the nutrient deficiency treatments in both genotypes (Table I). Noticeably, a specific micronutrient deficiency induced a higher uptake of other micronutrients, leading to twice as high Fe, Zn, and Cu concentrations in Mn-deficient plants compared with control plants.
Similarly, in Fe- and Cu-deficient plants, the Mn and Zn concentrations were more than doubled. The stress level, measured as the quantum yield efficiency of PSII caused by individual nutrient deficiencies, was determined by measuring the chlorophyll $a$ fluorescence induction kinetics. The resulting $F_v/F_m$ values (for maximum photochemical efficiency of PSII in the dark-adapted state) within each treatment were similar between the genotypes, being 0.8, 0.5, 0.5, 0.7 and 0.8 for control and Mn-, Fe-, Cu-, and Zn-deficient conditions, respectively, indicating similar stress levels between the genotypes (data not shown).

**DISCUSSION**

Differences in Mn efficiency among barley genotypes are related to the high-affinity uptake system for Mn$^{2+}$ operating in the low nanomolar concentration range (Pedas et al., 2005). However, the molecular basis for this phenomenon is not known. In this study, we report on the cloning and characterization of a barley Mn$^{2+}$ transporter, HvIRT1, belonging to the ZIP family. ZIP members have been cloned from other plant species (Eide et al., 1996; Eckhardt et al., 2001; Bughio et al., 2002; Lombi et al., 2002; Cohen et al., 2004; Li et al., 2006; Enomoto et al., 2007; Waters et al., 2007), but so far only AtIRT1 from Arabidopsis has been documented to be involved in Mn$^{2+}$ uptake in planta (Vert et al., 2002). Sequence analysis of the known IRT1 proteins revealed that the predicted HvIRT1 protein contains all of the characteristic features of the ZIP family (Fig. 1). HvIRT1 is predicted to have eight transmembrane domains, with the highly conserved ZIP signature in the fourth transmembrane domain (Eng et al., 1998) and a putative metal-binding His-rich region between domains III and IV (Guerinot, 2000). However, a few additional modifications to the ZIP signature are proposed (Fig. 1), including those in HvIRT1.

IRT1 homologs have been identified for other plant species (Eckhardt et al., 2001; Vert et al., 2002; Ishimaru et al., 2006), and searching public databases has identified several barley EST sequences with high homology to IRT1 (TC145252, TC151006, TC138364, and TC153445), but to our knowledge, no full-length IRT1 homolog has been identified from barley.

HvIRT1 restored the growth of the Δsmt1 yeast mutant deficient in Mn$^{2+}$ uptake (Fig. 3), suggesting...
that HvIRT1 is an integral plasma membrane protein, at least when expressed in yeast. This was further substantiated by transient expression of a HvIRT1:GFP fusion protein in onion epidermal cells, showing that the protein was localized to the ER and the plasma membrane (Fig. 6). HvIRT1 was predicted to contain a signal peptide (Fig. 1) targeting the protein to the secretory pathway, and the relatively high abundance in the ER suggests accumulation in the secretory system before reaching the final destination in the plasma membrane. OsIRT1 and AtIRT1 are also localized in the plasma membrane (Vert et al., 2002; Ishimaru et al., 2006), whereas the positions of the other IRT1 proteins remain to be identified.

All IRT1 proteins appear to have specificity for transporting Fe\(^{2+}/Fe^{3+}\) except in Thlaspi caerulescens and tobacco (Nicotiana tabacum), where the elemental preferences remain to be determined. In addition, IRT1s from Arabidopsis, tomato, and pea have the ability to transport Mn\(^{2+}\) (Korshunova et al., 1999; Eckhardt et al., 2001; Lombi et al., 2002; Vert et al., 2002; Cohen et al., 2004; Enomoto et al., 2007), but HvIRT1 is the first transport protein with a proven ability to transport Mn\(^{2+}\) that has been isolated from a graminaceous plant species. Functional expression of HvIRT1 cDNA in the yeast double mutants \(\Delta f e t 4 \Delta f e t 4\) and \(\Delta z r t 1 \Delta z r t 2\), defective in Fe\(^{2+}\)/Fe\(^{3+}\) and Zn\(^{2+}\) uptake, respectively, show that the HvIRT1 protein has the ability to transport, besides Mn\(^{2+}\), Fe\(^{2+}/Fe^{3+}\) and Zn\(^{2+}\) (Fig. 3).

The transport specificity of HvIRT1 was further examined in a yeast uptake assay using ICP-MS (see “Materials and Methods”) for analysis of 31 different metals and metalloids. This constituted a sensitive screen for all tested elements, even those in the subnanomolar range, such as cadmium, strontium, antimony, lanthanum, thorium, and uranium. However, only Mn\(^{2+}\), Fe\(^{2+}/Fe^{3+}\), Zn\(^{2+}\), and Cd\(^{2+}\) transport were affected by the expression of HvIRT1 in yeast (Figs. 4 and 5). AtIRT1 has previously been shown to transport Fe\(^{2+}/Fe^{3+}\), Mn\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\), and Cd\(^{2+}\) (Korshunova et al., 1999; Vert et al., 2002), while IRT1 from rice only is able transport Fe\(^{2+}/Fe^{3+}\) and Cd\(^{2+}\) (Buglio et al., 2002; Nakanishi et al., 2006). An interesting observation was made with the yeast uptake assay, as the empty vector transformants showed a higher uptake of Mn and Fe than HvIRT1-transformed cells when expressed in the \(\Delta z r t 1 \Delta z r t 2\) yeast mutant (Fig. 5B). However, studies have shown that Zn deficiency in yeast induces an up-regulation of several genes involved in various cell processes (Lyons et al., 2000; De Nicola et al., 2007). One gene highly up-regulated is \(f e t 4\), and \(f e t 4p\) being localized to the plasma membrane is suggested to be involved in the transport of Fe\(^{3+}\), Mn\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\), and Cu\(^{2+}\) (Li and Kaplan, 1998; Hassett et al., 2000; Waters and Eide, 2002). This can potentially explain the difference in

Table I. Element concentration (\(\mu g \, g^{-1} \, dry \, weight\)) in the youngest leaf of the Mn-efficient genotype Vanessa and the Mn-inefficient genotype Antonia plants grown under Mn-, Fe-, Cu-, and Zn-deficient and control conditions.

<table>
<thead>
<tr>
<th>Genotype and Element</th>
<th>Growth Condition</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Vanessa</td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>13.24 ± 0.5</td>
</tr>
<tr>
<td>Fe</td>
<td>58.54 ± 1.5</td>
</tr>
<tr>
<td>Cu</td>
<td>13.24 ± 0.6</td>
</tr>
<tr>
<td>Zn</td>
<td>37.76 ± 1.3</td>
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<tr>
<td>Antonia</td>
<td></td>
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<tr>
<td>Mn</td>
<td>13.64 ± 0.9</td>
</tr>
<tr>
<td>Fe</td>
<td>37.04 ± 4.2</td>
</tr>
<tr>
<td>Cu</td>
<td>8.68 ± 0.6</td>
</tr>
<tr>
<td>Zn</td>
<td>26.24 ± 3.1</td>
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Fe$^{2+}$/Fe$^{3+}$ and Mn$^{2+}$ uptake rates between HolIRT1- and empty vector-transformed Δzrt1Δzre2 yeast cells, as the HolIRT1-transformed yeast cells have a higher Zn supply and thereby a lower expression level of Fet4 and consequently a lower capacity for Fe$^{2+}$ and Mn$^{2+}$ uptake.

The transcript level of HolIRT1 was mainly influenced by Mn and Fe deficiency (Fig. 7). Similar responses have been observed for OsIRT1 (Bugbio et al., 2002; Ishimaru et al., 2006), while AtIRT1 was unaffected by micronutrient deficiencies other than Fe (Eide et al., 1996; Korshunova et al., 1999). The HolIRT1 expression level was significantly ($P < 0.01$) higher in the Mn-efficient genotype Vanessa compared with the Mn-inefficient genotype Antonia in all treatments except Cu deficiency (Fig. 7). The genotypic difference in HolIRT1 transcript abundance corresponded to the observed higher Mn$^{2+}$ uptake rates in Vanessa relative to Antonia both under steady-state Mn provision (Fig. 8) and during resupply of Mn$^{2+}$ to Mn-deficient plants (Fig. 9). The genotypic differences in HolIRT1 transcript levels also fit with the fact that Vanessa has a considerably higher $V_{\text{max}}$ value for Mn$^{2+}$ uptake than Antonia (Pedas et al., 2005). Also, transcript levels of IRT1-G in T. caerulescens correlated markedly with the $V_{\text{max}}$ for root Cd$^{2+}$ influx and shoot cadmium accumulation and were related to genotypic differences (Lombi et al., 2002). The substrate specificity of TcIRT1-G has not yet been determined, but the putative ability to transport other IRT1 homologs.

Resupply of Mn$^{2+}$ to Mn-deficient plants caused lower Mn$^{2+}$ uptake rates (Fig. 9), indicating a down-regulation of Mn$^{2+}$ transport proteins at ample Mn$^{2+}$ supply. Also, the root-to-shoot translocation of Mn$^{2+}$ seems to be reduced under Mn deficiency (Tsukamoto et al., 2006). OsIrt1 has not yet been documented to transport Mn$^{2+}$, but an OsIRT1 promoter-GUS experiment showed GUS activity in root phloem cells, particularly in the companion cells, and in root epidermis and exodermis. This indicates an important role for IRT1 in primary Fe$^{2+}$/Fe$^{3+}$ uptake, phloem loading, and long-distance transport to sink organs (Ishimaru et al., 2006). The leaf Mn concentration in Fe-deficient barley plants was up to 150% higher than in control plants (Table I), suggesting a strong up-regulation of Mn$^{2+}$ transport proteins involved in both primary Mn$^{2+}$ uptake and root-to-shoot translocation during Fe deficiency. The high similarity with OsIrt1 suggests that HolIRT1 may also be involved in Mn$^{2+}$-translocation during Fe deficiency. This is supported by the parallel increases in HolIRT1 expression, root Mn$^{2+}$ uptake, and foliar Mn concentration during Fe deficiency. However, further analysis of the specific cell tissue localization of HolIRT1 is needed before this can be confirmed.

The genotypic differences in Mn$^{2+}$ uptake kinetics shown previously (Pedas et al., 2005) might be due to the expression of different isoforms of Mn$^{2+}$ transporters rather than differential expression levels of Mn$^{2+}$ transport proteins. However, screening of the barley root cDNA library for genes encoding putative plasma membrane-localized Mn$^{2+}$ transport proteins only resulted in one isolated gene, HolIRT1. The observed difference in induction level upon deficiency treatments between the expression level of HolIRT1 and the Mn$^{2+}$ uptake capacity might be related to the transport capacity of the HolIRT1 protein, as a higher protein level can be followed by an even higher transport capacity. The IRT1 homolog from Arabidopsis is shown to be posttranscriptionally regulated, and in addition, a transcription factor is also involved in regulating the protein level of AtIRT1 (Connolly et al., 2002; Colangelo and Guerinot, 2004). Whether such regulation mechanisms exist in barley is currently unknown.

Therefore, HolIRT1 represents the only plasma membrane-localized Mn$^{2+}$ transport protein that has been found in barley, and it is concluded that HolIRT1 is important for Mn$^{2+}$ uptake in barley roots. Moreover, HolIRT1 seems to be a central component in differential Mn efficiency among barley genotypes.

**MATERIALS AND METHODS**

**Yeast Strains**

A wild-type *Saccharomyces cerevisiae* strain (BY4741) and five deletion mutants in the same genetic background were used in this study (Table II). Two double mutants were produced by crossing haploid single-deletion mutants: Y06461 (ΔYM1313c) crossed with Y16192 (ΔYMRO588y), and Y06422 (ΔYLR130c) crossed with Y10487 (ΔYLR130c). Subsequent sporulation according to standard procedures generated the Δfet1Δfet1 and Δzrt1Δzre2 double mutants.

**Functional Complementation in Yeast**

Yeast strains were transformed with HolIRT1, *AIRT1* vector construct, or the empty vector pFL61. Transformants were selected on uracil-deficient medium and grown in synthetic medium containing 2% Glc, 50 mM succinic acid/Tris base, pH 5.5, 0.7% yeast nitrogen base (YNB) without amino acids (Difco), and 0.3% appropriate amino acids. Agar was added to 2% for solid plate medium (Sherman, 1991). For medium deprived of Mn, Zn, or Fe, YNB without metals (BIO 101 Systems) supplemented with various metals was used, and various additions of the chelators bathophenanthroline disulfonic acid (Fluka) and EDTA (Sigma) were added, as specified in “Results.” Plates were incubated at 30°C for 3 to 5 d.

**Identification of the Mn$^{2+}$ Transporter Protein**

A cDNA library inserted into the vector pFL61 (Minet et al., 1992) was produced from mRNA extracted from the barley (*Hordeum vulgare*) genotypes Vanessa and Antonia supplied with varying levels of Mn$^{2+}$, ranging from sufficient to insufficient levels, achieving all scenarios potentially important for Mn efficiency. The yeast strain *S. cerevisiae* Δmof1 (Table II) was transformed by electroporation as described (http://www.bio.kuleuven.be/dp/logt/protocol/yeastelectroporation.htm) with the barley root cDNA library. The pFL61 vector was modified with the Gateway system (Invitrogen), and it contains the replication origin of the yeast 2 μ plasmid, the Ura3 gene, and the promoter and terminator of the yeast phosphoglycerate kinase gene for the expression of the foreign cDNA in *S. cerevisiae*. Transformants were selected on uracil-deficient synthetic medium with 2% Glc, 50 mM succinic acid/Tris base, pH 5.5, 0.7% YNB without amino acids (Difco), and 0.3% amino acids His, Leu, and Met. Transformants were harvested from the plates, pooled, and resuspended in glycerol. From this pool, aliquots were replated on medium without Mn$^{2+}$ to select for yeast cells complementing the Mn$^{2+}$-defective mutant. Mn$^{2+}$-deficient medium was composed as above except that the YNB
(Difco) were replaced with YNB without metals and without amino acids (BIO 101 Systems) supplemented with 0.9 mM CaCl₂, 2 mM MgSO₄, 160 mM CuSO₄, 740 mM FeCl₃, and 1.4 μM ZnSO₄. The agar used contained a significant amount of Mn²⁺; therefore, the ion activity of Mn²⁺ was decreased using 10 to 15 mM EGTA. Plasmid DNA from yeast transformants was extracted (Hoffman and Winston, 1987) and amplified in Escherichia coli by standard procedures. Plasmids, which after retransformation still were unable to complement Δmrt1, were sequenced, and the gene sequences were BLASTed against various databases to identify similarities to known gene families.

Cloning of AtIRT1 into Yeast Expression Vector

AtIRT1 (Eide et al., 1996) was subcloned into the pFL61 vector to conduct yeast complementation as a positive control. A PCR-based cloning strategy was used to clone AtIRT1 and insert the cDNA into the Gateway-adapted pFL61 vector. Primers with attB1 and attB2 sequences including gene-specific sequences were used to amplify the open reading frame: AtIRT1 forward, 5’-ACAGTGGTGTACAAAAAAGACGGTACGTAAGAAAGAACATCTCCCCTCGTATA-3’; AtIRT1 reverse, 5’-GACCACCTTGTACAAAGAAGCTGGTCTTAAGGCCATTTAGCCTAAAT-3’. The PCR product was amplified using LA Taq (Takara), and the in vitro BP clonase recombinant reaction into the pDONR221 vector (Invitrogen) was carried out according to the manufacturer’s directions followed by sequencing. The coding sequence of AtIRT1 from cDNA from Arabidopsis (Eide et al., 1996) was subcloned into the pFL61 vector to conduct a reverse transcriptase polymerase chain reaction according to the manufacturer’s instructions into the pFL61 vector, identical to the vector used for the cDNA library bar code.

Yeast Metal Uptake Studies

Yeast transformants were precultured overnight in 2 mL of the medium prepared as above, and a liquid culture was inoculated overnight, achieving an optical density value measured at 600 nm (OD₆₀₀) in the range of 1.0 to 1.2. A growth assay was made for observing growth rates between yeast strains transformed with different constructs, so the harvesting was done at the optimal time point for the individual transformants. Cells were then pelleted by centrifugation and washed three times in ice-cold Milli-Q water, and the resulting pellets were resuspended in Milli-Q water. Ten milliliters of the cell suspensions with similar content of cells rates between yeast strains transformed with different constructs, so the IC-MS calibration standard; CPI International). The OD₆₀₀ was 1.6 (cells up-

Plant Material

Seeds of two barley (Hordeum vulgare) genotypes differing in Mn efficiency were germinated at 21°C in vermiculite. After 5 d, uniform seedlings were selected and transferred to light-impermeable 4-L black buckets with four plants per bucket. The buckets were filled with a chelate-buffered solution prepared in double ionized water as specified by Pedas et al. (2005), with the exception of Mn²⁺; pH was kept at 6.0 using 0.5 mM MES-Tris, pH 6.0. To induce deficiencies of Zn, Mn, or Cu, plants were grown for 4 weeks in solutions without these nutrients. For the Fe-deficient treatment, 2-week-old control plants were transferred to nutrient solution without Fe and grown for 2 weeks more. With the exception of plants exposed to Mn deficiency, 22 and 44 μM of Mn²⁺ (resembling Mn²⁺ concentrations of 100 and 200 nM, respectively) was added every second day in the form of MnCl₂ for the first and last 2 weeks of growth, respectively. The plants used for Mn²⁺ uptake experiments did not receive any Mn²⁺ addition on the same day that the measurements were performed. A number of plants were induced with Mn deficiency, and they were used to examine how the Mn²⁺ uptake rates were influenced by changing the Mn²⁺ available concentration compared with plants with continuous Mn deficiency. These plants were grown as follows. For 2 weeks, plants were grown at control conditions. Then, the plants were without Mn²⁺ additions for 19 d, inducing Mn deficiency, controlled by measuring chlorophyll a fluorescence. At this point, the experiment started by taking plants to analyze the Mn²⁺ uptake rate as described below. A Mn²⁺ addition of 4.4 μM per bucket (achieving 20 nM Mn²⁺) was then added to half of the plants, and every morning for the following 3 d, an additional Mn²⁺ addition of 22 μM was added to same plants, leaving half of the plants with continuous Mn deficiency. Sample times were at day zero (before adding Mn²⁺) and at 12 h, 1, 2, 3, and 4 d after the first Mn addition. Plants were grown in a controlled growth chamber with a 250 to 280 μmol m⁻² s⁻¹ photon flux density, 75% to 80% humidity, and a 20°C/15°C (16 h/8 h) day/night temperature regime.

Chlorophyll a Fluorescence

Induction of the individual micronutrient deficiency and its effect on PSII were recorded with chlorophyll a fluorescence measurements (Kriedemann et al., 1985) using a Handy PEA (Plant Efficiency Analyzer; Hansatech Instruments) on four leaves per bucket. A flash of saturating light (3,000 μmol photons m⁻² s⁻¹) lasting 2 s applied to leaves that had been dark adapted for 25 min. The fluorescence data were analyzed, and the Fv/Fm ratio was calculated using the Handy PEA software (version 1.30).
54Mn$^{2+}$ Uptake Measurements

Experiments were started at times corresponding to the photoperiod of the plants, except for the sample time 12 h after Mn$^{2+}$ resupply. The barley plants were gently removed from the nutrient solution. The roots were rinsed in 18.2 M2 Milli-Q water for 10 min and placed in 700-mL polypropylene beakers containing a pretreatment solution (2 mM MES-Tris, pH 6.0, 0.2 mM CaSO$_4$, 12.5 mM H$_2$BO$_3$, and 0.5 mM MnCl$_2$) for 30 min. A new set of beakers was prepared with 700 mL of uptake solution consisting of 5 mM MES-Tris, pH 6.0, 0.2 mM CaSO$_4$, 12.5 mM H$_2$BO$_3$, and 10 nM MnCl$_2$. The radioactivity of 54Mn$^{2+}$ (Christ Alpha 2-4; Martin Christ), and weighed. The roots were dissolved in 10 mL of concentrated HNO$_3$. The radioactivity of 54Mn$^{2+}$ was analyzed by ICP-MS as described previously (Pedas et al., 2005).

Prior to PCR, cDNA was diluted 1:5 in sterile water (BIBCO; Invitrogen). The threshold limits found by Reuter et al. (1997). The plant samples were freeze-dried (Christ Alpha 2-4; Martin Christ), digested using ultrapure acids, and multielemental analyses of leaf tissue were performed and compared with the desired total Mn$^{2+}$ concentration of 50 and 100 nm for the steady-state uptake measurements (control and Mn- and Fe-deficient conditions) and for the response experiments (resupply of Mn$^{2+}$ to Mn-deficient plants), respectively. Before initiating the uptake measurements, the Mn$^{2+}$ concentration in the uptake solutions was checked by ICP-MS. At the end of the 2-h uptake period, plants were gently moved and rinsed for 10 min in Milli-Q water to remove the nutrient solution-water film around the roots. All of the solutions were aerated, and the experiments were carried out at 20°C under artificial light with a photon flux density of 250 to 280 m$\mu$m$^{-2}$ s$^{-1}$. In parallel, similar experiments were carried out in a cooling room (4°C–5°C) with no light in cold (2°C) solutions mimicking the Mn$^{2+}$ absorption in the root apoplas.

This fraction was subtracted from the data obtained under 20°C and light, estimating the actual uptake during the 2-h uptake period. After the final rinse in Milli-Q water, the roots were blotted dry with paper towels, freeze dried (Christ Alpha 2-4; Martin Christ), and weighed. The roots were dissolved in 10 mL of concentrated HNO$_3$. The radioactivity of 54Mn$^{2+}$ taken up by the roots was measured by $\gamma$ spectrometry using a Ge(Li) detector (Princeton Gamma-Tech; 28% relative efficiency, 1.8-kV energy resolution) placed in a 10-cm lead shield and connected to a Canberra multiprotnulchannel analyzer. The spectra were evaluated with Canberra Genie 2000 software.

Determination of Trace Element Concentrations in Plant Material

To ensure that the micronutrient deficiency had been induced in the plants, multielemental analyses of leaf tissue were performed and compared with the threshold limits found by Reuter et al. (1997). The plant samples were freeze dried (Christ Alpha 2-4; Martin Christ), digested using ultrapure acids, and analyzed by ICP-MS as described previously (Pedas et al., 2005).

Analysis of HvIRT1 Expression in Plants

Total RNA was extracted from approximately 250 mg of fresh plant tissues using the FastRNeasy Pro Green Kit (Q BIoGene) and a Fast Prep (FP210). Total RNA was treated with RQI RNase-free DNase (Promega) to remove contaminating genomic DNA. The RNA was checked for purity, integrity, and quantity using RNA gel electrophoresis and spectrophotometry. One microgram of total RNA was used as a template for cDNA synthesis using Moloney murine leukemia virus reverse transcriptase with $\delta$-10 oligonucleotide primers according to the manufacturer’s directions (New England Biolabs). Prior to PCR, cDNA was diluted 1:5 in sterile water (BIBCO; Invitrogen). The PCR program used consisted of 27 cycles (45 s at 94°C, 1 min at 55°C, and 1.5 min at 72°C), and the control reactions specific for the HvACTIN and HvGAPDH transcripts were allowed to proceed for only 25 cycles. The PCR product was amplified using LA Taq (Takara), and in the vitro BP clonase recombinant reaction into the pDONR221 vector (Invitrogen) was carried out according to the manufacturer’s directions followed by sequencing. After sequencing, HvIRTI DNA was transferred by LR recombinant reaction according to the manufacturer’s instructions into the pMDCS8 vector, consisting of 2°C CaMV 35S promoter, a NOS terminator, and the GFP6 gene (Curtis and Grossniklaus, 2003). The pMDCS8-HvIRT1 vector was then digested with EcoRI and HindIII, resulting in the 2°C CaMV 35S promoter-HvIRT1-GFP-NOS terminator fragment. A construct of the plbPTA9-GFP vector harboring the CaMV 35S promoter, the GFP6 gene, and the 35S terminator was kindly provided by Dr. Michael Krogh Jensen (University of Copenhagen). The construct contains an EcoRI site 5’ of the promoter and a HindIII site 5’ of the terminator. A sequential digestion of the plbPTA9-GFP vector was made by EcoRI and HindIII, and the 2°C CaMV 35S promoter-HvIRT1-GFP-NOS terminator fragment from pMDCS8-HvIRT1 was then ligated into the plbPTA9 vector. plbPTA9-GFP and plbPTA9-HvIRT1-GFP were used to transiently express GFP and HvIRT1-GFP, respectively, in onion (Allium cepa) epidermal cells. The transient expression and localization were done as described by Jensen et al. (2007) with a few modifications. Briefly, 1 × 1 cm$^2$ of onion bulb scales was placed on agar (1× Murashige and Skoog medium, 0.7% agar, and 3% Suc; pH 5.8) with the inner epidermis facing up. They were bombarded using the PDS-1000/He biolistic particle delivery system (Bio-Rad). Approximately 6 μg of expression vector was coated onto 1.5 mg of 1-μm gold particles and transferred into the cells. After bombardment, petri dishes containing the onion bulb squares were placed in the dark at room temperature for 18 to 24 h. Where indicated, the cells were treated with 10 μg mL$^{-1}$ cycloheximide (Sigma) for 5 h after only 5 h in the dark. After treatment, the cells were washed in water before the epidermis was peeled, and transformed cells were visualized using a Leica TCS SP2/MP confocal laser scanning microscope (Leica Microsystems). Excitation for GFP was 488 nm, and emission was detected between 500 and 575 nm. For plasmolysis, the cells were incubated in a 1% mannitol solution for 30 min.

Accession Numbers

The GenBank accession numbers for the sequences described in this article are as follows. Barley: HvIRTI, EU548802; HvACTIN, TC131547; and HvGAPDH, X06343; Arabidopsis: AiIRTI, NM_118089; tobacco: NiIRTI, AB263746; pea: PoIRTI, AFO54444; tomato: LiIRTI, A264266; rice: OsIRTI, A0707226; Malus xiaojinensis: MsIRTI1, AIY193886; Cucumis sativus: CsIRTI, AA104114; T. caerulescens: TcIRTI-G, AJ320253.

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LITERATURE CITED


HvIRTI, a Metal Ion Transporter Contributing to Mn Efficiency

A PCR-based cloning strategy was used to generate HvIRTI DNA with a mutated stop codon for C-terminal fusion to the GFP6 gene. The primers used

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