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Ethylene could influence ferric reductase, iron transporter, and H$^+$-ATPase gene expression by affecting *FER* (or *FER*-like) gene activity

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Abstract

In previous works, it has been shown, by using ethylene inhibitors and precursors, that ethylene could participate in the regulation of the enhanced ferric reductase activity of Fe-deficient Strategy I plants. However, it was not known whether ethylene regulates the ferric reductase gene expression or other aspects related to this activity. This paper is a study of the effects of ethylene inhibitors and precursors on the expression of the genes encoding the ferric reductases and iron transporters of *Arabidopsis thaliana* (*FRO2* and *IRT1*) and *Lycopersicon esculentum* (= *Solanum lycopersicum*) (*FRO1* and *IRT1*) plants. The effects of ethylene inhibitors and precursors on the activity of the iron reductase and the iron transporter have been examined in parallel. Also studied were the effects of ethylene inhibitors and precursors on the expression of the H$^+$-ATPase genes of cucumber (*CsHA1* and *CsHA2*) and the transcription factor genes of tomato (*LeFER*) and *Arabidopsis* (*AtFRU* or *AtFIT1*, an *LeFER* homologue) that regulate ferric reductase, iron transporter, and H$^+$-ATPse activity. The results obtained suggest that ethylene participates in the regulation of ferric reductase, the iron transporter, and H$^+$-ATPase gene expression by affecting the *FER* (or *FER*-like) levels.

Keywords: *Arabidopsis*, cadmium, cucumber, ethylene, ferric reductase, H$^+$-ATPase, iron, iron transporter, tomato

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AOA, amino-oxyacetic acid; AVG, aminoethoxyvinylglycine; STS, silver thiosulphate

Introduction

Iron (Fe) is essential for the survival and function of plants and most other organisms. In dicots and non-grass monocots (Strategy I plants), some Fe-regulated proteins, ferric reductases, iron transporters, and H$^+$-ATPases, play key roles in Fe uptake by the roots (Hell and Stephan, 2003). Most soil Fe is sparingly soluble and occurs in the Fe(III) form. Fe(III) reductases, or ferric reductases, are integral plasma membrane proteins that transfer electrons from cytoplasmic NAD(P)H to reduce Fe(III) to Fe(II) outside the cell. The reduced Fe is then a substrate for integral plasma membrane Fe(II) transporter, or iron transporter, proteins. In recent years, several genes that encode these proteins have been identified. Ferric reductases *AtFRO2* of *Arabidopsis thaliana* (Robinson et al., 1999), *PsFRO1* of pea (Waters et al., 2002), and *LeFRO1* of tomato (Li et al., 2004) have been characterized, and cDNA sequences with significant similarity can be found in genome databases. The first iron transporter gene to be identified, and a founding member of the ZIP (*Z*RT/*I*RT like *P*roteins) family of metal transporters, was *Arabidopsis IRT1* (*I*ron-*R*egulated *T*ransporter; Eide et al., 1996). ZIP family plant iron transporter genes include *AtIRT2* (Vert et al., 2001),

LeIRT1 and *LeIRT2* (Eckhardt et al., 2001), *OsIRT1* (Bughio et al., 2002), and *PsRIT1* (Cohen et al., 2004). In relation to H^+-ATPases, responsible for rhizosphere acidification, some Fe-regulated genes identified are the *AtAHA7* of *Arabidopsis* (Colangelo and Guerinot, 2004) and the *CsHA1* of cucumber (Santi et al., 2005). These latter authors also identified another H^+-ATPase gene in cucumber roots, *CsHA2*, which did not respond to Fe deficiency (Santi et al., 2005). Genes for ferric reductase, iron transporter, and H^+-ATPase proteins are up-regulated in Fe-deficient roots and, in some cases, in other tissues, in order to maintain Fe homeostasis within the plant (Vert et al., 2001, 2002, 2003; Connolly et al., 2002, 2003; Henriques et al., 2002; Bauer et al., 2004; Li et al., 2004; Santi et al., 2005). Additional Fe deficiency-stress responses include development of transfer cells and subapical root hairs (Romera and Alcántara, 2004).

Although up-regulation of Fe acquisition genes under Fe deficiency has been well established, the signals for plant Fe status and the mechanisms of their regulation remain elusive. Bienfait et al. (1987) found that isolated potato roots developed Fe deficiency-stress responses (i.e. enhanced ferric reductase activity, acidification, root hairs, and transfer cells) when grown in a Fe-free medium. This result suggested that the root itself can control the development of Fe deficiency stress responses. However, other results indicate that the control of the Fe deficiency-stress responses does not depend only on the root Fe content, but that their regulation is far more complex and probably involves signals coming from the aerial part. Grusak and Pezeshgi (1996) showed, by grafting the pea mutant *dgl* (*deg*enerated *l*eaflets) onto its wild-type DGV (Dippes Gelbe Viktoria), that the reductase activity of the grafted plants was up-regulated, which suggests that the *dgl* shoot transmits a signal compound that acts as a promoter of this response. In addition, several split root experiments have shown that a root half growing in Fe-replete media induced Fe-stress responses when the other root half was growing without Fe (Romera et al., 1992; Li et al., 2000; Schikora and Schmidt, 2001; Schmidt et al., 2003; Vert et al., 2003). To explain these results, it has also been proposed that systemic signal(s) exist that could move within the plant (Romera et al., 1992; Vert et al., 2003).

As systemic signals involved in the regulation of Fe deficiency- stress responses, some authors have proposed the plant hormones auxin (Landsberg, 1984) or ethylene (Romera and Alcántara, 1994). Ethylene production is increased under Fe deficiency in several Strategy I plants (Romera et al., 1999; Waters and Blevins, 2000; Li and Li, 2004; Molassiotis et al., 2005). Moreover, treatment of several Strategy I plants with inhibitors of ethylene synthesis or action greatly decreased their ferric reductase activity, while treatment with precursors of ethylene synthesis enhanced it (Romera and Alcántara, 1994, 2004; Romera et al., 1999; Molassiotis et al., 2005). Despite these results, normal induction of ferric reductase activity occurs in some Fe-challenged ethylene-insensitive mutants, putting into question the role of ethylene in the regulation of this response (Schmidt et al., 2000; Li and Li, 2004). However, and according to what is already known about ethylene perception and signaling, such questioning is not adequate because ethylene may use different receptors and transduction pathways to regulate different responses (Larsen and Chang, 2001; Moshkov et al., 2003; Stepanova and Alonso, 2005). Five ethylene receptors have been described (ETR1, ETR2, EIN4, ERS1, and ERS2) in *Arabidopsis* (Stepanova and Alonso, 2005) and six ethylene receptors in tomato (Klee and Tieman, 2002). Therefore, a particular mutation in one receptor may render plants unable to induce some of the ethylene-triggered responses, like the development of subapical root hairs, while conserving a wild-type regulation for others, like the enhancement of the ferric reductase activity (Romera and Alcántara, 2004, and references therein).

In relation to the up-regulation of Fe acquisition genes under Fe deficiency, it is also known that the FER protein, recently identified in tomato as a bHLH transcription factor (Ling et al., 2002), is necessary for the transcription of *LeFRO1* and *LeIRT1*, since the tomato mutant *fer* fails to up-regulate both genes (Ling et al., 2002; Bereczky et al., 2003; Li et al., 2004). Similarly, the FER protein is also necessary for the acidification response, due to enhanced H^+-ATPase activity (Dell'Orto et al., 2000), since the tomato mutant *fer* did not acidify the nutrient solution under Fe-deficient conditions (Brown et al., 1971). The FRU protein (also named FIT1), a homologue of the tomato FER protein recently identified in *Arabidopsis*, is also necessary for the activation of *AtFRO2* (Colangelo and Guerinot, 2004; Jakoby et al., 2004) and *AtIRT1* transcription (Jakoby et al., 2004). *LeFER* and *AtFRU* expression is induced in roots in response to Fe deficiency (Bauer et al., 2004; Colangelo and Guerinot, 2004; Jakoby et al., 2004; Brumbarova and Bauer, 2005). However, until now no relationship between LeFER, or AtFRU, and ethylene has been established.

The aim of this work was to study the involvement of ethylene on the expression of the ferric reductase, the iron transporter, and the H^+-ATPase genes in *Arabidopsis*, tomato, and cucumber plants, as well as on the expression of the transcription factor genes of tomato (*FER*) and *Arabidopsis* (*FRU*). Plant species, in which ferric reductase, iron transporter, H^+-ATPase, and FER (or FER-like) genes had already been isolated, were chosen. Also studied were the *Arabidopsis* ethylene-insensitive mutant *etr1* and the *Arabidopsis frd3-3(man1)* mutant. The *etr1* mutant did not develop subapical root hairs either under Fe deficiency or upon treatment with 1-aminocyclopropane-1-carboxylic acid (ACC), while it enhanced its ferric reductase activity under both treatments (Romera and Alcántara, 2004). The *frd3* mutant shows constitutive

expression of *AtFRO2*, *AtIRT1*, and *AtFRU* genes despite high levels of Fe in their roots (Green and Rogers, 2004). The expression of *Arabidopsis AtFRO2*, *AtIRT1* and *AtFRU*; tomato *LeFRO1*, *LeIRT1*, and *LeFER*; and cucumber *CsHA1* and *CsHA2*, is examined in response to plant Fe status, ethylene inhibitors and ethylene precursors. In addition, the basic chitinase gene (*AtCHIT-B*), which is known to be regulated by ethylene, has been studied in some experiments with *Arabidopsis* plants.

Materials and methods

Plant materials, growth conditions, and treatments

Arabidopsis, cucumber, and tomato plants were grown in a growth chamber at 22°C day/20°C night temperatures, with relative humidity between 50% and 70%, and a 14 h photoperiod (8 h for *Arabidopsis*, to postpone flowering) at a photosynthetic irradiance of 300 μmol m^{-2} s^{-1} provided by fluorescent tubes (Sylvania Cool White VHO). Seeds of *Arabidopsis thaliana* (L.) Heynh ecotype Columbia, the ethylene-insensitive *etr1-1* mutant, the *frd3-3* (*man1*) mutant, and the transgenic lines 3-18H (overexpressing IRT1; Connolly et al., 2002) and 15G (overexpressing FRO2; Connolly et al., 2003) were germinated in black peat. When plants were 30-d-old, they were inserted in plastic lids and held in the holes of a thin polyurethane raft floating on aerated nutrient solution. After 10–15 d in this hydroponic system, the plants were individually transferred to 70 ml plastic vessels and given different treatments. Seeds of cucumber (*Cucumis sativus* L. cv. Ashley) were germinated in the dark within papers moistened with 5 mM $CaCl_2$. After 2–3 d, the seedlings were transferred to a plastic mesh held over half-strength nutrient solution, and kept in the dark for 2 d. Cucumber seedlings were then transplanted individually to 70 ml plastic vessels containing continuously aerated nutrient solution with 20 μM FeEDDHA. After 2–3 d in this nutrient solution, cucumber plants were transferred to the different treatments. Seeds of tomato [*Lycopersicon esculentum* (= *Solanum lycopersicum*) Mill. cv. Tres Cantos, T3238FER, and T3238fer] were germinated and grown in sand until they were *c.* 15-d-old. Tomato seedlings were transplanted to 70 ml plastic vessels containing continuously aerated nutrient solution with 20 μM FeEDDHA or 100 μM FeHEDTA (for tomato *fer*; it did not grow very well in nutrient solution with FeEDDHA). After 5–7 d in this nutrient solution, tomato plants were transferred to the different treatments. The nutrient solution (without Fe) had the following composition: 2 mM $Ca(NO_3)_2$, 0.75 mM K_2SO_4, 0.65 mM $MgSO_4$, 0.5 mM KH_2PO_4, 50 μM KCl, 10 μM H_3BO_3, 1 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.05 μM $(NH_4)_6Mo_7O_{24}$. Fe-EDDHA was added at different concentrations depending on the experiments. The pH was adjusted to 6.0 with 0.1 N KOH.

The treatments imposed were: +Fe, nutrient solution with Fe-EDDHA; +Fe + ACC, same as +Fe treatment but with ACC addition during the previous 24 h; –Fe, nutrient solution without Fe; –Fe + ACC, –Fe treatment with ACC addition during the previous 24 h; –Fe + Co, –Fe + aminoethoxyvinylglycine (AVG), –Fe + aminooxyacetic acid (AOA); or –Fe + silver thiosulphate (STS), –Fe treatment (1, 2, or 3 d) with $CoSO_4$, AVG, AOA, or STS added during the previous 24 h. Stock solutions of ACC and AVG (Sigma) were prepared in deionized water. Stock solution of STS was prepared as previously described (Romera and Alcántara, 1994). After treatment, root ferric reductase activity was determined as described below. Finally, the roots were collected and kept at –80°C for later analysis of mRNA.

Ferric reductase activity determination

Intact plants were pretreated for 30 min in plastic vessels with 50–70 ml of a nutrient solution without micronutrients, pH 5.5, and then placed into 50–70 ml of a Fe(III) reduction assay solution for 30 min. This assay solution consisted of nutrient solution without micronutrients, 100 μM Fe(III)-EDTA and 300 μM Ferrozine, pH 5.0 (adjusted with 0.1 N KOH). The environmental conditions during the measurement of Fe(III) reduction were the same as the growth conditions described above. The ferric reductase activity was determined spectrophotometrically by measuring the absorbance (562 nm) of the Fe(II)–Ferrozine complex and using an extinction coefficient of 29 800 M^{-1} cm^{-1}. After the reduction assay, roots were excised and weighed, and the results were expressed on a root fresh weight basis. In some treatments, the location of the ferric reductase activity along the roots was visualized in agar plates with ferric reduction assay solution.

Cd uptake capacity determination

The method for Cd uptake determination was based on the method used by Cohen et al. (1998). Tomato [*Lycopersicon esculentum* (= *Solanum lycopersicum*) Mill. cv. Tres Cantos] seedlings, 22-d-old, were transferred to either nutrient solution with 40 μM Fe-EDDHA or without Fe for 3 d. $CoSO_4$, at 10 μM, or STS, at 200 μM, were added to the nutrient solution of some of the Fe-deficient plants during the previous 24 h, prior to Cd uptake determination. To determine Cd uptake capacity, intact plants were pretreated for 30 min in a solution with 0.2 mM $CaSO_4$ and then transferred for a period of 30 min to 70 ml of a solution with 0.2 mM $CaSO_4$, 25 μM $Cd(NO_3)_2$ at pH 5.5, buffered with 5 mM MES. After a desorption period of 15 min in a solution with 5 mM $CaSO_4$ and 5 mM MES, pH 5.5, roots were separated from the aerial part and Cd content was analyzed by acid digestion and atomic absorption spectrophotometry.

RT-PCR analysis

Roots were ground to a fine powder in a mortar and pestle in liquid nitrogen. Total RNA was extracted using the Tri Reagent solution (Molecular Research Center, Inc. Cincinnati, OH, USA) according to the manufacturer's instructions. M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA) was used to generate cDNA with 3 μg of total RNA from roots as template and random hexamers or oligo dT(20) as primers. Negative controls included all reaction components except M-MLV enzyme. One-tenth of each RT reaction was used as PCR template. Primer pairs AtFRO2F/AtFRO2R and AtIRT1F/AtIRT1F were designed to amplify specifically *Arabidopsis AtFRO2* and *AtIRT1* cDNA, respectively (table 1). Oligonucleotides designed by Li et al. (2004) were used to amplify *LeFRO1* and *LeIRT1* cDNA. Primers from Brumbarova and Bauer (2005) were used to amplify *LeFER* cDNA. *AtFRU* cDNA was amplified with the primers described in Jakoby et al. (2004). *CsHA1* and *CsHA2* cDNA was amplified with the primers designed by Santi et al. (2005). Actin cDNA was amplified with primers ActPsCsF and ActCSPcR (table 1) as an internal control in some RT-PCR experiments. Alternatively, 18S cDNA was amplified using Quantu*mRNA* Universal 18S Standards primer set (Ambion, Austin, Texas, USA) as internal control to another set of experiments. The thermalcycler program was one initial cycle of 94°C, 5:00; followed by cycles of 94°C, :45; 55°C, :45; 72°C, 1:00, with 27 cycles for *AtFRO2*, *AtIRT1*, *AtFRU*, *AtCHI-B*, *LeFRO1*, *LeIRT1*, *LeFER*, *CsHA1*, and *CsHA2*, and 25 cycles for actin and 18S, all followed by a final 72°C elongation cycle of 7:00.

Northern analysis

Samples containing 20 μg total RNA were separated by electrophoresis, blotted onto nylon filters and hybridized to radioactively labeled probes according to standard procedures (Sambrook et al.,

Table 1. Primers designed for this study

Primer name	Sequence (5′–3′)	Purpose
AtFRO2F	TGG TTG CCA CAT CTG CGT AT	*AtFRO2* forward
AtFRO2R	GAG AGC GAT GAA GGA GAA ACT T	*AtFRO2* reverse
AtIRT1F	CAT TGC AAG CAT GAT TGG TGT TGG	*AtIRT1* forward
AtIRT1R	ACC CGA GAA GAG CCG CGA TT	*AtIRT1* reverse
AtCHIBF	ATG AAA CTA CAG GTG GAT GGG CTA CA	*AtCHI-B* forward
AtCHIBR	GGA AAT AAA ATC GCA ACA TAA ACA GTG	*AtCHI-B* reverse
ActPsCsF	AGA TGA CGC AGA TAA TGT TTG AGA C	Actin forward
ActPsCsR	TCA GGA AGT TCA TAG TTC TTC TCA A	Actin reverse

1989). Hybridization was performed at 42°C overnight, in solutions containing 50% formamide and the corresponding probe. Probe for *LeIRT1* was prepared by labeling the full-length *LeIRT1* (a generous gift from Dr. Y. Wang) cDNA. The probe was labeled by random priming with the Rediprime II Random Prime Labeling System kit (Amersham Biosciences, Amersham, Bucks, UK). After hybridization, filters were washed twice for 15 min in 0.2 × SSC, 0.1% (w/ v) SDS at 65°C. Results of the northern analyses were revealed by autoradiography after exposing X-ray films (Kodak X-Omat AR) to the filters for 5 d at –80°C.

Results

Regulation of the ferric reductase, the iron transporter, and the H⁺-ATPase genes in Arabidopsis*, tomato, and cucumber plants*

Ethylene has been involved in triggering several physiological and morphological responses to Fe-deficiency in Strategy I plants (Romera and Alcántara, 2004). However, its ability to regulate key genes for Fe acquisition, like *FER*, *FRO*, *IRT*, or *HA*, has not yet been proved. In this work, inhibitors of ethylene synthesis or ethylene action were tested to find out if they affect message levels of *AtFRO2*, *AtIRT1*, and *AtFRU* genes in response to Fe deficiency. In both the *Arabidopsis* wild-type cultivar Columbia (fig. 1) and its ethylene-insensitive mutant *etr1* (fig. 2), *FRO2*, *IRT1*, and *FRU* transcript levels were greatly increased when plants were challenged with Fe-deficiency stress (+Fe40 versus –Fe). However, when roots were treated for 24 h with Co (ethylene synthesis inhibitor; Yang and Hoffman, 1984), message levels of all three genes were induced to lower levels in both WT (fig. 1) and *etr1* (fig. 2). Treatment with STS (ethylene action inhibitor; Yang and Hoffman, 1984) also led to lower transcript levels in WT (fig. 1) and *etr1* (fig. 2). These ethylene inhibitors also attenuated the enhanced ferric reductase activity of both Fe-deficient Columbia and *etr1* (figs. 1, 2). However, the addition of ethylene inhibitors (AOA, Co, or STS) to the *Arabidopsis* transgenic lines 3-18H (that overexpress IRT1) and to the 15G (that overexpress FRO2) did not inhibit the mRNA accumulation of either IRT1 or FRO2, respectively (fig. 3).

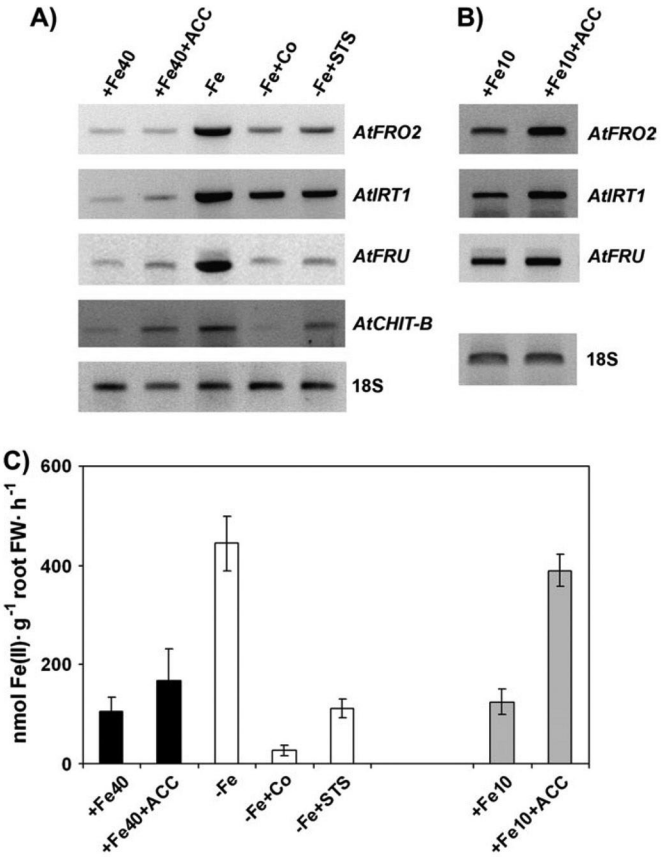

Figure 1. Regulation of *AtFRO2*, *AtIRT1*, *AtFRU*, and *AtCHIT-B* expression (A, B) and ferric reductase activity (C) by ethylene inhibitors and precursors in *Arabidopsis* Columbia plants. Some plants grown in nutrient solution with 40 μM Fe (+Fe40), 10 μM Fe (+Fe10), or the previous day without Fe (–Fe) were treated during the previous 24 h with 1 μM ACC, 50 μM Co, or 200 μM STS. After treatment, the ferric reductase activity (C) was determined (values are the means ± standard error of six replicates) and total root RNA extracted. RT-PCR (A, B) was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs of *AtFRO2*, *AtIRT1*, *AtFRU*, and *AtCHIT-B*. 18S cDNA was amplified as a positive control.

To test whether ethylene alone could induce Fe deficiency- responsive genes, the ethylene precursor ACC (Yang and Hoffman, 1984) was added to the growth medium. In Columbia, *AtFRO2*, *AtIRT1*, and *AtFRU* expression was not substantially increased following ACC treatment in high-Fe plants (+Fe40 + ACC; fig. 1A), nor was ferric reductase activity substantially increased by this treatment (fig. 1C). In low-Fe plants (+Fe10 + ACC; fig. 1B), transcripts of *FRO2*, *IRT1*, and *FRU* were increased, and ferric reductase activity was induced to levels nearly as high as those in the –Fe treatment. In the *etr1* genotype, the effect of ACC was similar to the one in Columbia plants, inducing higher levels of *FRO2*, *IRT1*, and *FRU* message (fig. 2B) in the lower-Fe plants but hardly in the high-Fe ones (fig. 2A).

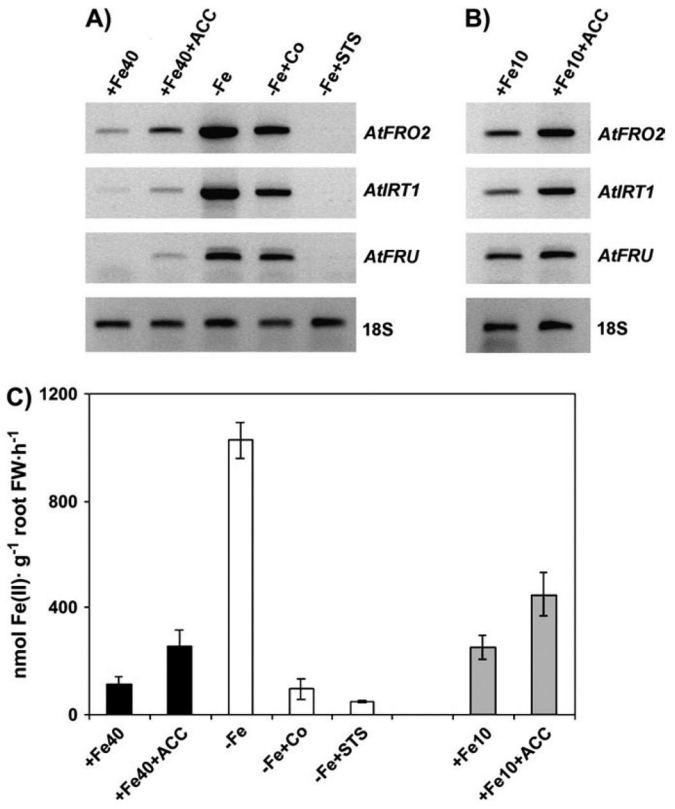

Figure 2. Regulation of *AtFRO2*, *AtIRT1*, and *AtFRU* expression (A, B) and ferric reductase activity (C) by ethylene inhibitors and precursors in the *Arabidopsis* ethylene-insensitive mutant *etr1*. Experimental conditions as in figure 1.

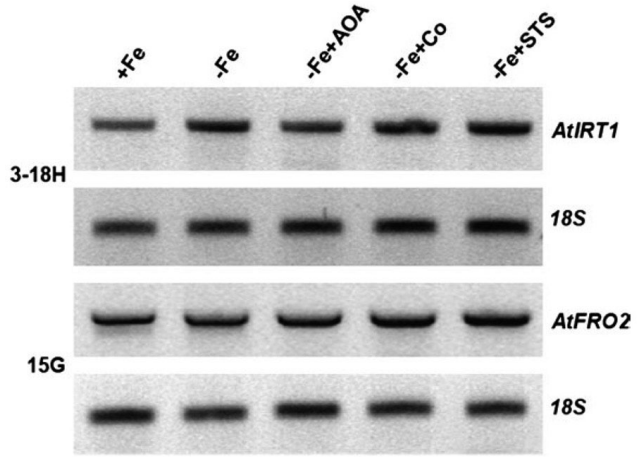

Figure 3. Effects of Fe deficiency and ethylene inhibitors on *AtIRT1* and *AtFRO2* expression in the *Arabidopsis* transgenic lines 3-18H (that overexpress *IRT1*) and 15G (that overexpress *FRO2*). Plants were grown in nutrient solution with 40 μM Fe (+Fe) or the previous 2 d without Fe (–Fe). Some of the –Fe plants were treated during the previous 24 h with 50 μM AOA, 50 μM Co, or 200 μM STS. RT-PCR was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs of *AtIRT1* and *AtFRO2*. 18S cDNA was amplified as a positive control.

The present study was extended to tomato to further confirm the involvement of ethylene in the regulation of *FER*, *FRO*, and *IRT* genes. Again, ethylene inhibitors (Co, AVG, AOA, and STS; Yang and Hoffman, 1984) efficiently prevented the accumulation of *LeFER*, *LeFRO1*, and *LeIRT1* mRNA (figs. 4A, 5) and prevented the normal upregulation of ferric reductase activity (fig. 4C) and the enhanced Cd uptake capacity of Fe-deficient plants (fig. 5). Iron-deficient plants have an increased uptake capacity for a broad range of metals, resulting from the increased expression of relatively low-specificity IRT1 transporters. As an indirect measurement of Fe-uptake capacity, the accumulation of Cd in roots was measured. Fe-deficient tomato plants had enhanced capacity for Cd uptake in relation to the Fe-sufficient ones, while this enhanced capacity was greatly inhibited in Fe-deficient Co-treated and STS-treated plants (fig. 5). The differences in capacity for Cd uptake were consistent with the observed *LeIRT1* expression in the different treatments (fig. 5). Treatment of low-Fe plants (10 μM) with ACC resulted in higher *LeFRO1*, *LeIRT1*, and *LeFER* message levels (fig. 4B) and a slight increase in ferric reductase activity (fig. 4C). The enhanced

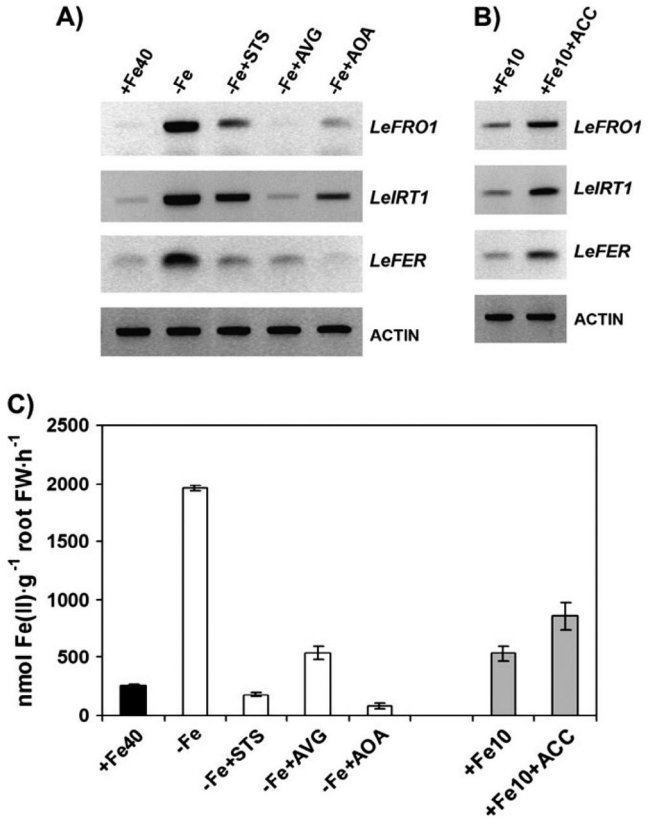

Figure 4. Regulation of *LeFRO1*, *LeIRT1*, and *LeFER* expression (A, B) and ferric reductase activity (C) by ethylene inhibitors and precursors in tomato plants. Some plants grown in nutrient solution with 40 μM Fe (+Fe40), 10 μM Fe (+Fe10) or the previous 3 d without Fe (–Fe) were treated during the previous 24 h with 1 μM ACC, 200 μM STS, 10 μM AVG, or 25 μM AOA. After treatment, the ferric reductase activity (C) was determined (values are the means ± standard error of six replicates) and total root RNA extracted. RT-PCR was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs of *LeFRO1*, *LeIRT1*, and *LeFER*. 18S cDNA was amplified as a positive control.

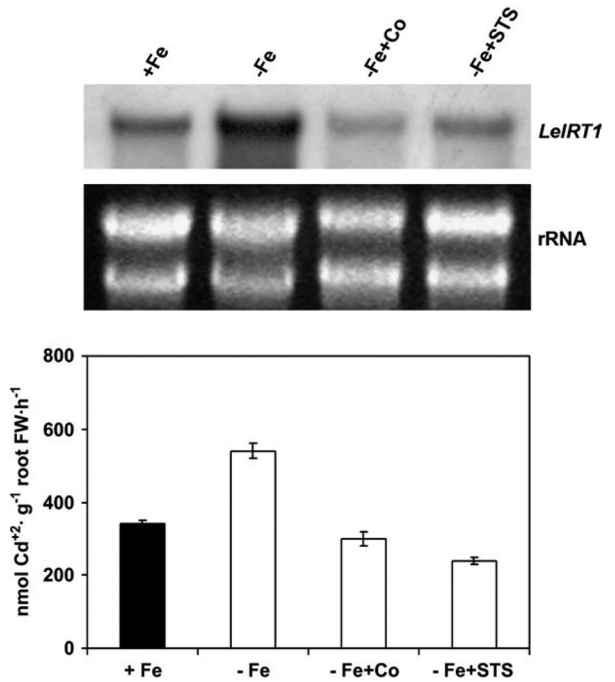

Figure 5. Effects of the Fe status and the ethylene inhibitors Co and STS on *LeIRT1* expression and Cd uptake capacity of tomato plants. Plants were grown in nutrient solution with Fe (+Fe). Some of them were transferred to nutrient solution without Fe during the previous 3 d (–Fe). Some of the Fe-deficient plants were treated with 10 μMCo (–Fe + Co) or 200 μMSTS (–Fe + STS) 24 h before Cd uptake determination. Cd uptake capacity was determined without the presence of ethylene inhibitors in the Cd uptake medium. Values are the means ± standard error of six replicates. For northern blot analysis, *LeIRT1* probe was hybridized to a blot containing 20 μg of total RNA extracted from roots of tomato plants.

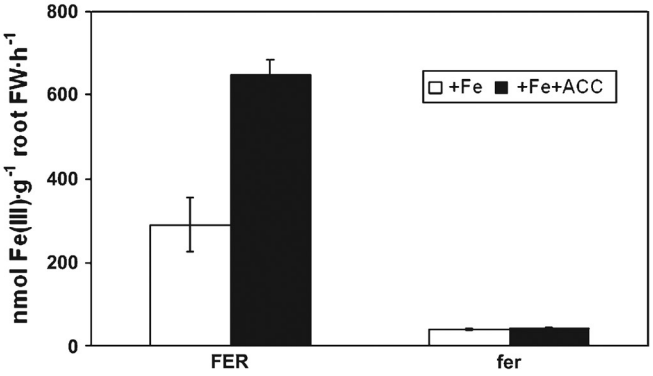

Figure 6. Effect of ACC on the ferric reductase activity of tomato FER and *fer* plants. Tomato plants were grown in nutrient solution with 20 μM Fe and during the previous 24 h half of the plants were treated with 0.6 μM ACC. After treatment, the ferric reductase activity was determined (values are the means ± standard error of six replicates).

reductase activity provoked by the ACC addition was not observed in the tomato mutant *fer* (fig. 6). Similar results to the ones found in *Arabidopsis* and tomato plants with ethylene inhibitors and precursors have also been found in cucumber and pea plants (FJ Romera, C Lucena; MJ García, and R. Pérez-Vicente, unpublished results).

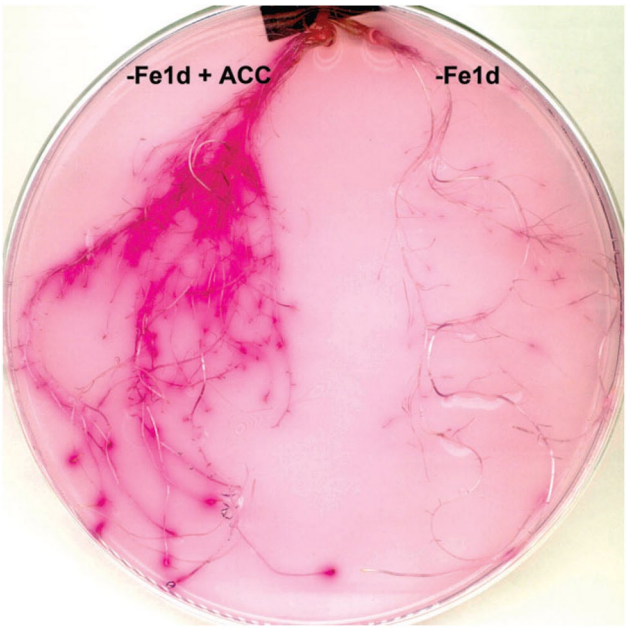

Figure 7. Localization of ferric reductase activity induction by ACC in cucumber roots. Cucumber plants were grown in nutrient solution without Fe in a split-root experiment: half of the roots was treated with 10 μM ACC addition (left) while the other half was not (right). After 24 h, the ferric reductase activity was determined in agar plates. The more intense color around the subapical region of the roots is due to the Fe(II)–ferrozine complex formed (red) and denotes higher ferric reductase activity. This intense red color is not presented in the half root not treated with ACC (right).

In cucumber plants, upon treatment of half of the root with ACC and 1 d of Fe deprivation, there was an enhanced ferric reductase activity in that half root, located in the subapical region of the roots (fig. 7) and associated with the development of subapical root hairs, as occurred in Fe-deficient plants (Romera and Alcántara, 1994, 2004). The study of the expression of the *CsHA1* and *CsHA2* H^+-ATPase genes showed that the *CsHA1* gene was upregulated by Fe deficiency, enhanced by ACC treatment, and inhibited by Co treatment, similarly to the other Fe-acquisition genes of *Arabidopsis* and tomato, while the *CsHA2* gene was not affected by any of these treatments (fig. 8).

The *Arabidopsis* mutant *frd3* shows constitutive activation of *AtFRO2*, *AtIRT1*, and *AtFRU* gene expression even when grown in Fe-sufficient conditions and accumulates high levels of Fe in their roots. However, it presents leaf chlorosis because the Fe cannot reach the leaves properly (Green and Rogers, 2004). To test whether the foliar application of Fe could block the up-regulation of the *AtFRO2*, *AtIRT1*, and *AtFRU* genes, Fe was sprayed onto the leaves of this mutant and after that the expression of the genes was determined. As shown in figure 9, the foliar application of Fe caused the regreening of the leaves and blocked the expression of the *AtFRO2*, *AtIRT1*, and *AtFRU* genes. Furthermore, the addition of ACC to the Fe-sprayed plants did not enhance the expression of these three genes, although it did enhance that of the basic chitinase gene (fig. 9).

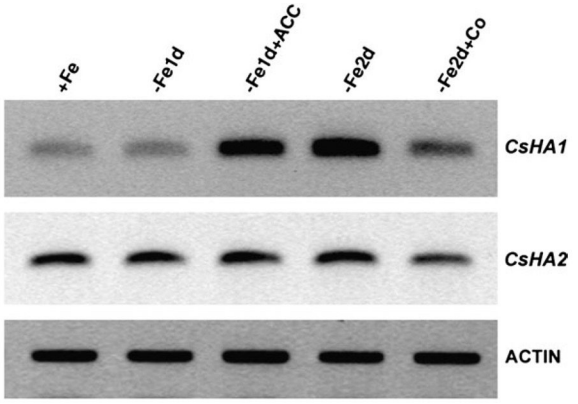

Figure 8. Regulation of *CsHA1* and *CsHA2* expression by ethylene inhibitors and precursors in cucumber plants. Plants were grown in nutrient solution with 60 µM Fe (+Fe) or no Fe (–Fe). Fe-deficient plants (–Fe) were grown in nutrient solution without Fe during the previous 1 d (–Fe1d) or 2 d (–Fe2d). Some of the –Fe1d plants were treated during the previous 24 h with 1 µM ACC, and some of the –Fe2d plants were treated during the previous 24 h with 10 µM Co. RT-PCR was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs of *CsHA1* and *CsHA2*. *ACTIN* cDNA was amplified as a positive control.

Discussion

In previous works it has been shown that the addition of ethylene inhibitors to Strategy I plants greatly inhibited their ferric reductase activity while ethylene precursors enhanced it (reviewed in Romera and Alcántara, 2004). However, it was not known whether ethylene affected *FRO* expression or other components involved in the ferric reductase activity. Here, it is shown that the addition of ethylene inhibitors (Co, AVG, AOA, or STS), for only 24 h, to Fe-deficient *Arabidopsis* (both wild-type Columbia and its ethylene-insensitive mutant *etr1*) and tomato plants negatively affected *AtFRO2* and *LeFRO1* (ferric reductase genes) expression, and ferric reductase activity (figs. 1, 2, 4), and also decreased *AtIRT1* and *LeIRT1* (iron transporter genes) expression (figs. 1, 2, 4, 5), and Cd uptake capacity (fig. 5). The inhibition of *LeIRT1* expression by the ethylene inhibitors Co or STS would explain the lower Cd uptake capacity of the Fe-deficient Co-treated or STS-treated plants in relation to the Fe-deficient ones (fig. 5), since IRT1 has been considered as a Cd transporter in Fe-deficient plants (Eide et al., 1996; Cohen et al., 1998, 2004; Korshunova et al., 1999; Eckhardt et al., 2001). The ethylene inhibitor Co also inhibited the mRNA accumulation of *CsHA1* (Fe-regulated H+-ATPase gene; Santi et al., 2005) but not that of *CsHA2* (H+-ATPase gene not regulated by Fe; Santi et al., 2005) (fig. 8). All these results indicate that ethylene could be involved in the regulation of *AtFRO2*, *LeFRO1*, *AtIRT1*, *LeIRT1*, and *CsHA1* transcription.

Ethylene could affect mRNA stability instead of transcription of the genes. However, results shown in figure 3, obtained with *Arabidopsis* transgenic lines overexpressing either *IRT1* or *FRO2*, suggest that ethylene affects transcription rather than mRNA stability. The mRNA levels of the *35S-IRT1* transgenic line 3-18H, overexpressing *IRT1*, and those of the *35S-FRO2* transgenic line 15G, overexpressing *FRO2*, were not decreased by ethylene inhibitors (fig. 3), as was observed in the wild-type cultivar (fig. 1). These results clearly suggest that ethylene mainly affects *FRO2* and *IRT1* transcription.

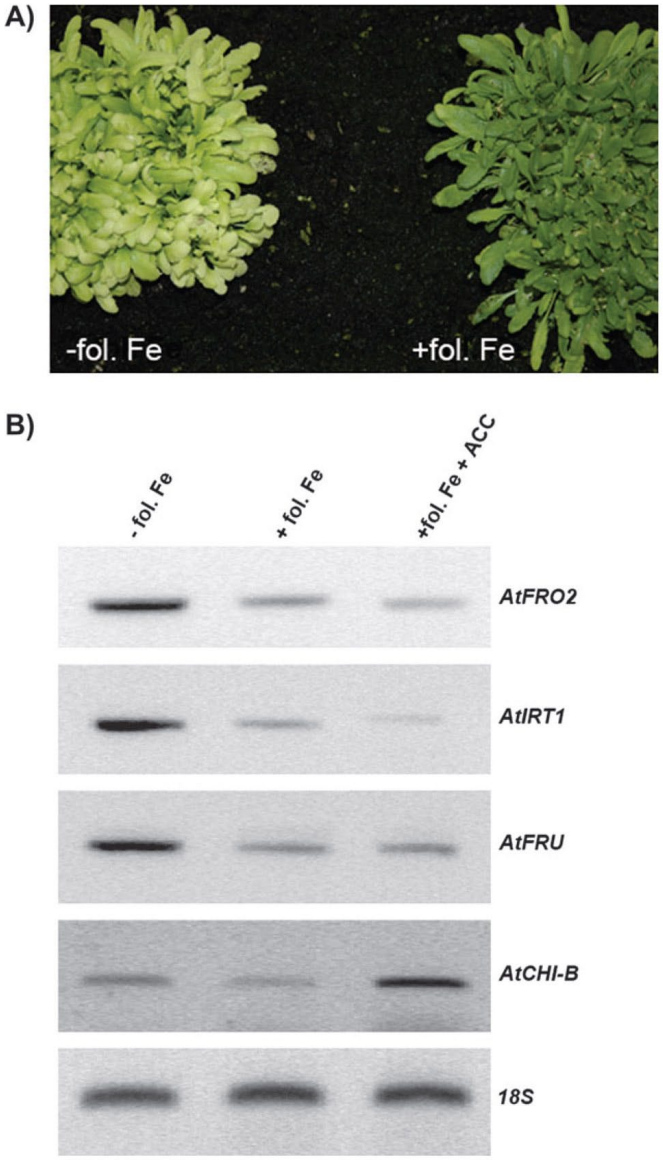

Figure 9. Effects of foliar application of Fe on leaf regreening (A) and *AtFRO2*, *AtIRT1*, *AtFRU*, and *AtCHIT-B* expression (B) of the *Arabidopsis* mutant *frd3* (*man1*). Plants were grown either in black peat (A) or in nutrient solution with 20 µM Fe (B). Some of the plants were sprayed with $FeSO_4$ [0.03% (w/v)] for 2 d (twice a day). After treatment of plants grown in nutrient solution, roots were harvested and total RNA was extracted. RT-PCR (B) was performed using total RNA from roots as template and gene-specific primers to amplify partial cDNAs of *AtFRO2*, *AtIRT1*, *AtFRU*, and *AtCHIT-B*. 18S cDNA was amplified as a positive control.

The addition of the ethylene precursor ACC for 24 h to either *Arabidopsis*, tomato, or cucumber plants grown in low-Fe conditions enhanced the expression of *FRO*, *IRT1*, and *HA1* (but not *HA2*) genes (figs. 1, 2, 4, 8), and ferric reductase activity (figs. 1, 2, 4, 6, 7), which also suggests that ethylene is involved in their regulation. It is important to note that ACC enhanced the ferric reductase activity in the subapical region of cucumber roots after 1 d of Fe deprivation, as eventually occurred in Fe-deficient roots after 2–3 d of Fe deprivation (fig. 7; Romera and Alcántara, 1994, 2004). When ACC was applied to plants grown in high-Fe conditions, there was little enhancement of induction of either gene (figs. 1, 2). Similar results have been found in cucumber and pea plants (FJ Romera, C Lucena; MJ García, and R Pérez-Vicente, unpublished data). These results imply that ethylene alone, whose production is increased in Fe-deficient roots (Romera et al., 1999; Waters and Blevins, 2000; Li and Li, 2004; Molassiotis et al., 2005), is not sufficient to induce *FRO* and *IRT1* transcription. It is suggested that ethylene acts as enhancer, and Fe acts as inhibitor, either directly or indirectly, of Fe stress genes. Probably, the enhancement of expression by ethylene would not be possible until some repressing factor related to Fe is removed.

The mechanism of ethylene enhancement of *FRO*, *IRT*, and *HA1* expression is not yet understood, but results in this work suggest that ethylene could affect Fe-stress genes by regulating FER (or FER-like) transcription factor levels. The addition of ethylene inhibitors to Fe-deficient tomato plants diminished their *FER* mRNA levels (fig. 4) and similar results with *FRU* mRNA levels were found in both *Arabidopsis* Columbia and *etr1* plants (figs. 1, 2). On the other hand, ACC enhanced *FRU* mRNA levels (or *FER* mRNA levels) in plants grown under low-Fe conditions but hardly in those grown under high-Fe conditions (figs. 1, 2, 4). The hypothesis that ethylene could affect *FRO*, *IRT*, and *HA1* expression by acting through the *FER* (or *FER*-like) transcription factor is supported by the fact that the tomato mutant *fer* (defective in the FER transcription factor; Brumbarova and Bauer, 2005) did not enhance its ferric reductase activity upon ACC treatment while its wild-type cultivar did (fig. 6). Furthermore, an 8 bp AWTTCAAA motif has been identified as an ethylene-responsive element in the promoter region of several ethylene-induced genes, like the tomato E4 gene (Montgomery et al., 1993), the carnation *GST1* gene (Itzhaki et al., 1994), and the *TLC1.1* retrotransposon from *Lycopersicon chilense* (Tapia et al., 2005). Analysis of *AtFRU* promoter region (locus AT2G28160) revealed the presence of AWTTCAAA motives at –303 and –771 bp (result not shown) which is in agreement with an ethylene regulation of this key regulatory gene.

A model, which has been proposed to explain the interaction of Fe deficiency, ethylene, FER (or FER-like) transcription factor, FRO, IRT1, and HA1, is shown in figure 10. According to this model, Fe-deficiency would cause an increase in ethylene production (and perhaps increased sensitivity) in the roots, possibly affected by ACC or other signals (auxin, etc.) coming from the shoots or its own roots. Ethylene would then promote *FER* (or *FER*-like) transcription or *FER* mRNA stability in the roots. Sufficient plant Fe status could block the ethylene effect on FER, if a high Fe level ultimately acts as an inhibitor of expression of Fe-stress genes. This combinatorial control would provide Fe-specificity to the system, and both Fe deficiency and ethylene action are necessary for full transcriptional activation. A precedent for combinatorial control over regulation is the FET4 Fe transporter of yeast, which is transcriptionally repressed by Rox1, and transcriptionally activated by Aft1 and/or Zap1. Removal of Rox1 repression is needed for full expression (Waters and Eide, 2002). The model proposed in figure 10 would explain several experimental results. First, it would explain why ethylene production increases in Fe-deficient plants (Romera et al., 1999; Waters and Blevins, 2000; Li and Li, 2004; Molassiotis et al., 2005), which agrees with the enhanced expression of the ethylene-regulated basic chitinase gene in these plants (fig. 1). Secondly, it would explain why ethylene inhibitors partially block *FRO1* (or *FRO2*), *IRT1*, and *HA1* expression, and ferric reductase activity and Cd uptake capacity (figs. 1, 2, 4, 5, 8; Romera and Alcántara, 2004). Thirdly, it would explain why ACC application to Fe-sufficient plants did not induce ferric reductase activity on all occasions (figs. 1, 2, 4, 9; Romera and Alcántara, 2004) since, depending on the internal Fe level, plants may or may not have enough Fe to prevent it. Fourthly, this model would explain (taking also into account the post-transcriptional regulation of AtFRO2 by Fe; Connolly et al., 2003) why ferric reductase is not

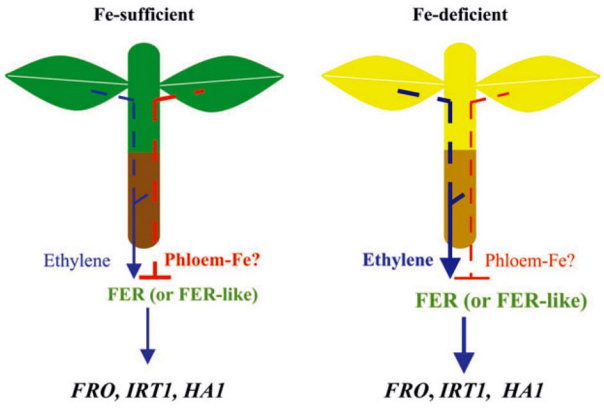

Figure 10. Schematic representation of a model proposed to explain the coordinated regulation of *FRO1* (or *FRO2*), *IRT1*, and *HA1* expression by ethylene. According to this model, Fe-deficiency would cause an increase in ethylene production in the roots, perhaps influenced by some signals (like ACC or auxin) coming from the shoots or their own roots. Ethylene then would promote *FER* (or *FER*-like) transcription (or mRNA stability), which subsequently would affect *FRO1* (or *FRO2*), *IRT1*, and *HA1* transcription in the roots. Fe (phloem–Fe?) would inhibit, either directly or indirectly, the activation of *FER* transcription by ethylene, as well as its post-transcriptional regulation.

constitutively activated in the constitutive ethylene-response mutant *ctr1*, or in the ethylene-overproducer mutant *eto*, of *Arabidopsis*, when grown in Fe-sufficient conditions (Schmidt et al., 2000).

According to the model described above (fig. 10), Fe would act to block *FER* (or *FRU*), *FRO1* (or *FRO2*), *IRT1*, and *HA1* transcription even in the presence of ethylene. But, is it the total symplastic Fe in the root which blocks transcription? We think not. Very recently, Green and Rogers (2004) have found that the *frd3 Arabidopsis* mutant, which has constitutively up-regulated *FRU*, *FRO2*, and *IRT1* expression, is able to accumulate enough symplastic Fe in its root when grown under Fe-sufficient conditions, as suggested by ferritin accumulation (Green and Rogers, 2004). Consequently, it can be deduced that the total symplastic Fe in the root would not be the inhibitor of *FRU*, *FRO2*, and *IRT1* expression. It is possible that the Fe that acts as inhibitor of *FRO2* and *IRT1* expression in the root is the Fe that recirculates from the leaves to the root via the phloem, as suggested by Maas et al. (1988). In the *frd3* mutant there would be less Fe recirculating back in the phloem sap from leaves to roots since Fe cannot reach leaf cells properly (Green and Rogers, 2004). When *frd3* plants were sprayed with Fe, their leaves regreened and *FRU*, *FRO2*, and *IRT1* expression was blocked (fig. 9), which suggests that the Fe provided by the leaves was the inhibitor of the expression of these Fe-acquisition genes. The Fe in the phloem has special characteristics since it is complexed with some proteins of the LEA family (Krüger et al., 2002). It should be noted that the addition of ACC to *frd3* Fe-sprayed plants induced *CHIT-B* expression but not *FRU*, *FRO2*, or *IRT1* expression (fig. 9), which agrees with the idea that the expression of these latter genes does not depend only on ethylene (fig. 10).

Ethylene has also been involved in the regulation of the development of subapical root hairs under Fe deficiency by Strategy I plants (reviewed in Romera and Alcántara, 2004). The results obtained in this work with the *Arabidopsis* ethylene-insensitive mutant *etr1* suggest that the subapical root hairs and the physiological responses (ferric reductase and iron transporter) are probably regulated by ethylene through different ways. This mutant is unable to develop subapical root hairs either under Fe deficiency or upon ACC treatment (Romera and Alcántara, 2004). However, it responds to ethylene inhibitors and ACC similarly to the wild-type cultivar Columbia in relation to the *FRO2*, *IRT1*, and *FRU* expression (figs. 1, 2). These results indicate that ethylene may use different receptors and transduction pathways to regulate different responses (Larsen and Chang, 2001; Moshkov et al., 2003). Therefore, particular mutations (like the ETR1 mutation) may render plants unable to induce some of the ethylene-triggered responses, like the development of subapical root hairs, while conserving a wild-type regulation for others, like the enhancement of the ferric reductase activity (Romera and Alcántara, 2004, and references therein).

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