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Research article

Ethylene involvement in the regulation of the H^+-ATPase *CsHA1* gene and of the new isolated ferric reductase *CsFRO1* and iron transporter *CsIRT1* genes in cucumber plants

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Abstract

In previous works using ethylene inhibitors and precursors, it has been shown that ethylene participates in the regulation of several Fe deficiency stress responses by Strategy I plants, such as enhanced ferric reductase activity, rhizosphere acidification and subapical root hair development. Furthermore, recent evidence suggests that ethylene could regulate the expression of both the ferric reductase and the iron transporter genes of Strategy I plants by affecting the FER (or FER-like) transcription factor. Recently, two H^+-ATPase genes have been isolated from cucumber roots, *CsHA1* and *CsHA2*. *CsHA1* is up-regulated under Fe deficiency while *CsHA2* is constitutively expressed. In this work we have cloned and characterized the sequences of the ferric reductase (*CsFRO1*) and the iron transporter (*CsIRT1*) genes from cucumber (*Cucumis sativus* L. cv Ashley). Expression of *CsHA1*, *CsFRO1* and *CsIRT1* is diminished in Fe-deficient roots by treatment with ethylene inhibitors, like Co (cobalt) or AOA (aminooxyacetic acid). Treatment with ethylene precursors, like ACC (1-aminocyclopropane-1-carboxylic acid) or Ethephon (2-chloroethylphosphonic acid), resulted in increased *CsHA1*, *CsFRO1* and *CsIRT1* transcript levels and increased ferric reductase activity during early stages of Fe deficiency. These results suggest that ethylene is involved in the regulation of *CsHA1*, *CsFRO1* and *CsIRT1* gene expression.

Keywords: Cucumber; Ethylene; Ferric reductase; H^+-ATPase; Iron; Iron transporter; Iron deficiency

1. Introduction

In dicots and non-grass monocots (Strategy I plants), several Fe-regulated proteins, like ferric reductases (EC 1.16.1.7), iron transporters and H^+-ATPases (EC 3.6.3.6), play key roles in Fe uptake by the roots [10,16]. In recent years, several genes that encode these proteins have been identified. Ferric reductases *AtFRO2* of *Arabidopsis* [25], *LeFRO1* of tomato [20] and *PsFRO1* of pea [36]; and iron transporters *AtIRT1* [13] and *AtIRT2* [32] of *Arabidopsis*, *LeIRT1* and *LeIRT2* of tomato [12] and *PsRIT1* of pea [6] have been characterized, and cDNA sequences with significant similarity can be found in genome databases. In relation to H^+-ATPases, some Fe-regulated identified genes are the *AHA2* [14] and *AHA7* [7] of *Arabidopsis* and the *CsHA1* of cucumber [30]. These latter authors also identified another H^+-ATPase gene in cucumber roots, *CsHA2*, that did not respond to Fe deficiency [30].

Genes for ferric reductase, iron transporter and H^+-ATPase proteins are up-regulated in Fe-deficient roots and, in some

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AOA, aminooxyacetic acid; AVG, aminoethoxyvinylglycine.

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cases, in other tissues, in order to maintain Fe homeostasis within the plant [8,9,17,20,30,32–34]. However, several results indicate that the control of the expression of these genes 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. As systemic signals involved in the regulation of these genes, and of other Fe deficiency stress responses, some authors have proposed the plant hormone ethylene [26,27]. Ethylene production increased under Fe deficiency in several Strategy I plants [21,24,28,35]. Moreover, treatment of several Fe-deficient Strategy I plants with inhibitors of ethylene synthesis or action greatly decreased both ferric reductase and iron transporter activity, and the expression of their corresponding *FRO* and *IRT* genes, while treatment with precursors of ethylene synthesis enhanced it [23,24,26–28]. Similarly, treatment of several Fe-deficient Strategy I plants with inhibitors of ethylene synthesis greatly decreased their acidification response [19,27].

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 [22], is necessary for the transcription of *LeFRO1* and *LeIRT1*, since the tomato *fer* mutant fails to up-regulate both genes [2,20,22]. In addition, the FER protein is necessary for the acidification response (due to enhanced H^+-ATPase activity [11]), since the tomato *fer* mutant did not acidify the nutrient solution under Fe-deficient conditions [3]. The *Arabidopsis* FRU protein (also named FIT1), a homolog of the tomato FER protein, is necessary for the activation of the ferric reductase *AtFRO2* [7,18], the H^+-ATPase *AtAHA7* [7] and the iron transporter *AtIRT1* [18] gene expression, although in the regulation of the iron transporter there is discrepancy between the results of Colangelo and Guerinot [7] and those of Jakoby et al. [18]. *LeFER* and *AtFRU* expression is induced in roots in response to Fe deficiency [1,5,7,18]. Very recently, Lucena et al. [23] have shown that ethylene could regulate *FRO* and *IRT* gene expression by affecting the *FER* (or *FER*-like) gene activity. These authors found that the treatment of Fe-deficient tomato plants with inhibitors of ethylene synthesis or action greatly decreased the *FER* mRNA accumulation while treatment of tomato plants grown in low Fe conditions with ACC (ethylene precursor) enhanced it. Similarly, the treatment of Fe-deficient *Arabidopsis* plants with inhibitors of ethylene synthesis or action greatly decreased the *FRU* mRNA accumulation while treatment of ACC on *Arabidopsis* plants grown in low Fe conditions enhanced it [23]. However, when the *Arabidopsis* plants were grown in high Fe conditions, ACC hardly affected the *FRU* mRNA accumulation [23]. Based on these results, a model has been proposed in which ethylene acts as an activator of the *FER* (or *FER*-like) gene, and consequently of the *FRO* and *IRT* genes, while Fe (probably phloem Fe) acts as inhibitor of it [23].

The aim of this work was to study the involvement of ethylene on the expression of the ferric reductase, the iron transporter and two H^+-ATPase genes (*CsHA1* and *CsHA2*) of cucumber plants. We have chosen cucumber because it has been extensively used as a model in several studies relating ethylene to iron nutrition. In addition, the identification of the *CsHA1* and *CsHA2* genes allowed us to study whether both genes had similar response to ethylene or not. For this, we have isolated a ferric reductase cDNA, named *CsFRO1*, and an iron-regulated transporter cDNA, named *CsIRT1*, from cucumber (*Cucumis sativus* L.) plants. The expression of cucumber *CsFRO1, CsIRT1*, *CsHA1* and *CsHA2* is examined in response to plant Fe status, ethylene inhibitors and ethylene precursors.

2. Methods

2.1. Plant materials, growth conditions and 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 days, the seedlings were transferred to a plastic mesh held over half strength nutrient solution, and kept in the dark for 2 days. Cucumber seedlings were transplanted to 70 ml plastic vessels containing continuously aerated nutrient solution with 20 μM Fe-EDDHA. After 3–5 days in this nutrient solution, cucumber plants were transferred to the different treatments. Cucumber 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 at a photosynthetic irradiance of 200 $\mu mol\ m^{-2}\ s^{-1}$ provided by fluorescent tubes (Sylvania Cool White VHO). 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 60 μM Fe-EDDHA; +Fe + ACC: same as +Fe treatment but with 1 μM ACC addition during the last 24 h; −Fe: nutrient solution without Fe; −Fe + ACC or −Fe + Eth: −Fe treatment (1 day) with 1 μM ACC or 14 μM Ethephon (ethylene releasing substance) addition during the last 24 h; −Fe + Co, −Fe + AOA, −Fe + AVG: −Fe treatment (1, 2, 3 or 4 days) and 10 μM $CoSO_4$, 20 μM AOA, or 10 μM AVG addition during the last 24 h. Stock solutions of ACC, Ethephon, AOA and AVG (Sigma) were prepared in deionized water. After treatments, root ferric reductase activity was determined as described below. Finally, roots, stems and leaves (depending on the experiments) were collected and kept at −80 °C to later analyze the mRNA.

2.2. Ferric reductase activity determination

To determine ferric reductase activity, 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^{3+}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^{2+}–Ferrozine complex formed. After the reduction assay, roots were excised and weighed, and the results were expressed on a root fresh weight basis. Reduction rates were calculated using an extinction coefficient of 29,800 $M^{-1}\,cm^{-1}$.

2.3. RNA extraction and cloning

The tissues of roots, stems (hypocotyls), and leaves were isolated and ground to a fine powder in a mortar and pestle in liquid nitrogen. Total RNA was extracted using the Qiagen RNeasy plant mini-kit. The cDNAs for cucumber genes responsible for ferric reductase and iron transporter proteins were isolated by using protein sequences of known FRO and IRT proteins to design degenerate primers to be used in RT–PCR to initially amplify partial cDNAs. The amino acid sequences used for cucumber FRO1 (CsFRO1) were LQWHPFT, which corresponds to an FAD binding site, and EGPYGP, which corresponds to a NADPH binding site, both of which are conserved motifs necessary for Fe reduction [25]. For cucumber IRT1 (CsIRT1), the sequences used to design the primers were TGFMHVLP and AGLLIYMALVD. Primers were obtained from IDT (Coralville, IA). M-MLV reverse transcriptase (Promega, Madison, WI) was used to generate cDNA with 2 µg of total RNA from Fe-deficient roots as template, using random hexamers as primers. One tenth of the RT reaction was used as PCR template with DF3 and DR3 primers (Table 1) for *CsFRO1*, and DF1IRT and DR2IRT primers for *CsIRT1*. The RT–PCR products were cloned using pGem-T Easy Vector System (Promega). Plasmids were isolated using Wizard Plus SV Minipreps (Promega). The cloned DNA was sequenced by MCLab (South San Francisco, CA), and the sequences were submitted to BLAST (*B*asic *L*ocal *A*lignment *S*earch *T*ool) searches (http://www.ncbi.nlm.nih.gov/BLAST). Based on the high degree of similarity to FRO and IRT proteins, nested gene specific forward and reverse primers were designed to be used for RACE RT–PCR to generate DNA for 5′ and 3′ end containing clones (Table 1). Following manufacturer's instructions (GeneRacer, Invitrogen Co., Carlsbad, CA), PCR fragments that corresponded to the 5′ and 3′ ends of each cDNA were amplified, cloned, and sequenced. Both *CsFRO1* and *CsIRT1* 5′ end sequences contained an ATG with upstream in-frame stop codons. By overlapping the 5′ and 3′ clones, these ATG codons were revealed to be in frame with a long DNA sequence that ended with a stop codon. The deduced amino acid sequences from the ATG to the TGA were submitted to BLAST searches, and were of high similarity and comparable length to known *FRO* and *IRT* genes. Both *CsFRO1* and *CsIRT1* 3′ end sequences contained a polyA tail and untranslated regions following the in-frame stop codon. On this basis, we are confident that the entire coding sequence was identified. PCR primers were designed that contained the start and stop codons, and a full length cDNA was amplified and cloned by RT–PCR. The sequences were confirmed and submitted to GenBank (*CsIRT1* accession no. AY590764, *CsFRO1* accession no. AY590765).

2.4. RT–PCR analysis

M-MLV reverse transcriptase (Promega, Madison, WI) was used to generate cDNA with 2 µg of total RNA from roots, stems, and leaves as template and random hexamers

Table 1
Primers used in this study

Primer name	Sequence (5′–3′)[a]	Purpose
DF3	CGCGAATTCYTICARTGGCAYCCITTYAC	*CsFRO1* degenerate forward
DR3	CGCAAGCTTGGICCRTAIGGICCYTC	*CsFRO1* degenerate reverse
CsGSP1	TACAATCACATCTCACAGCGATTTAGAACC	*CsFRO1* outer forward GSP[b]
CsGSP2	AGAAAAGCTTTCCGTTGTGATCAAATGCG	*CsFRO1* nested forward GSP
CsGSP3	TGGGCCCTAGGGGCCTTCGTGGG	*CsFRO1* outer reverse GSP
CsGSP4	ACTTGAAAATGGTCGTTAATGGCGG	*CsFRO1* nested reverse GSP
CsFROF1	ATGGATAGAGAGAGGGTTTTGAGC	*CsFRO1* forward full length
CsFROR1	TCACCAAGTGAACCTGATGGAGTG	*CsFRO1* reverse full length
DF1IRT	ACIGGITTYATGCAYGTIYTICC	*CsIRT1* degenerate forward
DR2IRT	TCIACIARIGCCATRTADATIARIARICCIGC	*CsIRT1* degenerate reverse
CsGSP5	TTGCCTGACTCTTTCGATATGCTTTGGTCG	*CsIRT1* outer forward GSP
CsGSP6	AATTGCCTCAAGGAGAATCCATGGC	*CsIRT1* nested forward GSP
CsGSP7	GCCTGAAGAATACAGCCACCGAGACCC	*CsIRT1* nested reverse GSP
CsGSP8	AAACCTATTCCAAGTGCTATCCCAAATGGGG	*CsIRT1* outer reverse GSP
CsIRTF1	ATGGCATCCTTCACTAAACCC	*CsIRT1* forward full length
CsIRTR1	TCAAGCCCACTTTGCCATTAATGAC	*CsIRT1* reverse full length
ActF	AGA TGA CGC AGA TAA TGT TTG AGA C	Actin forward
AcR	TCA GGA AGT TCA TAG TTC TTC TCA A	Actin reverse

[a] Y = C or T; R = A or G; D = A, G, or T; H = A, C, or T; I = deoxyinsosine.

[b] GSP, gene specific primer.

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 CsFROF1/CsGSP4 and CsIRTF1/CsGSP8 (Table 1) were used to amplify *CsFRO1* and *CsIRT1* cDNA, respectively. Primer pairs 113F2/113R1 and 127F1/127R1 [30] were used to amplify *CsHA1* and *CsHA2* cDNA, respectively. Actin cDNA was amplified with primers ActF and ActR (Table 1) as an internal control in some RT–PCR experiments. Alternatively, *18S* cDNA was amplified using Quantum*mRNA* Universal 18S Standards primer set (Ambion, Austin, TX) as internal control to other set of experiments. The thermal cycler program was one initial cycle of 94 °C, 5:00; followed by cycles of 94 °C, :45; 56 °C, :30; 72 °C, 1:30, with 28 cycles for *CsFRO1* and *CsIRT1* genes, and 25 cycles for actin and *18S*, all followed by a final 72 °C elongation cycle of 7:00.

2.5. Sequence analysis

Sequences similar to the CsFRO1 and CsIRT1 deduced amino acid sequence were identified by BLAST searches. To graphically represent this similarity, several of these were aligned with ClustalW 1.8 software running on the Baylor College of Medicine Search Launcher website (http://searchlauncher.bcm.tmc.edu). Following the multiple sequence alignment, BOXSHADE was utilized to indicate similar and identical residues (http://www.ch.embnet.org/software/BOX_form.html). CsFRO1 and CsIRT1 were placed in phylogenetic trees with several similar protein sequences, which were constructed using Phylip software on the TreeTop site (http://www.genebee.msu.su/services/malign_reduced.html) with a ClustalW 1.8 multiple sequence alignment as input. The TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) was used to construct the phylogenetic tree graphic.

3. Results

3.1. CsFRO1 and CsIRT1 cloning and sequence characterization

A multiple sequence alignment of CsFRO1 with LeFRO1, PsFRO1 and AtFRO2 is shown in Fig. 1. CsFRO1 has 48% identity and 69% similarity to Arabidopsis FRO2, and 56% identity and 74% similarity to pea FRO1, two previously characterized ferric reductases. CsFRO1 was placed in a phylogenetic tree with similar protein sequences (Fig. 3A). The most closely related proteins are FRO1 of tomato, pea and *Medicago truncatula*; and FRO2 of *Arabidopsis*. Sequence comparisons of CsIRT1 to known plant iron transporters by BLAST revealed significant similarity with several proteins. Among other plant Fe transporters, CsIRT1 is most similar to LeIRT1 from tomato. These two proteins share 74% identity and 85% similarity. Fig. 3B is a phylogenetic tree of several iron transporters with significant similarity to CsIRT1. CsIRT1 is grouped most closely with LeIRT1 and LeIRT2.

To test the function of CsIRT1, the IRT1 cDNA was cloned into a yeast expression vector and transformed into the *fet3fet4* strain of *Saccharomyces cerevisiae*, which lacks both high- and low-affinity Fe uptake systems and has impaired growth in low Fe conditions. As has been observed with AtIRT1 and LeIRT1, CsIRT1 complements the fet3fet4 growth defect (Fig. 4).

3.2. Regulation of the ferric reductase, the iron transporter and H^+-ATPase gene expression in cucumber plants

The relative abundance of *CsFRO1*, *CsIRT1*, *CsHA1* and *CsHA2* transcripts in cucumber roots under either Fe-sufficient or Fe-deficient conditions was determined by RT–PCR. A low amount of *CsFRO1*, *CsIRT1* and *CsHA1* transcripts was detected in roots of Fe sufficient plants (Figs. 5–7). Transfer of plants to media lacking Fe for 2–4 days induced the accumulation of mRNA from these three genes in roots (Figs. 5–7). *CsIRT1* transcripts were also detected in stems and leaves, where Fe deficiency induced a slight increase of its level (Fig. 5). On the contrary, *CsFRO1* mRNA was not detected in leaves or stems from either Fe-sufficient or Fe-deficient plants (Fig. 5). *CsHA2* transcripts were not appreciably affected by Fe deficiency (Figs. 6 and 7).

Ethylene inhibitors (Co and AOA [37]) efficiently prevented the accumulation of *CsFRO1* and *CsIRT1* transcripts in roots of Fe-deficient plants for the 3-day course of the experiment, while *CsHA1* transcript levels were somewhat diminished as compared to −Fe treatments (Figs. 6 and 7). While roots of −Fe plants showed little increase in *CsFRO1*, *CsIRT1*, or *CsHA1* transcript levels after 1 day, treatment with ACC greatly enhanced the expression of these three genes at this early stage of Fe deficiency, with transcript levels comparable to those of −Fe at 2 days and 3 days (Fig. 6). Ethephon had a slight effect on *CsFRO1* expression but no significant effect on *CsIRT1* and *CsHA1* expression (Fig. 6). Both ACC and Ethephon caused the development of subapical swollen root tips where the enhanced ferric reductase activity was located (Fig. 8). ACC did not affect *CsFRO1*, *CsIRT1* or *CsHA1* expression in +Fe plants (Fig. 6). The results of ferric reductase activity of the cucumber plants were related to the levels of *CsFRO1* expression (Figs. 6 and 7) while those of the acidification response (Table 2) were related to *CsHA1* expression (Figs. 6 and 7). *CsHA2* transcripts were not affected by ethylene inhibitors or precursors (Figs. 6 and 7).

4. Discussion

In this work, we have isolated the *CsFRO1* and *CsIRT1* genes from cucumber plants. Several results support the conclusion that *CsFRO1* and *CsIRT1* genes encode ferric reductase and iron transporter proteins, respectively. The high degree of conservation between CsFRO1 and the previously characterized ferric reductase proteins is illustrated by the multiple sequence alignment (Fig. 1) and the phylogram (Fig. 3A). The amino acid sequence LQWHPFT, which

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LeFRO1    1 --MAQTSSSSPSLKASSSVTNDLQATIMALILIVTIGYFLLLIVTPTNMYRQIWTPKIKAHTTNSTYFGA
PsFRO1    1 -----MAQENVKRSPSQIKYTMVKSIIRLFVFLVFLGLLFIFIMMPVTTFKQKWIPKIQAKTN-STYFGV
AtFRO2    1 MEIEKSNNGGSNPSAGEEFKDMIKGVTKFLMMVIFLGTIMLWIMMPTLTYRTKWLPHLRIKFGTSTYFGA
CsFRO1    1 ----------------MDRERVLSLAIRVLVLILFLGWIFLWVMMPTNTYRKKWLPKVREKSYKSTYFGS

LeFRO1   69 QGRTLLMNTFPLIFIAVLGCVYLHLWKKSNNKNINR--FEKKQKLAIWRRPIIMKG-LGIVSRIELGFFV
PsFRO1   65 QGFRILIYTFPLLLIATLGCVYIHIAKKSNQIDN-----GKKHETTIWKRPMLVKGPLGIVSITEIAFLL
AtFRO2   71 TGTTLFMYMFPMMVVACLGCVYLHFKNRKSPHHIDR--ETKGGVWSKLRKPMLVKGPLGIVSVTEITFLA
CsFRO1   55 EGTSLLMYTFPILFIAILGCVYNHLEKKRSDPNVKTRKGTKHHGLAMWKQPAIVKGPLGIVSWTELTLLT

LeFRO1  136 MFIALLVWTFASYLHIIFPTITPKSVANSGEKVWEAKLEDSGLRLGLVGNICLTFLFVPVTRGSSVLQVF
PsFRO1  130 MFIALLVWTLATYLHNDFSSIASSPEEEHGPKVWQEKLESVGLRLGLVGNICLVLLFFPVTRGTSVLPMF
AtFRO2  139 MFVALLLWCFITYLRNSFATITPKSAAAHDESLWQAKLESAALRLGLIGNICLAFLFLPVARGSSLLPAM
CsFRO1  125 MFIILLVWSFATYLHNSFITEK----------MWEIRLGSAGFWLGIVGNICLVFLFFPVARGSSLLPLL

LeFRO1  206 GLTSEASVKYHIWLGHIVMTLFSAHGICYIIYWASTHQLSEMLKWGKTDISNLAGELALLSGLVLWIATF
PsFRO1  200 GLTSEGSIKYHIWLGHVLMTIFTLHGVCYIIYWISTNQISQMLKWNKIGVSNLAGEISLLAGLFLWVATI
AtFRO2  209 GLTSESSIKYHIWLGHMVMALFTVHGLCYIIYWASMHEISQMIMWDTKGVSNLAGEIALAAGLVMWATTY
CsFRO1  185 GLTSEGCIKYHIWLGHMTMAFFSAHGICFLIYWAATNNISQMIKWAKTDISNIAGELALVFGLIMWATTI

LeFRO1  276 PKIRRKMFELFFYTHHFYILFVVFFVFHVGVSYACIMLPGFFLFMVGRFLRFLQSRSNVRLVSARVLPCE
PsFRO1  270 PKLRRKFFELFFYTHNLYIIFVIFFVFHVGISFANIMLPGFYLFMVDRYLRFLQSRRGVRLVSARVLPCE
AtFRO2  279 PKIRRRFFEVFFYTHYLYIVFMLFFVLHVGISFSFIALPGFYIFLVDRFLRFLQSRENVRLLAARILPSD
CsFRO1  255 PRIRRKFFELFLYTHYLYILFIVFFIFHVGISYACVMLPGFYLFVIDRYLRFLQSRRRVRLLSARLLPCQ

LeFRO1  346 TLELNFSKTKGLSYTPTSIMFVNVPSISKLQWHPFTITSSSNLEPEKISVAIKGEGSWSKKLYQMISSPN
PsFRO1  340 AVELNFSKGHELSYNPTSVMFINVPSISKLQWHPFTITSNSNLEQDKLSVVIKSEGTWTKKLYKLLSNPS
AtFRO2  349 TMELTFSKNSKLVYSPTSIMFVNIPSISKLQWHPFTITSSSKLEPEKLSIVIKKEGKWSTKLHQRLSSSD
CsFRO1  325 TLELNFSKHPGLKYNPTSTMFINIPSISKLQWHPFTITSHSDLEPEKLSVVIKCEGTWSSKLYKTLSSSS

LeFRO1  416 S---VDRLNVSVEGPYGPPSTHFLRHDLLVMISGGSGITPFISIIRELIHTS-ESQKCKTPEILLISVFK
PsFRO1  410 P---IDRLQISVEGPYGPASTNYLRHDTLVMISGGSGITPFISIIRELIYLS-TTFKCKTPNIVLICSFK
AtFRO2  419 Q---IDRLAVSVEGPYGPASADFLRHEALVMVCGGSGITPFISVIRDLIATS-QKETCKIPKITLICAFK
CsFRO1  395 SSAINDHFQVSLEGPYGPVSTSFLQFDTLIMISGGSGITPFISIIKHIIHNSSYSHHNKTPKLLLISAFK

LeFRO1  482 NSEDLTMLDLLLPISGAPSETC-KLGLQIEAFVTREKQPVSTEDKKNVRTIWFKPNPSDKPITPILGQNN
PsFRO1  476 NTSSLSMLDLILPISSTPQDIS-DMQLQIEAYITRDKE-FKSDIPIHPQTLWFKPNPTDAPIHAILGPNG
AtFRO2  485 KSSEISMLDLVLPLSGLETELSSDINIKIEAFITRDNDAGDEAKAGKIKTLWFKPSLSDQSISSILGPNS
CsFRO1  465 TTADLTFLHLLQTP-------TTPHNLQIEAYVTREKS--PQNENPQIRSVTFKSHIEESAAAGILGRNG

LeFRO1  551 WLWLGAIISCSFLIFLISLGVLNRYYIYPIDNNTNDIFSYPIKAVLNMLIICISIVITSSAAFVWNKRQS
PsFRO1  544 WIWLGAIISSSFIIFLIIIGIITRYYIFPIDHNTNKIFSYPLRSFIHMLAICVSIVVVASVAVLSNKKQN
AtFRO2  555 WLWLGAILASSFLIFMIIIGIITRYYIYPIDHNTNKIYSLTSKTIIYILVISVSIMATCSAAMLWNKKKY
CsFRO1  526 WMWLAGVICSSFGIFLIFIGVLNRYYIYPIDGNTNDVFALGLKSFLHMLGLCFGVFTAATAAVLWNKRET

LeFRO1  621 -GTDAKQIQNMEGATPMASPNS-WFYNADREMESLPQQSLFQSTNLHFGERPDLKRILFERKESSVGVLV
PsFRO1  614 -AREAKQIQNMEGSTPTVSPNS-MIYNADRELESFPYQSLVETTNVHYGARPDLSRLLLEIKGSSVGVFA
AtFRO2  625 GKVESKQVQNVDRPSPTSSPTSSWGYNSLREIESTPQESLVQRTNLHFGERPNLKKLLLDVEGSSVGVLV
CsFRO1  596 -AKEERQIQNVEGATPNGSPCA-IGCEKEIELESDPFQVLNQSVNVHYGERPNLPRMIEECKGENIGVMA

LeFRO1  689 CGPKRMRHEVANICSSGLASNLHFESISFSW
PsFRO1  682 SGPKQLRQNVATICSSGLVENLHFESISFTW
AtFRO2  695 CGPKKMRQKVAEICSSGLAENLHFESISFSW
CsFRO1  664 SGPKKLRQEVAAICGSALPKNLHYHSIRFTW
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Fig. 1. Multiple sequence alignment of CsFRO1 and similar proteins. The deduced amino acid sequence of CsFRO1 was submitted to a BLASTp search. Proteins with significant similarity were aligned with ClustalW and graphic was made with BOXSHADE. Residues shaded in black are identical, those shaded in gray are similar. Ferric reductase conserved domains involved in FAD (QWHPFT) and NADPH (EGPYGP) binding, and NAD(P)H oxidoreductases signature sequence (MISGGSGITPFISI), are boxed. Aligned proteins are *Lycopersicon esculentum* FRO1 (LeFRO1, accession no. AY224079), *Pisum sativum* FRO1 (PsFRO1, accession no. AAK95654), *Arabidopsis thaliana* FRO2 (AtFRO2, accession no. CAA70770), and *Cucumis sativus* FRO1 (CsFRO1, accession no. AAT01415).

corresponds to an FAD binding site, and EGPYGP, which corresponds to a NADPH binding site, both showed 100% conservation in LeFRO1, PsFRO1, AtFRO2 and CsFRO1 (Fig. 1). CsFRO1 also contains a signature sequence associated with NAD(P)H oxidoreductases (MISGGSGITPFISI; Fig. 1). Similarly, CsIRT1 has a high degree of conservation with previously characterized iron transporter proteins of tomato and *Arabidopsis*, which belong to the ZIP family of metal transporters (Figs. 2 and 3B), and complementation of the yeast *fet3fet4* strain provides functional evidence for Fe transport (Fig. 4). In plant Fe-transporting ZIPs, a variable region between transmembrane domains III and IV contains a potential metal binding domain rich in histidine residues [15]; in AtIRT1, this sequence is HGHGHGH. CsIRT1 also contains a histidine-rich region with the sequence of HFHGHHH (Fig. 2); this could be an orthologous domain for CsIRT1.

Both *CsFRO1* and *CsIRT1* are induced by Fe deficiency (Figs. 5–7) similarly to other ferric reductase and iron

Table 2
Effects of ethylene inhibitors and precursors on the acidification response of Fe-deficient cucumber plants

Treatment	pH nutrient solution
+Fe	7.2 ± 0.1
−Fe	4.5 ± 0.3
−Fe + Co	6.8 ± 0.1
−Fe + AOA	7.0 ± 0.1
−Fe + AVG	6.2 ± 0.3
−Fe + AVG + ACC	4.3 ± 0.2

Cucumber plants 7 days old were transferred to nutrient solution either with Fe (+Fe) or without Fe (−Fe) adjusted to pH 6.0. Some plants without Fe were treated with ethylene inhibitors (10 μM $CoSO_4$; 20 μM AOA or 10 μM AVG) during the last 24 h. At the moment of the treatment with AVG, some of the AVG-treated plants were also treated with 10 μM ACC during 8 h. The pH of the nutrient solution was determined after 72 h without Fe. Values are means ± S.E. of six replicates.

transporter genes [8,9,17,20,32–34]. When root ferric reductase activity is elevated, so is *CsFRO1* message (Figs. 6 and 7). Moreover, *CsFRO1* and *CsIRT1* are regulated by ethylene in the same way as *AtFRO2*, *AtIRT1*, *PsFRO1*, *PsRIT1*, *LeFRO1* and *LeIRT1* [23]: ethylene inhibitors block the expression of both kind of genes in Fe-deficient plants while ethylene precursors, ACC or Ethephon, enhanced their expression in plants suffering the first stages of Fe deficiency but not in plants grown in high Fe conditions (Fig. 6). It should be noted that ACC, or Ethephon, enhance the ferric reductase activity in the subapical region of the roots, as occurred in Fe-deficient plants (Fig. 8). Furthermore, bicarbonate and anaerobiosis, two factors known as inducers of Fe deficiency, similarly influence *FRO1* (or *FRO2*) and *IRT1* (or *RIT1*) expression in cucumber, *Arabidopsis*, pea and tomato plants, which suggests that CsFRO1 is the main ferric reductase and CsIRT1 the main iron transporter of cucumber roots ([29]; F.J. Romera et al., unpublished results).

We examined expression of *CsFRO1* in different seedling tissues. There was no evidence of mRNA in the stems or the leaves in plants grown under either Fe-sufficient or Fe-deficient conditions (Fig. 5), suggesting that *CsFRO1* is not expressed in these tissues, at least under our experimental conditions. In contrast, *PsFRO1* [36] and *AtFRO2* [1] were detected in leaves of pea and *Arabidopsis* plants, respectively, by RT–PCR. It is believed that a ferric reduction step is needed for uptake of Fe into leaf cells [4]. Therefore, it seems likely that additional, unidentified, *FRO* genes are present in cucumber.

RT–PCR analysis indicated increased expression of *CsIRT1* in roots, stems, and leaves of Fe-deficient cucumber plants (Figs. 5–7). Iron regulation of expression has been observed for *LeIRT1* in the roots of tomato plants [12,20] and for *AtIRT1* in the roots [1,34] and leaves [1] of *Arabidopsis* plants. *CsIRT1* expression was noted in both leaves and stems (Fig. 5). This result suggests that an IRT-type transporter may be required for leaf cell Fe uptake.

In relation to the H^+-ATPase genes, the response of each gene to Fe deficiency and ethylene inhibitors and precursors

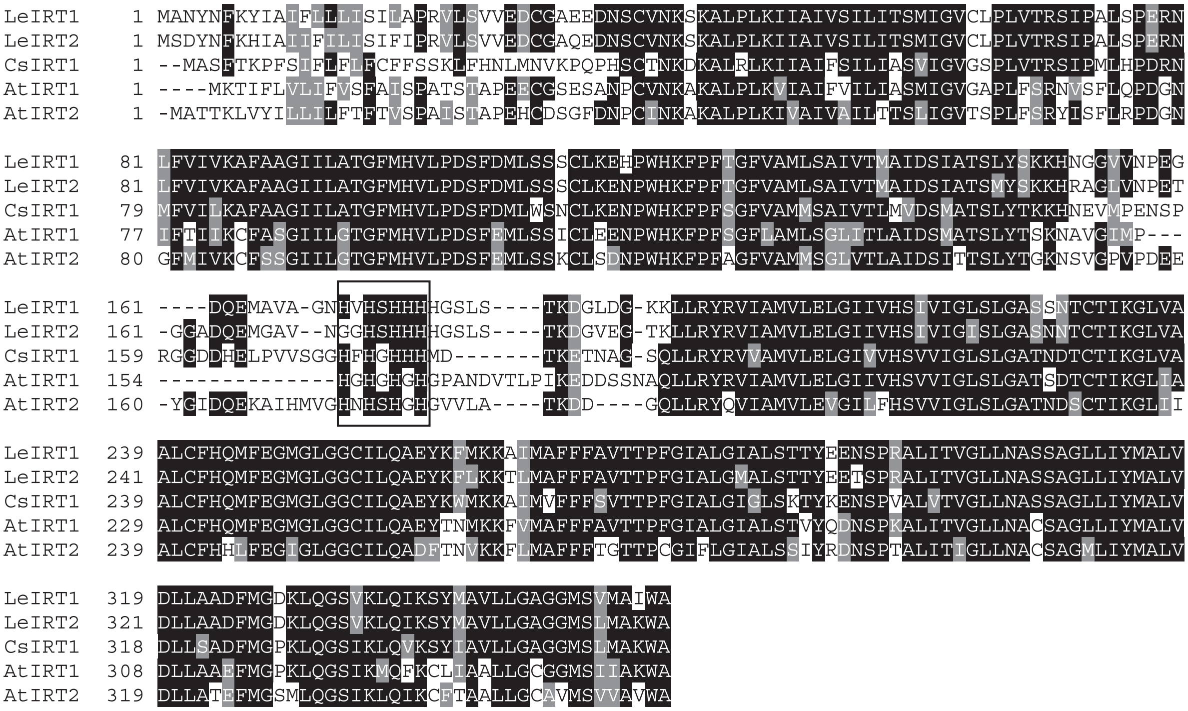

Fig. 2. Multiple sequence alignment of CsIRT1 and similar proteins. The deduced amino acid sequence of CsIRT1 was submitted to a BLASTp search. Proteins with significant similarity were aligned with ClustalW and graphic was made with BOXSHADE. Residues shaded in black are identical, those shaded in gray are similar. Histidine-rich domain conserved in Fe-transporter is boxed. Aligned proteins are *Lycopersicon esculentum* IRT1 (LeIRT1, accession no. AAD30548) and IRT2 (LeIRT2, accession no. AAD30549), *Cucumis sativus* IRT1 (CsIRT1, accession no. AAT01414), *Arabidopsis thaliana* IRT1 (AtIRT1, accession no. AAB01678) and IRT2 (AtIRT2, accession no. O81850).

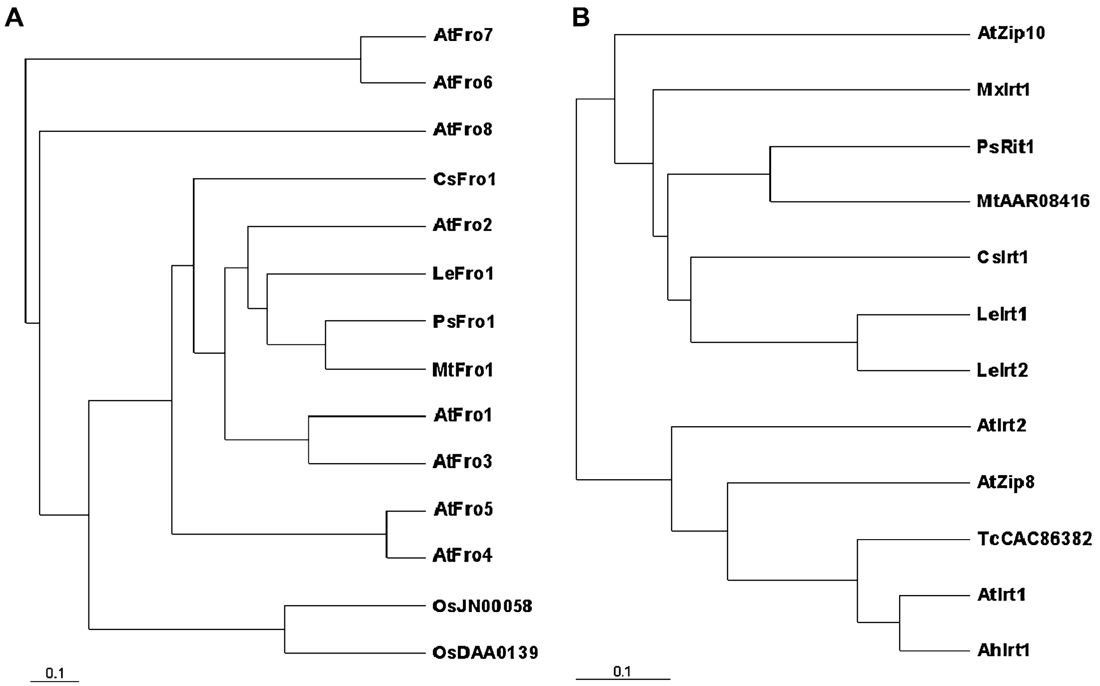

Fig. 3. Phylogenetic trees of cucumber FRO1 and IRT1 and similar proteins. The deduced amino acid sequences of CsFRO1 and CsIRT1 were submitted to a BLASTp search. Proteins with significant similarity were aligned using ClustalW, and the alignment was used an input for Phylip software. The tree graphics were constructed using TreeView. A) Tree including CsFRO1. Proteins shown are *Lycopersicon esculentum* FRO1 (LeFRO1, accession no. AY224079), *Pisum sativum* FRO1 (PsFRO1, accession no. AAK95654), and *Arabidopsis thaliana* FRO1–8 (AtFRO1, accession no. CAA70769; AtFRO2, accession no. CAA70770; AtFRO3, accession no. AAB72168; AtFRO4, accession no. BAB08721; AtFRO5, accession no. NP_197787; AtFRO6, accession no. NP_199784; AtFRO7, accession no. NP_199785; AtFRO8, accession no. NP_199827), *Medicago truncatula* FRO1 (MtFRO1, accession no. AAR15416), *Oryza sativa* loci OSJN0058 (OsJN0058, accession no. CAE03257) and DAA01390 (OsDAA01390, accession no. DAA01390). B) Tree including CsIRT1. Proteins shown are *Arabidopsis thaliana* IRT1 (AtIRT1, accession no. AAB01678), IRT2 (AtIRT2, accession no. O81850), ZIP8 (AtZIP8, accession no. Q8S3W4), and ZIP10 (AtZIP10, accession no. NP_174411), *Malus xiaojinensis* IRT1 (MxIRT1, accession no. AAO17059), *Lycopersicon esculentum* IRT1 (LeIRT1, accession no. AAD30548) and IRT2 (LeIRT2, accession no. AAD30549), *Pisum sativum* RIT1 (PsRIT1, accession no. T06385), *Arabidopsis halleri* IRT1 (AhIRT1, accession no. CAE30485), *Medicago truncatula* AAR08416 (MtAAR08416, accession no. AAR08416), and *Thlaspi caerulescens* CAC86382 (TcCAC86382, accession no. CAC86382).

was different. The *CsHA2* gene did not respond to Fe deficiency, which agrees with results of Santi et al. [30]. In addition, this gene did not respond to either ethylene inhibitors or precursors (Figs. 6 and 7). The *CsHA1* gene, however, responded to both Fe deficiency and ethylene treatments similarly to the *CsFRO1* and *CsIRT1* genes: it was induced under Fe deficiency (as found by Santi et al. [30]), and ethylene inhibitors blocked its expression in Fe-deficient plants while ACC enhanced it in plants suffering the first stages of Fe deficiency (Fig. 6). The inability of ACC to enhance *CsHA1* gene expression in +Fe plants (Fig. 6) should

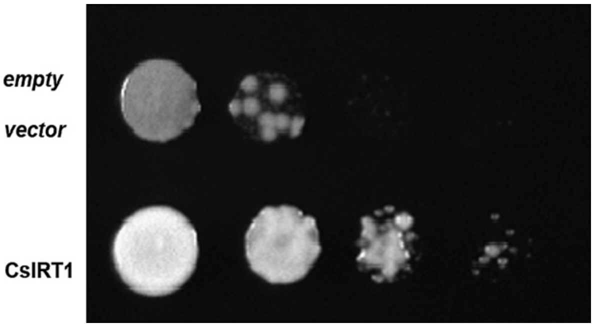

Fig. 4. CsIRT1 is a functional Fe(II)-transporter when expressed in yeast. The top row is *fet3fet4* transformed with empty vector (pYES2.1, Invitrogen), the bottom row is CsIRT1 cloned into this same vector, also in *fet3fet4*. The spots from left to right are 1.0, 0.1, 0.01, and 0.001 OD in distilled water, plated on SD-ura media.

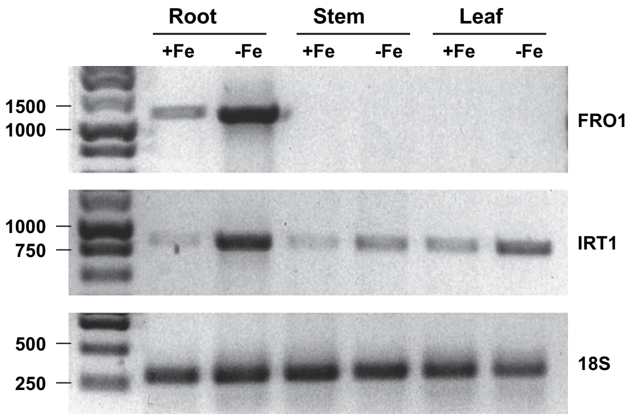

Fig. 5. Tissue specific expression of *CsFRO1* and *CsIRT1*. Total RNA was extracted from Fe-sufficient (+Fe) and Fe-deficient (−Fe) roots, stems, and leaves. Fe-deficient plants were grown the last 4 days in nutrient solution without Fe. RT–PCR was performed using the RNA extracted as template, and gene specific primers were used to amplify partial cDNAs of *CsFRO1*, *CsIRT1*, or *18S* as positive control.

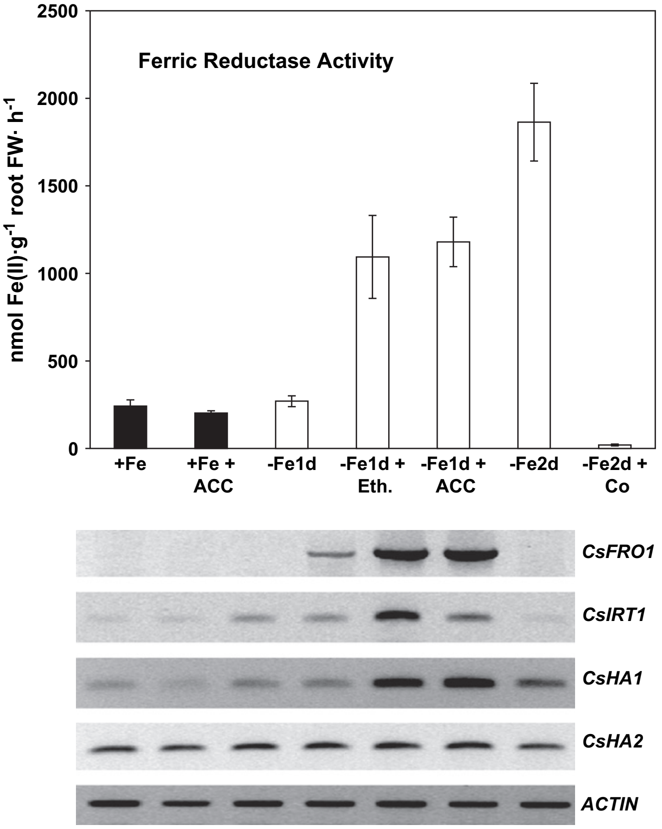

Fig. 6. Regulation of *CsFRO1*, *CsIRT1*, *CsHA1* and *CsHA2* expression, and ferric reductase activity, by ethylene inhibitors and precursors in cucumber plants. Some Fe-sufficient (+Fe) and Fe-deficient (−Fe1d or −Fe2d) cucumber plants were treated during the last 24 h with 1 μM ACC, 14 μM Ethephon (Eth) or 10 μM $CoSO_4$. Fe-deficient plants were grown during the last 1 day (−Fe1d) or 2 days (−Fe2d) in nutrient solution without Fe. After treatments, the ferric reductase activity was determined (values are the means ± S.E. 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 *CsFRO1*, *CsIRT1*, *CsHA1* and *CsHA2*. Actin partial cDNA was amplified as positive control.

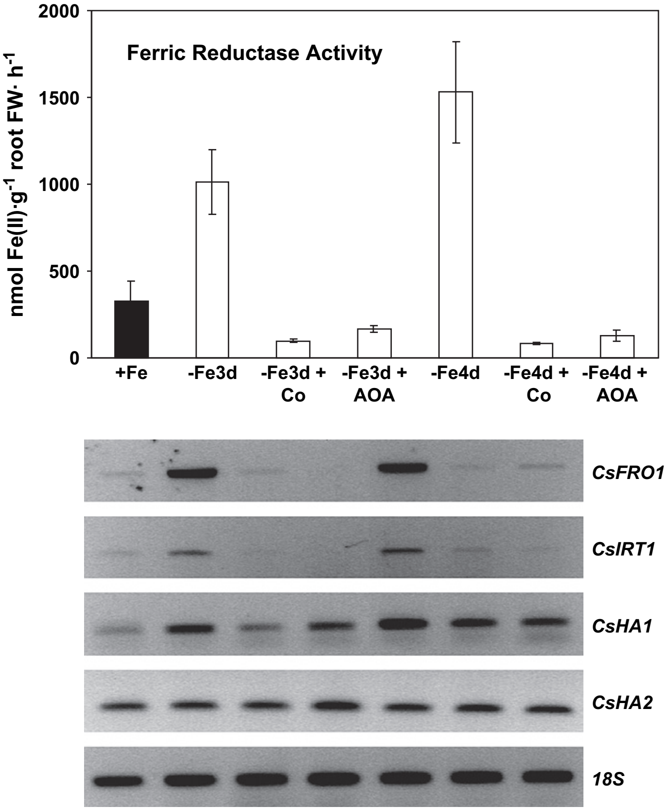

Fig. 7. Regulation of *CsFRO1*, *CsIRT1*, *CsHA1* and *CsHA2* expression, and ferric reductase activity, by ethylene inhibitors and precursors in cucumber plants. Experimental conditions as described in Fig. 6, except that Fe-deficient plants were grown 3 (−Fe3d) or 4 (−Fe4d) days without Fe and *18S* was used as positive control.

explain the results of Schmidt et al. [31], who found no increase of immunologically detectable H^+-ATPase upon treatment of +Fe tomato plants with ACC. The inhibition of the *CsHA1* gene expression was reflected in the lower acidification response of the Fe-deficient plants treated with ethylene inhibitors (Table 2). It should be noted that the inhibition provoked by the AVG treatment was reversed by the simultaneous addition of ACC (Table 2). Since AVG blocks the synthesis of ACC [37], this result also supports a role for ethylene in the acidification response, as suggested by Romera and Alcántara [27]. Taken together, these results suggest that ethylene could be involved in the acidification response by affecting the regulation of the H^+-ATPase *CsHA1* gene. To our knowledge, this is the first time that ethylene has been implicated in the regulation of any H^+-ATPase gene involved in the acidification response of Fe-deficient plants. Very recently, Lucena et al. [23] have found that ethylene could regulate *FRO* and *IRT* transcription by affecting the FER (or FER-like) transcription factor, necessary for the up-regulation of both genes [18,20]. Since FER (or FER-like) is also a transcription factor necessary for the acidification response induced by Fe deficiency [3], our results suggest that ethylene could regulate ferric reductase *CsFRO1*, iron transporter *CsIRT1* and H^+-ATPase *CsHA1* gene expression by affecting CsFER-like transcription factor activity.

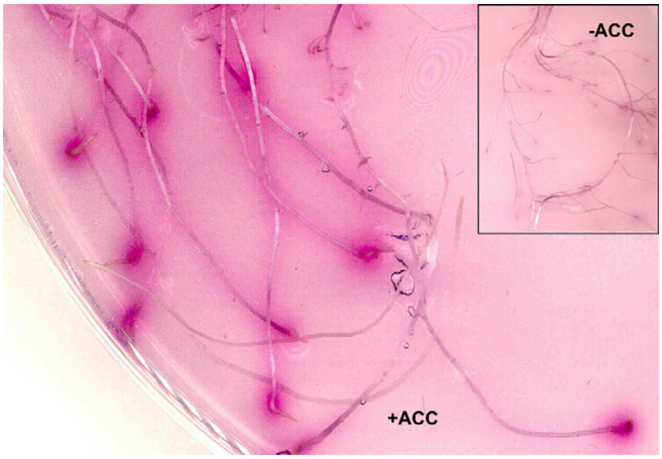

Fig. 8. Localization of ferric reductase activity induction by ACC in cucumber plants. Cucumber plants were grown the last 24 h without Fe and with or without 1 μM ACC. The more intense color around the subapical region of the roots is due to the Fe(II)-ferrozine complex formed (red) and denotes higher ferric reductase activity. This intense color was not presented in roots of plants grown during 24 h without Fe but with no ACC addition (inside).

Acknowledgments

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