Overexpression of the FRO2 Ferric Chelate Reductase Confers Tolerance to Growth on Low Iron and Uncovers Posttranscriptional Control[1]

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The Arabidopsis *FRO2* gene encodes the low-iron-inducible ferric chelate reductase responsible for reduction of iron at the root surface. Here, we report that *FRO2* and *IRT1*, the major transporter responsible for high-affinity iron uptake from the soil, are coordinately regulated at both the transcriptional and posttranscriptional levels. *FRO2* and *IRT1* are induced together following the imposition of iron starvation and are coordinately repressed following iron resupply. Steady-state mRNA levels of *FRO2* and *IRT1* are also coordinately regulated by zinc and cadmium. Like *IRT1*, *FRO2* mRNA is detected in the epidermal cells of roots, consistent with its proposed role in iron uptake from the soil. *FRO2* mRNA is detected at high levels in the roots and shoots of *35S-FRO2* transgenic plants. However, ferric chelate reductase activity is only elevated in the *35S-FRO2* plants under conditions of iron deficiency, indicating that *FRO2* is subject to posttranscriptional regulation, as shown previously for *IRT1*. Finally, the *35S-FRO2* plants grow better on low iron as compared with wild-type plants, supporting the idea that reduction of ferric iron to ferrous iron is the rate-limiting step in iron uptake.

Iron is an essential element for plants. It functions to accept and donate electrons and thus serves as an important cofactor for a number of metalloenzymes involved in respiration and photosynthesis. Although iron is abundant in the earth's crust, it is often unavailable to plants because it tends to form insoluble ferric hydroxide complexes in aerobic environments at neutral or basic pH (Guerinot and Yi, 1994). In addition to the solubility problem, the chemical properties of iron require cells to place limitations on its accumulation. Fe(II) and Fe(III) act catalytically to generate hydroxyl radicals that can damage cellular constituents such as DNA and lipids (Halliwell and Gutteridge, 1992). Thus, iron uptake (Eide et al., 1996; Robinson et al., 1999; Connolly et al., 2002) and storage (Lescure et al., 1991; Briat and Lobréaux, 1997; Wei and Theil, 2000) are carefully regulated processes.

Dicots and non-grass monocots employ the strategy I response to mobilize iron from the soil (Marschner and Römheld, 1994). This response includes release of protons and subsequent acidification of the rhizosphere, which serves to drive more Fe(III) into

solution, reduction of Fe(III) to Fe(II) at the root surface, and transport of Fe(II) across the root epidermal cell membrane. We have previously identified the genes in Arabidopsis that encode the iron-regulated ferric chelate reductase (*FRO2*) and the ferrous iron transporter (*IRT1*) that function to take up iron from the soil as part of the strategy I response (Eide et al., 1996; Yi and Guerinot, 1996; Robinson et al., 1999; Vert et al., 2002). *FRO2* is predicted to encode a polypeptide of 725 amino acids, with conserved FAD- and NADPH-binding sites. FRO2 has six hydrophobic domains within its amino-terminal region along with two additional carboxyl-terminal hydrophobic domains, all of which are predicted to form trans-membrane helices. This suggests that an intracellular region of FRO2, which contains the deduced cofactor-binding sites, is anchored at both ends by membrane-spanning regions. Two pairs of His residues that are thought to coordinate heme in FRO2 lie on two similarly oriented trans-membrane helices and are in equivalent locations to His residues in superfamily members FRE1 and gp91phox (Robinson et al., 1999). The *FRO2* gene belongs to an eight-member gene family in Arabidopsis; the other seven *FRO* genes have not yet been functionally characterized.

Here, we report the results of studies aimed at further characterizing expression of *FRO2*. Previously, we showed that *IRT1* expression is induced when plants are transferred to iron-deficient medium and is repressed upon iron resupply (Connolly et al., 2002). In this report, we show coordinate control of the expression of *FRO2* and *IRT1*. Because previous

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work suggested that reduction of ferric iron to ferrous iron by ferric chelate reductase is the rate-limiting step in iron uptake (Grusak et al., 1990), we hypothesized that overexpression of *FRO2* in transgenic plants might lead to enhanced iron accumulation. *35S-FRO2* transgenic plants do not accumulate iron when compared with wild type; analysis of these plants revealed that *FRO2* is subject to posttranscriptional regulation by iron, as previously shown for *IRT1* (Connolly et al., 2002). However, *35S-FRO2* plants do display tolerance to growth on low-iron medium. This result suggests that expression of *FRO2* in crop plants may yield lines with improved iron efficiency.

RESULTS

Coordinate Regulation of *FRO2* and *IRT1* by Iron and Zinc

FRO2 mRNA is detected in the roots of Arabidopsis plants after 3 d of growth on iron-deficient medium (Robinson et al., 1999). Previously, we examined the induction of *IRT1* expression over time and showed that *IRT1* mRNA is detectable in roots 24 h after plants are transferred to iron-deficient media (Connolly et al., 2002). *IRT1* mRNA levels peak 3 d after plants are transferred to iron-deficient media. Because strategy I plants require reduction of iron before transport, we reasoned that expression of *FRO2* and *IRT1* would either be coordinately controlled or that the induction of *FRO2* expression would precede the induction of *IRT1* expression. We performed RNA gel-blot analysis using the *FRO2* cDNA as a hybridization probe; this analysis revealed that the induction of *FRO2* expression exactly parallels that of *IRT1* (Fig. 1). Moreover, after the transfer of plants from iron-deficient medium to iron-sufficient medium, both *FRO2* and *IRT1* mRNA levels decline rapidly (Fig. 2). The two transcripts are undetectable 24 h after iron resupply.

In addition, we previously showed that *IRT1* transcript abundance is affected by zinc (Connolly et al., 2002). It is logical that *IRT1* expression is repressed when high amounts of zinc are present in the medium, because accumulation of zinc is toxic and IRT1 mediates zinc transport (Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2002). FRO2 is not known to play a role in zinc uptake or homeostasis. However, because *FRO2* and *IRT1* are coordinately regulated by iron, we wondered whether the coordinate control would extend to other metals. Both *FRO2* and *IRT1* were detected in the roots of plants grown for 3 d on either iron-deficient medium or iron-deficient medium supplemented with 100 μM zinc (Fig. 3). However, neither transcript was detectable in roots when plants were grown on iron-deficient medium supplemented with 500 μM zinc, confirming that zinc mediates coordinate control of *FRO2* and *IRT1* transcript abundance. Although 500
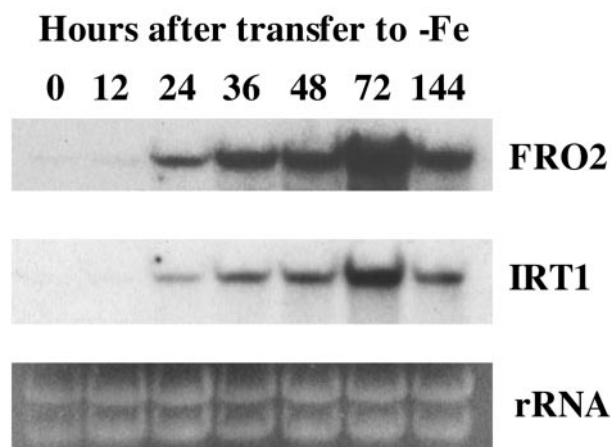

Figure 1. Time course of *FRO2* and *IRT1* mRNA abundance patterns in roots exposed to iron deficiency. Wild-type plants were grown for 14 d on standard medium and transferred to iron-deficient medium. Roots were harvested 0, 12, 24, 36, 48, 72, and 144 h after the transfer to iron-deficient medium. RNA was isolated from the samples and used to prepare RNA gel blots. The blots were hybridized with radiolabeled *FRO2* and *IRT1* cDNA probes. Ethidium bromide-stained rRNA is shown as a control for loading.

μM zinc does cause zinc toxicity, previous work with *IRT1* showed that the decrease in *IRT1* mRNA transcript abundance after zinc exposure is dependent upon the *IRT1* promoter (Connolly et al., 2002). Similar experiments with cadmium confirm that high levels of cadmium in the growth medium also mediate coordinate repression of *FRO2* and *IRT1* expression (data not shown). Together, these results show
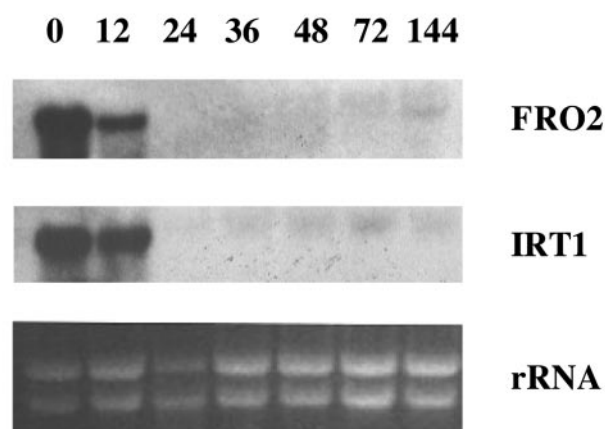

Figure 2. Time course of *FRO2* and *IRT1* mRNA abundance patterns in roots after iron resupply. Wild-type plants were grown for 14 d on standard medium, transferred to iron-deficient medium for 3 d, and then transferred a second time to iron-sufficient medium. Roots were harvested 0, 12, 24, 36, 48, 72, and 144 h after the second transfer. RNA was isolated from the samples and used to prepare RNA gel blots. The blots were hybridized with radiolabeled *FRO2* and *IRT1* cDNA probes. Ethidium bromide-stained rRNA is shown as a control for loading.

500 μM Zn   -   -   +   -   +
100 μM Zn   -   -   -   +   -
Fe   +   -   +   -   -

FRO2

IRT1

rRNA
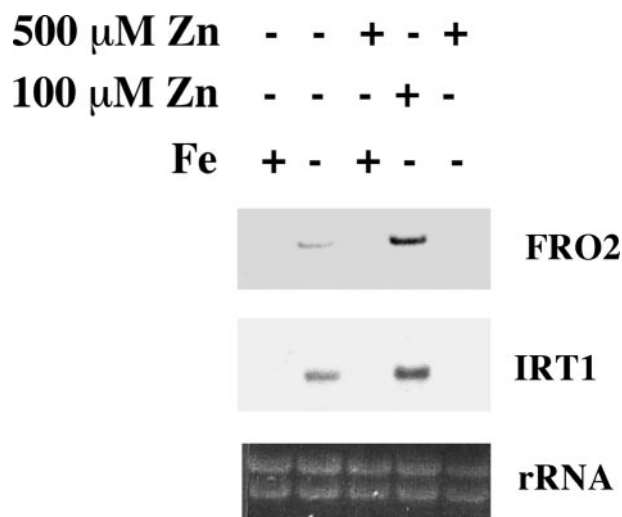
Figure 3. Coordinate control of *FRO2* and *IRT1* expression by zinc. Wild-type plants were grown for 14 d on standard medium. Plants were subsequently transferred and grown for 3 d on iron-sufficient medium (lane 1), iron-deficient medium (lane 2), iron-sufficient medium supplemented with 500 μM zinc (lane 3), iron-deficient medium supplemented with 100 μM zinc (lane 4), or iron-deficient medium supplemented with 500 μM zinc (lane 5). RNA was isolated from roots and used to prepare RNA gel blots. The blots were hybridized with radiolabeled *FRO2* and *IRT1* cDNA probes. Ethidium bromide-stained rRNA is shown as a control for loading.

FRO2 Is Expressed in the Root Epidermis and Flowers

Next, we investigated the localization of *FRO2* mRNA in the roots of iron-deficient plants. Previous studies suggested that FRO2 functions in iron uptake from the rhizosphere (Robinson et al., 1999; Yi and Guerinot, 1996). *IRT1* is known to be expressed in epidermal root cells of iron-deficient plants (Vert et al., 2002). For these reasons, we hypothesized that *FRO2* expression would be observed in the root epidermis. In situ hybridization analysis with a *FRO2* antisense probe on longitudinal sections of iron-deficient roots showed that *FRO2* mRNA is present in the epidermal layer (Fig. 4A). Hybridization of a sense probe to similar sections yielded no detectable signal (Fig. 4B). These results are consistent with the proposed role of FRO2 in iron uptake from the soil and further support coordinate regulation of *IRT1* and *FRO2*.

We constructed transgenic plants in which the *FRO2* promoter drives expression of the *uidA* gene, which encodes β-glucuronidase (GUS). Histochemical staining of 10 independent transgenic lines demonstrated that the *FRO2* promoter is much more active in iron-deficient roots than in iron-sufficient roots and is highest in lateral roots of iron-deficient plants (data not shown). GUS activity was observed
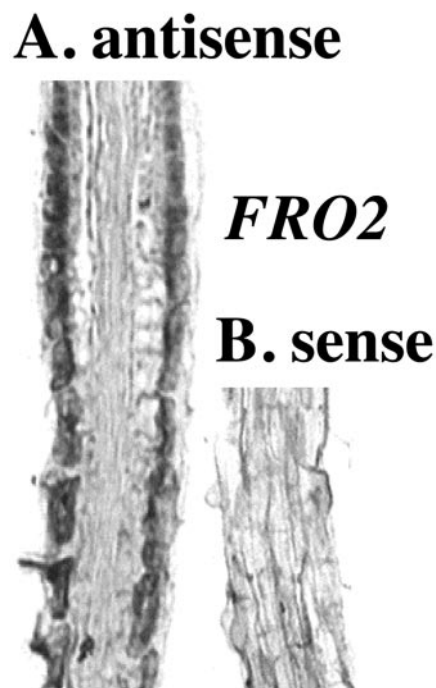

A. antisense

FRO2

B. sense

Figure 4. Localization of the *FRO2* transcript in longitudinal sections of iron-deficient roots. A, In situ hybridization experiments were performed on longitudinal sections (10 μM) of iron-deficient roots using a *FRO2* antisense probe. B, In situ hybridization experiments were performed on longitudinal sections (10 μM) of iron-deficient roots using a *FRO2* sense probe.

in the outer tissues of iron-deficient roots including root hairs and epidermal cells (data not shown); this data confirms the results obtained with in situ hybridization.

The *FRO2* promoter is active in flowers (Fig. 5A). Histochemical staining of *FRO2-GUS* flowers revealed GUS activity in the anther filaments (Fig. 5A).
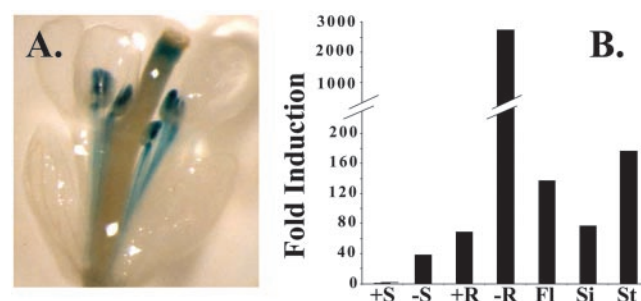

Figure 5. *FRO2* is expressed in flowers. A, Histochemical staining of flowers from *FRO2-GUS* transgenic plants grown in soil. A representative individual is shown. No significant GUS activity was detected in wild-type plants (data not shown). B, Real time RT-PCR analysis of *FRO2* expression in various tissues. Plants were grown for 14 d on standard medium and then transferred to either iron-deficient or -sufficient medium as indicated or were grown in soil (flowers, siliques, and stems). *FRO2* values were normalized and expressed as -fold induction relative to +Fe shoots. Due to the ratio calculation, SDs are not shown in this figure.

that the expression of two genes that are required for iron uptake from the soil are coordinately regulated by metals.

The *IRT1* promoter also is active in filaments (Vert et al., 2002). In addition, we observed *FRO2* promoter activity in the anthers and the style (Fig. 5A). To confirm *FRO2* expression in flowers, we examined *FRO2* transcript abundance in various tissues using real time reverse transcriptase (RT)-PCR. *FRO2* transcript abundance is greatest in iron-deficient roots, but *FRO2* mRNA also is detected in other tissues tested (Fig. 5B). Our results suggest a role for FRO2 in the delivery of iron to developing pollen grains.

Characterization of *35S-FRO2* Plants

Previous studies suggested that reduction of ferric iron to ferrous iron, as opposed to transport of ferrous iron, is the rate-limiting step for iron uptake (Grusak et al., 1990). Thus, we reasoned that overexpression of *FRO2* in transgenic plants would lead to enhanced iron uptake and accumulation. We used the cauliflower mosaic virus *35S* promoter to drive expression of *FRO2* in Arabidopsis and identified six independent, homozygous transgenic lines. We examined *FRO2* steady-state RNA levels in all six lines; the results for three lines are shown in Figure 6. RNA was isolated from the roots and shoots of T5 seedlings grown for 3 d on either iron-sufficient or -deficient medium. In wild-type plants, *FRO2* mRNA was detected only in the roots of iron-deficient plants, as has been shown previously (Fig. 6). In contrast, all three transgenic lines showed altered accumulation of *FRO2* mRNA. Lines 11E and 15G display high levels of *FRO2* mRNA in roots and shoots of iron-deficient and -sufficient plants. Line 5A accumulates lower, but detectable, levels of *FRO2* mRNA in iron-sufficient and -deficient shoots as well as in iron-sufficient roots. Quantification of *FRO2* mRNA levels in iron-deficient roots showed that lines 5A, 11E, and 15G have 2.1, 1.9, and 2.3 times more *FRO2* mRNA, respectively, than wild type. Three additional *35S-FRO2* transgenic lines examined contained detectable levels of *FRO2* mRNA only in iron-deficient roots and thus resembled wild-type plants. None of the lines displayed a visible phenotype when grown under normal growth conditions (data not shown).
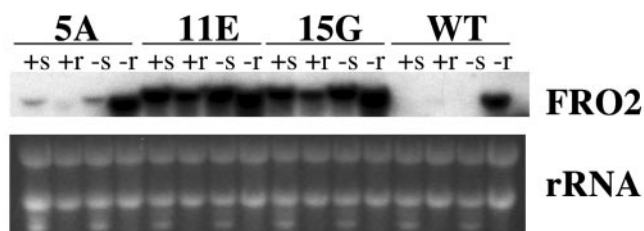

Figure 6. Northern-blot analysis of wild-type and transgenic *35S-FRO2* plants. RNA was prepared from the roots and shoots of 17-d-old plants grown for 3 d on either iron-sufficient or -deficient medium. The radiolabeled *FRO2* cDNA was used as a probe. Ethidium bromide-stained rRNA is shown as a control for loading.
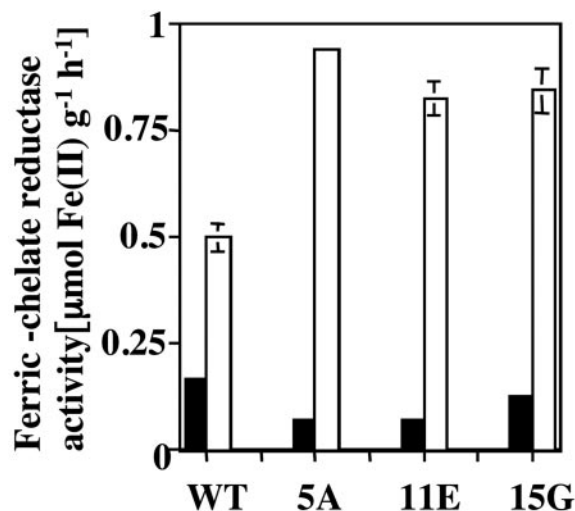

Figure 7. Assays of ferric chelate reductase activity in wild-type and *35S-FRO2* transgenic lines. Plants were grown on standard medium for 14 d and then transferred to either iron-deficient (open bars) or -sufficient (filled bars) medium for 3 d before the assay. Values are the mean of five assays. The means of wild-type and *35S-FRO2* (lines 5A and 15G) plants grown on iron-deficient medium are statistically different at $P < 0.05$.

It is interesting to note that *FRO2* mRNA levels are higher in iron-deficient roots than in iron-sufficient roots of *35S-FRO2* transgenic plants. Lines 5A, 11E, and 15G have 13, 1.3, and 2.7 times more *FRO2* mRNA in iron-deficient roots than iron-sufficient roots, respectively. This result indicates that the *FRO2* transcript may be more stable when plants are iron deficient. However, at least for lines 11E and 15G, *FRO2* mRNA levels in iron-deficient roots can be explained by expression of *FRO2* from the endogenous gene and the transgene resulting in elevated levels in iron-deficient roots as compared with iron-sufficient roots (compare wild-type −Fe root with 15G −Fe root). Because line 5A is the only line obtained thus far that contains disproportionately high levels of *FRO2* mRNA in iron-deficient roots, whether the *FRO2* transcript is stabilized under conditions of iron deficiency is unclear and warrants further investigation.

Next, we examined ferric chelate reductase activity in the transgenic plants as compared with wild type. Plants were grown for 2 weeks on standard medium and then transferred to iron-deficient or -sufficient medium for 3 d. Ferric chelate reductase assays showed that the transgenic plants have elevated ferric chelate reductase activity only when plants are grown on iron-deficient medium (Fig. 7). Lines 5A, 11E, and 15G displayed 1.9, 1.7, and 1.7 times higher activity than wild type, respectively. These values correspond well with the mRNA levels (Fig. 6). These results indicate that expression of *FRO2* is controlled at the posttranscriptional level. Previously, we showed that *IRT1* is subject to posttranscriptional regulation; IRT1 protein only accumulates in the

roots of iron-deficient plants even when *IRT1* expression is driven from the cauliflower mosaic virus *35S* promoter. We have been unable to generate antibodies that recognize FRO2 protein, despite numerous attempts, and thus are unable to determine whether FRO2 protein levels correlate with activity levels. Regardless, it is clear that FRO2 and IRT1 are coordinately regulated at the posttranscriptional level, as they are at the level of transcript accumulation.

FRO2 is known to function in the reduction of copper; *frd1-1* mutants lack low-iron-inducible copper chelate reductase activity. However, *frd1-1* mutants do not show alterations in copper accumulation, making the role of FRO2 in copper mobilization and uptake unclear (Yi and Guerinot, 1996). We measured copper chelate reductase activity in the *35S-FRO2* transgenic plants and wild-type plants. Copper chelate reductase activity is elevated in the roots of iron-deficient *35S-FRO2* plants but not in roots of iron-sufficient *35S-FRO2* plants (Fig. 8). Lines 5A, 11E, and 15G show levels that are 2.6, 1.3, and 2.5 times that of wild-type iron-deficient roots, respectively. Thus, this result also is consistent with posttranscriptional regulation of *FRO2*. To determine whether copper chelate reduction by FRO2 is physiologically relevant, we measured root growth of wild-type and *35S-FRO2* plants grown vertically on plates that were iron-deficient and supplemented with varying concentrations of copper. We reasoned that, if reduction of copper by FRO2 is physiologically important, then the *35S-FRO2* lines might show sensitivity to growth on high-copper medium, but only when plants were grown on iron-deficient me-
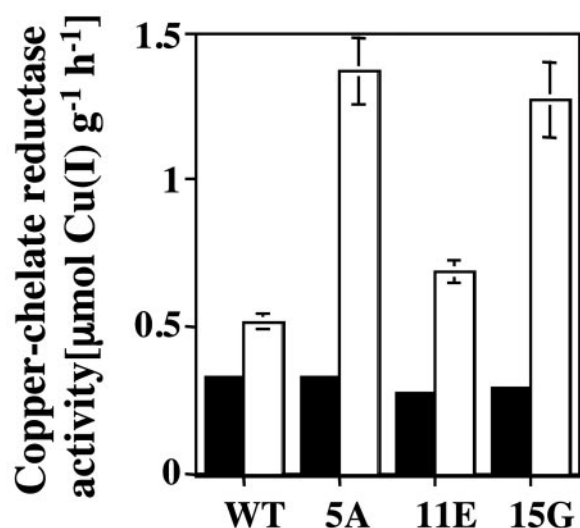
dium, because this is the only growth condition that permits higher copper chelate reductase activity. We were unable to detect any differences in root growth between wild-type and *35S-FRO2* plants over a range of copper concentrations (iron-deficient minimal medium supplemented with 0, 1, 10, 50, 75, 100, 150, and 200 μM $CuSO_4$; data not shown). Root growth of all plants was inhibited by copper concentrations greater than 100 μM. This result suggests either that reduction of copper by FRO2 is not physiologically relevant or that copper, like iron, causes posttranscriptional regulation of *FRO2* when present at high concentrations.

We also examined metal levels in the *35S-FRO2* transgenic plants. Elemental analysis was performed on 13 individual plants each of wild type and *35S-FRO2* lines 5A, 11E, and 15G grown in soil. No significant trends were observed when the three transgenic lines were compared with wild type (data not shown). Elemental analysis also was performed on roots and shoots of wild type and *35S-FRO2* transgenic lines 5A, 11E, and 15G grown on iron-deficient and -sufficient medium. We reasoned that because FRO2 activity is elevated only in the roots of iron-deficient plants, we might detect differences in metal ion content only when plants were grown on iron-deficient medium. We did not detect any differences in metal accumulation between wild type and the three *35S-FRO2* transgenic lines grown on iron-deficient medium (data not shown). It is not surprising that we were unable to detect elevated iron levels because iron is not available for reduction and uptake when the plants are grown on iron-deficient media. The elemental analysis data is consistent with posttranscriptional regulation of *FRO2*; elevated *FRO2* mRNA levels result in elevated FRO2 activity only when plants are starved for iron. Thus, posttranscriptional regulation of *FRO2* may serve to protect against the accumulation of potentially toxic amounts of iron.

We germinated and grew wild type and the transgenic lines on media that contained varying amounts of iron. First, we grew plants on minimal media that was not supplemented with iron, but that also did not contain an iron chelator. Thus, the small amount of iron that remains following water purification or that contaminates the agar may be available to seedlings growing in the media. Each of the three transgenic lines displayed an enhanced ability to tolerate growth on low-iron media with line 15G showing the greatest tolerance (Fig. 9). The enhanced growth of *35S-FRO2* plants on low-iron medium is pH dependent, consistent with the pH optima of ferric chelate reductase (data not shown; Susin et al., 1996). Presumably, the *35S-FRO2* plants show enhanced growth on low-iron medium due to enhanced iron reduction leading to higher rates of uptake. This result, therefore, supports previous work that

Figure 8. Assays of copper chelate reductase activity in wild-type and *35S-FRO2* transgenic plants. Plants were grown on standard medium for 14 d and then transferred to either iron-deficient (open bars) or -sufficient (filled bars) medium for 4 d before the assay. Values are the mean of five assays. The means of wild-type and *35S-FRO2* (lines 5A and 15G) plants grown on iron-deficient medium are statistically different at $P < 0.05$.
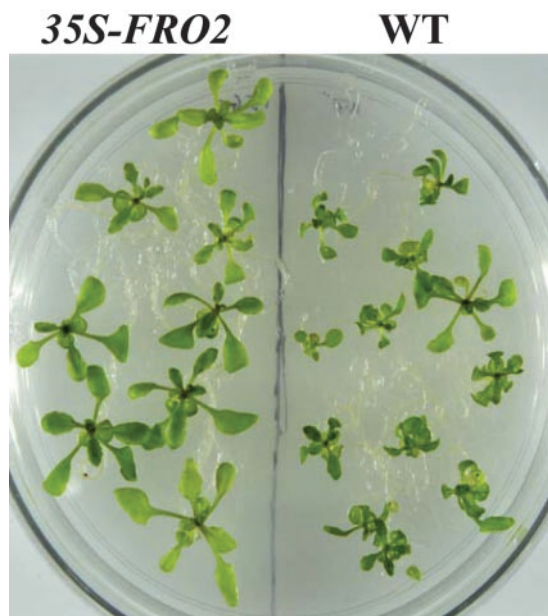
35S-FRO2   WT

Figure 9. *35S-FRO2* plants (line 15G) tolerate growth on low-iron medium. Seedlings were germinated on B5 medium and then were transferred and grown for 2 weeks on low-iron medium before being photographed.

showed that iron reduction is the rate-limiting step for iron uptake (Grusak et al., 1990).

We also grew wild type and the transgenic lines on high-iron media. Germination and growth of plants on minimal media supplemented with 100, 250, or 500 μM Fe(III)-EDTA did not reveal any difference in tolerance between wild type and the transgenic plants. Both wild-type and *35S-FRO2* plants displayed iron toxicity symptoms when grown in the presence of 250 or 500 μM Fe(III)-EDTA (data not shown). Moreover, we observed no difference in growth between the *35S-FRO2* transgenic plants and wild type when plants were grown on standard medium, which contains 50 μM Fe(III)-EDTA. These results are consistent with posttranscriptional regulation of *FRO2*.

DISCUSSION

The data presented here provide evidence for coordinate regulation of two of the major components of the strategy I response used by dicots and nongrass monocots, FRO2 and IRT1. Our results suggest that expression of *FRO2* and *IRT1* is coordinately regulated at the level of transcript accumulation by iron, zinc, and cadmium. It makes sense that induction of *FRO2* and *IRT1* would be coordinately controlled, because FRO2 activity is required to provide the ferrous iron that is a substrate for IRT1. This regulatory mechanism may be acting either at transcription initiation or at the transcript stability level; at this time, we are unable to eliminate either one of these possibilities.

Coordinate control of transcription initiation likely would be mediated through common cis-acting elements and trans-acting proteins. Recently, the tomato (*Lycopersicon esculentum*) *FER* gene was isolated; *FER* encodes a putative transcription factor that controls iron uptake responses in roots (Ling et al., 2002). Both root ferric chelate reductase activity and *LeIRT1* expression are altered in the *fer* mutant. A FER ortholog is present in the Arabidopsis genome and may be responsible for coordinate regulation of *FRO2* and *IRT1*.

IRT1 mediates uptake of both zinc and cadmium (Eide et al., 1996; Korshunova et al., 1999; Connolly et al., 2002; Vert et al., 2002), and both metals can cause toxicity when they accumulate to high levels. Therefore, repression of *IRT1* expression when plants are grown in the presence of high amounts of zinc and cadmium would avoid accumulation of either metal to toxic levels (Connolly et al., 2002). However, it is surprising that *FRO2* expression is controlled by zinc and cadmium because there is no evidence that FRO2 functions in the uptake of either of these metals. One possibility is that FRO2 and IRT1 form a complex that is only stable when both proteins are present in the membrane. Thus, when a cell represses *IRT1* expression, it would make sense that *FRO2* expression would be repressed as well. In *frd1-1*, the *FRO2* gene encodes a protein with a stop codon at position 21; western analysis of these plants showed that IRT1 protein is detected at normal levels (data not shown). In addition, inducible root ferric chelate reductase activity is elevated in an IRT1 KO line (Vert et al., 2002). These results suggest that the accumulation of neither IRT1 nor FRO2 protein is dependent upon the presence of the other protein.

In situ hybridization with a *FRO2* antisense probe showed that *FRO2* mRNA is detected at high levels in the epidermal cells of the root. This pattern is consistent with the proposed role of FRO2 in iron reduction at the root surface (Yi and Guerinot, 1996; Robinson et al., 1999) and again is consistent with the idea that *FRO2* and *IRT1* are coordinately regulated. IRT1 also is expressed in root epidermal cells (Vert et al., 2002). In tomato, *FER* mRNA is detected in root epidermal cells and in the outer cortical cell layers of roots (Ling et al., 2002). However, *FER* mRNA also is detected in the vascular cylinder of the mature root-hair zone of the root. A gene encoding ferric chelate reductase has been cloned from pea (*Pisum sativum*), and in situ hybridization experiments have shown that it is expressed throughout the root, including the epidermis, cortex, and vascular cylinder (Waters et al., 2002). Pea *FRO1* also is expressed in nodules as well as leaf mesophyll and parenchyma cells. In situ hybridization failed to show that *FRO2* and *IRT1* mRNA are present in the vascular cylinder of Arabidopsis roots (this study; Vert et al., 2002). However, staining of *FRO2-GUS* plants did reveal *FRO2* promoter activity in the central cylinder (data not shown). Our real time RT-PCR results show that

FRO2 is expressed at relatively low levels in tissues other than iron-deficient roots and support the results obtained with *FRO2-GUS* plants. However, because histochemical staining of promoter-GUS plants is unreliable and because we could not confirm *FRO2* expression in the central cylinder using in situ hybridization, a role for FRO2 in the vasculature remains unsubstantiated.

The Arabidopsis genome contains eight putative *FRO* genes; it is possible that other FROs function in the vascular cylinder and/or in the aerial portions of the plant. It is thought that iron is transported in the xylem as a Fe(III)-citrate complex (Tiffin, 1966) and that ferric chelate reductase is required in the aerial portions of the plant to reduce ferric iron before transport into the leaf cells. Ferric chelate reductase activity has been detected in leaves of sunflower (de la Guardia and Alcantara, 1996) and *Vigna unguiculata* (Brüggemann et al., 1993). Ferric chelate reductase activity also appears to be developmentally regulated, with ferric chelate reductase activity increasing during seed fill to supply the developing seeds (Grusak, 1995). The Arabidopsis *FRO3* gene is expressed in both root and leaf cells in response to low iron (Robinson et al., 1997).

Our data suggest that, like *IRT1*, expression of *FRO2* is controlled posttranscriptionally; IRT1 protein accumulates only in the roots of iron-deficient *35S-IRT1* lines despite the accumulation of high levels of *IRT1* mRNA in all tissues (Connolly et al., 2002). Here, we show that *FRO2* mRNA accumulates in roots and shoots of iron-sufficient and iron-deficient *35S-FRO2* transgenic plants, yet root ferric chelate reductase activity is elevated only when plants are starved for iron. In addition, *35S-FRO2* plants do not accumulate more metals than wild-type plants.

There certainly are precedents for metal control of gene expression. Zinc uptake in yeast is tightly regulated in response to changing zinc levels (Guerinot and Eide, 1999). Expression of *ZRT1*, the high-affinity zinc transporter in yeast, is controlled both at the level of transcription, via the ZAP1 transcription factor (Zhao et al., 1998; Zhao and Eide, 1997) and posttranslationally, via zinc-mediated ubiquitination and protein turnover (Gitan et al., 1998; Gitan and Eide, 2000). This system allows the rapid turnover of ZRT1 when cells come in contact with high zinc, thus preventing the accumulation of toxic amounts of this metal. Arabidopsis IRT1 and yeast ZRT1 belong to the ZIP family of metal transporters and thus share a high degree of sequence similarity; in addition, the two proteins share the same predicted membrane topology. For these reasons, it seems possible that IRT1 is regulated posttranslationally via ubiquitination and turnover, like ZRT1. It is harder to predict why FRO2 is regulated at the posttranscriptional level. FRO2 functions to reduce iron at the cell surface before transport. Thus, overexpression of FRO2 should not lead directly to the accumulation of toxic

amounts of iron because it does not transport iron itself. Because posttranscriptional regulation of IRT1 expression prevents the accumulation of the transporter when iron and zinc are present, it seems unnecessary to regulate FRO2 posttranscriptionally as well. However, it would be inefficient to continue to express FRO2 protein if the major ferrous transporter is not present.

35S-FRO2 plants display elevated copper chelate reductase activity when plants are grown on iron-deficient medium. However, the *35S-FRO2* plants did not display enhanced sensitivity to growth on iron-deficient medium supplemented with copper, nor did *35S-FRO2* plants accumulate copper when grown on iron-deficient medium. Together, these data suggest that either copper reduction is not required before transport, or that copper reduction is not the rate-limiting step in copper uptake. Although loss-of-function mutations in FRO2 result in a loss of low-iron-inducible copper chelate reductase activity, FRO2 mutant plants do not show reduced copper accumulation. Moreover, *FRO2* transcript abundance does not increase when plants are deprived of copper (Robinson et al., 1999). The yeast FRE1 and FRE2 proteins are known to function in the reduction of iron and copper (Georgatsou and Alexandraki, 1994; Georgatsou et al., 1997). *FRE1* and *FRE2* belong to family of genes in yeast that are thought to encode metal reductases. Two of these, *FRE1* and *FRE7*, are regulated by copper through the Mac1 transcriptional regulator (Martins et al., 1998). It is possible that other Arabidopsis FRO family members function in copper reduction and, in turn, may be regulated by copper.

35S-FRO2 transgenic plants grow better than wild type on low-iron medium. This result is likely due to the fact that these plants display elevated levels of ferric chelate reductase activity under iron starvation; therefore, the transgenic plants may be better able to acquire the low amounts of iron present in the medium. Alternatively, the low-iron tolerance phenotype of the *35S-FRO2* plants may be due to enhanced storage of iron in the seed. In agreement with our data that suggests that expression of *FRO2* is controlled posttranscriptionally, *35S-FRO2* plants are not sensitive to growth on high iron. Our results are significant in that they suggest that overexpression of *FRO2* may be useful for the construction of plants capable of thriving on low-iron soils. This is particularly exciting because approximately one-third of the world's soils are considered iron deficient.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis wild-type (ecotype Columbia *gl-1*) and transgenic seeds (*35S-FRO2* and *FRO2-GUS*) were surface sterilized, sown on plates of Gamborg's B5 medium (Sigma-Aldrich, St. Louis) supplemented with 2% (w/v) Suc, 1 mm MES, and 0.6% (w/v) agar, pH 5.8, and placed in the dark for 2 to 4 d at 4°C. Transgenic plants were grown on plates supplemented with

either 50 μg mL^{-1} kanamycin (*35S-FRO2*) or 25 μg mL^{-1} hygromycin (*FRO2-GUS*). Plates were incubated at 22°C under constant illumination for 12 to 14 d until they reached the 4- to 6-true-leaf stage. Yellow filters (acrylic yellow-2208, Cadillac Plastic and Chemical, Pittsburgh) were placed between the lights and plants to prevent the photochemical degradation of Fe(III)-EDTA (Hangarter and Stasinopoulos, 1991).

Seedlings were transferred to either iron-sufficient [50 μM Fe(III)-EDTA] or iron-deficient {300 μM FerroZine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate]; HACH Chemical, Ames, IA} medium. The medium contained 0.6% (w/v) agar, 1 mM MES, pH 6.0, and macronutrients and micronutrients as described previously (Marschner et al., 1982). Plants were incubated for various times after transfer in a growth chamber as described above. For growth on low-iron medium, plants were transferred to plates that contained no added iron but that also did not contain the iron chelator, Ferrozine; thus any iron present in the agar or following purification through a Barnstead Nanopure Diamond water purification system was available to the plants. For growth on zinc supplemented medium, iron-sufficient or -deficient medium was supplemented with either 100 μM or 500 μM ZnSO$_4$.

Construction of the Chimeric *FRO2-GUS* Gene

An *Eco*RV/*Pst*I genomic DNA fragment containing 1,204 bp of *FRO2* 5' sequence and 63 bp of *FRO2* coding sequence was cloned into *Sma*I/*Pst*I digested pCAMBIA1381Xa (GenBank accession no. AF234303) creating pELC207. Translation is initiated from the *FRO2* ATG.

Construction of the Chimeric *35S-FRO2* Gene

The Arabidopsis *FRO2* cDNA was cloned into the *Bam*HI site of pCGN18 creating pELC215. pCGN18 was the kind gift of Dr. Thomas Jack (Department of Biological Sciences, Dartmouth College, Hanover, NH; Connolly et al., 2002). The *FRO2* cDNA contains 22 bp of sequence 5' of the FRO2 ATG and 270 bp of sequence downstream of the stop codon.

Plant Transformation

The *FRO2-GUS* (pELC207) and *35S-FRO2* (pELC215) constructs were used to transform *Agrobacterium tumefaciens* strains GV3101and ASE, respectively. *A. tumefaciens*-mediated transformation of Arabidopsis plants (Columbia *gl-1*) was accomplished using the floral dip protocol (Clough and Bent, 1998). T$_1$ seeds obtained from self-fertilization of the primary transformants were surface sterilized and plated on Gamborg's B5 medium supplemented with either kanamycin (*35S-FRO2*) or hygromycin (*FRO2-GUS*). Resistant plants were transferred to soil, and the T$_2$ seeds resulting from self-fertilization were collected. The T$_2$ seeds were surface sterilized and plated on the appropriate medium; transgenic lines that displayed 3:1 segregation for antibiotic resistance to antibiotic sensitivity in the T$_2$ generation and that were 100% antibiotic resistant in the T$_3$ generation were selected for further analysis. All further experiments were performed using T$_4$ or T$_5$ seeds.

Isolation of RNA, Northern-Blot Analysis, and Real Time RT-PCR

Total RNA was prepared (Verwoerd et al., 1989) from the roots and shoots of 17-d-old plants grown on plates that were either iron sufficient or deficient or from various tissues of soil-grown plants. Northern-blot analysis was performed as described previously (Connolly et al., 2002). An *Eco*RI fragment containing the 2.47-kb *FRO2* cDNA was used as a probe for RNA gel-blot analysis; a *Not*I fragment containing the 1.4-kb *IRT1* cDNA also was used a probe. Previous work demonstrated the specificity of the *IRT1* probe (Vert et al., 2002). Arabidopsis contains eight *FRO* genes; thus, we performed high-stringency Southern-blot hybridization analysis to determine whether the *FRO2* probe hybridizes to additional *FRO* sequences. The analysis revealed that the *FRO2* probe hybridizes to a single band in *Eco*RI- and *Bam*HI-digested genomic DNA and to three bands in *Hind*III digested genomic DNA, as predicted (data not shown). DNA was radiolabeled according to the random-primer method (Feinberg and Vogelstein, 1984). Quantification of signal resulting from *FRO2* hybridization was performed

using a Molecular Dynamics Storm phosphoimager. *FRO2* values were normalized to *rRNA* levels; *rRNA* levels were quantified using the Quantity One gel documentation software package (Bio-Rad Laboratories, Hercules, CA).

For real time RT-PCR, cDNA was prepared from total RNA according to the manufacturer's instructions (SuperScript First-Strand Synthesis System for RT-PCR kit, Invitrogen, Carlsbad, CA). Total RNA was treated with DNase I before cDNA synthesis to remove trace amounts of genomic DNA. Primers were designed to amplify a 103-bp fragment of the *FRO2* transcript (FRO2RTFOR, 5'GCCACATCTGCGTATCAAGTT3'; FRO2RTREV, 5'TCCCAAACAAGCTACGACCA3'). The primers amplify a fragment that spans intron 1, allowing the detection of genomic DNA contamination. BLAST searches using the *FRO2* primers verified that the primers will anneal only to *FRO2* sequences. Primers corresponding to *UBQ10* were designed for use as an internal control (UBQ10RTFOR, 5'TGGTGGTATG-CAGATTTTCG3'; UBQ10RTREV, 5'GGCTTTCACGTTATCAATGG3').

Real time RT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the iCycler thermocycler (Bio-Rad Laboratories). To quantify *FRO2* and *UBQ10* levels in unknown reactions, standards were prepared from serial dilutions of *FRO2* and *UBQ10* cDNA clones. The standards were run in parallel with the cDNA prepared from the unknown samples and then used to generate a standard curve. Upon completion of the amplification reactions, a threshold level is set; the threshold level is arbitrarily set at a level where the amplification curves are parallel to one another. The threshold values for *FRO2* and *UBQ10* unknown samples were compared with their corresponding standard curves and assigned an initial starting concentration in femtomoles. To normalize the data, the *FRO2* concentrations were divided by the *UBQ10* concentrations from each cDNA sample to obtain a relative value for each sample. The lowest *FRO2* concentration was set to 1×, and the rest of the samples were assigned relative values based on the 1× sample. Finally, following the real time PCR reaction, melt curves were performed to verify that a single product was amplified; samples were run on agarose gels to confirm the presence of a single band.

Assay of Ferric Chelate and Copper Chelate Reductase Activities

Wild-type (Col *gl-1*) and *35S-FRO2* transgenic plants were grown for 2 weeks on B5 medium and then transferred to iron-sufficient or -deficient medium for 3 d. Ferric-chelate reductase and copper-chelate reductase activities were determined as described previously, except that copper-chelate reductase activity was determined after plants had been grown for 4 d on either iron-sufficient or -deficient medium (Yi and Guerinot, 1996). Two seedlings were pooled and assayed as a group. Each value is the mean of five independent trials. The entire experiment was repeated four times. Statistical analysis of activity data was performed using a Student's *t* test.

Elemental Analysis

The concentrations of various minerals in the roots and shoots of wild type and three independent *35S-FRO2* transgenic lines was determined using inductively coupled argon plasma spectroscopy in the Nutrient and Element Analysis Lab in the Department of Horticulture at Cornell University (Ithaca, NY). Plants were grown for 2 weeks on B5 medium and then transferred to iron-sufficient or -deficient medium for 6 d. Roots and shoots were separated and dried overnight in a 65°C oven. Approximately 50 to 100 plants were pooled for each sample. Metal ion content of wild type and three independent *35S-FRO2* transgenic lines (13 individual plants each) grown on soil was determined in the lab of Dr. David Salt (Purdue University, West Lafayette, IN).

GUS Histochemical Staining

Wild type and 10 independent, single-insertion, homozygous *FRO2-GUS* transgenic lines were grown for 2 weeks and then transferred to either iron-sufficient or -deficient medium. After 3 d, plants were subjected to GUS histochemical staining using 5-bromo-4-chloro-3-indolyl β-D-glucoronide as a substrate (Jefferson et al., 1987). GUS histochemical staining was later performed on wild type and six independent *FRO2-GUS* lines grown either on plates for various times before transfer to iron-sufficient or -deficient medium for 3 d or on soil.

In Situ Hybridization

The plasmid pELC214 (*FRO2* cDNA in *Bam*H1 site of pBluescript) was used to generate sense and antisense probes for in situ hybridization. Sense and antisense probes were labeled with digoxigenin-11-UTP (Roche Diagnostics, Indianapolis) according to the manufacturer's protocol. For antisense probes, pELC214 was linearized with *Eco*R1 and transcribed with T3 RNA polymerase. For sense probes, pELC214 was linearized with *Sac*II and transcribed with T7 RNA polymerase. Tissue samples were fixed and embedded as described (Vert et al. 2002), and in situ hybridization was performed according to previous protocols (Long et al., 1996; Long and Barton, 1998).

Distibution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Accession Number

The GenBank accession number for the *FRO2* genomic clone is Y09581.

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