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## Application of the split root technique to study iron uptake in cucumber plants

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## Abstract

The regulation exerted by the Fe status in the plant on Fe deficiency responses was investigated in *Cucumis sativus* L. roots at both biochemical and molecular levels. Besides the two activities strictly correlated with Fe deficiency response, those of the Fe(III)-chelate reductase and the high affinity Fe transporter, we considered also H<sup>+</sup>-ATPase (EC 3.6.3.6) and phospho*enol*pyruvate carboxylase (EC 4.1.1.31), that have been shown to be involved in this response. Both enzymatic activities and gene expression were monitored using a split root system. Absence of Fe induced the expression of the four transcripts, accompanied by an increase in the corresponding enzymatic activities. The application of the split root technique gave some information about the regulation of Fe uptake. In fact, 24 h after split root application, transcripts were still high and comparable to those of the -Fe control in the Fe-supplied half side, while in the -Fe side there was a drop in the expression and the relative enzymatic activities. Major changes occurred after 48 and 72 h. The coordinated regulation of these responses is discussed.

## Highlights

► The split root technique was used to study the response to Fe deficiency in cucumber roots. ► Important morphological changes at root level after split root application were observed. ► Enzyme activity of FRO1, H<sup>+</sup>-ATPase and PEPC and immunolocalization of H<sup>+</sup>-ATPase and PEPC were determined. ► Different coregulation among FRO1, IRT1, HA1 and PEPC1 genes was found.

## Keywords

*Cucumis sativus* Fe(III)-chelate reductase H<sup>+</sup>-ATPase Fe deficiency response Phospho*enol*pyruvate carboxylase

## 1. Introduction

Plants require Fe to complete their life cycle. The importance of Fe is due to the existence of two stable, inter-convertible forms of this metal, which take part in fundamental processes involving electron transfer reactions, including respiration and photosynthesis [1].

Generally, there is a high quantity of Fe in the soil, but in aerobic and sub-alkaline pH environments its solubility is strongly restricted. To cope with this constraint and to enhance metal bioavailability, plants have evolved adaptation strategies to face low Fe concentrations in the environment [2]. These include morphological changes in the root architecture and specific biochemical and molecular responses serving to increase rhizosphere Fe availability and uptake [1,3].

While the uptake system through which root acquires Fe is well characterized, its regulation, as well as the flux of signals inducing or repressing these responses are not yet completely clarified. Strategy I plants (dicotyledonous and non-graminaceous) respond to lack of Fe mainly through increases in the reduction and uptake activities, by inducing trans-plasma membrane proteins in the rhizodermal cells [i.e. Fe(III)-chelate reductase [FC-R] and Iron Regulated Transporter 1 (IRT1), respectively] [1]. In Strategy I plants, an induction of genes encoding the FC-R has been observed in response to Fe starvation [4–7]. After reduction, the Fe<sup>II</sup> form is taken up across the plasma membrane by a specific Fe transporter (IRT1) [7–11]. In most of the Strategy I plants, acidification of the rhizosphere occurs through the induction of a plasma membrane-located P-type H<sup>+</sup>-ATPase [3,12–15]. A multigene family encoding different isoforms and tissue specific expression of H<sup>+</sup>-ATPase has been demonstrated [16–19]. Moreover, significant metabolic

changes occur in roots to sustain the energy requests for FC-R and H<sup>+</sup>-ATPase activities [20]. In particular, phosphoenolpyruvate carboxylase (PEPC) activity plays a crucial role [21,22]. Fe deficiency leads to an up-regulation of the expression of many genes involved in Fe acquisition, transport and homeostasis. Among these genes, several transcription factors in different Strategy I plants have been characterised [23 and references therein]. The regulation of genes induced by Fe deficiency is not completely elucidated, but recently new evidence has been presented to support a role for ethylene and nitric oxide in their activation [23–25]. These findings suggest that the Fe deficiency responses are modulated by a complex mechanism acting at different levels and through different signals.

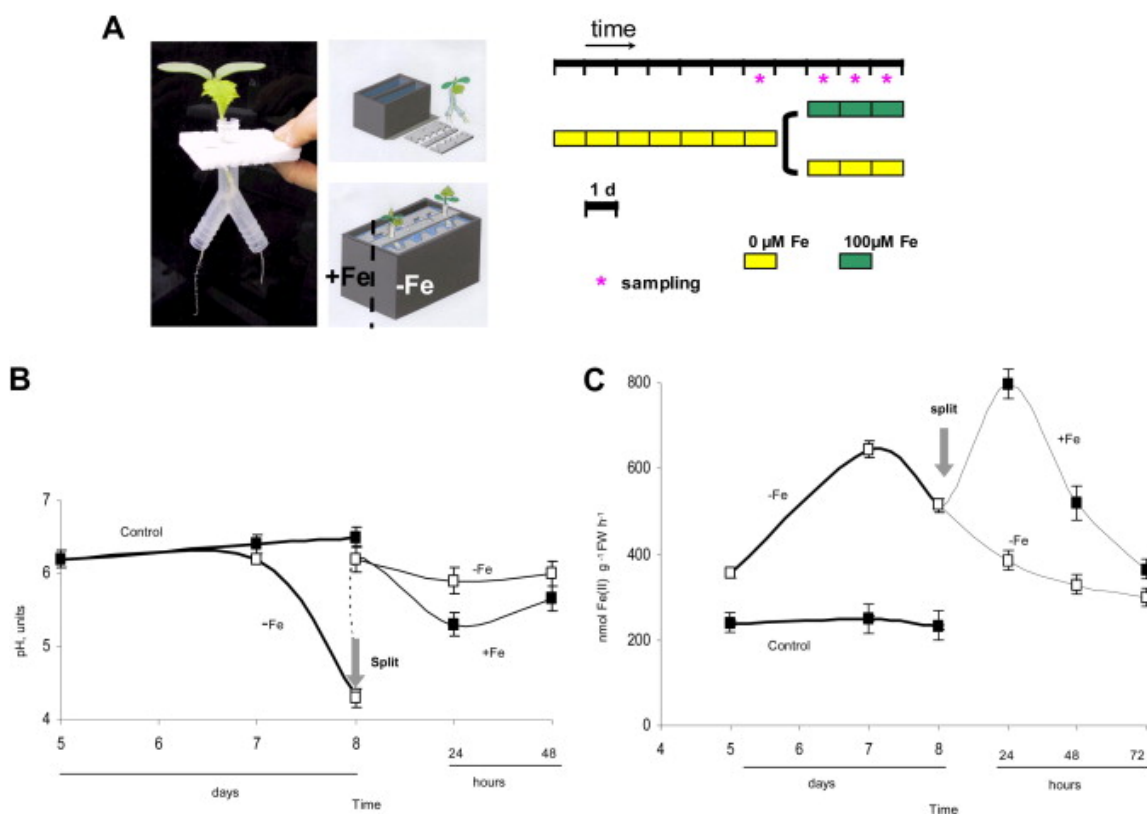
In *Arabidopsis thaliana*, Vert et al. [26] showed that *IRT1* and *FRO2* are controlled both in a local and a systemic way and that these genes are over expressed during the day and down regulated at night. Despite this temporal and spatial coordination, no evidence has been reported yet about the control of the whole *iron-uptake multiple system* that also includes metabolism components [20,27].

This work was aimed to identify the timing and coordination of biochemical and molecular responses in Strategy I plants. It was suggested [26] that two different signals, local and systemic, are involved in the regulation of the Fe status in Strategy I plants. The split-root technique allows to study the regulation of Fe-deficiency responses in cucumber roots and discriminating the roles of the systemic and localized signals involved in this regulation.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus* L. cv Marketmore 76) were sown in Agriperlite, watered with 0.1 mM CaSO<sub>4</sub>, allowed to germinate in the dark at 26 °C for 3 d, and then transferred to a nutrient solution (30 plants/10 L) with the following composition: 2 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.75 mM K<sub>2</sub>SO<sub>4</sub>, 0.65 mM MgSO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 10 μM H<sub>3</sub>BO<sub>3</sub>, 1 μM MnSO<sub>4</sub>, 0.5 μM CuSO<sub>4</sub>, 0.5 μM ZnSO<sub>4</sub>, 0.05 μM (NH<sub>4</sub>)<sub>2</sub>MoO<sub>7</sub> and 100 μM Fe(III)-EDTA (when added). The pH was adjusted to 6.0-6.2 with NaOH [28]. Aerated hydroponic cultures were maintained in a growth chamber with a day/night regime of 16/8 h and 24°/18 °C and a PPFD of 200 μmol m<sup>-2</sup> s<sup>-1</sup> at the plant level. Plants showed chlorotic symptoms after approximately seven days of culture in the absence of Fe. For split-root experiments only Fe deficient plants were used. The root system was then split into two parts and kept in separated compartments filled with a complete medium containing or not 0.1 mM Fe(III)-EDTA (Fig. 1A).



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Fig. 1. Graphical representation of plant growing conditions (A) and time courses of acidification (B) and reduction (C) capacity of cucumber roots grown in the presence (closed squares) or in the absence of Fe (open squares). The split root treatment (arrows in B and C) was applied to 8-d-old Fe-deficient plants. The medium of the two compartments had the same nutrient composition plus or minus 100 μM Fe, pH 6.2.

## 2.2. In vivo localization of the acidification and reduction capacities

Visualization and localization of acidification and Fe(III) reduction was performed by embedding the roots in an agar medium, added with the pH-sensitive dye Bromocresol Purple or with Fe(III)-EDTA and BPDS as a chelating Fe(II) agent, respectively.

## 2.3. Isolation of plasma membrane vesicles and determination of H<sup>+</sup>-ATPase and FC-R activities

Enriched plasma membrane (PM) vesicles were obtained using the two-phase partitioning procedure as previously described [28]. Final pellets were resuspended in a medium containing 2 mM MES, pH 7.0, 1 mM PMSF and 330 mM sucrose.

H<sup>+</sup>-ATPase activity was assayed by a spectrophotometric method, coupling ATP hydrolysis to NADH oxidation, as reported elsewhere [28]. The reaction was started by the addition of 20–50 μl aliquots of plasma membrane preparation and the absorbance changes at 340 nm were monitored over a 5 min period.

The FC-R activity was assayed at 26 °C in 1 ml volume containing 250 mM sucrose, 15 mM MOPS-BTP (pH 6.0), 0.25 mM Fe(III)-EDTA, 0.25 mM NADH and 0.01% Lubrol. The reaction was started by the addition of 20–50 μl aliquots of plasma membrane preparation and the NADH oxidation was monitored over a 5 min period at 340 nm.

## 2.4. PEPC assay

The enzyme was extracted from whole or split plant roots grown in the presence or in the absence of Fe as reported by De Nisi and Zocchi [21]. The reaction was started by adding aliquots of protein extract and the enzymatic assay was performed at 25 °C in 1.5 ml final volume. Oxidation of NADH was followed spectrophotometrically at 340 nm as already described.

## 2.5. Immunolocalization of PEPC and PM H<sup>+</sup>-ATPase

Eight-d-old Fe-deficient and split (after 24 and 48 h) apical (new grown, a) and proximal

(corresponding to the old apices preceding root splitting, p) root segments were excised and fixed at 4 °C overnight in 100 mM Na phosphate buffer (pH 7.4) containing 4% paraformaldehyde. Samples were then dehydrated through an ethanol-tertiary butanol series and embedded in paraffin (Paraplast plus, Sigma) as described by Dell'Orto et al. [17]. Immunological detection was performed on serial 6 µm sections according to Dell'Orto et al. [17] with some modifications. Polyclonal antibodies raised against a central domain of the *A. thaliana* PM H<sup>+</sup>-ATPase (kind gift by Dr. R. Serrano) and against a PEPC isoform of sorghum (kind gift by Dr. J. Vidal) were diluted 1:250 in TBS with 0.5% BSA and incubated overnight at 4 °C on the sections. The biotinylated second antibody (anti-rabbit IgG biotin conjugate developed against goat, Sigma Aldrich) was diluted 1:200 in TBS with 0.5% BSA and incubated for 2 h at room temperature. Sections were stained through the Immuno Pure ABC Peroxidase Staining Kit-Standard-Pierce, mounted, observed by Optical Microscope (Leica DMR) and images acquired by a Leica DC300F Digital Camera. Immunological determination of FC-R was not carried out since no antibody is yet available.

#### 2.6. Semiquantitative RT-PCR

Total RNA isolation from 8-d-old Fe-deficient and split (after 24 and 48 h) apical and proximal root segments and semiquantitative RT-PCR analysis were performed according to [27,29].

#### 2.7. Protein determination

Protein was determined by using the dye-binding method of Bradford [30], using γ-globulin as a standard.

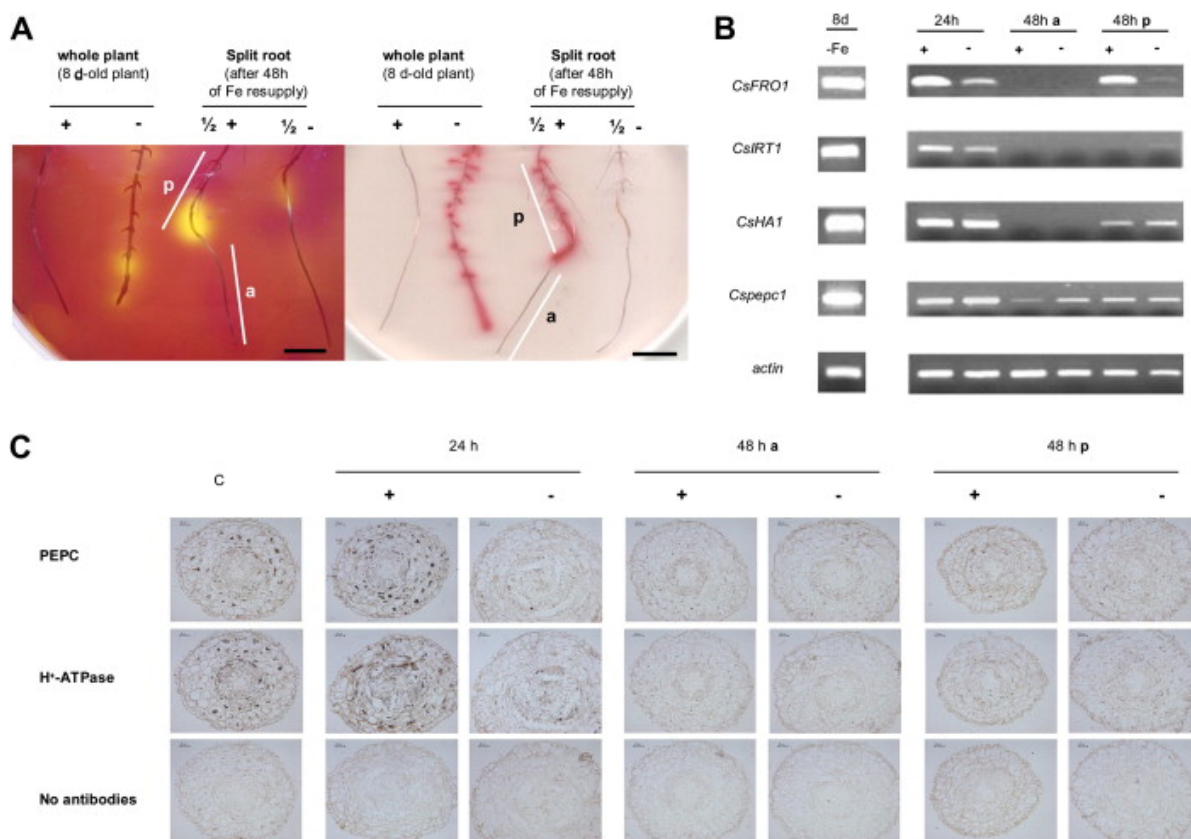
#### 2.8. Fe and starch determinations

Roots and leaves were collected and mineralized at 120 °C with HNO<sub>3</sub>. Prior to mineralization, apoplastic Fe was removed by washing roots for 10 min in a Fe-free medium containing 10 mM Na dithionite and 1 mM BPDS and rinsed in distilled water. Iron was determined by inductively coupled plasma-mass spectrometry (ICP-MS). Starch was determined by enzymatic analysis using a Starch Assay Kit (Sigma SA-20) according to the manufacturer's instructions.

### 3. Results

#### 3.1. Effect of Fe availability on acidification and reduction activities

The whole plant root showed the general behaviour observed in previous studies: *in vivo* and *in vitro* assays of FCR and H<sup>+</sup>-ATPase activities showed an increase in -Fe plants compared to + Fe ones (Fig. 1B, C, 2A). The increase in FC-R preceded that in H<sup>+</sup>-ATPase, as it also occurs in *Medicago truncatula*[31].



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Fig. 2. (A) Visualization of acidification (left panel) and Fe reduction (right panel) capacity along primary roots. Excised primary roots were incubated in 0.1% agar medium and the acidification was detected as pH changes of the indicator Bromocresol Purple (yellow), while the reduction was determined as the FeII-(BPDS)3 complex formation (red). The left part of each plate represents the 8-day-old root grown with (+Fe) and without (-Fe) Fe; the right part of each plate shows the split root system after 48 h from treatment +Fe and -Fe. For the split roots it is possible to observe two different response zones: a, apical region and p, proximal region, which corresponds to the *de novo* growth and the old root segments, respectively. Bar = 1 cm. (B) Fe deficiency-dependent expression of Strategy I and PEPC genes. Total RNA was extracted from whole (8d) or split roots after 12, 24, 48 h treatment. The transcript levels of *CsFRO1*, *CsIRT1*, *CsHa1*, *Cspepc1* and *actin* of roots grown under different Fe nutritional status were monitored by semi-quantitative RT-PCR. Expression pattern in split roots after 24 and 48 h of treatment are shown. At 48 h, transcript levels were monitored in two different morphological zones: a (apical) and p (proximal) region, respectively (see panel A). The experiment was performed 3 times with identical results. A representative experiment is shown. (C) Localization of PEPC and PM H<sup>+</sup>-ATPase in transverse sections of cucumber roots grown for 8 days in the absence of Fe (C) and 48 h after the resupply of Fe to the split roots. In a, roots were cut in the apical zone (0–5 mm from the tip), in p, roots were cut in the subapical zone, corresponding to the old apical portion (during growth in Fe deficiency). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The 8-day-old roots of -Fe plants were split and one half was maintained in -Fe whereas the second half was supplied with Fe (+Fe) (Fig. 1A). After split root application, the -Fe half still retained the capacity to decrease the pH for the first 24 h but to a lower extent compared to the +Fe half, which showed a larger pH decrease. After 48 h, the pH raised to higher values (Fig. 1B). Additionally, the *in vivo* acidification activity tested by agar embedding after 48 h of split root application showed that the +Fe half root still retains acidification capacity only in the former distal zone (p) (Fig. 2A, left panel). Accordingly, the *in vitro* H<sup>+</sup>-ATPase activity, measured on

plasma membrane preparations isolated from split roots, increased in the first 24 h only in the +Fe half and then, after 72 h, there was a decline to a value comparable to that of the whole control root (Table 1).

Table 1. Effect of Fe nutritional status on FCR, H<sup>+</sup>-ATPase and PEPC activities of plant grown in the presence or absence of Fe and in split roots and after Fe resupply. H<sup>+</sup>-ATPase, Fe(III)-chelate reductase (FC-R) and PEPC activities were determined in the root apical segments of 8-day-old plants grown in Fe sufficient or Fe deficient nutrient solution or in split roots. Data are the mean of three independent experiments. SE never exceeds 8%. All data are expressed as nmol NADH mg prot<sup>-1</sup> min<sup>-1</sup>.

	Time	Plasma membrane enriched fraction						Cytosolic fraction PEPC		
		H <sup>+</sup> -ATPase		FC-R						
		+Fe	-Fe	+Fe	-Fe	+Fe	-Fe			
Whole plant	8 d	103 ± 6.3	157 ± 8.2	40.2 ± 4	142.5 ± 8			82 ± 4	2	
Split root			+Fe	-Fe	+Fe	-Fe			+Fe	+
	24 h		280 ± 4	160 ± 11	196.3 ± 11	61.8 ± 4			3	
	72 h		116 ± 6	105 ± 3	37.6 ± 5	40.8 ± 7			1	

Similarly to the acidification, the *in vivo* Fe reduction by apices showed that after split root application, in the -Fe half the reducing activity continued to decrease, while the +Fe half increased its capacity to reduce Fe(III) (Fig. 1C). This sharp increase lasted for 24 h after Fe application and then the reduction activity decreased at 72 h to values which were lower than those detected in the whole -Fe roots. Additionally, the activity of Fe(III) reduction in the proximal root part (p in Fig. 2A, left panel) is also visible in Fig. 2A (right panel) where the roots were embedded in agar containing Fe(III)-EDTA and BPDS. Fig. 2A also shows the morphological changes occurring under Fe deficiency: starved roots show an increase in the amount of lateral roots and swollen tips (roots of whole plant). Accordingly, the highest FC-R *in vitro* activity, detected on plasma membrane preparations isolated from split roots was detected in the half roots supplied with Fe (Table 1).

### 3.2. Effect of Fe availability on PEPC activity

In previous papers we have demonstrated the implication of the PEPC in the response to Fe deficiency, and several hypotheses were proposed to explain the correlation existing between induction of Fe deficiency responses and PEPC activation [21,32]. We studied the changes in PEPC activity in root extracts from the split root experiment. Table 1 shows that 24 h after roots were split there was an increase only in the +Fe half with respect to the -Fe control and then the activities in the extracts of both root parts declined towards the control level. Seventy two hours later, the FC-R, H<sup>+</sup>-ATPase and PEPC activities in both halves behaved in the same way, i.e. they were similar to those of the Fe-sufficient roots.

### 3.3. Expression of Fe deficiency response genes in cucumber split roots

Twenty four h after split root application the pattern of mRNA expression in the two halves of the roots changes with respect to the 8-d-old Fe-deficient ones (Fig. 2B). In fact, for the *CsFRO1* transcript there was a decrease in the -Fe with respect to the correspondent +Fe half, which maintained the same enhanced expression as the 8-d-old Fe-deficient roots. This is consistent with the large enzymatic activity measured for the FC-R (Table 1). Also for *CsIRT1* the amount of transcript decreased at 24 h more in the -Fe split roots, indicating a loss of a local induction exerted by the presence of Fe. No difference was found for the *CsHA1* and the *Cspepc1* gene expression between the two halves at this time, and both showed decreases compared to the expression in the 8-d-old Fe-deficient roots.

To study the effect exerted by the Fe status in plants 48 h after split root application, we divided the roots not only in plus and minus Fe sides, but also in apical new grown segment, (a) and in proximal corresponding to the old apices preceding root splitting, (p) zones (Fig. 2A). The induction of transcripts was quite different (Fig. 2B); in the new grown apical segments (a) from both the +Fe and -Fe halves, the level of the mRNAs for all the activities considered was back to the +Fe control level [Supplemental Fig. 1]. In the tissue corresponding to the old tip left behind by the root



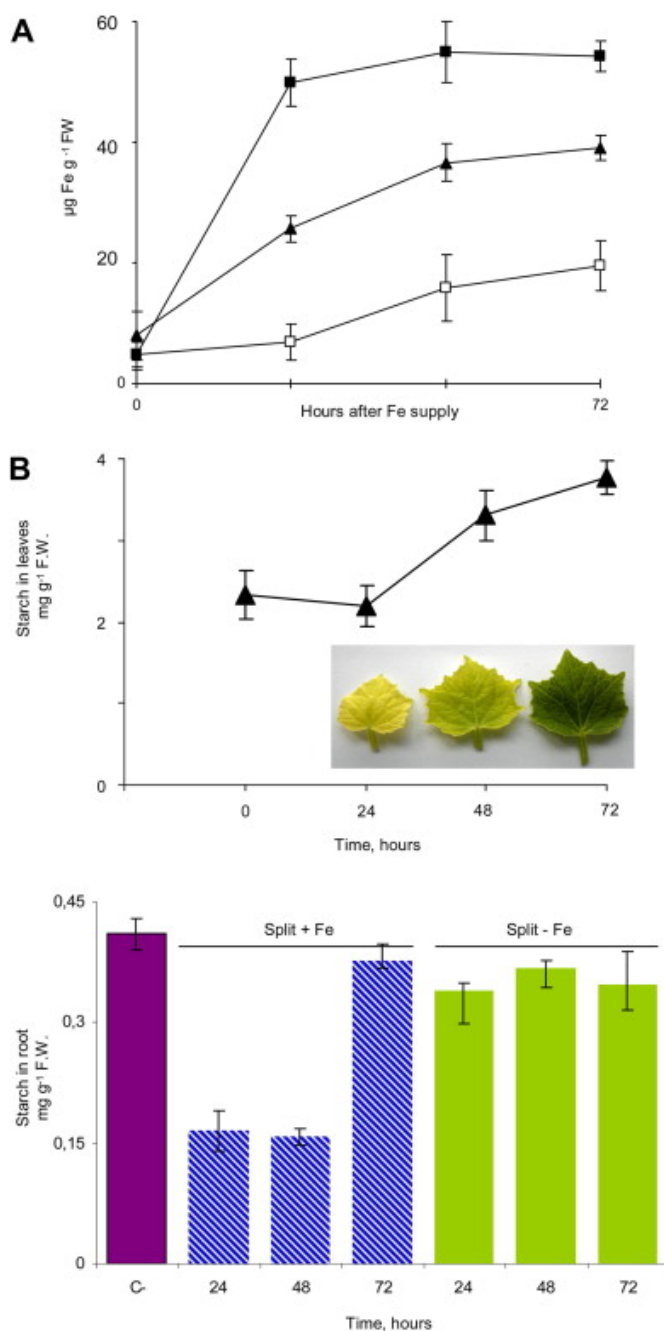
growth (p), the expression of *CsHA1* and of *Cspepc1* was still appreciable in both the halves. *CsFRO1* was still present in the +Fe half, whilst it had almost completely disappeared from the -Fe half. *CsIRT1* transcript was undetectable in all conditions and root zones.

#### 3.4. Immunolocalisation of H<sup>+</sup>-ATPase and PEPC

Cross sections were subjected to immunoreaction with polyclonal antibodies against H<sup>+</sup>-ATPase and PEPC (Fig. 2C). Overall, the expression of these two proteins exhibited the same pattern. Twenty four hours after split root application, the +Fe apical segment showed a high expression of both proteins, even increased with respect to the 8-d-old Fe-deficient segment (8 d -Fe), while in the -Fe apical segment the signal decreased markedly. As was done for the results of gene expression, we divided the roots not only in plus and minus Fe sides, but also in apical (a) and in proximal (p) zones (Fig. 2A). After 48 h root apices (a) almost completely lose the accumulation of H<sup>+</sup>-ATPase and PEPC proteins in both the -Fe and +Fe halves. The same occurred also in the p zones.

#### 3.5. Fe concentration in roots and leaves of split root plantlets

We determined the concentration of Fe in the roots and leaves in root splitting experiments (Fig. 3A). Within 24 h, the amount of Fe reached the maximum in the +Fe half root, indicating that the starved roots respond rapidly to promote Fe uptake once it is resupplied. The consequence of this rapid uptake was the increase in the Fe translocation to the leaves to satisfy the Fe request of the shoot. It is interesting to note that also in the -Fe half there was an increase in Fe content, likely as a consequence of Fe reallocation (although to a lower extent).



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Fig. 3. (A) Fe uptake and translocation. Total Fe concentration ( $\mu\text{g g}^{-1}$  FW) in roots and leaves collected from 8-day-old plants grown under Fe deficiency (time 0) and 24, 48 and 72 h after split root application. -Fe, the half part grown without Fe (open squares); +Fe, the half part supplied with  $100 \mu\text{M}$  Fe (closed squares); leaf (closed triangles). Bars = SE. (B) Starch concentration and re-greening of leaf after Fe resupply (upper panel) and starch content in +Fe and -Fe split roots (lower panel). Time 0 correspond to the 8-day-old Fe-deficient plant. The amount of starch in the control roots was  $0.85 \text{ mg g}^{-1}$  FW. Bars = SE.

In the meantime, the leaves turned green, showing that the Fe status of the shoot had been restored. The colour changed from a pale yellow to an intense green after 72 h of Fe re-supply (inset in Fig. 3B). Restoration of chlorophyll content was accompanied by an increase in the starch content in the leaves (Fig. 3B), indicating that photosynthesis and  $\text{CO}_2$  fixation was also recovered. In split root experiment while the -Fe half does not show any significant change in the starch content, the +Fe half shows a 50% decrease in the following 48 h and successively the starch concentration increased back to the levels found in the -Fe roots (Fig. 3B lower panel).

There is a rationale for these results: in the first 24 h after split root application in the +Fe side there was an enhancement of enzymatic activities of FC-R, H<sup>+</sup>-ATPase and PEPC and, more likely, of protein synthesis [33] so that the need of substrates and energy would greatly increase leading to a major consumption of carbohydrates.

#### 4. Discussion

The split root technique has been used to study the regulation mechanism of the Fe deficiency-induced responses in the last ten years by some researchers. Among them Vert and co-workers [26] casted new light on the regulation (local and systemic) of the high-affinity Fe uptake system. In this work, by making use of split root systems, we have added new information on the mechanism(s) which control(s) Fe uptake in response to the Fe status of the plant, investigating the principal Fe deficiency-induced responses at the different levels: gene expression, immunohistochemical localization and enzymatic activity in cucumber root tips. With respect to Vert's work, we have performed our experiments starting with Fe-deficient culture to minimize any contamination of the apoplast with residual Fe, then Fe was supplied to half of the split roots. It has been observed that cucumber roots respond to Fe deficiency by inducing acidification of the culture medium and reduction of Fe(III) within 7 d [28]. In the same time period, *CsFRO1*, *CsIRT1*, *CsHA1* and *Cspepc1* transcripts are increased under Fe deficiency [29]. Furthermore, in the same work, a correlation between the induction of specific genes for Fe uptake like *CsFRO1* and *CsIRT1* and that of *CsHA1* and *Cspepc1* was observed [29]. It has been hypothesised that there is a sequential coupled regulation which involves these four genes: direct or primary for the response of *CsFRO1* and *CsIRT1*, to promote Fe uptake, and secondary or metabolic for *CsHA1* and *Cspepc1*. Thus, the activation of *CsHA1* and *Cspepc1* transcripts seems to be stimulated as a metabolic consequence of Fe starvation rather than by a direct system.

Application of the split root technique provided us with some more information about the regulation of the Fe uptake system. In fact, 24 h after Fe resupply to half of the root system the expression of the transcripts was still high only in the root side supplied with Fe, while for the -Fe side there was a drop only for the *CsFRO1* and *CsIRT1* transcripts. This is in agreement with the enzymatic activities assayed at this time point (see Table 1). The presence of the substrate acts as a local inducer, and therefore only occurs in the half side of the roots supplied with Fe. The lack of response in the half side of the roots left without Fe could be related to the fact that these roots were still growing in the absence of Fe. Forty-eight h after Fe resupply to half of the root system there was a further decrease in these activities in the -Fe half root. We could ascribe this drop to a cross-talk signal that in less than 24 h is able to reach the -Fe half of the roots, signalling a recovery of Fe uptake in the +Fe side, so preventing energy waste in the -Fe side. This kind of cross-talk should be rationale in the open field, where roots of the same plant can experience different Fe availability due to the non-homogeneity of the soil, without necessarily developing Fe deficiency response, at least until the Fe status of the plant is not compromised yet, and limiting the response only to the root portions in which it can actually result in increased Fe uptake. But, what happens after 48 h of split root application? Interestingly, Fig. 2A showed that after 48 h the new grown apical segments of +Fe and -Fe halves behave in the same way: they do not show any morphological response to Fe deficiency (compared with the whole plant roots in the same plate) lacking the swollen tips and the capacity to produce lateral roots. The Fe concentration in the half side of the roots left in the absence of Fe increased after 48 h (Fig. 3A), indicating that a reallocation of Fe occurred in the whole plant and, perhaps, this was the main reason why both sides of the roots behaved similarly.

The gene expression analysis in the apical segments (Fig. 2B, lane 48h a) revealed that both halves of the split roots, irrespective of the treatment, behaved in the same way, repressing the transcription of the genes involved in the Fe deficiency response. This was consistent with the decrease in the corresponding enzymatic activities (Table 1). Only the proximal segments (Fig. 2B, lane 48h p) of the side grown in the presence of Fe and containing the old tip portion still retained some activities that were also visible in the agar plates (Fig. 2A). Our data are consistent with the local and systemic regulatory model of the root Fe uptake proposed by Vert et al. [26]. In addition

to the transcript levels of *CsFRO1* and *CsIRT1*, we showed that also other transcripts considered to be linked to the Fe deficiency response, *CsHA1* (in particular) and *Cspepc1*, may be under the same regulation. According to this hypothesis, low shoot Fe content might produce a signal triggering the expression of genes in the roots bringing to the activation of the corresponding enzymes. Restoration of a normal Fe content in the shoot will stop the signal release with a consequent inactivation of the gene expression and a decrease in the activity of the corresponding enzymes. A transient increase in both gene expression and enzymatic activities could be seen when Fe starved roots are supplied with Fe, showing a local control by substrate.

The application of the split root techniques could be very helpful to see how effective is the presence of Fe to evoke local controlling signals and how the Fe status of the shoot may systemically regulate the expression and the enzymatic activities. In fact, the half side of starved roots treated with Fe showed that in the presence of the local inducer (Fe itself), all the enzyme activities assayed were increased in the first 24h (Table 1). On the contrary, the expression of all the genes considered decreased in both root halves after 24 h (Fig. 2B). In particular, *CsFRO1* and *CsIRT1* decreased more in the -Fe half, while for *CsHA1* and *Cspepc1* we could not appreciate any substantial difference between the two halves of the root. This contrasting results can be ascribed to a direct modulation of the enzymes (local signal) due to the presence of substrate (Fe), which, at the same time, de-induces the transcription of the corresponding genes (systemic signal). After 48 h the decreases in the transcript levels in both sides of the root seems to be consistent with a long distance signal (systemic) coming, more likely, from the shoot, as suggested by the experiments of Grusak and Pezeshgi [34]. At this stage, leaves already showed higher Fe concentration and an active chlorophyll biosynthesis (Fig. 3). Since at this point the plant behaves as a normal Fe-sufficient plant, growth is resumed and a repressive signal(s) is sent, or alternatively, the promotive signal ceased to be sent to the roots which now, irrespective of the presence or absence of Fe in the nutrient solution, switch off all the Fe deficiency responses (see Fig. 2 and Table 1).

We have discussed in a previous work which signals might be responsible for the promotive action. Several molecules, such as IAA [2,35], ethylene [24,36], sugar [37], Fe complexed by a ligand [38], nitric oxide [24,39] and phloem Fe as inhibitory signal [25] have been proposed until now. The data obtained in this work support that Fe itself, through its movements in the xylem and phloem saps, may signal the Fe status of the plant. It can act, according to the dual regulation model proposed by Vert et al. [26], both as a local inducer signal and as a repressive systemic signal once the concentration in the plant reaches a satisfactory level. This is consistent with the model proposed by García et al. [25] in which phloem Fe would act as an inhibitory signal in the regulation of Fe acquisition genes. On the other hand, rescued leaves increase the synthesis of carbohydrates (Fig. 3B). As shown in *Arabidopsis* [40,41], several genes involved in the metabolism and export of carbohydrates are strongly up-regulated in Fe deficiency, in particular the phosphate/triose phosphate translocator and the sucrose transporter, suggesting an increased energy requirement outside the shoot. In fact, the energy demand in the Fe-deficient roots is very high [20 and references therein]. Indeed, an increase in the sugar concentration was found in the phloem of Fe-deficient bean plants [42]. Whether sugars, or other molecules transported along with them in the phloem, are responsible for a systemic signal in Fe deficiency response is still unknown.

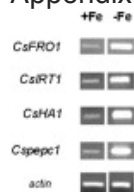
## 5. Conclusions

In this work, we used the split root technique to characterize the transcript expression, the immunohistochemical localization and the enzymatic activity of some responses involved in the Fe uptake mechanism in cucumber plants. We confirmed that FRO1 and IRT1 genes are co-regulated as already well documented in other Strategy I plants. Furthermore, in this work we showed that H<sup>+</sup>-ATPase, being another typical enzyme involved in the Strategy I mechanism in cucumber, is not regulated at the same time as for the FRO1 and IRT1, but it is regulated together with the PEPC, which is a typical metabolic enzyme induced by this stress, at the transcript, protein and enzymatic activity levels.

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Appendix A. Supplementary data



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Supplemental Fig. 1. RT-PCR semiquantitative analysis of *CsFRO1*, *CsIRT1*, *CsHA1* and *Cspepc1* genes. Total RNA was extracted from apex root of 8-day-old plants grown in the presence (+Fe) and in the absence (-Fe) of Fe.

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