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***Arabidopsis* IRT2 gene encodes a root-periphery iron transporter**

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Summary

Iron uptake from the soil is a tightly controlled process in plant roots, involving specialized transporters. One such transporter, IRT1, was identified in *Arabidopsis thaliana* and shown to function as a broad-range metal ion transporter in yeast. Here we report the cloning and characterization of the *IRT2* cDNA, a member of the ZIP family of metal transporters, highly similar to IRT1 at the amino-acid level. *IRT2* expression in yeast suppresses the growth defect of iron and zinc transport yeast mutants and enhances iron uptake and accumulation. However, unlike IRT1, IRT2 does not transport manganese or cadmium in yeast. *IRT2* expression is detected only in roots of *A. thaliana* plants, and is upregulated by iron deficiency. By fusing the *IRT2* promoter to the *uidA* reporter gene, we show that the *IRT2* promoter is mainly active in the external cell layers of the root subapical zone, and therefore provide the first tissue localization of a plant metal transporter. Altogether, these data support a role for the IRT2 transporter in iron and zinc uptake from the soil in response to iron-limited conditions.

Keywords: iron uptake, zinc, transport, IRT, *Arabidopsis*, root.

Introduction

Metal ions, especially iron, are of major importance in living cells as co-factors for many enzymatic reactions. Consistent with the fundamental role of these elements, deficiency causes severe disorders in all living organisms. Although abundant in nature, iron is often unavailable because it precipitates into its insoluble oxidized form (Guerinot and Yi, 1994). Organisms, and especially plants, have therefore evolved efficient processes to take up iron from their environment. However, overload of iron in the cell can cause serious damage due to iron's redox properties and its ability to react with reduced forms of oxygen. Uptake of iron by cells must therefore be strictly regulated.

Except for grasses, plants take up iron in a similar way to *Saccharomyces cerevisiae* (Eide, 1998), including (i) acidification of the rhizosphere in order to increase solubilization of Fe³⁺ (ii) reduction of Fe³⁺ in Fe²⁺ and (iii) specific transport of Fe²⁺ across the root plasma membrane. Over the past 5 years, knowledge about the molecular basis of iron transport systems in plants has greatly increased. The *FRO2* gene from *Arabidopsis thaliana*, encoding an Fe³⁺-chelate reductase, was cloned based on the sequence of its

yeast homologue's *FRE1* and *FRE2* genes (Robinson *et al.*, 1999). In addition, functional complementation of the *fet3fet4* yeast mutant, defective in both high- and low-affinity iron transport systems, allowed the cloning of a new type of metal transporter of *A. thaliana* named IRT1. IRT1 mediates Fe²⁺ transport when expressed in yeast, and its expression in plants is upregulated under iron-deficient conditions in roots (Eide *et al.*, 1996). In addition to iron, *IRT1*-expressing yeast possesses manganese and zinc uptake activities (Korshunova *et al.*, 1999), and shows increased sensitivity to external cadmium (Rogers *et al.*, 2000). Therefore IRT1 is likely to be a major iron uptake system in plant roots, allowing plants to respond to iron deficiency, and capable of transporting a broad range of cations.

Other systems able to take up metal ions have been described. Among the most studied are members of the NRAMP family of divalent cation transporters, which have been found in bacteria as well as throughout the animal and plant kingdoms (Fleming *et al.*, 1997; Gunshin *et al.*, 1997; Nelson, 1999). In *A. thaliana*, for example, six genes encode NRAMP proteins, three of which, *AtNramp1*,

AtNramp3 and *AtNramp4*, were shown to be involved in iron homeostasis and to be upregulated by iron starvation in plants (Curie *et al.*, 2000; Thomine *et al.*, 2000). In addition, we recently reported on the cloning of the maize *yellow stripe 1* (*ys1*) gene encoding the major Fe³⁺-uptake system of grasses (Curie *et al.*, 2001) and found that eight *ys1* homologues exist in *A. thaliana* as well, thus defining a novel family of plant transporters.

IRT1 belongs to a large family of metal transporters, the ZIP family, that comprises over 25 members, from *Trypanosoma* to humans and plants (Eng *et al.*, 1998; Guerinot, 2000). Common features shared by ZIP proteins include eight putative transmembrane domains (TM) and a variable intracellular loop between TM3 and TM4, rich in histidine, potentially involved in metal binding (Guerinot, 2000). *A. thaliana* ZIP1 and ZIP3 are able to transport zinc, but not iron, when expressed in yeast, and their expression in plants is upregulated by zinc deficiency (Grotz *et al.*, 1998). One sequence, belonging to the ZIP family and most closely related to *IRT1*, and therefore called *IRT2*, was initially identified in the *A. thaliana* EST database (Eide *et al.*, 1996). Here we report the cloning of the *IRT2* cDNA from *A. thaliana*. Expression of *IRT2* in yeast strains defective in either iron or zinc uptake suppressed the growth defect of these mutants and enhanced ⁵⁵Fe uptake. However, *IRT2* was not able to complement the $\Delta smf1$ mutant affected in divalent cation uptake, and did not confer sensitivity to cadmium when expressed in a wild-type strain. *IRT2* transcripts, although present in iron-replete conditions, accumulate under iron deficiency in roots. Finally, we show that the *IRT2* promoter drives GUS expression exclusively in the cells located at the periphery of the root, in the subapical zone, and that this expression is enhanced by iron deficiency.

Results

IRT2 is a member of the ZIP family of metal transporter

A PCR strategy was used to clone the *IRT2* cDNA from *A. thaliana* (see Experimental procedures). The amplification product obtained was sequenced, and this sequence was validated by comparison with the *IRT2* genomic sequence from BAC T16H5 available in GenBank. The *IRT2* cDNA contains an open reading frame 1053 bp in length, encoding a predicted polypeptide of 350 amino acids. *IRT2* is highly similar to the previously identified *A. thaliana* iron transporter *IRT1* (Figure 1) (Eide *et al.*, 1996), showing 69% identity and 82% similarity at the amino-acid level. The *IRT2* protein harbours features characteristic of the members of the ZIP family (reviewed by Guerinot, 2000). Like most known ZIP family members, *IRT2* contains eight predicted transmembrane domains (Eng *et al.*, 1998). Moreover, *IRT2* possesses a so-called variable cytosolic

loop, rich in histidine, between TM3 and TM4 (Grotz *et al.*, 1998), which contains the putative metal-binding site. Within TM4, *IRT2*, like all the ZIP proteins, possesses a fully conserved histidine residue, proposed to be involved in the formation of an intramembranous heavy metal binding site involved in the transport pathway (Eng *et al.*, 1998; MacDiarmid *et al.*, 2000; Rogers *et al.*, 2000). Intracellular localization prediction using PSORT I software indicates that *IRT2* is likely to be a plasma membrane protein.

IRT2 expression in yeast mediates high-affinity iron uptake

Because of the strong homology between *IRT1* and *IRT2*, and because expression of *IRT1* in yeast mediates high-affinity uptake of Fe²⁺ (Eide *et al.*, 1996), we tested the iron transport properties of *IRT2*. The *fet3fet4* yeast mutant is defective in both high- and low-affinity iron uptake and is extremely sensitive to iron limitation (Dix *et al.*, 1994). We examined the ability of *IRT2* to suppress the growth defect of the *fet3fet4* strain. *IRT2* coding sequence was subcloned in the yeast expression vector pFL61, under the control of the strong PGK promoter. The *A. thaliana* metal transporter *IRT1* was used as a positive control in the yeast experiments. On a medium containing as little as 10 μ M iron citrate, both *IRT2* and *IRT1* expressing yeast grew whereas the pFL61 control did not (Figure 2a). These data establish that, like *IRT1*, *IRT2* can functionally complement the phenotype of the *fet3fet4* mutant in iron-limited conditions.

To determine whether *IRT2* encodes an iron transporter, we examined Fe²⁺ short-term accumulation levels in the *fet3fet4* strain expressing *IRT2*. Incubation of *IRT2*-expressing cells with 2 μ M ⁵⁵Fe²⁺-ascorbate resulted in a sixfold increased ⁵⁵Fe uptake over 5 min (Figure 2b). A comparable value was measured when expressing *IRT1* (Figure 2b). Moreover, long-term accumulation experiments showed a 1.7-fold increase in cellular iron content as a result of either *IRT1* or *IRT2* expression (Figure 2c).

IRT2 restores the growth of a Zn uptake-defective yeast mutant

We next examined whether *IRT2* is a multispecific transporter, as *IRT1* was shown to mediate high-affinity uptake of Zn²⁺ and Mn²⁺ (Korshunova *et al.*, 1999) and to increase yeast sensitivity to external cadmium (Rogers *et al.*, 2000). Functional complementation of the yeast *zrt1zrt2* mutant, defective in both high- and low-affinity zinc uptake, was examined. This mutant grows poorly on zinc-limited medium (Zhao and Eide, 1996). The expression of *IRT2* in the *zrt1zrt2* strain enabled the cells to grow in the absence of added zinc, whereas complementation by *IRT1* could be



Figure 1. Comparison of IRT2 deduced amino-acid sequence with plant ZIP proteins shown to complement the growth defect of the *fet3fet4* yeast mutant. Lines under the sequences indicate the position of the putative transmembrane domains. The putative metal-binding domain is boxed. *A. thaliana* IRT1 and IRT2 (Genbank accession numbers U27590 and T04324), *Lycopersicon esculentum* LeIRT1 and LeIRT2 (Genbank accession numbers AF136579 and AF136580), and *Pisum sativum* PsRIT1 (Genbank accession number AF065444).

observed only at 50 μM ZnCl_2 (Figure 3a). This experiment shows that both IRT2 and IRT1 can complement the phenotype of the *zrt1zrt2* mutant on zinc-limited medium. However, growth restoration by IRT2 is dramatically more efficient on extremely low zinc content than it is by IRT1, indicating that at least in yeast, IRT2 and IRT1 possess different kinetic properties relative to zinc.

Smf1p is one of the three homologues of the NRAMP family of transporters expressed in *S. cerevisiae*. Because Smf1p functions in cellular accumulation of manganese (Portnoy *et al.*, 2000; Supek *et al.*, 1996), the Δsmf1 yeast mutant cannot grow on medium containing EGTA (Supek *et al.*, 1996). To test the possibility that IRT2 is also able to transport manganese, IRT2 cDNA was expressed in the Δsmf1 mutant and examined for growth restoration on EGTA-containing medium. Although IRT1 expression greatly improves Δsmf1 growth in the presence of 5 mM EGTA, we failed to observe a change in the growth rate due to IRT2 expression (Figure 3b). Finally, expression of

IRT2 in the wild-type yeast strain BY4742 does not alter cadmium sensitivity of the yeast, whereas the same strain expressing IRT1 shows a dramatic increase in sensitivity (Figure 3c). Therefore our results demonstrate that IRT2 can transport iron. They suggest that IRT2 may also transport zinc, but not manganese or cadmium. Thus IRT1 and IRT2 have distinct substrate specificities.

IRT2 expression is induced in response to iron deficiency

We examined the expression pattern of IRT2 in response to iron status. Detection of transcripts was carried out by high-stringency Northern blot hybridization using total RNA from roots and shoots of *A. thaliana* plants grown under iron-sufficient or iron-deficient conditions. Roots of iron-deficient plants accumulate large amounts of IRT1 mRNA (Eide *et al.*, 1996), and we therefore used an IRT1 probe as a positive control for the iron-deficiency status of the plants. We detected IRT2 mRNA exclusively in the

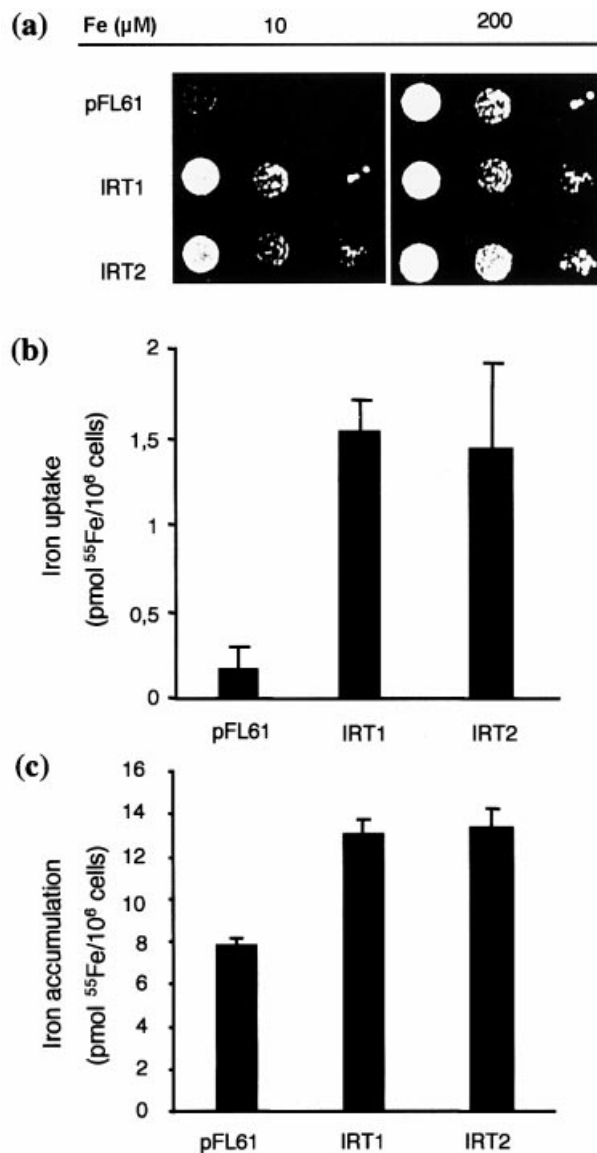


Figure 2. Iron-transport properties of IRT2.

(a) Complementation of the *fet3fet4* mutant (DEY1453 strain) on synthetic medium supplemented with 10 or 200 μM Fe^{3+} -citrate. (b) Assays were performed during 5 min with 3 OD units of yeast in MG medium containing 2 μM $^{55}\text{FeCl}_3$ and 1 mM sodium ascorbate. Values represent the mean of six independent assays. (c) Exponentially growing yeast (10^6 cells ml^{-1}) were grown in synthetic medium containing 5 μM $^{55}\text{FeCl}_3$ at 30°C for 22 h. Values represent the mean of four independent assays.

roots, where it accumulates under iron deficiency (Figure 4a). *IRT2* expression was highest after 1 day of culture in iron-depleted medium (Figure 4a, lane -1), and decreased thereafter (3 or 5 days). 48 h re-supply of iron following a 3 day iron-deficiency period enabled *IRT2* expression to go down to its basal level (Figure 4a, lane +), showing that iron is indeed responsible for the differential expression observed here. Like *IRT2*, *IRT1* was specifically expressed in roots of iron-starved plants. However, unlike *IRT2*, *IRT1*

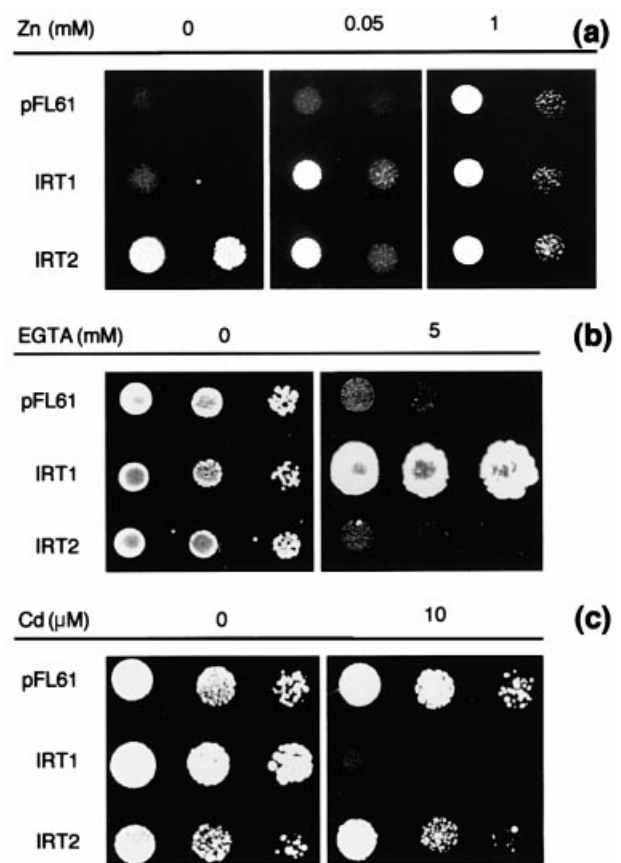


Figure 3. Concentration-dependent complementation of metal uptake-defective yeast mutants.

(a) Complementation of the *zrt1zrt2* mutant (ZHY3 strain) on synthetic medium supplemented with 0, 0.05 or 1 mM ZnCl_2 . (b) Complementation of the Δsmf1 mutant (SLY8 strain) on synthetic medium containing 0 or 5 mM EGTA. (c) Effect of 10 μM CdCl_2 on the growth of the wild-type strain BY4742. 0.2, 0.02 (a) or 0.2, 0.02, 0.002 (b,c) OD unit drops of overnight cultures were spotted on synthetic medium, and plates were incubated at 30°C for 4 days.

transcripts were not detected under iron-sufficient conditions in roots. Moreover, *IRT1* expression level was dramatically higher than *IRT2* after induction by iron deficiency. Consequently, *IRT1* and *IRT2* mRNA amounts are increased by a factor of 28 and 3.5, respectively, in response to iron deficiency (Figure 4b, upper and lower). Therefore, although the kinetics of *IRT2* and *IRT1* responses to iron starvation in the plant are qualitatively identical, there is a major quantitative difference between the two genes, as both the level of expression and the induction factor by iron deficiency are several-fold lower for *IRT2*.

IRT2 promoter activity is upregulated by the iron status in roots

One kilobase pairs of the *IRT2* promoter/5' UTR region was fused to the GUS reporter gene in the binary vector pBIN19

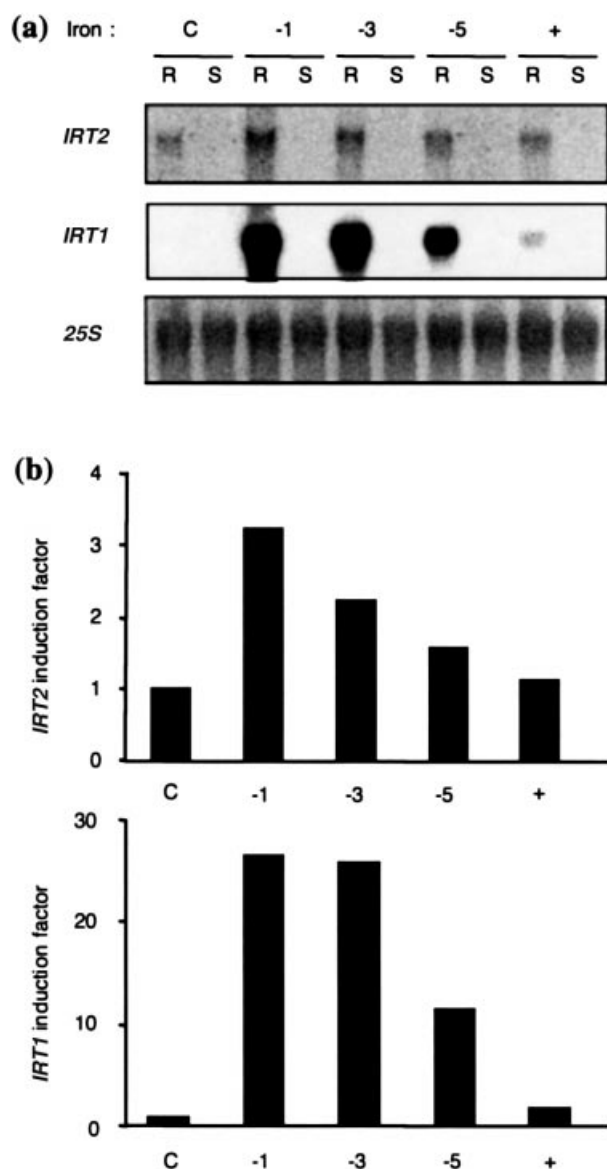


Figure 4. Regulation of *IRT2* and *IRT1* mRNA level by iron status. (a) Northern blot analysis of *IRT2* gene. *IRT2* coding sequence was hybridized to a blot containing 14 μ g total RNA extracted from roots (R) and shoots (S) of *A. thaliana*. Plants were grown under iron-sufficient conditions (C), in absence of iron for 1 (-1), 3 (-3), or 5 (-5) days, or in absence of iron for 3 days followed by re-supply of iron for 48 h (+). (b) Quantification of *IRT2* (upper) and *IRT1* (lower) induction factor under iron deficiency relative to the 25S rRNA hybridization signal.

(Bevan, 1984), and the resulting fusion was introduced into *A. thaliana* plants by *Agrobacterium*-mediated transformation (see Experimental procedures). Transgenic plants carrying this fusion were obtained. GUS enzymatic activity was assayed in transformed lines grown under iron-sufficient (+Fe) or iron-deficient (-Fe) conditions. No significant activity could be detected in the shoots (data not shown). Under iron deficiency, the activity of the *IRT2*

promoter was increased in roots (Figure 5a), from 112 (+Fe) to 582 (-Fe) pmol MU min⁻¹ mg⁻¹ protein, values corresponding to the mean of 16 independent transgenic lines. Taken separately, each of the 16 transgenic lines represented in Figure 5(a) showed an increase in root GUS activity under -Fe conditions, resulting in an average induction factor of 4.4 ± 2.7 . This value is consistent with the 3.5-fold increase in mRNA accumulation measured by Northern blotting (Figure 4b). We therefore concluded that the regulation of *IRT2* expression by iron status essentially occurs at the transcriptional level.

In order to determine in which cell layer of the root *IRT2* gene is expressed, GUS histochemical staining was performed in roots of 12-day-old, iron-starved plantlets. We observed GUS activity in the youngest part of the root, including a strong staining in root hairs, except for the meristem (Figure 5c,d). The area stained corresponds to morphologically defined territories – the elongation, differentiation and root hair zones (Figure 5b,c). GUS activity is not detected in older parts of the root, that is, the lateral emerging zone (Figure 5b,d). Cross-sections of the roots in the region of GUS activity showed staining mainly in the epidermis, as well as faintly in the cortex (Figure 5e). Roots of plants grown under iron-replete conditions harboured GUS staining in the same territories as roots grown under iron deficiency, albeit more faintly (data not shown). Thus our results establish that *IRT2* promoter activity is specific to the external cell layers of the root, and is enhanced by iron deficiency. Both characteristics are consistent with a role of *IRT2* in the uptake of iron from the rhizosphere.

Discussion

By expressing the *IRT2* cDNA in a mutant yeast strain defective in iron uptake, and monitoring the growth restoration and short-term accumulation levels obtained, we have demonstrated that the *IRT2* gene encodes a protein able to transport iron in yeast. Increased iron accumulation in *IRT2*-expressing *fet3fet4* strain further proves that suppression of the *fet3fet4* growth defect results from iron uptake rather than from iron remobilization inside the cell. Comparison of the ability of *IRT1* and *IRT2* to complement various mutants shows that the spectrum of divalent cations transported by the two transporters is overlapping but different. Both *IRT2* and *IRT1*, when expressed in *fet3fet4* and *zrt1zrt2* yeast strains, defective in iron and zinc uptake, respectively, are able to complement the transport functions of these mutants. On the contrary, *IRT1* but not *IRT2* expression can restore the growth defect of the $\Delta smf1$ yeast mutant (Korshunova *et al.*, 1999) affected in divalent cation transport (Chen *et al.*, 1999). Furthermore, expression of *IRT2* in a wild-type yeast strain does not confer sensitivity to cadmium, as does *IRT1*. Therefore *IRT2* exhibits a higher substrate

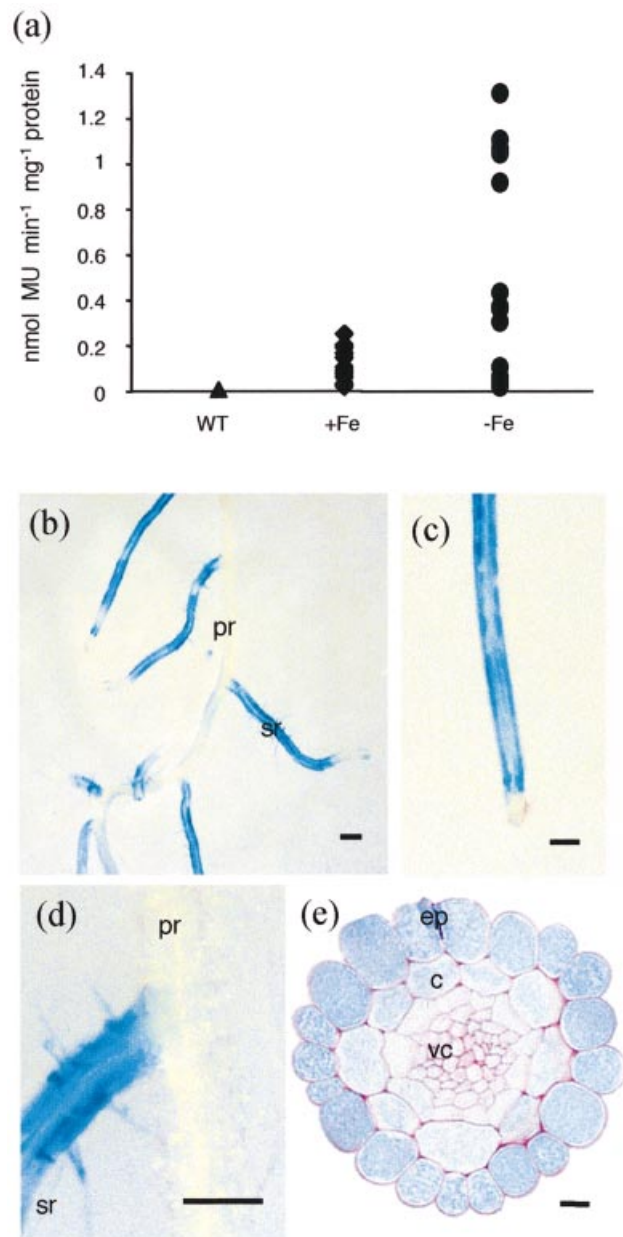


Figure 5. GUS expression analysis and localization of *IRT2* expression. (a) Sixteen transgenic lines expressing the *uidA* gene of *E. coli* under control of the *IRT2* promoter were assessed for GUS activity under iron-sufficient (+Fe) and iron-deficient (-Fe) conditions. (b) Tissue localization of GUS activity in the secondary root-branching zone. Bar = 100 μ m. (c) Primary root subapical zone. Bar = 100 μ m. (d) Branching zone between primary and secondary roots. Bar = 100 μ m. (e) Cross-section of a primary root in the subapical zone. Bar = 20 μ m. pr, primary root; sr, secondary root; e, epidermis; c, cortex; vc, vascular cylinder.

specificity than *IRT1*. Likewise, many of the characterized members of the ZIP family of metal transporters are able to transport a variety of cations (Guerinot, 2000). *LelIRT1* and *LelIRT2* are two tomato ZIP proteins whose sequence is

most closely related to *IRT1* and *IRT2* in the ZIP family (Figure 1). These transporters were shown to restore the growth rate of mutant yeast strains affected in Fe^{2+} , Zn^{2+} , Mn^{2+} and Cu^{2+} uptake (Eckhardt *et al.*, 2001). Their pea homologue, *PsRIT1* (Figure 1), can also transport Fe^{2+} , Zn^{2+} and Cd^{2+} in yeast (Cohen *et al.*, 1998).

Cadmium poses a serious threat to human health, and uptake by roots represents the main way in which cadmium enters the plant. Plants grown under iron deficiency are known to accumulate cadmium (Cohen *et al.*, 1998; Yi and Guerinot, 1996). *IRT1* and *AtNRAMP3*, which are both able to transport Cd and are upregulated under iron deficiency, are likely to be responsible for Cd accumulation in plants (Eide *et al.*, 1996; Rogers *et al.*, 2000; Thomine *et al.*, 2000). *IRT2*, however, although its expression is induced under iron deficiency, is unlikely to participate in Cd accumulation. Because of the high homology that exists between *IRT1* and *IRT2* proteins, it may be possible to swap domains between the two proteins in order to pinpoint the residues involved in heavy metal transport. Such a strategy could lead to the engineering of a hybrid transporter with improved substrate selectivity, allowing us to manipulate plant resistance to toxic metals. Pursuing the same goal, a slightly different approach was reported recently in which alteration of the metal-transport selectivity of *IRT1* was obtained following single amino-acid changes (Rogers *et al.*, 2000).

Apart from substrate specificity, understanding how these genes are regulated in response to nutrient availability, as well as determination of the cellular localization of their expression, may help gain insight into their physiological role *in planta*. *IRT2* mRNA accumulates in roots transiently and rapidly after 1 day under iron-deficient conditions. This pattern of expression is consistent with a role in iron uptake. Because our functional complementation studies in yeast suggest that *IRT2* gene encodes a root multispecific transporter, we analysed its pattern of expression *in planta* in response to zinc, copper or manganese deficiency in the culture medium. As opposed to the effect of iron deficiency, none of these growth conditions resulted in variation in *IRT2* expression (data not shown). Furthermore, *IRT2* promoter activity in *A. thaliana* transgenic lines did not increase when plants were grown under zinc deficiency (data not shown).

Although several plant metal transporters have been identified to date (Curie *et al.*, 2000; Curie *et al.*, 2001; Eide *et al.*, 1996; Grotz *et al.*, 1998; Thomine *et al.*, 2000), the results presented here show the first tissue localization of such a transporter. Expression of *IRT2* in root hairs, in epidermis and in the cortex strongly suggests a role in iron uptake from the soil solution. Moreover, *IRT2* expression was restricted to the primary and secondary root subapical zone. *IRT2*, like *IRT1*, transports the Fe^{2+} form of iron.

Therefore its action must be coupled to an acidification and a reduction step. Experiments carried out with cucumber roots indicate that H⁺ net extrusion and ferric reductase activities are clearly localized in the subapical zone of primary and secondary roots (Marschner *et al.*, 1986). Thus these results show that the IRT2 transporter could be part of the physiological response of dicotyledonous plants to iron starvation, as *IRT2* promoter activity co-localizes with the two other known responses of the root: rhizosphere acidification and iron reduction. The presence of an efficient iron uptake system near the tip of the root can easily be explained by the need of the developing root to explore new areas in the soil in search of higher iron concentrations.

Are IRT1 and IRT2 functionally redundant with regard to iron transport in the root? We have shown that *IRT1* and *IRT2* gene expression is restricted to the root and presents the same pattern in response to iron deficiency. The level of expression alone varies greatly between *IRT1* and *IRT2*, the latter being lower by two orders of magnitude (data not shown). It is possible that in the root, IRT1 and IRT2 are not present in the same tissue layer. We present evidence that IRT2 protein is expressed in the most external cell layers (epidermis, root hairs and cortex) of the root subapical zone, but the precise tissue localization of IRT1 in the root remains to be determined. However, it is also possible that both proteins share the same territories of expression in the root, albeit with a slightly different timing of expression, or responding to additional stimuli (including hormones, light, nutritional status, etc.) in order to satisfy different physiological and/or developmental needs. Alternatively, expression of *IRT2*, but not *IRT1*, in iron-replete plants suggests that IRT2 may play more of a housekeeping function in iron accumulation; IRT1, by virtue of its higher level of expression during deficiency, may play a greater role in scavenging available iron under limiting conditions. Finally, the roles of IRT2 and IRT1 *in planta*, as well as the integration of their function in the entire plant, will await the characterization of *A. thaliana* plants mutated in their genes.

Experimental procedures

Cloning of *IRT2* and sequence analysis

Computer databases searches using the *IRT2* EST sequence (Genbank accession number T04324) (Eide *et al.*, 1996) and the BLAST software (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>) identified a BAC clone (BAC clone T16H5, GenBank accession number AL024486) containing the *IRT2* genomic sequence. The *IRT2* coding sequence was cloned by PCR using primers *IRT2-forward* (5'-ataagaatgcggccgcTAATGGCTACTACCAAGCTC-3') and *IRT2-reverse* (5'-atagtttagcggccgcTTAAGCCACACGGCGACG-3'). The PCR experiment was performed using a cDNA library prepared from 15-day-old plantlets (Minet *et al.*, 1992) and the *pfu*

polymerase (Promega, Madison, WI, USA). The PCR product was entirely sequenced, and alignment analysis was performed using CLUSTALW (www2.ebi.ac.uk/clustalw) and BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Intracellular localization was predicted using the PSORT I software (<http://psort.nibb.ac.jp/form2.html>). Note that the *IRT1* cDNA identified by Eide *et al.* (1996) was not full-length. Indeed, another ATG was found 24 bp upstream of the previous one, which is located in a consensus context of translation initiation and therefore represents the likely translation initiation site of *IRT1* mRNA, generating a 347 amino-acid IRT1 protein (Figure 1).

Plasmid construct

Plasmid for expression in yeast

The 1.1 kb *NotI* PCR fragment, corresponding to the open reading frame of *IRT2* cDNA, was subcloned from pBluescript KS(+) into the yeast expression vector pFL61 digested by *NotI* (Minet *et al.*, 1992).

Plasmid for GUS expression analysis

1 kb of the *IRT2* gene 5' sequences located upstream of the ATG start codon were amplified by PCR using the *IRT2*-containing BAC clone T16H5 as a template, the *pfu* DNA polymerase and the oligonucleotides *IRT2* 5' (5'-gggggtacCTTCTCTGACTTTTAAACATCC-3') containing a *KpnI* restriction site and *IRT2* 3' (5'-gtagccatggGTATTGAGATTGTTTTATAATATATG-3') containing an *NcoI* restriction site. The 1 kb *KpnI*-*NcoI* fragment thus obtained was fused to the *uidA* gene open reading frame, encoding the β -glucuronidase (GUS), in the pGUS vector (Eyal *et al.*, 1995). The *KpnI*-*XbaI* fragment containing the *IRT2*-GUS fusion was excised from the resulting plasmid and subcloned in the pBIN19 vector (Bevan, 1984) digested by the same enzymes. The resulting construct was named pBIN19-*IRT2*-GUS.

Yeast experiments

The yeast strains *fet3fet4* DEY1453 (*MATa/MATa ade2/+ can1/can1 his3/his3 leu2 leu2 trp1/trp1 ura3/ura3 fet3-2::HIS3/fet3-2::HIS3 fet4-1::LEU2/fet4-1::LEU2*), *zrt1zrt2* ZHY3 (*MATa ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3*), Δ *smf1* SLY8 (*MATa ade2 his3 leu2 lys2 trp1 ura3 smf1::HIS3*), and wild-type BY4742 (*MATa his3 leu2 lys2 ura3*) were grown in synthetic defined media as described in (Eide *et al.*, 1996), supplemented with 20 g l⁻¹ glucose and necessary auxotrophic supplements. In growth-test experiments, 10 μ l-drops of yeast cultures at an optical density of 0.2, 0.02, and 0.002 were spotted onto synthetic media. These media were supplemented with several concentrations of Fe³⁺-citrate (1 : 20) for *fet3fet4*, ZnCl₂ for *zrt1zrt2*, with 50 mM MES pH 6.0 and 5 mM EGTA for Δ *smf1*, and with 10 μ M CdCl₂ for BY4742, as shown in the legends to Figures 2 and 3. Iron uptake experiments were performed using 2 μ M ⁵⁵FeCl₃ (Amersham, Little Chalfont, Bucks, UK) and 1 mM sodium ascorbate, over a period of 5 min (Eide *et al.*, 1996). In long-term iron accumulation assays, 5 μ M ⁵⁵FeCl₃ was added to a synthetic medium. Accumulation experiments were initiated by addition of 1 \times 10⁶ exponentially growing cells ml⁻¹ assay. The cells were grown for 22 h, filtrated, and counted with a Packard Tri-Carb 2100 TR scintillation counter.

Plant growth conditions

Culture of iron-starved plants

Seeds of *A. thaliana* (Columbia ecotype) were surface-sterilized and grown hydroponically in Magenta boxes in the presence of

sucrose (Touraine and Glass, 1997). Plants were grown for 15–20 days at 20°C under short-day conditions (8 h at 150 $\mu\text{E m}^{-2} \text{s}^{-1}$). The culture medium was regularly changed to allow optimal development. After washing the roots with 2 mM CaSO_4 , plants were transferred to either iron-sufficient (50 μM NaFe-EDTA) or iron-deficient medium (iron omitted), and cultivated as indicated in the legend to Figure 4.

Culture of GUS transgenic plants

Plants were grown on agar plates containing MS/2 medium (Murashige and Skoog, 1962) and 50 $\mu\text{g.ml}^{-1}$ kanamycin. Transformants were transferred after 7 days to either iron-sufficient (50 μM NaFe-EDTA) or iron-deficient medium (100 μM Ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate, Sigma, Saint Louis, MO, USA]) and cultivated for an additional 5 days before GUS assays.

Northern blot analysis

Total RNA was extracted (Lobreaux *et al.*, 1992) from root and shoot fractions of plants grown axenically on either iron-sufficient or iron-deficient media. Samples (14 μg) of RNA were denatured and electrophoresed on a 1.2%–MOPS–formaldehyde agarose gel, prior to being transferred to a nylon membrane (Hybond N+, Amersham). The same blot was sequentially hybridized to each probe in Church buffer (Church and Gilbert, 1984). The blot was first hybridized to the 1.1 kb *IRT2* probe, then stripped and hybridized to the full-length *IRT1* cDNA probe. Washes were performed at 65°C in $0.3 \times \text{SSC}$, 0.1% SDS. Filters were exposed to a PhosphorImager screen (Kodak) for 72 and 24 h for *IRT2* and *IRT1*, respectively, and to an X-O-mat film (Kodak) for 24 h for *IRT1* only. Quantification was performed using the PhosphorImager exposures (Molecular Dynamics, Sunnyvale, CA, USA) relative to the 25S rRNA hybridization signal.

Plant transformation

The MP90 strain of *Agrobacterium tumefaciens* carrying the pBIN19-*IRT2*-GUS construct was used to transform *A. thaliana* (Columbia ecotype) following the floral-dip protocol (Clough and Bent, 1998). Seeds obtained from the primary transformants were germinated on kanamycin and GUS activity was assayed on the resistant plants (F_2).

GUS expression analyses

Quantification of *IRT2* promoter activity

For GUS assays, roots and shoots of 16 kanamycin resistant F_2 lines were harvested separately and ground directly in Eppendorf tubes in 200 μl GUS extraction buffer (Jefferson *et al.*, 1987). GUS activity was measured fluorometrically using 1 mM 4-methylumbelliferyl- β -D-glucuronide (MUG) as substrate (Jefferson *et al.*, 1987). Total protein content of the samples determined according to (Bradford, 1976), was used to correct the GUS activity.

Localization of *IRT2* expression

GUS histochemical staining of 12-day-old plantlets using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide as substrate was carried out overnight to localize the tissue expression of *IRT2*, as described previously in (Jefferson *et al.*, 1987). Stained roots were embedded in hydroxyethylmethacrylate (technovit 7100, Heraeus-Kulzer GmbH, Wehrheim, Germany) prior to realizing thin cross-sections (2 μm) using a Leica RM 2165 microtome. Cross-

sections were counterstained with Schiff dye and observed with a microscope Olympus BH2.

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