The Plant Cell, Vol. 16, 3400–3412, December 2004, www.plantcell.org

The Essential Basic Helix-Loop-Helix Protein FIT1 Is Required for the Iron Deficiency Response

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Regulation of iron uptake is critical for plant survival. Although the activities responsible for reduction and transport of iron at the plant root surface have been described, the genes controlling these activities are largely unknown. We report the identification of the essential gene *Fe-deficiency Induced Transcription Factor 1* (*FIT1*), which encodes a putative transcription factor that regulates iron uptake responses in *Arabidopsis thaliana*. Like the Fe(III) chelate reductase *FRO2* and high affinity Fe(II) transporter *IRT1*, *FIT1* mRNA is detected in the outer cell layers of the root and accumulates in response to iron deficiency. *fit1* mutant plants are chlorotic and die as seedlings but can be rescued by the addition of supplemental iron, pointing to a defect in iron uptake. *fit1* mutant plants accumulate less iron than wild-type plants in root and shoot tissues. Microarray analysis shows that expression of many (72 of 179) iron-regulated genes is dependent on FIT1. We demonstrate that FIT1 regulates *FRO2* at the level of mRNA accumulation and IRT1 at the level of protein accumulation. We propose a new model for iron uptake in Arabidopsis where FRO2 and IRT1 are differentially regulated by FIT1.

INTRODUCTION

Iron is an essential element for most organisms, including plants, where it is required for cellular functions including photosynthesis and respiration. Iron deficiency poses an agricultural challenge because iron is one of the nutrients that most often limits plant growth. Iron deficiency also compromises human health because it is the leading human nutritional disorder worldwide, and plants are the most common source of dietary iron. Therefore, improving the iron content of plants will benefit both agriculture and human health. Engineering plants with increased iron levels requires an understanding of how plants cope with the challenges of acquiring iron from the soil and how these processes are controlled.

Plants overcome iron-deficient growth conditions in one of two ways. Nongraminaceous plants, including *Arabidopsis thaliana*, use the Strategy I response, which consists of the induction of three activities under low iron conditions (Römheld, 1987). A H^+-ATPase extrudes protons into the rhizosphere to lower the pH of the soil, thus making Fe(III) more soluble. The inducible ferric chelate reductase activity of FRO2 reduces Fe(III) to Fe(II) (Robinson et al., 1999), which was recently shown to be the rate limiting step for iron acquisition from the soil (Connolly et al., 2003). Fe(II) is then transported into the plant by IRT1 (Eide et al., 1996), which is the major iron transporter of the plant root (Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002). The grasses (Takagi et al., 1984), as well as species of bacteria and fungi (Guerinot, 1994), use the Strategy II response, which relies on chelation of Fe(III) rather than reduction. Phytosiderophores are released into the soil where they chelate Fe(III) and are then internalized in the iron-bound state via specific transporters (Curie et al., 2001).

Reduction and transport of iron into the plant root are the final steps of iron acquisition in Strategy I plants. Transcripts of the genes responsible for these activities are themselves induced by iron deficiency, providing a primary level of regulation. *FRO2* and *IRT1* transcript levels are undetectable by RNA gel blot analysis when plants are grown under iron sufficient conditions but are greatly induced 24 h after transfer to iron-deficient medium (Connolly et al., 2002, 2003). Protein levels are also under tight control because both FRO2 and IRT1 are subject to posttranscriptional control (Connolly et al., 2002, 2003), signifying the importance of controlling the uptake of this essential yet potentially toxic metal. In addition to the local induction by iron, signals that induce iron deficiency responses include shoot-derived signals as shown by reciprocal grafting experiments (Grusak and Pezeshgi, 1996) and split-root experiments (Schmidt et al., 1996; Schikora and Schmidt, 2001; Vert et al., 2003). The nature of this shoot-derived signal is not yet understood. Iron transport, homeostasis, and signaling have been recently reviewed (Curie and Briat, 2003; Hell and Stephan, 2003).

To date, the only description of a putative transcription factor involved in iron acquisition in plants is that of the *fer* protein in tomato (*Lycopersicon esculentum*). The FER mutant was initially characterized as being unable to induce Strategy I responses under iron deficiency (Brown et al., 1971; Brown and Ambler, 1974). Grafting experiments have shown that the *fer* gene is required in roots but not in shoots (Brown et al., 1971). Recent cloning of the *fer* gene reveals that it encodes a basic helix-loop-helix (bHLH) putative transcription factor (Ling et al., 2002).

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.104.024315.

Using microarray analysis, we have identified a putative bHLH transcription factor, *Fe-deficiency Induced Transcription Factor 1* (*FIT1*), which regulates iron deficiency responses in Arabidopsis. Of the 161 predicted bHLH proteins in Arabidopsis (Heim et al., 2003; Toledo-Ortiz et al., 2003), *FIT1* is the closest homolog to the tomato *fer* gene. Both genes appear to play similar, but not identical, roles in their respective systems (Ling et al., 2002). The superfamily of bHLH transcription factors is conserved from yeast to mammals. bHLHs are the second largest transcription factor family in plants and govern a wide range of biological processes (Riechmann et al., 2000). The conserved bHLH domain consists of ~18 hydrophilic and basic amino acids comprising the basic region, which permits binding to DNA at the hexanucleotide E-box sequence 5′-CANNTG-3′. Two stretches of hydrophobic residues separated by a loop region form two amphipathic α-helices and allow these proteins to form homodimers and/or heterodimers (Voronova and Baltimore, 1990; Toledo-Ortiz et al., 2003).

Here, we report the characterization of the essential gene *FIT1* and describe its role as it relates to iron deficiency responses in Arabidopsis. *FIT1* is required for proper regulation of ferric chelate reductase activity and iron transport into the plant root. This is achieved by regulating the Fe(III) chelate reductase *FRO2* at the level of steady state mRNA accumulation and by controlling protein accumulation of the Fe(II) transporter IRT1. *FIT1* also controls many genes implicated in iron homeostasis as well as many novel genes, as we demonstrate by microarray analysis of a *fit1* mutant.

RESULTS

Identification of *FIT1*

Microarray analysis was used to compare transcript abundance under varying iron conditions in wild-type and *frd3* mutant plants to identify novel iron-regulated genes involved in the uptake and distribution of iron. *frd3* plants exhibit constitutive iron deficiency responses independent of iron supply and may be defective in iron distribution or signaling (Rogers and Guerinot, 2002). The putative bHLH transcription factor *FIT1* was among many genes identified whose message is iron regulated in wild-type roots and deregulated in the *frd3* mutant (our unpublished data). To confirm the microarray data, RNA gel blot analysis was performed showing that *FIT1* message is more highly expressed in iron-deficient roots than in iron-sufficient roots and is undetectable in shoots of wild-type plants (Figure 1A). In *frd3* plants, levels of *FIT1* message are equivalent regardless of iron supply in roots and absent in shoots by RNA gel blot analysis (data not shown).

FIT1 is a putative transcription factor, and predictions of its DNA recognition sequence can be made. The identity of non-conserved amino acids in the basic region of bHLH transcription factors determine the affinity for specific E-box sequences in the promoters of regulated genes, the most common being the G-box 5′-CACGTG-3′ (Robinson et al., 2000). Upon examination of specific residues in the bHLH domain, FIT1 is predicted to belong to a subgroup of bHLHs that recognize the E-box 5′-CANNTG-3′, but not the G-box in the promoters of target genes (Toledo-Ortiz et al., 2003).

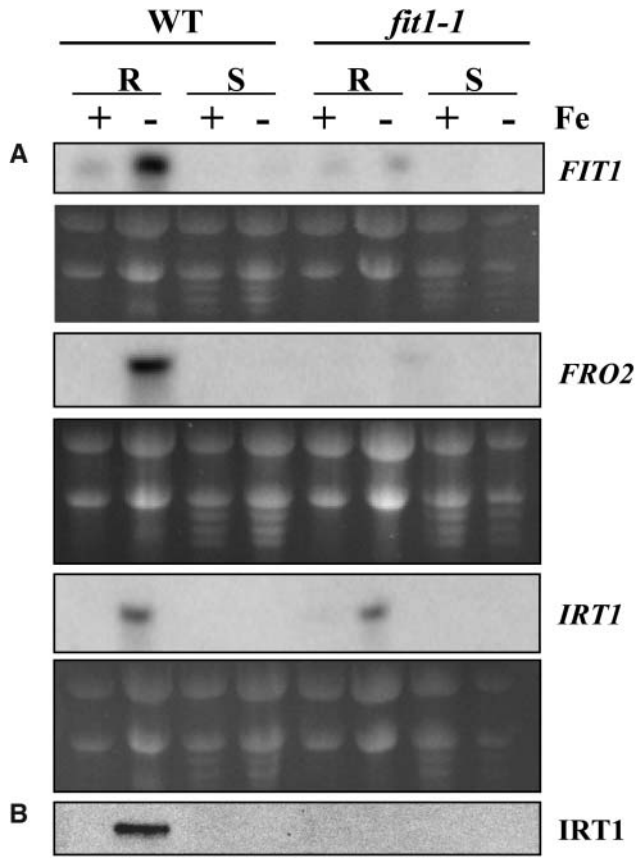

Figure 1. Steady State Levels of mRNA and Protein of Iron Uptake Genes in *fit1-1* Plants.

Wild-type and *fit1-1* plants were grown on B5 plates for 12 d, then transferred to iron sufficient (+) or iron deficient (−) media for 3 d. RNA and total protein samples were prepared from root (R) and shoot (S) tissues.

(A) *FIT1*, *FRO2*, or *IRT1* cDNAs were used to probe individual RNA gel blots, and the corresponding ethidium bromide–stained rRNA is shown as a loading control.

(B) An IRT1 affinity-purified peptide antibody was used to detect IRT1 protein at ~35 kD.

FIT1 is the Arabidopsis bHLH that shares the most homology outside of the bHLH domain with the *fer* gene in tomato, which was recently cloned and described as playing a role in regulating iron uptake (Ling et al., 2002). The FER and FIT1 proteins share 42.5% identity and 72% similarity (Ling et al., 2002). Indeed, these genes appear to have related functions because mutations in these genes result in similar growth phenotypes in planta. However, there are some striking differences between these two genes, such as expression pattern, localization, and effect on expression of iron uptake genes, as we describe below.

Localization of *FIT1* to Iron-Deficient Roots

To identify the regions within the root where *FIT1* mRNA is expressed, transgenic plants expressing the β-glucuronidase

(GUS) reporter gene fused to the 5′ end of *FIT1* under control of the endogenous *FIT1* promoter (*FIT1-GUS*) were analyzed for GUS staining. Seedlings from the four T3 transgenic lines examined showed GUS staining in the outer cell layers of the root as early as day 2 in plants germinated on iron-deficient plates (Figure 2A). GUS staining was observed in the differentiation zone but was absent from the elongation and meristematic zones of plants germinated on iron-deficient plates at days 2, 3, 4, 5, 7, and 9 post-germination (Figure 2B). Strong GUS staining was detected in the lateral roots of day 9 plants (Figure 2C) in a similar pattern as observed with the main root (Figure 2B). Staining of root hairs (Figure 2B) indicates *FIT1* expression in the epidermis. When plants were germinated on iron-sufficient plates, very weak GUS staining was observed only at day 7 and day 9, primarily in the lateral roots (data not shown). To further localize *FIT1* expression, RNA in situ hybridization was performed on longitudinal root sections. Hybridization of a *FIT1* antisense probe to day 7 iron-deficient roots showed that *FIT1* message is present in the outer cell layers (Figure 2D). No signal was observed in sections of day 7 iron deficient roots hybridized with a *FIT1* sense probe (Figure 2E). No signal was observed when sections of iron-sufficient roots were hybridized with either the sense or antisense probes (data not shown). *FIT1* message, like that of the Fe(III) chelate reductase *FRO2* (Connolly et al., 2003) and Fe(II) transporter *IRT1* (Vert et al., 2002), is localized to the outer cell layer of iron-deficient roots, making these iron deficiency response genes potential targets of FIT1 regulation.

FIT1 T-DNA Insertion Lines Indicate a Role for FIT1 in Iron Uptake

A T-DNA insertion line, *fit1-1*, was identified by a PCR-based screening approach from the Arabidopsis Knockout Facility's collection of 60,480 insertion lines (Krysan et al., 1999). A second

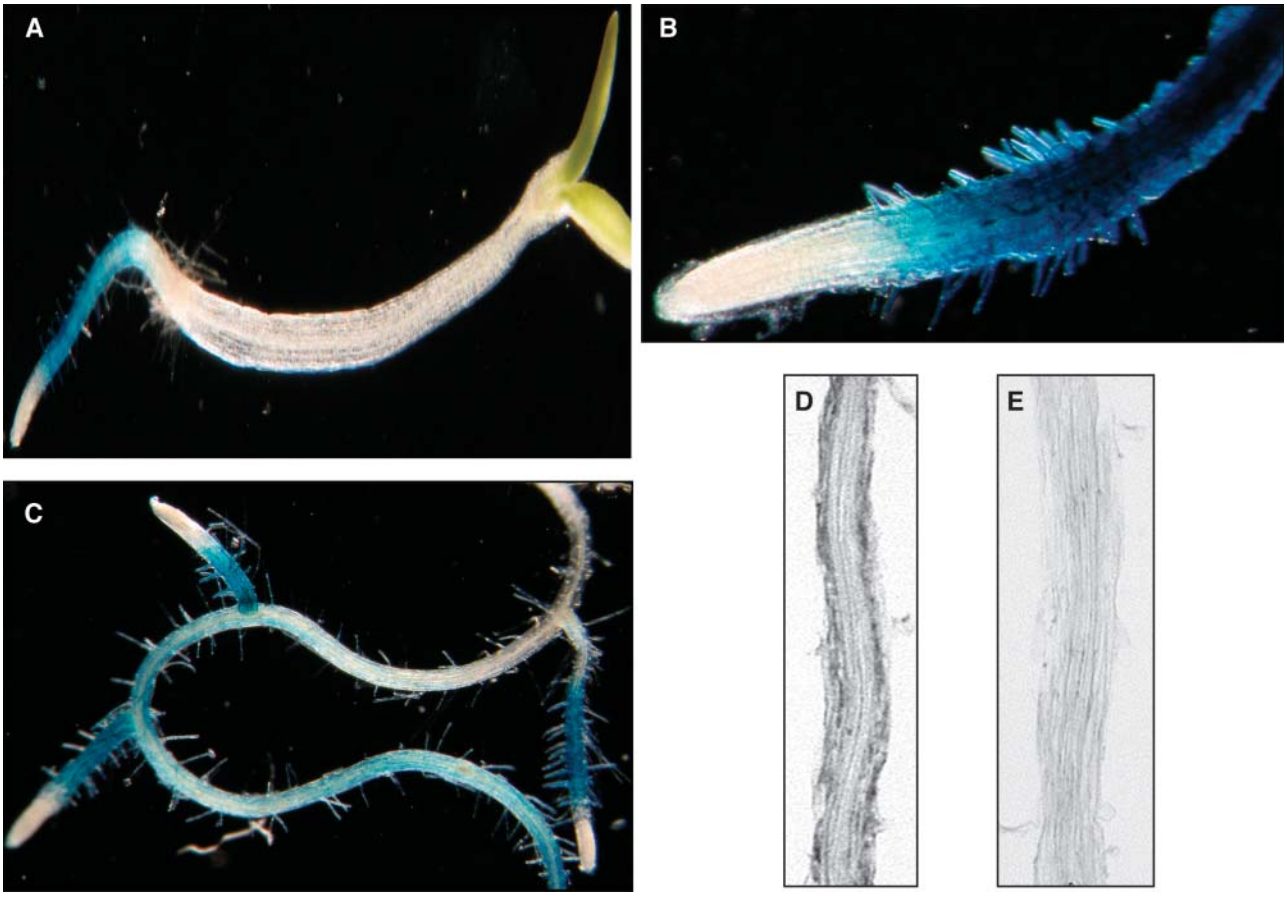

Figure 2. Localization of *FIT1* to Iron-Deficient Roots.

(A) to **(C)** GUS staining of a representative T3 transgenic line expressing a FIT1-GUS translational fusion protein. Plants were germinated directly on iron-deficient plates containing hygromycin (25 mg/mL).
(A) Staining of a day 2 seedling.
(B) The main root of a day 9 seedling.
(C) Main and lateral roots of a day 9 seedling.
(D) and **(E)** In situ hybridization was performed on 10-μm longitudinal root sections of day 7 plants grown on iron-deficient plates using a *FIT1* antisense probe **(D)** or a *FIT1* sense probe **(E)**.

T-DNA insertion line, *fit1-2*, was obtained from the Salk collection of insertion lines (Alonso et al., 2003). The T-DNA is inserted 106 bp upstream of the *FIT1* start codon in the *fit1-1* allele and 70 bp downstream of the start of the third exon in the *fit1-2* allele. RNA gel blot analysis of wild-type Wassilewskija (Ws) and *fit1-1* plants shows that *FIT1* mRNA is greatly reduced in *fit1-1* plants. In wild-type plants, *FIT1* mRNA is abundant in iron-deficient roots, at low levels in iron-sufficient roots, and absent in shoots regardless of iron supply (Figure 1A). In the *fit1-1* background, a very low amount of *FIT1* mRNA is detectable only in iron-deficient roots (Figure 1A). RNA gel blot analysis of *FIT1* in the wild-type Columbia and *fit1-2* backgrounds also shows reduced transcript accumulation in the mutant, and this transcript appears slightly larger than that of the wild type (data not shown).

Because heterozygous *fit1* insertion lines have no visible phenotype, the mutations in *FIT1* are recessive loss-of-function alleles. Homozygous insertion lines of both *fit1* alleles show a severe growth phenotype, indicating that *FIT1* is essential for survival. Disruption of the *FIT1* gene results in lethality at the seedling stage (Figure 3A). *fit1-1* and *fit1-2* seedlings are chlorotic, consistent with iron starvation, are smaller than their wild-type counterparts, and die 2 to 3 weeks post-germination. Watering *fit1* plants with supplemental iron overcomes lethality and permits *fit1* plants to reach the reproductive stage (Figure 3B), suggesting that *FIT1* is required for iron uptake. A genomic fragment consisting of the *FIT1* coding sequence and flanking 5′ and 3′ regions was used to complement the growth phenotype of both *fit1* insertion alleles (*fit1-1*:*FIT1* and *fit1-2*:*FIT1*) to show that lethality is a result of disruption of the *FIT1* gene. Seedling lethality was completely reversed in all 11 independent *fit1-1*:*FIT1* (Figure 3A) and *fit1-2*:*FIT1* T2 transgenic lines. Although a few plants appeared slightly chlorotic compared with the wild type, all plants were much healthier than *fit1* plants, indicating that disruption of *FIT1* is responsible for the observed phenotypes.

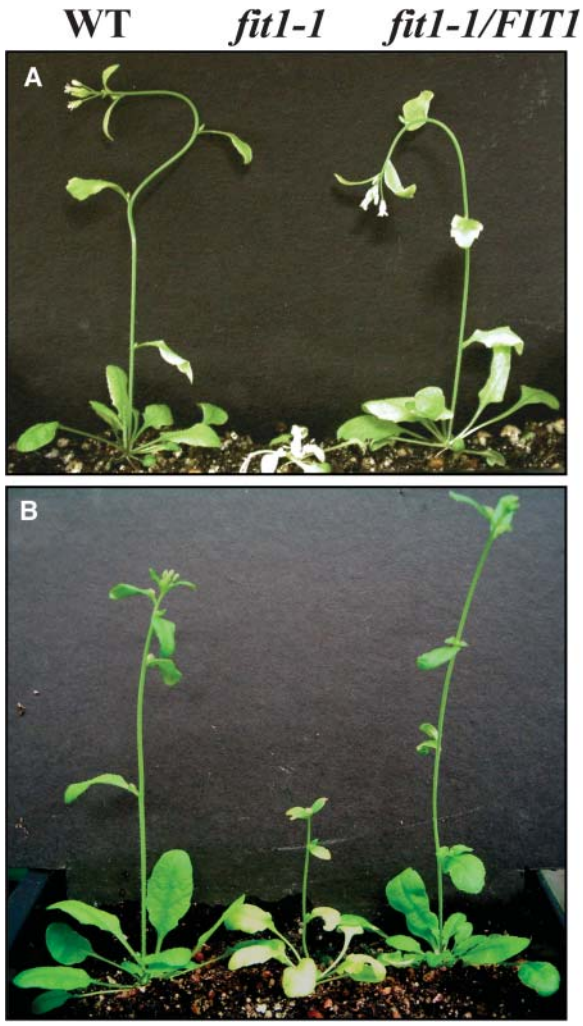

Figure 3. Growth Phenotype, Rescue, and Complementation of *fit1-1* Plants.

Wild-type and *fit1-1* plants were germinated on B5 plates. T2 *fit1-1* transgenic plants complemented with a genomic fragment containing *FIT1* (*fit1-1*:*FIT1*) were selected on B5 medium containing hygromycin (25 mg/mL). After 11 d, wild-type, *fit1-1*, and *fit1-1*:*FIT1* plants were transferred to soil for 3 weeks.

(A) No added iron.

(B) Plants were watered two times per week with 0.5 g/L of Sequestrene as an additional iron source.

Altered Iron Deficiency Responses in the *fit1* Mutant Background

To determine if FIT1 regulates the iron deficiency response genes *FRO2* and *IRT1*, transcripts of these genes were analyzed in the *fit1-1* background by RNA gel blot analysis. *FRO2* mRNA, which is detected in iron-deficient roots of wild-type plants, was not detectable in *fit1-1* plants (Figure 1A), indicating that FIT1 directly or indirectly regulates *FRO2* at the level of mRNA accumulation. The ferric chelate reductase activity of FRO2 was also measured using the ferrozine assay. Ferric chelate reductase activity, which is highly induced in iron-deficient roots of wild-type plants, was not induced under iron deficiency in *fit1-1* plants (Figure 4). This indicates that FRO2 protein activity is abolished in *fit1-1* plants, as expected from the lack of detectable *FRO2* mRNA. On the other hand, *IRT1* transcript was still detectable in the *fit1-1* background by RNA gel blot analysis (Figure 1A). IRT1 protein levels were then examined using an IRT1-specific peptide antibody to determine if IRT1 protein abundance is affected in the *fit1-1* background. IRT1 protein accumulates in iron-deficient roots of wild-type plants, but there was no detectable IRT1 protein in the roots of *fit1-1* plants (Figure 1B). Therefore, FIT1 controls both iron deficiency responses: *FRO2* at the level of mRNA accumulation and IRT1 at the level of protein accumulation.

Elemental Analysis of *fit1-1* Mutant Plants

To determine if *fit1* plants display altered iron accumulation, the iron content of two biological replicates of wild-type and *fit1-1* mutant 15-d-old seedlings was measured by inductively coupled plasma–mass spectrometry. The roots of *fit1-1* plants have an average of 43% less iron than the roots of wild-type plants when

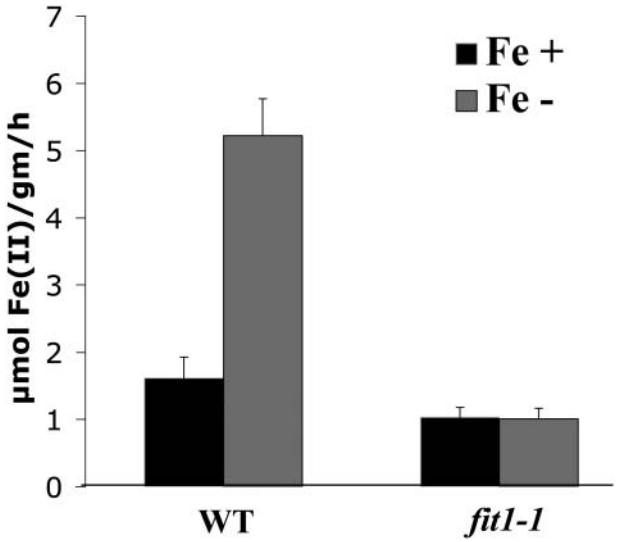

Figure 4. *fit1-1* Plants Lack Inducible Fe(III) Chelate Reductase Activity.

Wild-type and *fit1-1* plants were grown on B5 plates for 12 d, then transferred to iron-sufficient (Fe+) or iron-deficient (Fe−) plates for 3 d. Fe(III) chelate reductase activity of a pool of five plant roots was measured, in triplicate, using the ferrozine assay (Yi and Guerinot, 1996). Error bars indicate standard deviation.

grown under standard B5 conditions, iron-deficient conditions, and iron-sufficient conditions (Figure 5). This result was most pronounced under standard B5 growth conditions where *fit1-1* roots have 51% less iron than wild-type roots. Altered iron accumulation was also evident in the shoots, where *fit1-1* plants have 42% less iron when grown under B5 conditions and 21% less iron when grown under iron-sufficient conditions compared with the wild type (Figure 5). Each of these differences was statistically significant. However, there was no significant difference in shoot iron content when plants were grown under iron-deficient conditions (Figure 5).

35S:*FIT1* Transgenic Plants Have No Obvious Phenotype

Because decreasing the amount of *FIT1* mRNA has dramatic effects on plant survival, we wanted to determine if increasing *FIT1* copy number has an effect on plant growth or the expression of iron deficiency response genes. Transgenic plants expressing *FIT1* cDNA under control of the strong, constitutive 35S promoter were generated, and three independent homozygous single insertion lines were examined in the T4 generation. RNA gel blot analysis shows that *FIT1* mRNA was highly expressed regardless of iron supply in the roots and shoots of all three transgenic lines (Figure 6A). The expression patterns of both *FRO2* and *IRT1* are unchanged compared with the wild type in the three 35S:*FIT1* transgenic lines studied (Figure 6A). IRT1 protein accumulation was also unchanged in 35S:*FIT1* plants compared with the wild type (Figure 6B). There was no significant difference in iron content between wild-type plants (average Fe ppm 77.2) and three independent 35S:*FIT1* transgenic lines (average Fe ppm 79.0, 78.3, and 76.7), also supporting the conclusion that iron deficiency responses are not upregulated in 35S:*FIT1* plants. 35S:*FIT1* plants showed no obvious growth phenotype when grown on standard B5, iron-deficient, or iron-sufficient conditions or when grown on soil.

Identification of Genes under FIT1 Regulation by Microarray Analysis

Our expression analysis has identified *FRO2* as a potential direct target of FIT1 regulation. To identify additional FIT1 targets, we performed microarray analysis to compare expression levels in the roots of wild-type and *fit1-1* plants grown under iron-sufficient and iron-deficient conditions. Of particular interest are those genes that are iron regulated in the wild type and deregulated in the *fit1-1* mutant. Both of these conditions were tested for statistical significance, and genes with a Bayesian P value < 0.05 were studied. Of the 179 genes that are twofold upregulated in response to iron deficiency in wild-type roots, 72 are also deregulated at least twofold in *fit1-1* iron-deficient roots compared with wild-type iron-deficient roots. These 72 genes are grouped based on their regulation by FIT1 and on their predicted function. The signal intensities and fold changes in response to iron supply for the average of two replicates are presented in Table 1. Several genes in this group have previously been shown to be upregulated in response to iron deficiency, including *IRT1* (Eide et al., 1996; Connolly et al., 2002), *IRT2* (Vert et al., 2001; Wintz et al., 2003), *NAS1* (Wintz et al., 2003), and *NRAMP1* (Curie et al., 2000; Thomine et al., 2000).

Most genes, 59 out of 72, showed a near complete loss of iron regulation in the *fit1-1* mutant. We conclude that the increase in

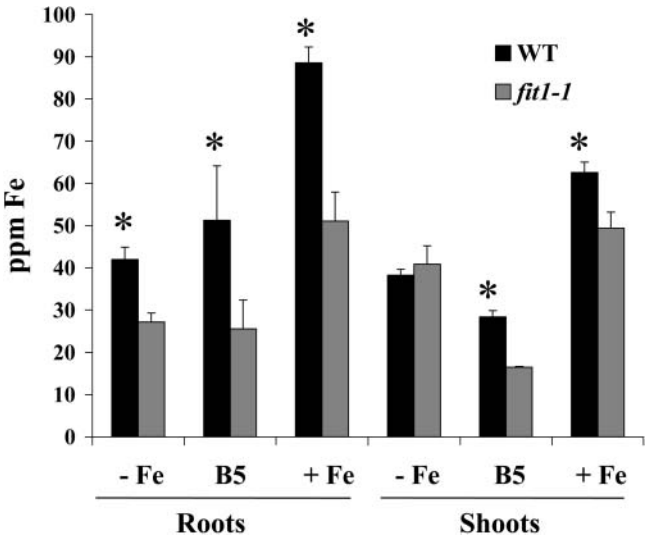

Figure 5. Iron Content of Wild-Type and *fit1-1* Plants.

Wild-type and *fit1-1* plants were grown on B5 plates for 12 d, then either harvested or transferred to iron-sufficient (+Fe) or iron-deficient (−Fe) media for 3 d. Plants were pooled and harvested into root and shoot samples, and two biological sets of tissue were subjected to elemental analysis. Standard deviations were calculated, and statistically significant differences are indicated (*).

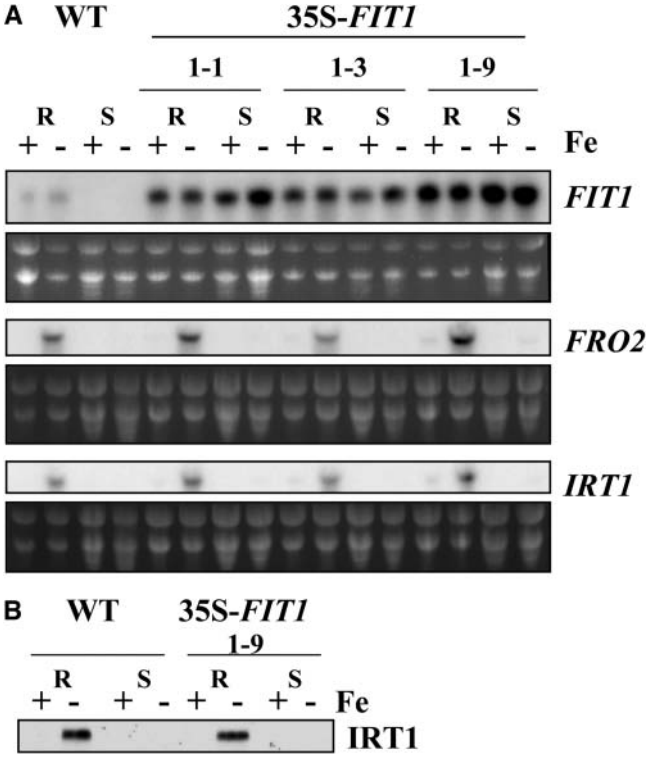

Figure 6. Expression of Iron Uptake Genes in 35S:*FIT1* Plants.

Wild-type plants and three independent homozygous 35S:*FIT1* transgenic lines were grown on B5 plates for 12 d, then transferred to iron-sufficient (+) or iron-deficient (−) media for 3 d.

(A) RNA was prepared from root (R) and shoot (S) tissues. *FIT1*, *FRO2*, or *IRT1* cDNAs were used to probe individual RNA gel blots, and the corresponding ethidium bromide–stained rRNA is shown as a loading control.

(B) Total protein was prepared, and an IRT1 affinity-purified peptide antibody was used to detect IRT1 protein in wild-type plants and a single 35S-*FIT1* line.

expression of these genes under iron deficiency in wild-type plants requires the presence of *FIT1*. Eight of the 72 transcripts retain partial iron regulation in *fit1-1* plants. We propose that FIT1 is involved in the regulation of such genes but is not the only regulatory factor. A third category of genes are those whose expression level is elevated under iron-sufficient conditions in *fit1-1* plants compared with the wild type. Five genes, including *IRT1*, fit this description. One explanation is that these genes are experiencing a relief from negative regulation in the *fit1-1* mutant. FIT1 would normally activate a negative regulator, but in the absence of FIT1, this repression is removed and expression levels are elevated in *fit1-1* plants. However, we favor the explanation that *fit1-1* plants are experiencing iron deficiency, even when grown under iron-sufficient conditions, because of a defect in iron uptake. This is supported by our data that *fit1-1* roots contain less iron than wild-type roots (Figure 5). Therefore, these genes may be responding to the iron-deficient conditions of the *fit1-1* mutant rather than to loss of *FIT1*. *IRT1* shows this expression pattern (elevated transcript levels in *fit1-1* plants compared with the wild type under iron-sufficient conditions). *IRT1* mRNA is known to be regulated by iron (Eide et al., 1996; Connolly et al., 2002; Vert et al., 2003), and we have shown that the IRT1 transcript still responds to iron deficiency in the *fit1-1* mutant (Figure 1). Therefore, *IRT1* would be predicted to fall into the category of genes responding to the iron-deficient conditions of the *fit1-1* mutant rather than to loss of FIT1.

Notably, several genes that are known to be iron regulated are not affected by loss of *FIT1*. RNA gel blot analysis of *NRAMP3* (Thomine et al., 2000) and recent microarray analysis of *FRO3* (Wintz et al., 2003) have shown that these genes are upregulated in response to iron deficiency, yet these transcripts are not affected by loss of *FIT1* (data not shown). Similarly, *Ferritin1* and *Ferritin4* have been shown to be downregulated in response to iron deficiency (Petit et al., 2001a; Wintz et al., 2003). Our analysis also demonstrates regulation of these genes by iron, but this expression pattern is independent of *FIT1* (data not shown). These results demonstrate that only a portion of iron-regulated genes are under FIT1 regulation. *FRO6* and *FRO8* showed very low expression under iron-sufficient and iron-deficient conditions in wild-type and *fit1-1* plants. *FRO2*, which is clearly iron regulated and deregulated in *fit1-1* (Figure 1), is not represented on the ATH1 chip. The remaining four members of the FRO family in Arabidopsis are also not represented on the ATH1 chip.

It is possible that groups of genes with similar expression patterns may be controlled by the same regulator(s). We analyzed 1000 bp of sequence upstream of the translational start of selected FIT1-regulated genes. The promoter regions were searched for occurrences of the bHLH recognition sequence 5′-CANNTG-3′ to determine if such sequences are overrepresented compared with genome-wide noncoding sequences. We used the calculation that noncoding sequences in the Arabidopsis genome have a GC content of 33% to determine if these recognition sequences occurred more frequently than would be predicted by chance (Arabidopsis Genome Initiative, 2000). We examined the upstream regions of 10 transporters, seven transcription factors, and the 20 iron-regulated genes showing the greatest deregulation in *fit1-1* from the list of 72 genes. We found that the bHLH recognition sequence was not significantly overrepresented in the upstream regions of the transcription factor group, the transporter group, or the top 20 genes showing the greatest deregulation in *fit1-1*. However, 35 of the 37 genes in this group contain at least one E-box in the 1000 bp of sequence upstream of the translational start (Figure 7).

DISCUSSION

Homozygous insertion alleles of *fit1* are seedling lethal, but plants can be rescued by watering with supplemental iron (Figure 3A). A similar growth phenotype was previously reported for the *irt1* mutant (Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002), suggesting that FIT1 may regulate IRT1 activity because the two mutants share the same growth phenotype. We show that *fit1-1* plants are unable to induce Fe(III) chelate reductase and Fe(II) transport activities (Figures 1 and 4). *fit1-1* plants accumulate less iron than their wild-type counterparts (Figure 5). However, when *FIT1* copy number was increased in 35S:*FIT1* transgenic plants, no obvious alteration in *FRO2* or *IRT1* expression

Table 1. Summary of Microarray Analysis

Locus Identifier	Wild Type +Fe Baseline Signal	Wild Type −Fe Chip Signal	Fold Change	*fit1-1* +Fe Baseline Signal	*fit1-1* -Fe Chip Signal	Fold Change	Annotation
Iron Regulation Depends Mainly on FIT1							
Transporter							
At5g38820	194	1300	6.8	189	218	1.2	Amino acid transporter family protein
At4g30120	144	794	5.5	162	173	1.1	**HMA3, cadmium-transporting ATPase**
At4g21680	122	570	4.7	120	111	−1.1	Oligopeptide transporter (POT) family
At1g80830	1531	6465	4.2	1592	2339	1.5	**NRAMP1, metal ion transporter**
At3g53480	1740	5282	3.1	1512	1464	−1.0	ABC transporter-like protein
At3g60330	261	780	3.0	313	125	−2.5	AHA7, plasma membrane H^+-ATPase
At4g33020	120	299	2.6	97	68	−1.5	**ZIP9, Fe(II) and Zn transport protein**
Transcription Factor							
At4g09110	50	308	6.2	46	48	1.0	Putative RING-H2 zinc finger protein
At3g13610	2131	8476	4.0	2355	2066	−1.2	Similarity to DNA binding protein zyxin
At2g28160	636	2244	3.5	251	280	1.1	**bHLH29/FIT1**
At5g06490	43	129	3.0	46	53	1.1	C3HC4 RING zinc finger protein-like
At2g20030	89	261	3.0	106	97	−1.1	Putative RING zinc finger protein
Signaling							
At1g34760	128	1097	8.4	153	131	−1.2	14-3-3 Protein
At3g61410	197	710	3.6	267	247	−1.1	Putative protein protein kinase
At2g19410	44	137	3.1	48	50	1.0	Putative protein kinase, Class 1
At1g77280	375	1125	3.0	376	427	1.1	Receptor-like protein kinase, Class 1
At1g51860	308	847	2.8	329	206	−1.6	Receptor-like protein kinase, Class 1
At5g35580	412	983	2.3	431	329	−1.3	Ser/Thr protein kinase-like, Class 1
At5g01060	130	278	2.1	91	79	−1.2	Putative protein kinase, Class 1
Metabolism							
At4g31940	32	1727	55.0	22	24	1.1	Cytochrome P450-like monooxygenase
At3g53280	27	519	19.4	27	27	1.0	Cytochrome P450 71B5
At5g02780	222	4245	19.1	227	415	1.8	Putative protein In2
At3g12900	96	1730	18.5	71	66	−1.1	Hypothetical, similar to oxidoreductases
At3g11750	41	433	10.5	44	56	1.3	Putative dihydroneopterin aldolase
At4g02330	64	398	6.3	50	50	1.0	Hypothetical, similar to pectinesterase
At5g36890	468	2784	6.2	373	369	−1.0	β-Glucosidase
At4g31950	25	118	4.7	23	24	1.0	Cytochrome P450-like monooxygenase
At2g01880	268	1041	4.0	358	385	1.1	Putative purple acid phosphatase
At2g02310	45	171	3.9	49	45	−1.1	Putative phloem-specific lectin
At1g09790	117	441	3.8	115	131	1.1	**Putative phytochelatin synthetase**
At3g47040	247	934	3.7	307	329	1.1	β-D-Glucan exohydrolase-like protein
At5g04950	1425	4951	3.5	1147	980	−1.2	**NAS1, nicotianamine synthase**
At3g47420	223	740	3.3	307	145	−2.1	Putative *sn*-glycerol-3-phosphate permease
At3g21240	1562	4646	3.0	1789	2142	1.2	Putative 4-coumarate:CoA ligase 2
At1g18910	663	1841	2.8	698	716	1.0	Similar to flavonol-induced pollen germ.
At1g60610	371	1025	2.8	361	493	1.4	Similar to *S*-ribonuclease binding protein
At4g29220	754	2027	2.7	871	840	−1.0	Phosphofructo-1-kinase-like
At3g31415	164	419	2.6	178	168	−1.1	Vetispiradiene synthase, putative
At2g05830	698	1761	2.5	693	666	−1.0	Putative translation initiation factor eIF-2B
At2g30670	74	181	2.4	57	74	1.3	Putative tropinone reductase
At2g40000	414	991	2.4	428	468	1.1	Putative nematode-resistance protein
At1g05530	57	133	2.3	53	50	−1.1	Indole-3-acetate β-D-glucosyltransferase
At4g12910	206	483	2.3	250	236	−1.1	Ser carboxypeptidase I precursor-like
At3g54580	876	2049	2.3	1084	592	−1.8	Extensin precursor-like protein
At2g37040	2089	4272	2.1	2145	2021	−1.1	Phe ammonia lyase (PAL1)
At4g38950	119	243	2.1	117	115	−1.0	Kinesin-like protein
At4g14680	288	595	2.1	243	222	−1.1	ATP-sulfurylase
At4g10510	75	154	2.1	59	72	1.2	Subtilisin-like Ser protease
Unknown							
At3g61930	69	2229	32.5	55	54	−1.0	Hypothetical protein
At4g19370	117	1157	9.7	148	199	1.3	Hypothetical protein
At3g58060	68	605	8.9	74	77	1.0	Putative protein
At3g51200	37	274	7.4	34	43	1.3	Putative protein

(Continued)

Table 1. (continued).

Locus Identifier	Wild Type +Fe Baseline Signal	Wild Type −Fe Chip Signal	Fold Change	*fit1-1* +Fe Baseline Signal	*fit1-1* -Fe Chip Signal	Fold Change	Annotation
At1g49820	603	2160	3.6	743	761	1.0	Unknown protein
At5g54790	76	261	3.4	97	72	−1.3	Unknown protein
At3g48450	274	790	2.9	307	375	1.2	Hypothetical protein
At3g06890	261	713	2.8	241	265	1.1	Hypothetical protein
At5g40590	777	1958	2.5	804	577	−1.4	Putative protein
At3g18560	393	907	2.3	473	346	−1.4	Unknown protein
At2g46740	822	1825	2.2	840	768	−1.1	Unknown protein
Iron Regulation Is Partially Dependent on FIT1							
Transporter							
At4g19680	103	2156	21.0	149	335	2.3	**IRT2, Fe(II) transport protein**
At3g46900	133	766	5.8	141	261	1.9	**COPT2, copper transport protein**
Transcription Factor							
At1g56160	75	1756	23.4	92	582	6.3	MYB72
At3g12820	70	790	11.7	94	222	2.4	MYB10
Signaling							
At1g05700	232	687	3.1	231	317	1.4	Putative light repressible receptor kinase,
Metabolism							
At1g14190	115	639	5.6	134	251	1.9	Putative mandelonitrile lyase
At5g47910	733	2763	3.8	785	1091	1.4	RbohD, respiratory burst oxidase protein
Unknown							
At1g73120	148	1548	10.5	78	391	5.0	Hypothetical protein
Baseline Expression Levels Higher in *fit1-1* +Fe versus Wild-Type +Fe							
Transporter							
At4g19690	368	6142	16.3	1224	2516	2.1	**IRT1, Fe(II) transport protein**
At3g58810	355	4257	11.9	1080	1864	1.7	**MTPa2, CDF family**
At5g03570	778	5097	6.6	1441	2041	1.4	**FERROPORTIN2, putative Fe transporter**
Metabolism							
At3g50740	437	3871	8.6	781	907	1.2	UTP-glucose glucosyltransferase-like
Unknown							
At3g07720	904	11666	12.9	3525	4078	1.2	Unknown protein

The locus identifiers are given for genes that are at least twofold upregulated in response to iron deficiency in the wild type and are also at least twofold downregulated in *fit1-1* iron-deficient roots compared to wild-type iron-deficient roots. Seventy-two genes met these two requirements and were found to be statistically significant. Wild-type and *fit1-1* chip signal intensities and fold changes are presented for the average of two biological replicates. Genes are grouped based on their regulation by FIT1 and secondly by predicted function. Genes known to be involved in metal transport or homeostasis are shown in bold.

or IRT1 protein accumulation was observed (Figure 6), and no growth phenotypes were revealed. Although we demonstrate that *FIT1* message is being overexpressed in 35S:*FIT1* plants, it is possible that FIT1 protein levels are not altered in the transgenic lines. Alternatively, overexpression of *FIT1* alone may not be sufficient to alter expression of target genes. This is likely because bHLH transcription factors have been shown to dimerize with other bHLHs and with members of other transcription factor families, such as the MYB family (Goff et al., 1992; Abe et al., 1997; Grotewold et al., 2000), and both partners may be required to affect transcription of target genes. We identified several iron-regulated transcription factors representing a variety of families, including the bHLH and MYB families, by microarray analysis. We will investigate the possibility that FIT1 interacts with one or more of these potential binding partners to regulate iron uptake. However, because only one partner of the dimer needs to be regulated by iron, interacting partners involved in iron homeostasis may not yet be identified.

Because of sequence similarity outside of the bHLH motif, FIT1 is the Arabidopsis protein most closely related to the FER protein of tomato. As described here, the chlorotic and lethal phenotype of *fit1* plants correlates with the description previously given for the *fer* mutant (Brown et al., 1971; Ling et al., 2002). We have shown that *FIT1* message accumulates to higher levels under iron-deficient growth conditions than under iron-sufficient growth conditions in roots (Figure 1A). This is in contrast with the expression pattern of *fer*, which was reported to be independent of the iron supply, although like *FIT1*, *fer* is also expressed in a root-specific manner (Ling et al., 2002). *fit1* and *fer* mutants both display an inability to induce ferric chelate reductase activity under iron deficiency. However, we have shown by RNA gel blot analysis that unlike *FRO2*, *IRT1* message does

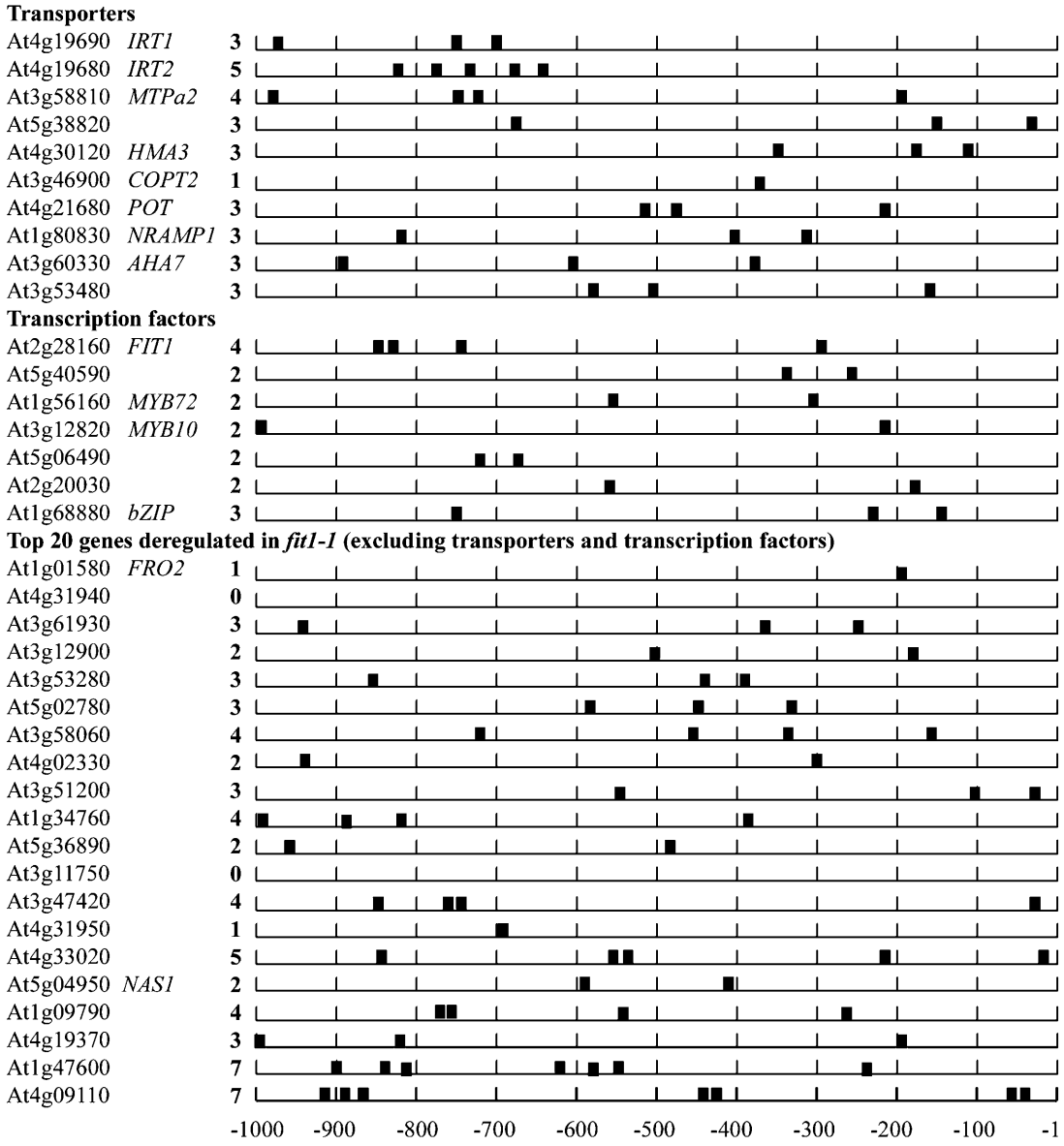

Figure 7. Promoter Analysis of Potential FIT1 Binding Sites.

The promoter regions of 37 iron-regulated genes that are also deregulated in *fit1-1* were analyzed for the occurrence of the E-box motif 5′-CANNTG-3′, which is a potential FIT1 binding site. The number of sites found in each upstream region is indicated to the left of the schematic, which represents 1000 bp of sequence upstream of the translational start. Approximate positions of E-box sites are indicated by filled squares, which are not drawn to scale.

accumulate in the *fit1-1* mutant and that *FIT1* controls IRT1 at the level of protein accumulation (Figure 1). This is in contrast with reports that *Leirt1* mRNA abundance is dependent on the *fer* gene (Ling et al., 2002; Bereczky et al., 2003). The *FIT1* and *fer* genes share similarities and differences in terms of their mRNA localization pattern. *fer* message was not detectable in the epidermal cells of the mature, root hair zone in tomato (Ling et al., 2002). We have shown in Arabidopsis that *FIT1* localizes to root hairs in the differentiation zone by histochemical staining of *FIT1-GUS* transgenic plants (Figure 2B). Because transcripts of the iron deficiency response genes *FRO2* and *IRT1* localize to the outer layers of the root (Vert et al., 2002; Connolly et al., 2003), it is possible that FIT1 is regulating these activities in a more direct manner, whereas FER may govern these activities indirectly, perhaps through another regulator(s). We have demonstrated that *FIT1* is expressed in the outer cell layers of the differentiation zone of wild-type roots (Figures 1 and 2). Our data is supported by large-scale microarray analysis (AtGenExpress, http://www.cbs.umn.edu/arabidopsis/) and expression analysis (Massively Parallel Signature Sequencing database, http://mpss.udel.edu/at/java.html), both indicating *FIT1* expression is highest in roots compared with other tissues. Birnbaum et al. (2003)

describe the localization of gene expression within the Arabidopsis root and report that *FIT1* is more highly expressed in the outer cell layers (epidermis and lateral root cap) than in the stele, endodermis, and cortex (Birnbaum et al., 2003). Expression is also lowest in stage 1 (at the root tip) and highest in stage 3 (higher up the root where root hairs are present) (Birnbaum et al., 2003), similar to our results.

Previous studies in plants have identified promoter elements involved in the regulation by iron. The *cis*-regulatory element, iron-dependent regulatory sequence (IDRS), was identified in the promoter region of Zm*Ferritin1* (Petit et al., 2001b). The IDRS is conserved in At*Fer1* and permits the induction of ferritin by iron by repressing Zm*Fer1* and At*Fer1* when iron levels are low (Petit et al., 2001b; Tarantino et al., 2003). Although the IDRS has been shown to control genes that respond to the presence of iron, it is likely that a distinct mechanism exists for controlling genes that respond to iron deficiency. Recently, two iron deficiency–responsive elements (IDE1 and IDE2) were identified in the promoter of the *IDS2* gene of barley (*Hordeum vulgare*) and shown to be required for iron deficiency–inducible expression of Hv*IDS2* in tobacco roots (Kobayashi et al., 2003). Several iron-inducible genes in barley, rice (*Oryza sativa*), and Arabidopsis contain sequences homologous to IDE1, including At*FRO2* and At*IRT1* (Kobayashi et al., 2003). Because FIT1 belongs to the bHLH family and is predicted to recognize a specific sequence in the promoters of regulated genes, we focused on the occurrence of the E-box motif in genes identified as potential FIT1 targets by microarray analysis. Although promoter analysis of the 10 transporters, seven transcription factors, and the 20 iron-regulated genes showing the greatest deregulation in *fit1-1* revealed that the E-box sequence was not overrepresented in either of these three groups, it is still possible that FIT1 may be a direct regulator in most cases. Thirty-five of the 37 promoters analyzed, including that of *FRO2*, contain at least one E-box sequence (Figure 7), supporting the possibility that they are direct targets of FIT1. Because the E-box sequence will also occur at random, we do not anticipate that all E-boxes identified in Figure 7 will serve as FIT1 DNA binding sites.

Our analysis addresses plant iron deficiency responses on the scale of the whole genome using the ATH1 Affymetrix chip, which represents ~24,000 genes. There have been several microarray studies that have addressed iron deficiency induced changes in gene expression. However, these studies are difficult to use for direct comparison with our data because different experimental growth conditions were employed in each. For example, Thimm et al. used a 6000 cDNA chip and studied expression in older plants of a different ecotype (Landsberg *erecta*) that were grown hydroponically. They identified a set of genes induced under iron deficiency, including several encoding cytochrome P450-like proteins and two encoding zinc finger proteins (Thimm et al., 2001). Although we did not identify these exact genes in our microarray analysis, we also reported cytochrome P450 proteins and zinc finger transcription factors whose transcripts accumulate in response to iron deficiency. A chip representing 8987 rice clones was used to analyze genes responding to iron deficiency in barley roots (Negishi et al., 2002). The *OsNAS1* gene and a zinc finger protein were identified in this way, which is consistent with our findings. In tomato, 1280 mineral nutrition-related genes were analyzed by microarray analysis, and *LeIRT2*, *LeNAS*, and a gene encoding a 14-3-3 protein were found to respond to Pi, K, and Fe deficiencies (Wang et al., 2002). As mentioned in Results, Wintz et al. reported *IRT2* and *NAS1* as being iron regulated using the Affymetrix DNA chip representing 8300 Arabidopsis genes (Wintz et al., 2003).

FRO2 and *IRT1* messages are coordinately regulated in response to iron. In plants transferred from iron-sufficient to iron-deficient conditions, both transcripts are detectable by RNA gel blot within 24 h, with transcript levels peaking 3 d after transfer (Connolly et al., 2002, 2003). When plants grown under iron-deficient conditions are transferred to iron-sufficient conditions, *FRO2* and *IRT1* mRNA levels quickly decrease and are undetectable by RNA gel blot 24 h after transfer (Connolly et al., 2002, 2003). Because these two genes with related activities have such similar expression patterns, it seems likely that they may be activated by the same regulator. However, our findings that FIT1 regulates *FRO2* at the level of mRNA accumulation and IRT1 at the level of protein accumulation suggests that regulation of *FRO2* and *IRT1* is more complicated than previously thought. Because *fit1-1* mutants die at the seedling stage and they lack *FRO2* mRNA and IRT1 protein, we can conclude that FIT1 plays a significant role in iron homeostasis. *FRO2* has been demonstrated to serve as the rate limiting step in iron acquisition (Connolly et al., 2003). Thus, its ability to regulate *FRO2* makes FIT1 an important factor in iron uptake. Although it is possible that FIT1 binds directly to the *FRO2* promoter to regulate transcription, there is likely an additional regulatory step(s) between FIT1 and *IRT1*. We now need to determine what regulators of *IRT1* exist downstream of FIT1. Previous studies have shown that IRT1 is subject to posttranscriptional control. Transgenic plants expressing *IRT1* under control of the 35S promoter accumulate *IRT1* mRNA in both roots and shoots under iron-sufficient and -deficient conditions, but IRT1 protein only accumulates in iron-deficient roots (Connolly et al., 2002). IRT1 protein could be controlled posttranslationally by ubiquitination and endocytosis. Ubiquitin-mediated protein turnover of ZRT1, a metal transporter belonging to the same family as IRT1, has been demonstrated in yeast and is dependent on a critical Lys

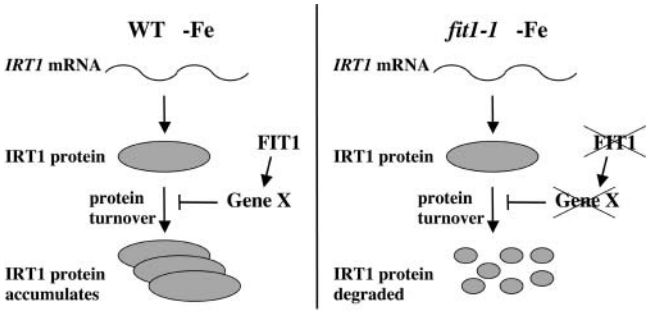

Figure 8. Model of IRT1 Protein Regulation by FIT1.

FIT1 may prevent IRT1 protein turnover through inhibition of an unknown factor (product of Gene X), allowing IRT1 protein to accumulate under iron-deficient conditions. Failure to activate Gene X in *fit1-1* plants would prevent IRT1 protein accumulation.

residue in the variable loop region (Gitan and Eide, 2000). IRT1 also contains Lys residues in the analogous region, which could serve as ubiquitination sites to mediate protein degradation (Connolly et al., 2002). One explanation for the loss of IRT1 in *fit1-1* plants is that FIT1 regulates a factor(s) involved in IRT1 protein turnover. A model depicting IRT1 protein regulation by FIT1 is presented in Figure 8. FIT1 may negatively regulate IRT1 protein turnover. In wild-type roots under iron-deficient conditions, IRT1 protein turnover could be inhibited by Gene X, which is positively regulated by FIT1. Therefore, in the *fit1-1* mutant, Gene X is not activated by FIT1, so IRT1 protein turnover ensues.

Finally, *FIT1* itself is iron regulated, so we will need to look for other iron-regulated transcription factors that control *FIT1* and may directly regulate *IRT1*. Upstream of *FIT1*, we would also expect to find an iron sensor that itself is not affected by iron status but can sense iron levels and communicate this message through the activation or repression of downstream targets.

METHODS

Identification of *FIT1* Loss-of-Function Mutants

The *FIT1* locus identifier is At2g28160 and was named *AtbHLH029* as reported (Heim et al., 2003). A *FIT1*-specific primer 5′-CAACAA-TCTCGGTTACATCATCACTAGAA-3′ and a T-DNA–specific primer 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′ were used to screen the Arabidopsis Knockout Facility's collection of T-DNA insertion lines (Ws ecotype) by PCR (Krysan et al., 1999). The *fit1-1* allele was identified and confirmed by DNA gel blot hybridization. The insertion site was confirmed by DNA sequencing using the T-DNA–specific primer. The *fit1-2* insertion mutant (Columbia-0 ecotype) was obtained from the Salk collection (Alonso et al., 2003). Both seed stocks were obtained from the ABRC (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm).

Plant Materials and Growth Conditions

Seeds were surface sterilized in 95% ethanol followed by gentle shaking in 25% bleach/0.2% SDS for 20 min. Seeds suspended in 0.15% agar were placed in the dark at 4°C for 2 to 4 d, then plated on Gamborg's B5 medium (Sigma, St. Louis, MO) with 2% sucrose, 1 mM Mes, and 0.7% agar, pH 5.8. At the four- to six-true-leaf stage, seedlings were transferred to iron-sufficient plates containing 50 μM Fe(III)-EDTA or iron-deficient plates containing 300 μM ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate] (HACH Chemical, Ames, IA) for 3 d. These media also contain macronutrients and micronutrients (Marschner et al., 1982), 0.7% agar and 1 mM Mes, pH 6.0. Plants were grown at 21°C under constant light (~90 $\mu E \cdot m^{-2} \cdot s^{-1}$) under a yellow filter (acrylic yellow-2208; Cadillac Plastic and Chemical, Pittsburgh, PA) to protect the Fe(III)-EDTA from photochemical degradation (Hangarter and Stasinopoulos, 1991). Plants used for in situ hybridization studies were germinated directly on iron-deficient or iron-sufficient plates and grown vertically. Plants used for GUS histochemical staining were germinated directly on iron-deficient or iron-sufficient plates. Ferric chelate reductase assays were performed as previously described (Yi and Guerinot, 1996). Pools of five plants were analyzed in triplicate, and standard deviations were calculated. Soil-grown *fit1* plants were watered with 0.5 g/L of Sequestrene (Helena Chemical, Spartanburg, SC) two times per week during the seedling stage. At the reproductive stage, every other watering was supplemented with 0.5 g/L of Sequestrene.

Plasmid Construction and Plant Transformation

35S:FIT1 Fusion

The *FIT1* cDNA, clone RZ108e05 (Asamizu et al., 2000), was subcloned into pGEM-TEasy (Promega, Madison, WI). The cDNA was excised using *Bam*HI and cloned into the *Bam*HI site of pCGN18. pCGN18 was the gift of T. Jack (Department of Biological Sciences, Dartmouth College, Hanover, NH; Connolly et al., 2002). The construct was moved into *Agrobacterium tumefaciens* strain ASE and transformed into wild-type Columbia plants. All plant transformations were done by the floral dip method (Clough and Bent, 1998).

FIT1-GUS Fusion

A 1924-bp PCR fragment was amplified from Ws genomic DNA using 5′-CGGGATCCCAACACCTAGATGGAATC-3′ and 5′-AACACTGCATC-TCCAACAATCCATGC-3′ primers and subcloned into pGEM-T Easy (Promega). A fragment containing 1333 bp of sequence upstream of the *FIT1* translational start and the 5′ 435 bp of coding sequence was excised by *Hin*dIII and *Bam*HI digestion and cloned into pCAMBIA1381Xa (GenBank accession number AF234303) at the *Hin*dIII and *Bam*HI sites creating an in-frame translational fusion to the *gusA* gene that was confirmed by DNA sequencing. The construct was moved into Agrobacterium strain GV3101 and transformed into wild-type Ws plants.

fit1 Complementation

A 2722-bp fragment containing the *FIT1* coding sequence, 1140-bp sequence upstream of the translational start, and 386-bp downstream of the stop codon was amplified by PCR with engineered *Xba*I sites and cloned into the *Xba*I site of pCAMBIA1300 (GenBank accession number AF234296). The plasmid was moved into Agrobacterium strain GV3101 and transformed into *fit1-1* and *fit1-2* plants.

Gel Blot Hybridization

Root and shoot tissues were harvested from plants grown under iron-sufficient or iron-deficient conditions. Total RNA was prepared by hot phenol extraction and treated with glyoxal (McMaster and Carmichael, 1977). Ten micrograms of total RNA was separated on a 1.2% agarose gel in 10 mM $NaPO_4$, transferred to a nylon membrane, and UV cross-linked. Hybridizations were performed at 42°C in 50% formamide according to standard procedures (Ausubel et al., 2004). Individual blots were probed with ^{32}P-labeled *FIT1*, *FRO2*, or *IRT1* cDNAs. Blots were visualized after 2 to 16 h exposure on a Typhoon Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA).

Immunoblot Analysis

Immunoblot analysis was performed as previously described (Connolly et al., 2002). Briefly, total protein was prepared from plants grown under iron-sufficient or iron-deficient conditions as described above. Approximately 10 μg of protein was separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Membranes were blocked, incubated overnight with affinity-purified IRT1 peptide antibody, washed, and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase for 1 h. Chemiluminescence was performed using a Western Lightning kit (Perkin-Elmer, Boston, MA).

Elemental Analysis

Wild-type and *fit1-1* mutant plants were grown on standard B5, iron-sufficient, or iron-deficient plates. Root and shoot tissues were harvested and dried overnight in a 65°C oven. Elemental analysis was performed

using inductively coupled plasma spectroscopy at Purdue University as described (Lahner et al., 2003). Standard deviations were calculated for two biological replicates.

In Situ Hybridization

The *FIT1* cDNA clone RZ108e05 (Asamizu et al., 2000) cloned into pBluescript II SK− at the *Eco*RI and *Xho*I sites (*FIT1*-SK) was used to generate sense and antisense probes for in situ hybridization. Probes were labeled with digoxigenin-11-UTP (Roche Diagnostics, Indianapolis, IN). Linearization of *FIT1*-SK was performed by *Apa*I digestion followed by transcription with T3 polymerase to obtain the sense probe. Linearization with *Sma*I followed by transcription with T7 polymerase was used to create the antisense probe. Tissue samples were fixed and embedded as described (Di Laurenzio et al., 1996), with the exception that after the 70% ethanol dehydration step, the tissue was embedded in 1% agarose. In situ hybridization was performed as previously described (Long et al., 1996; Long and Barton, 1998; Vert et al., 2002; Connolly et al., 2003).

GUS Histochemical Staining

GUS histochemical staining was performed on four independent T3 transgenic lines at day 4 and day 7 growth on iron-deficient and iron-sufficient plates containing hygromycin (25 mg/mL). One representative T3 line was examined under the same conditions at several time points between day 1 and day 9. Seedlings were incubated with the substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide as described (Jefferson et al., 1987).

Microarray Analysis

Total RNA from two biological replicates was reverse transcribed, labeled, and hybridized to individual ATH1 Affymetrix chips at the UCI DNA Array Core Facility (Irvine, CA). Expression data was analyzed using GeneTraffic (Iobion Informatics, La Jolla, CA). Determination of statistical significance was performed using the Cyber-T statistics program (http://visitor.ics. uci.edu/genex/cybert/).

ACKNOWLEDGMENTS

The authors thank Brett Lahner and David Salt for performing elemental analysis, Carol Ringelberg for help with microarray analysis, Arijit Chakravarty for discussion of promoter analysis, Sara Thiebaud for experimental assistance, and Rob McClung for critical reading of the manuscript. This work was supported by a National Science Foundation Plant Genome grant (0077378-DBI) to M.L.G.

Received May 18, 2004; accepted September 18, 2004.

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