Dual Regulation of the Arabidopsis High-Affinity Root Iron Uptake System by Local and Long-Distance Signals

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Regulation of the root high-affinity iron uptake system by whole-plant signals was investigated at the molecular level in Arabidopsis, through monitoring FRO2 and IRT1 gene expression. These two genes encode the root ferric-chelate reductase and the high-affinity iron transporter, respectively, involved in the iron deficiency-induced uptake system. Recovery from iron-deficient conditions and modulation of apoplastic iron pools indicate that iron itself plays a major role in the regulation of root iron deficiency responses at the mRNA and protein levels. Split-root experiments show that the expression of IRT1 and FRO2 is controlled both by a local induction from the root iron pool and through a systemic pathway involving a shoot-borne signal, both signals being integrated to tightly control production of the root iron uptake proteins. We also show that IRT1 and FRO2 are expressed during the day and down-regulated at night and that this additional control is overruled by iron starvation, indicating that the nutritional status prevails on the diurnal regulation. Our work suggests, for the first time to our knowledge, that like in grasses, the root iron acquisition in strategy I plants may also be under diurnal regulation. On the basis of the new molecular insights provided in this study and given the strict coregulation of IRT1 and FRO2 observed, we present a model of local and long-distance regulation of the root iron uptake system in Arabidopsis.

Iron is an essential element for all living organisms, including plants. As a transition metal, its ability to gain and lose an electron confers iron important properties for redox reactions. Although abundant in soils, iron often forms highly insoluble ferric-hydroxide precipitates that limit its availability for plants (Guerinot and Yi, 1994). Therefore, mechanisms allowing plants to solubilize and to efficiently take up iron have evolved. However, those mechanisms are carefully regulated to prevent oxidative damages due to iron overload. Angiosperms can be divided into two groups based on their strategies of iron uptake. Non-graminaceous strategy I plants rely on acidification of the rhizosphere to increase solubility of ferric iron complexes through activation of an H⁺-ATPase activity, transplasma membrane electron transfer to reduce iron into its more soluble ferrous form, and a transport activity to provide iron to root cells (Marschner et al., 1986). Grasses, also called strategy II plants, respond to iron insufficiency by releasing phytosiderophores, which have a strong affinity for ferric iron, and by transporting the Fe(III)-phytosiderophore complex across the plasma membrane of the root epidermal cell (Takagi et al., 1984).

A great effort has recently been made to isolate genes involved in this root response to iron deficiency, especially in Arabidopsis. In this species, the acidification function is likely to be performed by a member of the H⁺-ATPase AHA family (Palmgren, 2001), whereas the root ferric reductase that reduces ferric iron into ferrous iron in the rhizosphere is encoded by the FRO2 gene (Robinson et al., 1999). Arabidopsis apparently possesses a large number of iron transporters, either characterized or putative, among the ZIP, NRAMP, YSL, and IREG families (Eide et al., 1996; Curie et al., 2000, 2001; McKie et al., 2000; Thomine et al., 2000; Vert et al., 2001). Recent work using a reverse genetic approach has shown that IRT1, a ZIP family member, is the major iron transporter responsible for root iron uptake from the soil solution (Vert et al., 2002).

The uptake of mineral nutrients including iron relies not only on specific transport activities located on the plasma membrane of root epidermal cells, but also on powerful regulatory mechanisms controlling the abundance and activity of transporters to adjust the nutrient uptake to the demand of the whole plant. The existence of shoot-borne signals regulating the root response to iron starvation has first been identified with degenerative leaflets (dgl) and bronze (brz), two pea (Pisum sativum) mutants presenting constitutive iron deficiency responses while containing high amounts of iron in their tissues (Kneen et al., 1990; Welch and LaRue, 1990). Reciprocal grafting between dgl or brz and their parental genotypes indicated that the phenotype of the root is determined...
by the shoot genotype (Grusak and Pezeshgi, 1996). Moreover split-root experiments carried out on *Plantago lanceolata* and Arabidopsis showed that the ferric reductase activity is increased in the compartment supplied with iron, suggesting a long-distance signaling process (Schmidt et al., 1996; Schikora and Schmidt, 2001).

IRT1 and FRO2 play a key role in root iron uptake in response to iron deficiency (Robinson et al., 1999; Vert et al., 2002), and as such are likely to be terminal targets for the regulatory pathway that controls transport aspects of iron homeostasis in plants. Their expression is rapidly induced by iron starvation in roots (Eide et al., 1996; Robinson et al., 1999; Vert et al., 2001) as well as drastically turned off, via post-translational mechanisms, in iron sufficiency (Connolly et al., 2002). In an effort to understand the regulatory pathways leading to the root iron deficiency response, we have examined *IRT1* and *FRO2* expression in response to environmental changes including modulation of the iron nutrition in regular and split-root hydroponic cultures as well as during the day/night variation. We show that *IRT1* and *FRO2* are strictly coregulated in all conditions tested. We provide evidences for a dual control of *IRT1* and *FRO2* involving both a local induction by iron availability and a systemic regulation by the foliar iron status. Moreover, we show that *IRT1* and *FRO2* are regulated diurnally and that this additional control is overruled by iron starvation.

**RESULTS**

**IRT1 and FRO2 Are Coregulated in Response to Iron Deficiency**

Former studies showing the up-regulation of *IRT1* and *FRO2* in response to iron deficiency had been performed on plants grown in culture media containing Suc (Eide et al., 1992; Vert et al., 2001; Connolly and Guerinot, 2002). We chose to grow the plants hydroponically in a Suc-free medium (Lejay et al., 1999), which in addition to be more physiological, allows the setup of split-root cultures necessary to study signaling processes. The response of *IRT1* and *FRO2* to changes in plant iron status was re-examined in this hydroponic system. Fe-grown Arabidopsis plants were transferred to Fe-free solution for 1, 3, 5, or 7 d before harvest. Although detectable, *IRT1* mRNA is weakly abundant under +Fe conditions (Fig. 1A). Upon −Fe treatment, the level of *IRT1* transcripts transiently drops and then increases at 3 d with a maximum 5 d after the transfer to Fe-free solution. The exact same pattern of expression was observed for *FRO2* (Fig. 1A), demonstrating that these two genes involved in root iron uptake are coregulated. These data indicate that iron starvation-induced gene expression is roughly similar between the two culture systems. Differences are noticeable however such as (a) the absence of *IRT1* transcripts in +Fe conditions in Suc-containing media (Vert et al., 2001) as compared with the low but significant amount observed in hydroponics (Fig. 1A); and (b) the time-lag (about 24 h) for the induction after transfer to −Fe solution in hydroponics, whereas *IRT1* expression is immediately induced by an iron deficiency treatment in the former growth system (Vert et al., 2001).

Because *IRT1* is subject to posttranscriptional control in response to iron (Connolly et al., 2002), we wanted to verify whether protein accumulation followed mRNA accumulation in the same time-course experiment. The western blot indicates that both mRNA and protein increase transiently with a maximum 5 d after transfer to −Fe solution (Fig. 1B). We had previously shown through analysis of Arabidopsis transgenic plants expressing an *IRT1* promoter-GUS fusion that the control of iron deficiency responses occurs mainly at the transcriptional level (Vert et al., 2002). The present study demonstrates that, under the experimental conditions used, accumulation of transcripts in response to iron deficiency reflects the amount of proteins synthesized.

**Figure 1.** Time-course response of *IRT1* and *FRO2* expression to iron starvation in Suc-free medium. Plants were grown under iron-sufficiency (100 μM iron) for 5 weeks (lane C) and then washed for 5 min with fresh iron-deficient medium before being transferred to iron deficiency. Plants were harvested after 1, 3, 5, or 7 d of iron deficiency. Seven-day-starved plants were also resupplied with 100 μM iron for 24 h (lane R). A, Northern-blot analyses. *IRT1* and *FRO2* probes were sequentially hybridized to a blot containing 12 μg of total RNA extracted from roots of Arabidopsis. B, Immunoblot analysis. *IRT1* protein was detected by hybridizing the blot with an *IRT1*-specific antibody.
Iron Deficiency Response Gene Expression Is Induced by Iron

We performed a classical “recovery” experiment in which plants starved for 7 d were transferred back to a Fe-containing solution for 24 h. Interestingly, this iron resupply leads to an increase of \( IRT1 \) and \( FRO2 \) transcripts (Fig. 1A, R), which is also followed by accumulation of the \( IRT1 \) protein (Fig. 1B, R). This indicates that the presence of iron is required to turn on the iron deficiency response.

To investigate whether or not iron is required as an inducer of the iron deficiency response, we tested the effect of root apoplastic iron on \( IRT1 \) and \( FRO2 \) expression. Iron is known to aspecifically bind to cell wall components, such as carboxyl or hydroxyl residues, leading to the formation of large apoplastic iron pools that can be remobilized for plant growth under iron deficiency. By comparison with the standard experiment presented in Figure 1A, the pool of apoplastic iron was removed by washing the plants with the strong iron chelator bipyridyl before their transfer to iron-deficient medium (Fig. 2A). Whereas iron deficiency results in transient induction of \( IRT1 \) and \( FRO2 \) in standard iron nutrition (Fig. 1A), washing similarly grown plants with bipyridyl eliminates \( IRT1 \) and \( FRO2 \) transcripts accumulation (Fig. 2B).

This result is consistent with a local effect of iron that was embedded in the root apoplast after the 2 d of preculture at 100 \( \mu \)M Fe(III)-EDTA. This hypothesis is further supported by the fact that 7-d-starved plants, which are therefore highly deficient, are only able to efficiently induce \( IRT1 \) expression upon iron resupply (Fig. 2B, R).

Raising Fe(III)-EDTA concentration to 500 \( \mu \)M during the 2 d of preculture resulted in a transient induction of \( IRT1 \) and \( FRO2 \) that peaked around 3 d after transfer to iron deficiency (Fig. 2C), which is 2 d earlier than in the standard experiment (Fig. 1A). Plants cultivated with such a high Fe-EDTA concentration show large accumulation of iron in the apoplast (Bienfait et al., 1985), which is likely to be removed only partially by bipyridyl. Using a range of iron concentration in the 2-d preculture resulted in a progressive increase in the induction peak at 3 d (Fig. 2D). This reinforces our proposal that iron acts locally to induce \( IRT1 \) and \( FRO2 \) expression and probably allows the iron deficiency responses to occur in the root.

Dual Regulation of the Root Iron Deficiency Response

Although iron seems to play an inductive role on \( IRT1 \) and \( FRO2 \) expression, both transcripts are either weakly or not detected under standard +Fe conditions (Eide et al., 1996; Robinson et al., 1999; Vert et al., 2001; Connolly et al., 2002; this study). This suggests that, depending on the iron status, an additional level of control exists that modulates, together with local iron, the expression of the root genes involved in iron uptake. To further show this dual control, we performed a time-course experiment in which 7-d iron-starved plants were transferred to a solution containing excess amounts of iron for the times indicated on Figure 3 before harvest. We observed an increase in \( IRT1 \) and \( FRO2 \) mRNA accumulation 12 h after treating the plants with 500 \( \mu \)M iron. This could be explained by the inductive effect of iron on iron-starved plants. Between 12 and 24 h, however, the amount of \( IRT1 \) and \( FRO2 \) mRNA dramatically drops to reach undetectable levels at 48 h. Thus in the presence of a high iron concentration, expression of the genes encoding the high-affinity iron uptake is turned off, by a process that prevails on the mechanism of local induction by iron.

Expression of \( IRT1 \) and \( FRO2 \) Genes Is Systemically Regulated in Response to the Iron Status

The time lag between iron supply and the decrease of \( IRT1 \) and \( FRO2 \) expression (see Fig. 3) is compatible with a translocation of iron to the shoot where the modification of iron status is likely to be sensed and signaled to the root iron uptake machinery. The existence of a shoot-to-root signal regulating the iron uptake activity had been suggested earlier, mainly by grafting experiments (Grusak and Pezeshgi, 1996). To demonstrate at the molecular level the occurrence of such a systemic regulation, split-root experiments were carried out, in which one-half of the root system was submitted to iron deficiency. To ensure a drastic iron starvation on this side, roots were washed with bipyridyl before being transferred to an iron-deficient solution. Three days after transfer, \( IRT1 \) and \( FRO2 \) mRNA accumulation strongly decreased in the −Fe side and increased in the +Fe side of the roots (Fig. 4A). Quantification of the blot suggests that the increase of transcripts in +Fe compensates for the decrease in −Fe. A western blot performed on protein extracts from the same plants indicates that the \( IRT1 \) protein follows the same pattern of accumulation as \( IRT1 \) and \( FRO2 \) mRNAs (Fig. 4B). The disappearance of \( IRT1 \) and \( FRO2 \) expression in the −Fe side is consistent with the loss of local induction by iron. That expression is induced in the +Fe side demonstrates that the global iron status of the plant is sensed by the shoot, which in turn controls expression of the iron deficiency response genes in the part of the root supplied with iron. In molecular terms, either a derepression or an activation could lead to the elevated expression observed in the +Fe half of the root. This experiment proves the existence of systemic signals involved in controlling the plant iron content and identifies \( IRT1 \) and \( FRO2 \) as the first molecular targets of such signals.

Seven days after transfer to −Fe solution, \( IRT1 \) and \( FRO2 \) mRNA level in the +Fe half of the root is back down to the control level, whereas it has become undetectable in the −Fe side (Fig. 4C). It may appear
as though, thanks to the transient induction of IRT1 and FRO2 expression, the split-root plant has returned to a normal iron status, therefore turning off the iron deficiency response genes. However, monitoring the IRT1 protein accumulation by western blot (Fig. 4D) indicates that it remains higher than the control in the /H11001 Fe-grown root half. The IRT1 protein had been previously shown to be rapidly degraded in iron-sufficient conditions (Connolly et al., 2002). However this experiment shows that IRT1 can be stabilized in high iron supply when the shoot iron status is low, which suggests that IRT1 protein turnover is also controlled systemically.

Diurnal Regulation of the Root High-Affinity Iron Uptake System

The irt1-1 homozygous knock-out mutant was shown to be highly chlorotic due to its defect in iron uptake (Vert et al., 2002). The heterozygous mutant irt1-1 +/− is undistinguishable from the wild type when grown under long-day conditions (Vert et al., 2002), whereas it flowers earlier when grown under short-day conditions (data not shown). It is possible that plants carrying only one copy of the IRT1 gene take up iron more efficiently during the light period, which raises the question of whether iron uptake activity occurs mainly during the day. To address this question, we examined IRT1 and FRO2 expression during day and night. Both IRT1 and FRO2 gene expression show diurnal regulation, with a marked increase during the light period (Fig. 5A). The signals involved in this day/night regulation may be light itself, circadian rhythms, or photosynthesis products. Because IRT1 and FRO2 are up-regulated in response to iron deficiency, analysis of the day/night variation

Figure 2. Effect of apoplastic iron on IRT1 and FRO2 expression. A, Plant growth conditions. Plants were grown under iron-sufficiency (100 μM iron) for 5 weeks (lane C), transferred to a medium containing various concentrations of iron as indicated for 2 d, and then washed with bipyridyl before being transferred to iron-free medium. Roots were harvested 1, 3, 5, or 7 d after transfer to iron-free medium. When indicated, 7-d iron-starved plants were also resupplied with 100 μM iron for 24 h (B, lane R). B through D, Northern-blot analyses. IRT1 and FRO2 probes were sequentially hybridized to a blot containing 15 μg of total RNA extracted from roots of Arabidopsis grown as described in A, with a 2-d preculture in 100 μM iron (B), 500 μM (C), or 50 to 500 μM as indicated on the figure (D) before the root wash with bipyridyl.
was also carried out in iron-limited conditions. Interestingly, after transfer to iron deficiency, both IRT1 and FRO2 mRNA accumulate at a high level independently of the day/night variations (Fig. 5B). The diurnal signal regulating root iron deficiency responses that occurs in normal growth conditions appears to be bypassed by the iron nutritional status. In both cases, IRT1 and FRO2 gene regulation appears to be specific because the abundance of the EF-1α transcript is unchanged regardless of the time point and the condition considered (Fig. 5, A and B).

**DISCUSSION**

Here, we show that the root high-affinity iron uptake system involved in the response to iron deficiency is controlled by both local and systemic signals. First, iron itself seems to act locally as an inducer because (a) iron resupply to iron-starved plants promotes IRT1 and FRO2 transcripts as well as IRT1 protein accumulation within 12 to 24 h (Figs. 1 and 3), and (b) removing the apoplastic iron pool abolishes the transient induction of IRT1 and FRO2, whereas increasing apoplastic iron enhances this response (Figs. 2 and 4). Second, IRT1 and FRO2 are also under the control of a systemic regulation that occurs in iron replete conditions for at least two reasons. (a) Iron-deficient plants transferred to high iron concentration after a transient induction phase around 12 h show a drastic decrease in the amount of IRT1 and FRO2 transcripts around 24 h. Because this shut off of the expression occurs in the presence of...
the local inducer, it must involve a distinct regulatory mechanism. (b) In \(+\text{Fe}/-\text{Fe}\) split-root plants, the production of \(\text{IRT1}\) and \(\text{FRO2}\) transcripts and \(\text{IRT1}\) protein is enhanced in the \(+\text{Fe}\)-grown half of the root, demonstrating that the shoot is involved in regulating the iron deficiency response in the root. This result is consistent with earlier split-root experiments in which induction of the ferric-chelate reductase activity occurred in the compartment supplied with iron (Schikora and Schmidt, 2001; Schmidt et al., 1996) and agrees with an array of physiological evidences that favored an involvement of aerial parts in the control of iron deficiency responses (Grusak and Pezeshgi, 1996; Schmidt et al., 1996; Schikora and Schmidt, 2001). Furthermore, our results show for the first time that this long-distance signaling affects the response at the level of gene expression and not through modulation of the protein activity.

We found \(\text{IRT1}\) and \(\text{FRO2}\) genes to be strictly co-regulated over the variety of nutritional and environmental conditions tested in this study. This further supports the physiological dogma that ferric-chelate reductase and iron uptake functions are tightly associated in strategy I plants to perform the iron deficiency response. We can therefore state that, at least in Arabidopsis, the high-affinity iron uptake system in roots is substrate-induced and systemically regulated according to the shoot demand for iron. According to whether the shoot-borne signal is released in response to deficient iron status or to sufficient iron status, two opposite regulatory models can be envisaged that both require induction by local iron (Fig. 6). In the promotive model, the shoot-borne signal is released when the global iron status of the plant is low, leading to activation of the root iron uptake genes expression; upon increase of the iron content, no signal is released resulting in inactivation of gene expression. In contrast, in the repressive model, release of the signal occurs in response to

*Figure 5.* Diurnal regulation of \(\text{IRT1}\) and \(\text{FRO2}\). A, Northern-blot analyses. Wild-type plants were grown hydroponically in a medium containing 100 \(\mu\)M iron under short-day conditions. B, Northern-blot analysis. Plants grown as in A except that 24 h before the beginning of sample collection, plants were transferred to low-iron conditions (5 \(\mu\)M iron). A northern blot containing 15 \(\mu\)g of total RNA extracted from roots harvested at the time points indicated was sequentially hybridized with \(\text{IRT1}\), \(\text{FRO2}\), and \(\text{EF1}\) probes. Blots presented in this figure are representative of three independent experiments.

*Figure 6.* Schematic representation of two hypothetical models of regulation by the coordinated action of local and systemic signals. In the promotive model, the shoot-borne signal is produced in response to iron deficiency and, in conjunction with the local iron induction, results in activation of root iron uptake genes. In this scheme, iron-sufficient status in the shoot would prevent release of this systemic signal, and genes could not be expressed. Conversely, the repressive model accounts for a situation in which the systemic signal is produced in response to shoot-sufficient iron status. In that case, this signal could turn on a pathway leading to negative regulation of root iron uptake genes; its absence under iron deficiency would thus allow derepression of gene expression, given that iron molecules are present locally to trigger the response.
high symplastic iron and activates a pathway leading to repression of the root genes and eventually iron uptake shut off. In low iron conditions, the absence of the shoot signal would allow derepression of the expression of IRT1 and FRO2. In both models, the presence of iron in the vicinity of the root cells is required to induce gene expression because depleting the apoplast of iron results in total inhibition of IRT1 and FRO2 gene expression. Such a strategy, in which the nutrient is required locally to promote the uptake response, is common to the one described for the nitrate uptake system (Gansel et al., 2001) and may enable plants to save energy in case of extreme iron limitation.

The regulatory models presented fit our knowledge of the physiological disorders presented by the pea dgl and brz mutants. By reciprocal grafting with their wild-type parent, the phenotype of these mutants, i.e., constitutive activation of the root response to iron limitation, was shown to be determined by their shoot. This result could be equally explained by the lack of a repressive signal or by the release of a promotive signal by the shoot of the two mutants. If a negative shoot-borne signal is interrupted in the mutants, derepression of the response will be observed. Conversely, release of a promoting factor may be allowed by the mutation of DGL and BRZ, which would also result in activation of the response. A double-shoot graft, in which both dgl and DGV (its parental wild type) shoots were grafted on the same wild-type rootstock, exhibits an elevated rate of root Fe(III) chelate reductase (Grusak and Pezesghi, 1996). This result prompted the authors to conclude that the shoots probably release a promotive signal in iron deficiency on the ground that the lack of a repressive signal (under iron sufficiency) in dgl shoot would probably be compensated for by the DGV shoot. However, this experiment does not allow to discriminate between the two models presented because root Fe(III)-chelate reductase activity in the double-shoot graft plants is in fact intermediate between the graft plants DGV shoot/DGV and dgl shoot/DGV (Grusak and Pezesghi, 1996). This intermediate level of reductase activity is also compatible with the lack of half of a repressor signal in the double-shoot graft. Such a repressive model (see Fig. 6) could be more adapted to fine tune the iron uptake apparatus because it relies on a balance between a positive signal—the amount of inducer present in the root—and a negative signal—systemic repression resulting from the global iron status of the plant. Future experiments will be needed to demonstrate which of the two models, promotive or repressive, accounts for the regulation of the root iron deficiency response genes.

Regulation by local iron has also been shown to control root hair development, a morphological root response to iron deficiency (Schikora and Schmidt, 2001). In Arabidopsis split roots, root hairs develop in the +Fe-grown side. This morphological adaptation, but not the induction of ferric reductase activity, is impaired in ethylene mutants (Schikora and Schmidt, 2001), indicating that the two responses to iron deficiency are regulated in a different manner, as already suggested (Chaney et al., 1992; Moog et al., 1995). The fact that both expression of IRT1 and FRO2 and the iron deficiency-induced root hair formation respond to local iron levels indicates the requirement for an iron sensor in the root. Whether the sensing occurs in the environment and/or in the cell remains to be established. In yeast and mammals, an intracellular iron sensing process has been characterized involving, respectively, the AFT1/2 transcription factors (Yamaguchi-Iwai et al., 1996; Blaiseau et al., 2001; Rutherford et al., 2001) and the RNA binding proteins IRP1/2 (Samaniego et al., 1994; Hentze and Kuhn, 1996). In plants, the existence of a root extracellular iron sensor has been suggested by (Schmidt et al., 1996) who showed that in +Fe/−Fe split-root experiments, symplastic iron concentration in the root is identical in both sides even though the −Fe roots present an increased number of hairs. On the other hand, the fact that under iron sufficiency the irt1-1 knock-out mutant harbors a larger number of root hairs and a higher ferric reductase activity than the wild type while the two plants face the same extracellular iron concentration (Vert et al., 2002; data not shown) argues in favor of a sensor located inside the root cells.

A second important result of this work is that IRT1 and FRO2 transcripts accumulate specifically during the day under standard iron nutritional conditions, stressing once more the strict coregulation of these two genes. Photosynthetic products may mediate this diurnal regulation given that variation of sugar, and particularly Suc, during the day/night variations parallels the one of IRT1 and FRO2 expression (Kerr et al., 1985). The link between iron and sugar metabolisms has been revealed by a recent work from Thimm et al. (2001) who showed using a microarray analysis that many genes involved in gluconeogenesis and starch degradation are up-regulated by iron starvation in Arabidopsis. A role of sugar as activator of the iron uptake activity may explain why roots from a potato (Solanum tuberosum) tuber with sprout removed, which are thus connected to large stores of starch, are capable of developing a proper response to iron starvation (Bienfait et al., 1987). In addition, it may explain the discrepancy in the patterns of IRT1 and FRO2 expression observed between plants grown in vitro in Suc-containing solutions, and plants grown in hydroponic cultures without Suc. Interestingly, the diurnal regulation of IRT1 and FRO2 is overruled by iron nutritional growth conditions because we have found that starving plants of iron abolishes the day/night oscillation and restores a strong accumulation of the transcripts during the night. We conclude that the root iron uptake machin-
ery responds primarily to iron nutrition and that in iron replete conditions, it is additionally regulated by external signals, which could be sugars, other metabolites, or even light or circadian rhythm (Fig. 6). This is the first report of such a diurnal control of the iron deficiency response genes in strategy I plants. Strategy II plants also show a day/night-regulated response because secretion of phytosiderophores follows a distinct diurnal rhythm, with a peak rise after the beginning of illumination (Takagi et al., 1984). Further experiments will include the analysis of protein accumulation as well as functional activity during these treatments to determine whether or not root iron uptake is regulated diurnally.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis plants (ecotype Columbia) were grown hydroponically under non-sterile conditions for 5 to 6 weeks on individual Eppendorf tubes filled with sand, as described previously by Lejay et al. (1999). The basal nutrients common to all solutions were: 1 mM KH2PO4, 1 mM MgSO4 0.1 mM FeNa-EDTA, 50 μM KCl, 30 μM H3BO3, 5 μM MnSO4, 1 μM ZnSO4, 0.1 μM CuSO4 and 0.1 μM (NH4)6Mo7O24. Five-week-old plants were transferred to iron-deficient medium after washing the roots for 5 min either with Fe-free medium (Fig. 1) or with medium containing the reductant sodium dithionite (10 mM) and the chelator 2,2-bipyridyl (1.5 mM) as described (Bienfait et al., 1985; Figs. 2 and 4). Plants were then grown in iron-deficient conditions for 24 h. For day/night variation experiments, plants were cultivated in short-day conditions (light from 9 AM to 5 PM) in the presence of 100 μM Fe-EDTA (Fig. 5A). In the experiment described in Figure 5B, plants were further grown for 24 h in a solution containing 5 μM iron. Root plants were then harvested at the time points indicated in the legend to Figure 5. Time points performed the 2nd d are labeled with an apostrophe on Figure 5 and in the text (i.e. 8’ PM).

The split-root experiments were performed as previously described by Gansel et al. (2001). The localized supply treatments were initiated by transferring one part of the root system to the Fe-free solution after washing the roots with dithionite and bipyridyl, with the other part remaining supplied with Fe as before. At this stage, special care was taken to avoid cross-contamination between compartments.

Northern-Blot Analyses

Total RNA was extracted (Lebreux et al., 1992) from organs of plants cultivated as described above. Samples of RNA were denatured and electrophoresed on a 1.2% (w/v) MOPS-formaldehyde agarose gel before transfer to a nylon membrane (Biotrans, ICN, Irvine, CA). The same blot was sequentially hybridized with probes corresponding to the entire cDNA sequences of IRT1 and FRO2 (Eide et al., 1996; Robinson et al., 1999). Filters were exposed to a Phosphor Imager screen (Eastman Kodak, Rochester, NY) during 24 h in all experiments performed. Signal was revealed using a PhosphorImager Storm apparatus (Molecular Dynamics, Sunnyvale, CA). Quantification was performed relative to the 25S rRNA hybridization signal.

Western-Blot Analysis

Total protein was prepared from the roots of plants grown hydroponically that were either iron deficient or iron sufficient. Extracts were prepared by grinding tissue (2 mL buffer 1 g−1 wet tissue) on ice in extraction buffer (20 mM Tris, pH 8.0, 5% [v/v] glycerol, 4% [w/v] SDS, 1% [w/v] polyvinylpyrrolidone, and 1 mM phenylmethylsulfonyl fluoride) exactly as described by Connolly et al. (2002). Ten micrograms of total protein extracts were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes by electroblotting. The polyclonal IRT1 peptide antibody, used at the dilution of 1/1,000, was a generous gift from Dr Erin Connolly (Connolly et al., 2002).

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