Relationship between the Hormonal Balance and the Regulation of Iron Deficiency Stress Responses in Cucumber

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ABSTRACT. Some studies suggest that iron (Fe) stress root responses are regulated by variations in specific plant hormones. However, this question remains unclear. A time-course experiment dealing with the relationship between the expression of the Fe-stress root responses at transcriptional (*CsFRO1*, *CsIRT1*, *CsHAI*, and *CsHA2*) and enzymatic levels [root Fe(III)-chelate reductase and plasma membrane H⁺-ATPase], and the variation of phytohormone concentrations in the shoot and root of Fe-starved plants have been studied in a Fe-efficient cucumber cultivar (*Cucumis sativus* L. cv. Ashley). The results indicate that the expression over time of the physiological Fe-stress root responses at transcriptional and enzyme activity levels are consistent with significant increases in indole-3-acetic acid root (transient) and shoot (sustained) concentrations. Fe-starvation also caused transient changes in the root concentration of abscisic acid and nitric oxide. Finally, an increase in root ethylene production and a decrease in the root concentration of some cytokinins were observed under Fe starvation, but they were not clearly timely coordinated with the expression of Fe physiological root responses.

One of the most significant nutritional disorders affecting plant development in calcareous soils is Fe deficiency (Álvarez-Fernández et al., 2006; Hansen et al., 2006; Rombolà and Tagliavini, 2006). In this type of soil, the simultaneous presence of high concentrations of bicarbonate and high alkaline pH values causes Fe precipitation, which is associated with the unavailability of Fe for plants and microorganisms (Abadía et al., 2004; Lucena, 2006; Robin et al., 2008). To deal with this problem, plants have developed specific mechanisms aimed at increasing available rhizospheric Fe and the ability to absorb and assimilate Fe (Kawai and Alam, 2006; Schmidt, 2006). Dicots and nongrass monocots plants activate specific root responses to Fe deficiency. Among them are the acidification of the rhizosphere, the enhancement of the reduction of Fe(III) to Fe(II) at the root surface, the enhancement of the transport of Fe(II) into the root system, the release of reductants to the rhizosphere, and the development of morphological changes in the roots, principally subapical swelling and root hairs (Briat, 2008; Curie and Briat, 2003; Schmidt, 2006). Most of these responses are associated with the upregulation of several Fe-regulated genes such as the H⁺-ATPase, the ferric reductase, and the iron transporter genes (Curie and Briat, 2003).

Similarly, graminaceous plants have developed alternative mechanisms principally based on the synthesis and release to the rhizosphere of specific chelating agents, named phytosiderophores, capable of complexing Fe and other micronutrients, and on the production of Fe-phytosiderophore transporters to absorb them into the root system (Kawai and Alam, 2006).

Split-root assays show that development regulation in Fe-deficient plants is directly associated with physiological and biochemical responses related to Fe status in root and shoot (Vert et al., 2003). However, the nature of these regulation processes is poorly known. In fact, a previous work carried out in cucumber plants showed that the regulation of the expression of the physiological Fe deficiency stress root responses is greatly affected by the efficiency of the plants to optimize and use the available Fe in the shoot (Bacaicoa and García-Mina, 2009).

Certain studies involving specific hormonal precursors and functional inhibitors suggest that the regulation of Fe deficiency stress responses might be related to variations of the concentration of certain hormones in the roots (Romera et al., 2006; Rubio et al., 2009). Studies conducted by Landsberg and coworkers stressed the possible intervention of auxins in the regulation of Fe deficiency stress root responses (Landsberg, 1981a, 1981b, 1981c, 1984; Römhelt and Marschner, 1986). Likewise, the studies of Romera and coworkers strongly suggest an important role for ethylene in such a regulation (Lucena et al., 2006; Romera and Alcántara, 2004; Romera et al., 2006; Waters et al., 2007). Lamattina and coworkers (Graziano et al., 2002; Graziano and Lamattina, 2007) and others (Murgia et al., 2002) have noted the significant influence of nitric oxide (NO) in the regulation of Fe homeostasis in the

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shoot and in the expression of Fe stress responses in the roots. Finally, Séguéla et al. (2008) reported the capacity of different types of cytokinins (CKs) to repress the expression of *FRO2*, *IRT1*, and *FIT* genes in arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] by means of a mechanism different from that involved in the activation of these genes by Fe deficiency.

There are several studies that have questioned the determinant role of hormones in the regulation of Fe deficiency stress root responses. Schmidt et al. (2000b), in working with arabidopsis mutants and altering their sensitivity to specific hormones, observed that none of the hormones studied [including ethylene, indole-3-acetic acid (IAA), and abscisic acid (ABA)] appeared to have an important role in the regulation of Fe deficiency stress responses.

In principle, the mechanism by which plant hormones can affect biochemical processes involves different and complementary pathways. It can be expressed in changes in the local concentration of these hormones in specific areas of the root and/or shoots, or in the sensitivity of specific areas of the plant to hormone action. Although these changes may not always be related to measurable changes in the total concentration of the hormone in the whole plant, they are normally well correlated with changes in the concentration of the hormone in the plant area studied: root, stem, or leaf, principally in young plants (Davies, 2004). It also becomes clear that these events affecting hormone plant distribution must be timely coordinated with the functional expression of the hormone effect. However, the analysis of the differences between the distribution within the plant of the main plant regulators in Fe-starved and Fe-sufficient plants is scarce. Only two studies report data related to IAA levels in the roots of Fe-deficient plants, one in sunflower [*Helianthus annuus* L. (Römhelt and Marschner, 1986)] and the other one in apple [*Malus baccata* (L.) Borkh. and *M. baccata* var. *xiaojinensis* (M.H. Cheng & N.G. Jiang) Ponomar. (Han et al., 2005)]. In the case of ethylene, there are more studies reporting significant increases in this hormone in the roots of different Fe-deficient plant species (Romera et al., 1999, 2006). However, the synchronization in time between root ethylene increases and the expression of Fe deficiency root responses is still controversial (Li and Li, 2004). Finally, all these analytical studies have been carried out in roots, but if a shoot-to-root systemic signal is involved in the control of Fe deficiency responses, the analysis of the pattern of hormone variation in the shoots may be of great importance.

In this context, a complementary study dealing with the time-course relationship between the gene expression and enzyme activity of the main physiological Fe deficiency root responses and the hormonal balance in the whole plant (root and shoot) might clarify this controversy. This study could permit us to know what type of hormone is affected by Fe deficiency and if the change is compatible (timely synchronized) with a role in the induction of the main Fe deficiency root responses.

Thus, the objective of this work was to study the time-course variation in the concentrations of the main hormones [ethylene, IAA, ABA, gibberellins (GAs), NO, and CKs] in roots and shoots and the expression of the main Fe-regulated genes isolated in roots [Fe(III)-chelate reductase, Fe(II)-transporter, and H⁺-ATPase] in cucumber plants subjected to Fe starvation. In addition, we have analyzed in these plants the enzyme activity of the Fe(III)-chelate reductase and the H⁺-ATPase. An Fe-efficient cucumber cultivar, Ashley (Bacaicoa and García-Mina, 2009), was employed for the experiments.

Materials and Methods

PLANT MATERIAL AND GROWTH CONDITIONS. Seeds of 'Ashley' cucumber were germinated for 1 week in perlite moistened with 1 mM calcium sulfate solution. In the 10 d following post-germination, the plants were irrigated with an aerated nutrient solution having a pH of 6.0 described by Romera et al. (1999) as: 2 mM Ca(NO₃)₂, 0.75 mM K₂SO₄, 0.65 mM MgSO₄, 0.5 mM KH₂PO₄, 50 μM KCl, 10 μM H₃BO₃, 1 μM MnSO₄, 0.5 μM CuSO₄, 0.5 μM ZnSO₄, and 0.35 μM Na₂MoO₄ containing 1 μM Fe-HBED (Fe-N, N'-di-ortho hydroxybenzyl ethylene diamine diacetate). After 10 d, the plants [separated into two groups corresponding to the treatments: 40 μM Fe-HBED (+ Fe) and 0 μM Fe-HBED (-Fe)] were transferred to a continuously aerated solution. One hour before treatments and after 1, 2, 3, 4, 5, 7, 9, and 11 d from the onset of the treatments, the plants were harvested and samples corresponding to the root and shoot were collected to carry out the different analytical determinations as described below. The results concerning the different parameters analyzed were expressed from the first day after Fe starvation. There were not noticeable differences between the results corresponding to plants receiving Fe harvested 1 h before treatments and Fe-sufficient plants harvested the first day after treatments.

The plants were cultivated in a growth chamber [photosynthetic photon flux (PPF) = 250 μmol·m⁻²·s⁻¹] at a photoperiod of 15/9 h day/night, an average temperature of 23/21 °C day/night, and a relative humidity of 60% to 75%.

The whole experiment was carried out twice, and representative results are presented.

PARAMETERS EVALUATED IN THE EXPERIMENTS. The effect of Fe deficiency on Fe stress root plant responses was evaluated through the analysis of the following parameters.

FERRIC CHELATE REDUCTION BY INTACT ROOTS. Fe(III)-chelate reduction by roots of cucumber plants was measured as reported by Pinton et al. (1999) using the bathophenanthroline disulfonate (BPDS) reagent. Briefly, 1 g of roots from a single plant was incubated in darkness at 25 °C in 5.25 mL of the nutrient solution at pH 5.5, containing 0.387 mM Fe(III)-EDTA and 0.286 mM BPDS. After 30 min, the absorbance of the solution was measured at 535 nm using a spectrophotometer (model 8453; Agilent Technologies, Santa Clara, CA) running ultraviolet-visible Chemstation Software (Agilent Technologies). The quantity of Fe(III) reduced was calculated by the concentration of the Fe(II)-BPDS complex formed, using an extinction coefficient of 22.1 × 10⁻³ M⁻¹·cm⁻¹. We observed in previous experiments a good reproducibility of the results obtained using this method, as well as a good correlation between these results and those obtained using the whole root of the plant (data not shown).

REAL-TIME REVERSE TRANSCRIPTASE CHAIN REACTION (RT-PCR) ANALYSIS OF mRNA TRANSCRIPT. The roots of the plants were collected and disrupted with liquid nitrogen prior to RNA extraction. Total RNA was extracted from 60 mg of crushed root using 350 μL of guanidine-HCl lysis buffer of NucleoSpin RNA Plant Kit (Macherey-Nagel, Dierefn, Germany) and 3.5 μL of β-mercaptoethanol with a rotor-stator for 45 s following the manufacturer's instructions. The DNase-treated RNA was quantified by measuring the absorbance of the pooled elution at 260 nm. The RNA quality was confirmed as a 260/280 nm ratio above 1.8. The integrity of RNA was verified by electrophoresis on 1% formaldehyde agarose gels stained with SYBR Gold.

The absence of genomic DNA was initially checked in a real-time PCR containing 50 ng of total RNA (without reverse transcription) and α -tubulin-specific primers (*CsTua1*). The absence of inhibitors in 250 ng of RNA samples was also checked by SPUD assay with SYBR Green (Nolan et al., 2006). First-strand cDNA synthesis was carried out in 20- μ L reactions containing 1 μ g of RNA with RNase H⁺ MMLV reverse transcriptase iScript and a mix of oligo(dT) and random primers from iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The reverse transcription was made up for 5 min at 25 °C, 30 min at 42 °C, and ended by 5 min at 85 °C. The quality of cDNAs was verified by 5'-3' ratio (performing real-time PCR with α -tubulin-specific primers: *CsTua 4* in 5' strand and *CsTua 6* in 3' strand; Table 1). Real-time RT-PCR was performed on 50 ng of cDNA using iQ SYBR Green supermix containing hot-start iTaq DNA polymerase in an iCycler iQ (Bio-Rad Laboratories). Primer pairs used to amplify cucumber plasma membrane H⁺-ATPase genes (*CsHA1* and *CsHA2*), a Fe(III)-chelate reductase gene (*CsFRO1*), a Fe(II)-transporter gene (*CsIRT1*), and a α -tubulin (reference gene) were designed with Beacon Premier Biosoft Software and were synthesized by Sigma-Genosys (Cambridge, United Kingdom) (Table 1). The real-time PCR program consist of an iTaq DNA polymerase activation at 95 °C for 3 min, followed by 40 amplification cycles (denaturing step for 10 s at 95 °C; an annealing step for 10 s at 62 °C, and an elongation step for 10 s at 72 °C during which the fluorescence data were collected). To confirm PCR products, a melting curve was performed by heating the samples from 72 to 98 °C in 0.1 °C increments with a dwell time at each temperature of 10 s during which the fluorescence data were collected. The melting temperature of the products was determined with the iCycler iQ Optical System 3.1 Software (Bio-Rad Laboratories). The reaction efficiency of PCR (comprising between 1.99 and 2.02) was determined on the slope of standard dilution curves of pooled cDNAs with each specific primer pairs at harvest times. Target gene expression was normalized to α -tubulin expression. Relative expression (n-fold) of the normalized target gene in the Fe-starved plants was calculated with the REST© software [REST-MCS beta 2.0 (Pfaffl et al., 2002)] via comparison with

Fe-sufficient plants considering efficiency values according to the mathematical model proposed by Pfaffl et al. (2002).

ISOLATION OF PLASMA MEMBRANE VESICLES AND ATPASE ASSAYS. Plasma membrane (PM) vesicles were isolated from plant roots using a sucrose gradient technique as described previously by Pinton et al. (1999) and Santi et al. (2003). Briefly, the root segments corresponding to the different treatments were cut separately and ground with a pestle in an ice-cold homogenization medium containing 250 mM sucrose, 10% (v/v) glycerol, 10 mM glycerol-1-phosphate, 2 mM MgSO₄, 2 mM EDTA, 2 mM EGTA, 2 mM ATP, 2 mM DTT (dithiothreitol), 5.7% (w/v) chorine-iodine, 1 mM PMFS, 20 μ g·mL⁻¹ chymostatin, and 25 mM BTP {1,3-bis [TRIS(hydroxymethyl)-methylamino]propane} buffered to pH 6.7 with MES. About 2.5 mL per fresh weight of root tissues was used. The homogenates were filtered through four layers of cheesecloth and subjected to 3 min of centrifugation at 13,000 g_n at 4 °C (Microfuge 22R centrifuge; Beckman Coulter, Fullerton, CA). The pellets were discarded and the suspension was centrifuged for a further 25 min under the same conditions. The pellets were then recovered, gently resuspended in 400 μ L of homogenization medium, and loaded onto discontinuous density gradients made by layering 700 μ L of 25% (w/w) sucrose over 300 μ L of 38% (v/v) sucrose cushion in 1.5-mL tubes. Both sucrose solutions were prepared in 5 mM BTP-MES, pH 7.4, and contained all the protectants present in the homogenization medium. The gradients were centrifuged for 1 h at 13,000 g_n, and the vesicles banding at the 25%/38% interface were collected, diluted, and prepared for enzyme activity measurements.

The PM H⁺-ATPase activity was determined according to Santi et al. (2005) and Forbush (1983). Assays were performed at 38 °C in a 0.6 mL reaction volume containing 50 mM MES-BTP, pH 6.5, 5 mM MgSO₄, 100 mM KNO₃, 600 μ M Na₂MoO₄, 1.5 mM NaN₃, 5 mM ATP-BTP, pH 6.5, and 0.01% (w/v) Brij 58 (polyoxyethylene 20 cetyl ether) with or without 100 μ M V₂O₅. The reaction was started with the addition of membrane vesicles (0.5 μ g of total protein); after 30 min, the reaction was blocked and color developed as described in Varanini et al. (1993). Inorganic phosphate content was determined spectrophotometrically at 705 nm using an Agilent 8453 spectrophotometer running

Table 1. Primer sequences used in the gene expression studies of Fe-starved (0 μ M) and Fe-sufficient (40 μ M) cucumber plants.

Sequence definition	GenBank accession no.	Product length (bp)	Primer name	Primer sequence	
<i>Cucumis sativus</i> partial mRNA for proton-exporting ATPase (ha1 gene)	AJ703810	303	CsHA1	Sense	5'-GGGATGGGCTGGTGTAGTTTG-3'
				Antisense	5'-GCTATCTCTGCTCGTCTCTTGG-3'
<i>C. sativus</i> partial mRNA for proton-exporting ATPase (ha2 gene)	AJ703811	290	CsHA2	Sense	5'-TGAGCGACCTGGACTTCTATTG-3'
				Antisense	5'-GTGCCCATTGTGCTTCTCTTTC-3'
<i>C. sativus</i> iron-regulated transporter mRNA, complete cds	AY590764	259	CsIRT1	Sense	5'-TTCGCAGCAGGTATCATTCTCG-3'
				Antisense	5'-CACCACCTACTACAGGCAACTC-3'
<i>C. sativus</i> ferric reductase mRNA, complete cds	AY590765	152	CsFRO1	Sense	5'-AGCGGCGGCAGTGGAATC-3'
				Antisense	5'-GTTTGGAGGAGGTGGAGGAAGG-3'
<i>C. sativus</i> partial mRNA for α -tubulin (tua gene)	AJ715498	129	CsTUA1	Sense	5'-ACCGTTGGAAAGGAAATTGTTG-3'
				Antisense	5'-GGAGCCGAGACCAGAACC-3'
		141	CsTUA4	Sense	5'-ACTACACCGTTGGAAAGGAAATTG-3'
				Antisense	5'-AAAGGAGGGAGCCGAGACC-3'
		149	CsTUA6	Sense	5'-GACATTGAGCGACCTAACTAC-3'
				Antisense	5'-AACTGGATTCTGGGATATGGG-3'

ultraviolet-visible Chemstation Software (Agilent Technologies) and PM H⁺-ATPase activity was expressed as that inhibited by 100 μ M vanadate. Protein contents were determined following Bradford technique using BSA as standard (Bradford, 1976).

DETERMINATION OF RHIZOSPHERE ACIDIFICATION AS A RESPONSE TO IRON DEFICIENCY. The plant's capacity to acidify the rhizosphere was evaluated studying pH changes in the nutrient solution over time (Pinton et al., 1999; Romera and Alcántara, 2004).

ANALYSIS OF CHLOROPHYLL (CHL) CONCENTRATION. The leaf concentration of Chl was determined using the portable instrument SPAD-502 chlorophyll meter (Minolta, Osaka, Japan). Previously, a calibration curve was established measuring the Chl concentration corresponding to a selected group of SPAD readings. Chl leaf concentration was determined using the method described in Séstak et al. (1971).

ANALYSIS OF ETHYLENE. Cucumber plants corresponding to each treatment were separated into shoots and roots. Measurements were performed in root tissue because shoot analyses were below detection limits. Fresh root samples (1.5 g) were weighed and immediately transferred into 20-mL syringes with 200 μ L of deionized water. The syringes were sealed and dark incubated for 2 h at room temperature to allow ethylene production to subside. A 1-mL gas sample was withdrawn with a gas-tight syringe from the 20-mL syringe and analyzed for ethylene by gas chromatography using a gas chromatograph (Trace GC 2000 Series; ThermoQuest, Milan, Italy) equipped with a GS-GasPro 113–4332 column (30 m $\times$ 0.32 mm; Agilent Technologies) and flame ionization detector (FID). The rate of ethylene evolution was expressed as a function of per unit fresh weight. Data samples were processed using Xcalibur 1.0 Software (ThermoQuest).

DETERMINATION OF PLANT HORMONE CONCENTRATIONS. The concentration of the principal plant regulators in the shoot and root of treated plants was analyzed using high-performance liquid chromatography (HPLC)/mass spectrometry (MS)/MS as described below. The following hormones were studied: IAA, ABA, gibberellic acid (GA₃), gibberellin A₄ (GA₄), gibberellin A₇ (GA₇), *trans*-zeatin (Z), dihydrozeatin (DHZ), *trans*- and *cis*-zeatin riboside (t-ZR and c-ZR), dihydrozeatin riboside (DHZR), isopentenyladenine (iP), and isopentenyladenosine (iPR). The extraction and purification of the different plant regulators were carried out using the method described by Dobrev and Kamínek (2002) with some variations.

The analysis of IAA, ABA, and GAs content was as follows.

Frozen plant tissue (0.5 g; previously triturated in a mortar to a powder with liquid nitrogen) was homogenized with 5 mL of pre-cooled (–20 °C) methanol:water (80:20, v/v) and 2.5 mM sodium diethyldithiocarbamate. Deuterium-labeled internal standards {[²H₅] indol-3-acetic acid (D-IAA); [²H₆] (+)-*cis*, *trans*-abscisic acid (D-ABA); and [²H₂] gibberellic acid (D-GA₃) from Olchemim, Olomouc, Czech Republic} were added (100 μ L of a stock solution of 400 ng·mL^{–1} of each standard in methanol) to the extraction medium. After overnight extraction at –20 °C, solids were separated by centrifugation at

12,000 g_n for 10 min at 4 °C using a Centrikon T-124 centrifuge with an A8.24 rotor (Kontron Instruments, Cumbernauld, UK) and were re-extracted for 1 h with an additional 4 mL of extraction mixture. Supernatants were passed through a Strata C18-E cartridge (3 cm³, 200 mg, 8B-S001-FBJ; Phenomenex, Torrance, CA) preconditioned with 4 mL of methanol followed by 2 mL of extraction medium. After evaporation at 40 °C until aqueous phase using a Vortex Evaporator (Labconco, KS City, MO), 0.5 mL of 1 M formic acid was added. Afterward, the hormones were extracted successively with two portions of 5 and 4 mL of diethyl ether, and the organic phase was evaporated to dryness. The residue was redissolved in 250 μ L of methanol:0.5% acetic acid (40:60, v/v). The solution was centrifuged at 8,000 g_n for 5 min before its injection in the LC/MS/MS system. The hormones were quantified by HPLC (2795 Alliance HT; Waters, Milford, MA) linked to a 3200 Q TRAP LC/MS/MS System (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with an electrospray interface, using an reverse-phase column (Synergi 4 μ m Hydro-RP 80A, 150 $\times$ 2 mm; Phenomenex). A linear gradient of methanol (A) and 0.5% acetic acid in water (B) was used: 35% A for 1 min, 35% to 95% A in 9 min, 95% A for 4 min and 95% to 35% A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.20 mL·min^{–1}, the injection volume was 40 μ L, and the column and sample temperatures were 30 and 20 °C, respectively. The detection and quantification were performed by multiple reaction monitoring (MRM) in the negative-ion mode, employing multilevel calibration curves with deuterated hormones as internal standards. Compounds dependents parameters are listed in Table 2. The source parameters were: curtain gas: 172.37 kPa, GS1: 310.26 kPa, GS2: 413.69 kPa, ion spray voltage: –4000 V, and temperature: 600 °C. Data samples were processed using Analyst 1.4.2 software (Applied Biosystems/MDS Sciex).

The analysis of CKs content was as follows. Frozen plant tissue (0.5 g; previously triturated in a mortar to a powder with liquid nitrogen) was homogenized with 5 mL of pre-cooled (–20 °C) methanol:water:formic acid (15:4:1, v/v/v). Deuterium-labeled CKs internal standards {[²H₅] *trans*-zeatin (D-Z); [²H₅] *trans*-zeatin riboside (D-ZR); [²H₆] N⁶-isopentenyladenine (D-iP); and [²H₆] N⁶-isopentenyladenosine (D-iPR); from Olchemim} were added (40 μ L of a stock solution of 50 ng·mL^{–1} of each standard in methanol) to the extraction medium. After overnight extraction at –20 °C, solids were separated by centrifugation at 12,000 g_n for 10 min at 4 °C using

Table 2. Optimal parameters for the multiple reaction monitoring detection of indol-3-acetic acid, abscisic acid, and gibberellins used in the HPLC mass spectrometry analysis of Fe-starved (0 μ M) and Fe-sufficient (40 μ M) cucumber plants.

Compound ^z	Transition	Dwell (ms)	Ionization and collision parameters ^y				
			DP	EP	CEP	CE	CXP
IAA	174.0 > 130.1	150	–30	–3.0	–12	–14	–4
ABA	263.1 > 153.1	150	–25	–3.5	–20	–16	–4
GA ₃	345.1 > 143.1	150	–50	–4.0	–20	–36	–4
GA ₄	331.1 > 257.2	200	–55	–7.0	–16	–28	–5
GA ₇	329.1 > 223.2	200	–45	–5.0	–18	–26	–5
[² H ₅] IAA	179.0 > 135.2	150	–20	–7.0	–12	–14	–4
[² H ₆] ABA	269.1 > 159.2	150	–25	–12	–14	–14	–4

^zIAA = indol-3-acetic acid, ABA = abscisic acid, GA₃ = gibberellic acid, GA₄ = gibberellin A₄, GA₇ = gibberellin A₇, [²H₅] IAA = [²H₅] indol-3-acetic acid, [²H₆] ABA = [²H₆] (+)-*cis*, *trans*-abscisic acid.

^yDP = declustering potential, EP = entrance energy, CEP = collision cell entrance potential, CE = collision energy, CXP = collision cell exit potential.

a Centrikon T-124 centrifuge with an A8.24 rotor (Kontron Instruments) and were re-extracted for 1 h with an additional 4 mL of extraction mixture. Supernatants were passed through a Strata C18-E cartridge (3 cm³, 200 mg, 8B-S001-FBJ; Phenomenex) preconditioned with 4 mL of methanol followed by 2 mL of extraction medium. After evaporation at 40 °C until aqueous phase using a Vortex Evaporator (Labconco), 2 mL of 1 M formic acid was added and applied to an Oasis MCX column (3 cm³, 60 mg, 186000254; Waters) preconditioned with 4 mL of methanol and 2 mL of 1 M formic acid. The column was washed successively with 2 mL of 1 M formic acid, 2 mL of methanol, and 2 mL of 0.35 M NH₄OH, and the CKs bases, ribosides, and glucosides were eluted with 2 mL of 0.35 M NH₄OH in 60% (v/v) methanol. This eluted fraction was evaporated to dryness in the vortex evaporator and redissolved in 250 µL of methanol:0.05% formic acid (40:60, v/v). The solution was centrifuged at 8,000 g_n for 5 min before its injection in the LC/MS/MS system. The CKs were quantified by HPLC (2795 Alliance HT; Waters) linked to a 3200 Q TRAP LC/MS/MS System (Applied Biosystems/MDS Sciex) equipped with an electrospray interface, using an reverse-phase column (Tracer Excel 120 ODSA 3 µm, 100 × 4.6 mm; Teknokroma, Barcelona, Spain). A linear gradient of methanol (A) and 0.05% formic acid in water (B) was used: 35% to 95% A in 11 min, 95% A for 3 min and 95% to 35% A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.25 mL·min⁻¹, the injection volume was 40 µL, and the column and sample temperatures were 30 and 20 °C, respectively. Detection and quantification were performed by MRM in the positive-ion mode, employing multilevel calibration curves with deuterated CKs as internal standards. Compounds dependents parameters are listed in Table 3. The source parameters are: curtain gas: 172.37 kPa, GS1: 344.74 kPa, GS2: 413.69 kPa, ion spray voltage: 5000 V, and temperature: 600 °C. Data samples were processed using Analyst 1.4.2 software (Applied Biosystems/MDS Sciex).

ANALYSIS OF NO. Cucumber leaves and roots were harvested and washed with deionized water, removing the excess humid-

ity before measuring the NO. Cotyledons and stems were not used in this measure. Samples were ground with mortar and pestle in liquid nitrogen. Then, 0.5 g of ground samples (fresh weight based) were mixed with 2 mL of 40 mM HEPES buffer, pH 7.2, to achieve a 1:4 (w/v) proportion between sample and buffer (Corpas et al., 2004). Samples were kept on ice during the procedure to prevent sample degradation. The homogenized sample was filtered using Miracloth paper, and the filtered solution was then centrifuged at 39,000 g_n for 20 min at 4 °C. After that, 340 µL of supernatant and 10 µL of a 350 µM DAF-2 solution were then added into each well of a black 96-well plate (where the reaction took place). The plates were incubated for 30 min at 25 °C before reading the fluorescence intensities using a GENios Microplate Reader running Magellan2 Software (Tecan Group, Männedorf, Switzerland). A reference calibration curve was established using fluorescein at different concentrations. This curve provides reference intensities values to compare the fluorescence measured in samples. Negative controls (NC) with no DAF-2 were also used. The final result was expressed as fluorescence units per gram of fresh weight (FW).

STATISTICAL ANALYSIS. Four replications with three plants per replication were employed for the different analytical determinations described above. Significant differences ($P < 0.05$) among treatments were calculated by using one-way analysis of variance (ANOVA) and the Fisher's post hoc test. Statistical analysis of relative gene expression results was assessed using P-pairwise fixed reallocation randomization test.

Results

FE STARVATION AFFECTED DIFFERENTLY THE GENE EXPRESSION AND THE ACTIVITY OF THE MAIN PROTEINS INVOLVED IN THE PHYSIOLOGICAL FE STRESS ROOT RESPONSES. A clear increase in the root Fe(III)-chelate reductase activity was observed 1 d from the onset of treatments for Fe-starved plants with respect to Fe-sufficient plants (Fig. 1A). This increase was maintained along the experiment, although a decrease in intensity was noted at days 9 and 11. These results are in accordance with the expression of the gene that encodes the root Fe(III)-chelate reductase in cucumber (*CsFRO1*). Expression of this gene showed a significant increase after 1 d of Fe starvation (Fig. 1B). This increase was sustained along the time of the study, showing notable increases after 3, 5, 9, and 11 d from the onset of treatments. In relation to the gene encoding the Fe(II)-transporter (*CsIRT1*), it presented a significant increase after 1 d of Fe deficiency with maximum values at the end of the experiment (Fig. 1B).

The results concerning H⁺-ATPase activity and gene expression were different from those described above for Fe(III)-chelate reductase and Fe(II)-transporter. Two H⁺-ATPase genes have been isolated from cucumber roots: *CsHA1* and *CsHA2*. *CsHA1* is upregulated, while *CsHA2* is constitutively expressed

Table 3. Optimal parameters for the multiple reaction monitoring detection of cytokinins used in the HPLC mass spectrometry analysis of Fe-starved (0 µM) and Fe-sufficient (40 µM) cucumber plants.

Compound ^z	Transition	Dwell (ms)	Ionization and collision parameters ^y				
			DP	EP	CEP	CE	CXP
Z	220.3 > 136.2	150	36	3.5	16	25	4
DHZ	222.3 > 136.2	150	41	3.0	16	29	4
tZR	352.3 > 220.2	150	46	4.5	19	25	4
cZR	352.3 > 220.2	150	46	4.5	19	25	4
DHZR	354.3 > 222.2	150	36	4.0	19	27	4
iP	204.3 > 136.2	150	31	4.0	14	21	4
iPR	336.2 > 204.2	150	36	4.0	18	23	4
[² H ₅] tZ	225.1 > 136.9	150	31	3.5	16	27	4
[² H ₅] tZR	357.1 > 225.2	150	31	6.0	20	25	4
[² H ₆] iP	210.2 > 136.9	150	31	5.0	14	21	4
[² H ₆] iPR	342.2 > 210.2	150	61	4.5	20	25	4

^zZ = *trans*-zeatin, DHZ = dihydrozeatin, tZR = *trans*-zeatin riboside, cZR = *cis*-zeatin riboside, DHZR = dihydrozeatin riboside, iP = isopentenyladenine, iPR = isopentenyladenosine, [²H₅] tZ = [²H₅] *trans*-zeatin, [²H₅] tZR = [²H₅] *trans*-zeatin riboside, [²H₆] iP = [²H₆] N⁶-isopentenyladenine, [²H₆] iPR = [²H₆] N⁶-isopentenyladenosine.

^yDP = declustering potential, EP = entrance energy, CEP = collision cell entrance potential, CE = collision energy, CXP = collision cell exit potential.

and did not respond to Fe deficiency (Santi et al., 2005). Thus, although a significant and time-sustained increase in the gene expression of the *CsHAI* isoform was observed from the first day of the onset of treatments in Fe-starved plants (Fig. 2A), the H^+ -ATPase activity only experienced a significant increase after 5 and 9 d, reflected in a significant decrease of the pH of the nutrient solution at 11 d (Fig. 2, B and C). Thus, whereas the activation of *CsHAI* expression in Fe-starved cucumber plants was expressed similarly to *CsFRO1* and *CsIRT1* expression, the activity of the H^+ -ATPase enzyme was differently enhanced over time. These results indicate that the transcriptional control of H^+ -ATPase (*CsHAI* isoform) gene might be coregulated with that of Fe(III)-chelate reductase (*CsFRO1*) and Fe(II)-transporter (*CsIRT1*) genes. However, the regulation at the enzyme activity level of H^+ -ATPase is expressed differently from that of Fe(III)-chelate reductase and, probably, Fe(II)-transporter, in the root. These results suggest the possible involvement of different, although prob-

ably interrelated, regulatory systems for the transcriptional and post-transcriptional expression of the main physiological Fe stress root responses.

It is interesting to note that Fe-starved plants presented a slight but not significant decrease in the leaf concentration of chlorophyll in comparison with Fe-sufficient plants (Fig. 3).

FE STARVATION AFFECTED DIFFERENTLY THE GENE EXPRESSION AND THE ACTIVITY OF THE MAIN PROTEINS INVOLVED IN THE PHYSIOLOGICAL FE STRESS ROOT RESPONSES. The results show that there was a slight nonsignificant increase in ethylene production in the root after 1 d from the onset of Fe starvation in Fe-starved plants with respect to Fe-sufficient plants (Fig. 4). However, after 2 d, the increase in root ethylene production in Fe-starved plants was very clear and significant when compared with Fe-sufficient plants (Fig. 4). These results were systematically obtained in three independent experiments (data not shown).

Regarding IAA, a clear and sustained increase in its shoot concentration in Fe-starved plants with respect to Fe-sufficient plants was observed. This increase was slight after 1, 2, and 3 d, but very significant and sustained after 4 d (Fig. 5A). These changes in the shoots were correlated with a concomitant increase in the roots of Fe-starved plants after 1 and 2 d (Fig. 5B).

Fe starvation also affected ABA and NO concentrations. The results show that the variation pattern of ABA concentration in shoot and root changed significantly depending on Fe nutrition status (Fig. 6). Thus, whereas ABA concentration in the shoot of Fe-starved plants experienced a slight but significant decrease after 1, 4, 7, 9, and 11 d of Fe starvation (Fig. 6A), ABA root concentration presented a significant increase after 1, 2, and 3 d (Fig. 6B).

The results concerning NO concentration in shoots and roots (Fig. 7) also showed significant changes depending on Fe plant status. Thus, after 1 d of Fe starvation, a significant and transient increase in the root NO concentration (Fig. 7B) was noted in Fe-starved plants when compared with Fe-sufficient ones, which corresponded to a concomitant decrease in the shoot (Fig. 7A).

Finally, regarding the variation pattern of the different CKs studied under Fe starvation, the results obtained show that most of them tended to decrease in Fe-starved plants. These changes, along with those described above for IAA, caused significant decreases in auxin:cytokinin ratio values in the roots and shoots in Fe-starved plants when compared with Fe-sufficient plants (data not shown). Thus, whereas significant differences in Z concentration between treatments were not observed in roots or shoots (data not shown), the concentration of DHZR in the roots of Fe-starved plants was lower than that in Fe-sufficient plant roots after 3 d of Fe deficiency (Fig. 8B). These changes in root DHZR concentrations with Fe starvation were not accompanied by differences in the shoot (Fig. 8A). In the case of c-ZR, a lower concentration after 1, 2, 5, 7, and 11 d of Fe starvation in the shoots of Fe-starved plants was observed in comparison with Fe-sufficient plants (Fig. 9A), which was associated with higher concentrations in the root after 1 and 2 d, followed by lower concentrations after 3 and 11 d (Fig. 9B). However, the concentration of t-ZR did not present any significant differences between treatments (data not shown). Finally, whereas no significant differences between treatments were observed for iPR (data not shown), the root concentration of iPR in Fe-starved plants was lower than that for Fe-sufficient plants after 3 and 11 d from the onset of Fe-starvation (Fig. 10B). However, in the

Fig. 1. (A) Fe(III)-chelate reductase activity in roots of Fe-starved (□-) and Fe-sufficient (■-) cucumber plants. (B) Effects of Fe deficiency on *CsFRO1* (white striped bars) and *CsIRT1* (grey striped bars) gene expression in roots of cucumber plants (mean $\pm$ SE, $n = 4$). Asterisks indicate the presence of significant differences for $P < 0.05$ between +Fe (40 μ M) and -Fe (0 μ M) treatments based on one-way ANOVA and Fisher's post hoc test. Statistical analysis of relative gene expression results was assessed using P-pairwise fixed reallocation randomization test.

Fig. 2. (A) Effects of Fe deficiency on *CsHA1* (white striped bars) and *CsHA2* (grey striped bars) gene expression in roots of cucumber plants. (B) H^+ -ATPase activity in roots and (C) nutrient solution pH of Fe-starved ($\square$) and Fe-sufficient ($\blacksquare$) cucumber plants (mean $\pm$ SE, $n = 4$). Asterisks indicate the presence of significant differences for $P < 0.05$ between +Fe (40 μM) and -Fe (0 μM) treatments based on one-way ANOVA and Fisher's post hoc test. Statistical analysis of relative gene expression results was assessed using P-pairwise fixed reallocation randomization test.

shoot, there were not significant differences between treatments (Fig. 10A).

Regarding the gibberellins studied (GA3, GA4, and GA7), their concentrations were below the detection limits of our technique.

reductase activity, which presented significant activity before the ethylene root production increase. These results suggest that the variation in ethylene root production in Fe-deficient plants could be a consequence but not the cause of the

There is in the literature experimental evidence showing that the regulation of the main Fe deficiency root responses involves different and coregulated pathways at the local level (root region) and the systemic level (shoot to root region) (Curie and Briat, 2003; Schmidt, 2006; Vert et al., 2003). This coregulated system depends on the Fe status of the plant (Curie and Briat, 2003; Schmidt, 2006; Vert et al., 2003). Some authors have proposed specific hormones as candidates to control the expression of Fe deficiency responses: ethylene (Lucena et al., 2006; Romera et al., 2006), IAA (Landsberg, 1981a, 1981b, 1981c, 1984; Römheld and Marschner, 1986), and NO (Graziano and Lamattina, 2007). These studies have been fundamentally based on pharmacological approaches employing specific functional inhibitors or precursors. However, other studies employing different strategies (plant mutants and time-course patterns) questioned these conclusions (Li and Li, 2004; Schmidt et al., 2000b). In this context, a complementary study dealing with the relationship between the time-course expression of the main Fe-regulated genes in the roots and the hormonal balance in the whole plant (roots and shoots) might aid in clarifying this controversy.

A number of studies have reported that Fe deficiency is associated with an increment in the root production of ethylene (Romera et al., 2006). Further studies involving ethylene precursors and different inhibitors of ethylene synthesis or action indicated that ethylene could be directly involved in the regulation of the transcriptional expression of the main genes involved in Fe deficiency stress responses of Strategy I plants (Lucena et al., 2006). However, the results reported by Li and Li (2004) indicated that the increase in ethylene root production linked to Fe deficiency was not timely synchronized with the expression of the root Fe(III)-chelate

Fig. 3. Chlorophyll concentration in the newly formed leaves of Fe-starved ($\square$) and Fe-sufficient ($\blacksquare$) cucumber plants (mean $\pm$ SE, $n = 4$). Asterisks indicate the presence of significant differences for $P < 0.05$ between +Fe (40 μ M) and -Fe (0 μ M) treatments based on one-way ANOVA and Fisher's post hoc test.

Fig. 4. Ethylene released by roots of Fe-starved ($\square$) and Fe-sufficient ($\blacksquare$) cucumber plants (mean $\pm$ SE, $n = 4$). Asterisks indicate the presence of significant differences for $P < 0.05$ between +Fe (40 μ M) and -Fe (0 μ M) treatments based on one-way ANOVA and Fisher's post hoc test.

activation of root response. This conclusion agrees with other results obtained in experiments carried out with arabidopsis mutants insensitive or oversensitive to different hormones, including ethylene (Schmidt et al., 2000b). The results obtained in the present study indicate that the increase in ethylene root production in Fe-starved plants (Fig. 4) was not clearly timely synchronized with the transcriptional expression of the genes encoding the Fe(III)-chelate reductase (*CsFRO1*), the Fe(II)-root transporter (*CsIRT1*), and the H^+ -ATPase (*CsHAI*), or with the activity of the Fe(III)-chelate reductase (Figs. 1 and 2A). Thus, a slight, but not significant, increase in ethylene root production in Fe-starved plants was timely synchronized with the expression of Fe stress root responses. This small increase in ethylene root production was repeated in different independent experiments, but it was not significant due to data variability. Therefore, it is possible that this slight increase in ethylene root production is involved in the transcriptional control of the main

Fig. 5. Effects of Fe deficiency on indol-3-acetic acid (IAA) concentration in cucumber plants. (A) IAA concentration in shoot of Fe-starved plants ($\square$) compared with Fe-sufficient ones ($\blacksquare$). (B) IAA concentration in roots of Fe-starved ($\square$) and Fe-sufficient ($\blacksquare$) plants (mean $\pm$ SE, $n = 4$). Asterisks indicate the presence of significant differences for $P < 0.05$ between +Fe (40 μ M) and -Fe (0 μ M) treatments based on one-way ANOVA and Fisher's post hoc test.

physiological root responses, in agreement with the results reported by Lucena et al. (2006). However, it is noteworthy that Fe-sufficient plants also presented an increase in ethylene production up to the levels of Fe-starved plants, but 1 d later. This fact suggests that Fe starvation might accelerate a natural process involving root ethylene production, which also occurs in Fe-sufficient plants. In any case, studies carried out using Fe-sufficient plants indicated that the action of ethylene might be necessary but not enough to activate Fe stress physiological root responses (Lucena et al., 2006; Romera et al., 2006). In this context, studies on the transcriptional and post-transcriptional expression of the main physiological root responses to Fe deficiency in arabidopsis mutants insensitive to ethylene would be of great interest. In this sense, recent studies indicate that *FRO2* and *IRT1* expression is upregulated under conditions of Fe deficiency in several arabidopsis ethylene-insensitive mutants (*ert1*, *ein2*, and *ein3eil1*) (García et al., 2007). At first, these results could suggest that ethylene is not involved in the process. However, the application of ethylene precursors was

Fig. 6. Effects of iron deficiency on abscisic acid (ABA) concentration in cucumber plants. (A) ABA concentration in shoot of Fe-starved plants (□-) compared with Fe-sufficient ones (■-). (B) ABA concentration in roots of Fe-starved (□-) and Fe-sufficient (■-) plants (mean $\pm$ SE, $n = 4$). Asterisks indicate the presence of significant differences for $P < 0.05$ between +Fe (40 μ M) and -Fe (0 μ M) treatments based on one-way ANOVA and Fisher's post hoc test.

associated with the upregulation of both genes, whereas the application of ethylene inhibitors was associated with a down-regulation of these genes (García et al., 2007), thus indicating that ethylene could be involved in their transcriptional control, but through a singular and specific pathway. Likewise, a possible role of ethylene through changes in its local concentration, in specific root areas or in the cell sensitivity to ethylene action, cannot be ruled out.

The results obtained in this work also showed that Fe starvation was associated with significant increases in the IAA concentration in roots and, mainly, in shoots when compared with plants receiving Fe (Fig. 5). The effects of Fe starvation on IAA root concentration were observed on the same day as those related to root gene expression and enzyme activities, although it was transient and disappeared after 2 d. The effects observed on the shoot IAA concentration, however, were sustained and well correlated to those related to root gene expression and enzyme activities (Figs. 1 and 2). These results are compatible with a possible role of IAA in the regulation of

Fig. 7. Effects of Fe deficiency on nitric oxide concentration in shoot (A) and roots (B) of Fe-starved (□-) and Fe-sufficient (■-) cucumber plants (mean $\pm$ SE, $n = 4$). Asterisks indicate the presence of significant differences for $P < 0.05$ between +Fe (40 μ M) and -Fe (0 μ M) treatments based on one-way ANOVA and Fisher's post hoc test.

the plant responses to Fe deficiency at transcriptional and enzyme activity levels. Some authors have also suggested that the systemic shoot to root regulation of the main Fe stress root responses may be mediated by IAA (Landsberg, 1981a, 1981b, 1981c, 1984; Römheld and Marschner, 1986). In this way, some studies involving functional inhibitors of IAA polar transport and activity and the decapitation of plant shoot apex showed that IAA might be involved in the expression of morphological and physiological Fe stress root responses (De la Guardia et al., 1988; Landsberg, 1981a, 1981c, 1984; Li et al., 2000). Likewise, as in the case of ethylene, a number of studies using auxin compounds indicated that they were able to induce the main morphological root changes (subapical swelling, transfer cells, and root hairs) even in Fe-sufficient plants (Landsberg, 1981b, 1984; Schmidt and Bartels, 1996; Schmidt et al., 2000a). In this sense, some studies indicate that IAA and ethylene could act in a coordinated way in the regulation of the root morphological responses to Fe deficiency (Romera et al., 2006). However, as in the case of ethylene, the study carried out by Schmidt et al. (2000b) using arabidopsis mutants insensitive to IAA showed that the action of this hormone probably is not strictly necessary

Fig. 8. Effects of Fe deficiency on dihydrozeatin riboside (DHZR) concentration in shoot (A) and roots (B) of Fe-starved (□) and Fe-sufficient (■) cucumber plants (mean $\pm$ SE, $n = 4$). Asterisks indicate the presence of significant differences for $P < 0.05$ between +Fe (40 μ M) and -Fe (0 μ M) treatments based on one-way ANOVA and Fisher's post hoc test.

for the upregulation of the Fe(III)-chelate reductase. However, the results obtained by Schmidt et al. (2000b), and also the results obtained in this study, are compatible with an involvement of IAA in the regulation of some of the Fe stress root responses, at least at transcriptional level. In this sense, the IAA capacity to activate Fe stress root physiological responses in Fe-sufficient plants, although controversial, cannot be ruled out. Whereas Schmidt and coworkers did not observe an increase in root Fe(III)-chelate reductase activity (Schmidt, 1994) or rhizosphere acidification (Schmidt et al., 2003) upon 2,4-D application to Fe-sufficient plantago (*Plantago lanceolata* L.) and tomato (*Solanum lycopersicum* L.) plants, respectively, Li and Li (2004) noted that indol-3-butyric acid (IBA) caused an increase in root Fe(III)-chelate reductase activity in Fe-sufficient cucumber and bean (*Phaseolus vulgaris* L.) plants.

It was interesting to note the significant increase in shoot IAA concentration upon Fe starvation (Fig. 5). This fact supports the view that the increase in IAA root concentration observed under Fe deficiency might be directly linked to the transport to the root of IAA synthesized in the shoot (polar auxin transport) as proposed in previous studies (De la Guardia et al., 1988; Han et al., 2005; Landsberg, 1981a, 1981c, 1984;

Fig. 9. Effects of Fe deficiency on *cis*-zeatin riboside (c-ZR) concentration in shoot (A) and roots (B) of Fe-starved (□) and Fe-sufficient (■) cucumber plants (mean $\pm$ SE, $n = 4$). An asterisk indicates the presence of significant differences for $P < 0.05$ between +Fe (40 μ M) and -Fe (0 μ M) treatments based on one-way ANOVA and Fisher's post hoc test.

Li et al., 2000). Likewise, these results also suggest that IAA could be involved in the high efficiency of this cucumber cultivar to use the Fe available in the shoot (Bacaicoa and García-Mina, 2009). The possible enhanced shoot to root transport of IAA under conditions of Fe deficiency also support the hypothesis of a possible role of IAA in the systemic control of Fe stress root responses. In this context, further investigation into the transcriptional expression of the main Fe stress root responses involving arabidopsis IAA-insensitive mutants and functional IAA inhibitors is of great interest.

The pattern of variations found on the concentrations of ABA and NO in roots and shoots under Fe deficiency (Figs. 6 and 7) was also interesting. In both cases, the concentrations of these plant regulators in the root of Fe-starved plants were lower than those for Fe-sufficient plants, but these effects were transient and only expressed at the beginning of Fe starvation. In the case of ABA, these differences were clear for the first 3 d after Fe starvation. In the case of NO, they were expressed only the first day. Regarding the shoot, the effect of Fe starvation on ABA concentration was less clear, although it presented a tendency to decrease with respect to Fe-sufficient plants. In

Fig. 10. Effects of Fe deficiency on isopentenyladenine (iP) concentration in shoot (A) and roots (B) of Fe-starved (□) and Fe-sufficient (■) cucumber plants (mean $\pm$ SE, $n = 4$). An asterisk indicates the presence of significant differences for $P < 0.05$ between +Fe (40 μ M) and -Fe (0 μ M) treatments based on one-way ANOVA and Fisher's post hoc test.

the case of NO, its concentration in the shoot of Fe-starved plants was lower than that of Fe-sufficient ones at the first day from the onset of Fe starvation. The results concerning ABA and NO are compatible with an action of these compounds in the regulation of the studied Fe stress root responses at transcriptional and enzyme activity levels. Recently, Graziano and Lamattina (2007) reported that an accumulation of NO in root tissues was required for the expression of Fe(III)-chelate reductase (*LeFRO1*) and Fe(II)-transporter (*LeIRT1*) genes. As in the case of ethylene, NO seems to be necessary but not sufficient to induce these genes because its application to Fe-sufficient plants did not activate them (Graziano and Lamattina, 2007). Regarding ABA, the results of Schmidt et al. (2000b) employing arabidopsis ABA-insensitive mutants indicated that the action of this hormone was not necessary for the enhancement of Fe(III)-chelate reductase activity under Fe deficiency. However, these results are compatible with a possible action of this compound at transcriptional level. However, as in the case of ethylene, ABA changes in roots and shoots of Fe-starved and -sufficient plants suggest that Fe starvation induces the activation of a natural process involving ABA concentration in roots, which also occurs in Fe-sufficient plants, but later. This

fact could be related to Fe root to shoot transport and Fe storage in the shoot (Lobréaux et al., 1993).

Finally, it was also worthy to note the relationship between the development of Fe starvation and the reduction in the concentration of several CKs in the roots and shoots (Figs. 8–10). However, these effects were expressed one of several days (depending on the CK studied) after the increase in the expression of the studied genes under Fe starvation, thus suggesting that both phenomena may be complementary but not directly causally linked (CKs as regulators of Fe stress root responses under Fe deficiency). These results are in line with those reported by Séguéla et al. (2008) in arabidopsis. These authors observed that several CKs types down-regulated the expression of the Fe(III)-chelate reductase (*FRO2*), the Fe(II)-transporter (*IRT1*), and the master gene *FIT* at transcriptional level. However, this action was independent of Fe nutrition status and affected only one specific set of genes regulated by Fe deficiency. These results suggested that the repressive action of CKs on some of the physiological Fe deficiency root responses at the transcriptional level is probably mediated by a mechanism different from that involved in their induction. In this context, the reduction in the root and shoot concentration of some CKs observed in our study under conditions of Fe deficiency could favor the expression of Fe stress root responses to Fe starvation.

In summary, the time-course variations of the parameters studied and their relationships in Fe-sufficient and Fe-starved cucumber plants indicate that the transcriptional regulation of the expression of the studied genes (*CsFRO1*, *CsIRT1*, and *CsHAI*) under Fe starvation in cucumber are compatible with the participation of IAA in the regulation of Fe stress root responses, whereas the results concerning NO, ABA, and ethylene are less conclusive. Complementarily studies carried out on Fe-sufficient plant models indicated that the presence of ethylene and NO may be necessary but not sufficient for activating Fe stress root responses (Graziano and Lamattina, 2007; Romera et al., 2006). Preliminary studies carried out in our laboratory with ABA in Fe-sufficient cucumber plants suggested the same conclusion for this hormone. In this sense, it is more likely that the role of ABA and NO is related to Fe storage and remobilization within the plant (Lobréaux et al., 1993; Murgia et al., 2002). However, the results obtained concerning IAA, considered together with those reported by other authors in Fe-sufficient plants (Li and Li, 2004), deserve more in-depth studies. Further investigation on the action of this hormone on the transcriptional and post-transcriptional expression of root Fe stress responses in plant models involving arabidopsis IAA-insensitive and -oversensitive mutants, as well as functional inhibitors of IAA action, would be of great interest.

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