Modulation of iron responsive gene expression and enzymatic activities in response to changes of the iron nutritional status in *Cucumis sativus* L.

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Abstract

Regulation exerted by the iron status of the plant on the iron deficiency responses was investigated in cucumber roots (*Cucumis sativus* L.) both at the biochemical and molecular level. Absence of iron induced the expression of the *CsFRO1*, *CsIRT1*, *CsHA1* and the *Cspepc1* transcripts that was followed by an increase in the corresponding enzymatic activities. Supply of iron repressed gene expression, in particular those of the Fe(III)-chelate reductase and for the high affinity iron transporter and reduce the enzymatic activities. Our results confirm and extend the hypothesis of a coordinate regulation of these responses. Besides these two activities strictly correlated with iron deficiency adaptation, we considered also the H^+-ATPase and the phospho*enol*pyruvate carboxylase, that have been shown to be involved in this response.

Abbreviations: BPDS, bathophenanthrolinedisulphonate; BTP, 1,3-bis[tris(hydroxymethyl)-methylamino]-propane); MES, 2[N-morpholino]ethanesulphonic acid); MOPS, 3-(N-morpholino)propanesulfonic acid; PMSF, phenylmethylsulphonyl fluoride; PPFD, photosynthetic photon density flux

Introduction

Plants require iron to complete their life cycle. The importance of iron is due to the existence of two stable, but convertible forms, Fe(III), ferric, and Fe(II), ferrous, which take part in fundamental processes involving electron transfer reactions, mainly requested in both the oxidative (respiration) and biosynthetic (photosynthesis) pathways (Curie & Briat, 2003; Hell & Stephan, 2003). On the contrary, excess of iron can produce toxic oxygen compounds, for instance O_2^-, H_2O_2 and above all free hydroxyl radicals, produced by the Fenton reaction (Halliwell & Gutteridge, 1999; Briat, 2002). Consequently, balanced iron acquisition by the roots and control of the homeostatic mechanisms are necessary to prevent suffering or excess of this transition metal.

There is generally a high quantity of iron in the soil, but, in aerobic and sub-alkaline pH environment it is present mainly as Fe(III)-oxide and -hydroxide and its solubility is strongly restricted (Guerinot & Yi, 1994). To cope with this problem and to enhance the metal bioavailability, plants have evolved developmental and biochemical adaptation (Strategy I and Strategy II) to low iron concentration in the environment (Römheld & Marschner, 1986). Concerning Strategy I plants, evident responses reside at the root-soil interface involving morphological changes in the root architecture by increasing the number of secondary roots, root hair density and the formation of transfer cells at the root apex in order to enhance the absorbing root surface. In the meantime, primary

biochemical and molecular responses serve one main function: increase the rhizhosphere iron availability and its uptake (Schmidt, 1999; Curie & Briat, 2003; Hell & Stephan, 2003).

Strategy I plants (dicotyledonous and non-graminaceous plants) are able to respond to lack of iron mainly by increasing the reduction, the acidification and the uptake activities by inducing trans-plasma membrane proteins present in the rhizhodermal root cells {i.e. Fe(III)-chelate reductase [FC-R], H^+-ATPase and iron regulated transporters (IRT), respectively} directly involved in the iron uptake system (Curie & Briat, 2003). Extrusion of electrons and protons leads to an enhancement of soluble form of iron in the rhizosphere. It has been observed in Strategy I plants, that in response to iron starvation, there was an induction of the genes encoding for the Fe(III)-chelate reductase (*AtFRO2*, *PsFRO1* and *LeFRO1*) which were already characterized (Robinson *et al.*, 1999; Waters *et al.*, 2002; Li *et al.*, 2004). Enhancement of H^+ efflux, due to an increase in a P-type H^+-ATPase activity in response to iron deprivation, was demonstrated in many Strategy I plants (Schmidt, 1999; Zocchi, 2006). A multigene family encoding different isoforms for H^+-ATPase and tissue specific expression patterns have been demonstrated (Palmgren, 2001; Dell'Orto *et al.*, 2002; Santi *et al.*, 2005). After mobilization and reduction, the ferrous form, the unique form to be absorbed by these plants, needs to be taken up across the plasma membrane by a specific iron transporter (IRT1) that has been characterized in *A. thaliana* (Eide *et al.*, 1996) and successively in pea and tomato (Cohen *et al.*, 1998; Eckhardt *et al.*, 2001).

Micro-array analysis using *A. thaliana* grown under iron deficiency (Thimm *et al.*, 2001) has revealed that there were significant changes in the transcription of different genes, therefore reflecting the complexity of the molecular and metabolic response.

Many genes involved in Fe-starvation responses have been cloned and Fe responsiveness has demonstrated the importance of transcriptional control in the regulation of Strategy I mechanisms (Curie & Briat, 2003). To promote and sustain the increase in the release of electrons and protons in the rhizhosphere it has been shown that significant metabolic changes occurred in roots: enhancement of glycolitic pathway rate, cytosolic dehydrogenase activities, as well as respiration rate (Rabotti *et al.*, 1995; Espen *et al.*, 2000). Moreover, organic acid synthesis and CO_2 dark fixation increase under Fe-starvation during which phospho*enol*pyruvate carboxylase (PEPC) activity was shown to increase by four times or more (Rabotti *et al.*, 1995; De Nisi & Zocchi, 2000; Lopez-Millan *et al.*, 2000). The anaplerotic role of root PEPC has been characterized in root of cucumber grown under Fe- deficiency (De Nisi & Zocchi, 2000) and in other Strategy I species, both herbaceous and arboreous (Lopez-Millan *et al.*, 2000; Ollat *et al.*, 2003), which showed also an enhanced H^+-ATPase activity. So it is possible to consider the inducible enhancement of PEPC activity as one of the more important markers regarding metabolic responses to Fe deficiency.

In this work we were interested in identifying the coordination of biochemical and molecular responses in Strategy I plants, which are, more likely, the result of tightly controlled homeostatic mechanisms.

Materials and Methods

Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus* L. cv Marketer) were surface sterilized and sown in Agriperlite, watered with 0.1 mM $CaSO_4$, allowed to germinate in the dark at 26°C

for 3 d, and then transferred to a nutrient solution of the following composition: 2 mM $Ca(NO)_3$, 0.75 mM K_2SO_4, 0.65 mM $MgSO_4$, 0.5 mM KH_2PO_4, 10 µM H_3BO_3, 1 µM $MnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM $ZnSO_4$, 0.05 µM $(NH_4)Mo_7O_{24}$ and 100 µM Fe(III)-EDTA (when added). The pH was adjusted to 6.0-6.2 with NaOH. Aerated hydroponic cultures were maintained in a growth chamber with a day/night regime of 16/8 h and a PPFD of 200 µmol m^{-2} s^{-1} at the plant level. The temperature was 18°C in the dark and 24°C in the light. Plants showed chlorotic symptoms after approximately seven days of culture in the absence of Fe.

In vivo measurement and localisation of the acidification and reduction capacities

Medium acidification capacity was measured directly in the nutrient solution by measuring the pH every day with a pHM64 (Radiometer, Copenhagen) pHmeter. Fe(III)-reductase activity was measured by using the bathophenantrolinedisulfonate (BPDS) reagent (Chaney *et al.*, 1972). Ten apical root segments about 2 cm long were incubated in 10 ml of a solution containing 0.5 mM $CaSO_4$ and 0.5 mM K_2SO_4 pH 6.0, in the dark at 26°C under shaking. After 1 h incubation the solution was replaced with 5 ml of a solution with the following composition: 0.5 mM $CaSO_4$, 0.5 mM K_2SO_4, 0.1 mM Fe(III)-EDTA and 0.25 mM BPDS pH 6.0. After 3 h, 2 mL of the solution were withdrawn and the absorbance at 535 nm determined with a spectrophotometer. BPDS forms a stable, water soluble, red complex with Fe^{2+} and only a weak complex with Fe^{3+}. The amount of reduced Fe was calculated by the concentration of the formed $Fe^{2+}(BPDS)_3$ complex (ε of BPDS is 22.1 mM^{-1} cm^{-1}).

Visualization and localization of proton release and Fe(III) reduction was performed by embedding the roots in a agar medium as described in Marschner *et al.* (1982) in the presence of the pH indicator Bromocresole Purple and BPDS, respectively.

Isolation of plasma membrane vesicles

Enriched plasma membrane (PM) vesicles were purified using the two-phase partitioning procedure as previously described (Rabotti & Zocchi, 1994). Final pellets were resuspended in a medium containing 2 mM MES (2[N-morpholino]ethanesulphonic acid)-BTP (1,3-bis[tris(hydroxymethyl)-methylamino]-propane), pH 7.0, 1 mM PMSF and 330mM sucrose.

Assay of H^+-ATPase and FC-R activities in plasmalemma-enriched vesicles

H^+-ATPase activity was assayed with a spectrophotometric method (as described by Palmgren *et al.*, 1990), coupling ATP hydrolysis to NADH oxidation, at 25°C as already reported (Rabotti & Zocchi, 1994). The reaction was started by the addition of 20-50µl of PM preparation and NADH oxidation was followed spectrophotometrically at 340 nm in a V550 spectrophotometer (Jasco,Tokyo, Japan) as already described (Rabotti & Zocchi, 1994).

The NADH-dependent Fe(III)-reductase (FC-R) activity was assayed in the dark at 25°C in 1 ml volume containing 250 mM sucrose, 15 mM MOPS-BTP (pH 6.0), 0.25 mM FeEDTA, 0.25 mM NADH, 0.01% Lubrol. The reaction was started by the addition of 20-50µl of PM preparation and NADH oxidation was followed

spectrophotometrically at 340 nm in a V550 spectrophotometer (Jasco,Tokyo, Japan) as already described (Rabotti & Zocchi, 1994).

PEPCase assay

The PEPCase, soluble cytosolic enzyme, was extracted from roots of plants grown in the presence or in the absence of Fe as reported by De Nisi & Zocchi (2000). Reaction was started by adding aliquots of protein extracts and the enzymatic assays were performed at 25° C in 1.5 ml final volume. Oxidation of NADH was followed spectrophotometrically at 340 nm in a V550 spectrophotometer (Jasco,Tokyo, Japan) as already described (De Nisi & Zocchi, 2000).

Semiquantitative RT-PCR

Root and leaf tissues were pulverised in liquid nitrogen using mortar and pestle and total RNA was extracted using Trizol® reagent (Invitrogen), and first strand cDNA synthesis was carried out using iScript™cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. The gene-specific primers used to amplify: *CsFRO1* (accession No. AY590765) were 5'-GTATCACATATGCTTGGC-3'(forward) and 5'-CTACGAATGCGAGGAATAG-3'(reverse); *CsIRT1* (accession No. AY590764) primers used were 5'-CGCAGCAGGTATCATTCTCG-3' (forward) and 5'-TCTGCCTGAAGAATACAGCC-3' (reverse); *Cspepc1* (accession No. AJ417435) the primers used were 5'-GGACACAGACGAGATTCCATC-3' (forward) and 5'-CCAGTGTTCTGCATTCCCGC-3' (reverse); the primers used to amplify *actin* (accession No.AB010922) were 5'-GCCTGCTATGTATGTTGCCATC-3' (forward)

and 5'-CAAGAGCAACATATGCCAGCT-3' (reverse). To amplify *CsHA1* (accession No. AJ703810) the primers used were 113F2 (5'-CTCCAACCAGCACCAGAAA-3') and 113R1 (5'-TCCTTCATCTCTTTCTGCAACA-3') (Santi at al., 2005). The thermal cycle program was: one initial cycle of 94°C 5 min, followed by cycles of 94°C 30 sec; 56°-60°C 1 min (as annealing temperature we used 56°C for *CsFRO1* and 60°C for *Cspepc*, *CsIRT1*, *CsHA1*, and *Csactin*), 72°C 1 min, with 28 cycles for *CsFRO1*, *CsIRT1*, *CsHA1*, *Cspepc* and 26 cycles for *actin*, all followed by a final 72°C elongation cycle for 5 min. RT-PCR was carried out on the first-strand cDNA using *Taq* DNA polymerase (Promega) and the identity of the amplified fragments were verified by sequencing both the strands.

Protein determination

Protein was determined by the using dye-binding method of Bradford (1976), using γ–globulin as a standard.

Results

Effect of iron availability on acidification and reduction activities

To investigate the control exerted by the iron status on the Strategy I plant responses, we started with the induction of Fe deficiency at the whole plant level. Plants were grown for 8 days in the absence of iron to induce all the deficiency responses (Rabotti & Zocchi, 1994) and after this period iron was supplied at a concentration of 100 μM as FeEDTA . The time course of acidification before and after iron supply is reported in

Figure 1A. After 7d in the absence of iron, roots sharply decrease the pH of the medium by almost two pH units, while the control roots do not show any acidification capacity. After iron supply, roots still retain the capacity to decrease the pH for the first 24 h and then the pH raises to higher values. We have assayed the H^+-ATPase activity during this time course on a PM-enriched fraction isolated from the roots. The results are reported in Table 1. The H^+-ATPase activity measured confirms previous results (Rabotti & Zocchi, 1994; Dell'Orto *et al.*, 2000) showing a 50% increase under iron deficiency. After iron supply the this activity still remains higher for 48 h followed by a decrease, more or less, to the control level. This difference can also be visually appreciated in Figure 2A where roots are embedded in an agar containing the pH-sensitive dye Bromocresol Purple; the yellow colour around the – Fe roots denotes a decrease in the pH value (lower than 5.0).

Figure 1B shows the time course of the Fe^{3+} reduction before and after iron supply. Also in this case, the reduction activity is sharply increased under iron deficiency reaching the maximum after 7d of iron starvation, then the activity slowly decreases. After iron supply there is an second peak of increase in the reducing capacity of Fe^{3+} even greater with respect to the previous one. This sharp increase last for 24 h and is followed by a rapid decrease in the reduction capacity down to the value of the control. As for the H^+-ATPase activity, we have measured the Fe^{3+}-chelate reductase (FC-R) activity on a PM-enriched fraction. The results are reported in Table 1. This *in vitro* result confirms what was demonstrated in the time course experiment with a second peak of activity greater than that obtained in the -Fe roots. The increase in the reduction activity after Fe supply to Fe-starved roots, is a well known response and is referred to as a substrate induction effect, i.e. iron acts as a local inducer (Vert et al, 2003). This

difference in the activity of Fe(III) reduction is well visible in Figure 2B where the roots are embedded in an agar containing Fe^{3+}-EDTA and BPDS as a chelating Fe^{2+} agent [(the red colour is due to the formation of the complex $Fe^{2+}(BPDS)_3$]. Fig. 2 also shows the morphological changes occurring at the root level under Fe deficiency; starved plants show an increase in the amount of lateral roots and swollen tips (left part of each plate).

In previous papers we have demonstrated the implication of the PEPC in the response to Fe deficiency and several hypothesis were proposed to explain the strict correlation existing between the induction of iron deficiency responses and the PEPC activation (Rabotti *et al.*, 1995; De Nisi & Zocchi, 2000). Table 1 show the results of the time course of the PEPC activity. Under Fe deficiency the PEPC activity is increased by 4 time and also in this case there is an increase 24 h after Fe supply, consistent with those shown by the H^+-ATPase and FC-R activities.

Expression of iron deficiency response genes in cucumber plants

Gene expression analysis was performed using *CsFRO1*, *CsIRT1*, *CsHA1* and *Cspepc1* sequences. *CsFRO1* and *CsIRT1* were recently characterised by Waters et al. (2007) and shown to encode the ferric reductase and the iron transporter proteins, respectively. *Cs*FRO1 is hortologous to *At*FRO2 (Robinson et al., 1999), *Le*FRO1 (Li et al., 2004) and *Ps*FRO1 (Waters et al. 2002), while CsIRT1 is hortologous to *At*IRT1 (Eide et al., 1996) and *Le*IRT1 (Eckhardt et al., 2001). Concerning to the H^+-ATPase, we considered the differential expression level of *CsHA1* gene (Santi *et al.*, 2005). For PEPC we considered the *Cspepc1* gene expression level. In the database (http://www.ncbi.nlm.nih.gov) two partial mRNAs for PEPC are present for *Cucumis*

sativus (submitted by Santi et al, unpublished): *Cspepc1* (AJ417435) and *Cspepc2* (AJ417436). Preliminary phylogenetic analysis carried out on amino acidic sequences of cucumber and Arabidopsis PEPC isoforms [*Cs*pepc1 (CAD10147), *Cs*pepc2 (CAD10148), *At*ppc1 (CAD58725), *At*ppc2 (CAD58726), *At*pp3 (AAC24594), *At*ppc4 (CAC86034)] showed that only the *At*pcc4 was not related to all the other sequence considered (data not shown). For this reason, we performed the nucleotide sequence alignment of *Cspepc1*, *Cspepc2* and of the genes encoding for the three PEPC isoforms of *Arabidopsis,* and namely: *Atppc1* (AJ532901), *Atppc2* (AJ532902), *Atppc3* (AF071788) (Sanchez & Cejudo, 2003). *Cspepc1* showed 79% identity with *Atppc3*, 74%, with *Atppc2* and 78% with *Atppc1*, while *Cspepc2* showed 76% identity with *Atppc3*, 73% with *Atppc2* and 78% with *Atppc1*. We decided to use in this work the *Cspepc1* gene because its sequence showed the highest identity value with *Atppc3*, which is almost exclusively expressed in roots (Sanchez & Cejudo, 2003).

The presence of iron is believed to be an induction signal for the expression of the iron responsive genes, in particular for *AtFRO2* and *AtIRT1*, but these transcripts are often hardly detectable in the presence of iron (Vert et al, 2001, Connolly et al, 2002; Vert et al, 2003, this paper). On the contrary, it is in the absence of iron that genes encoding for *FRO2* and *FRO1* are up regulated (Waters *et al.*, 2002; Connolly *et al.*, 2003; Li *et al.*, 2004) and it is also in this condition that *IRT1* mRNA and protein accumulates in *A. thaliana* (Eide *et al.*, 1996; Connolly *et al.*, 2002; Vert *et al.*, 2002). In this work we confirm these data in cucumber as well and we extended them also to the expression of the *CsHA1* and the *Cspepc1* transcripts. In control and starved roots we observe a close coordination concerning the expression of these four activities. In fact, all of them are increased under Fe deficiency condition (Fig. 3A) and for the

CsFRO1, *CsIRT1* and *CsHA1*, in particular, the highest level of the mRNAs expression coincided with the maximum of their enzymatic activity (compare the –Fe lanes 5d and 8d, Fig. 3A and Table 1). For *AtFRO2* and *AtIRT1* a coordinate control of the expression was formulated (Connolly et al, 2003; Vert *et al.*, 2003). We may extend this hypothesis also to the *CsFRO1*, *CsIRT1*, *CsHA1* and the *Cspepc1* (Fig. 3). In fact, when starved roots are re-supplied with iron there is a decrease in the expression of all the transcripts and after 48 h they are almost undetectable (Fig. 3B). This is particularly true for what concern the *CsFRO1* and *CsIRT1*, that are directly involved in iron acquisition, while for *CsHA1* and *Cspepc1* even after 48 h the transcripts are still present. On the other hand, these two last enzymes are not involved solely in the Fe deficiency responses but participate in many other cellular events. Western blot analysis of the protein extracted from plants grown in the same conditions had shown a similar pattern of accumulation for the H^+-ATPase and the PEPC (Dell'Orto *et al.*, 2000; De Nisi & Zocchi, 2000) and IRT1 (Connolly *et al.*, 2002; Vert *et al.*, 2003).

Discussion

Cucumber roots respond to iron deficiency by inducing acidification of the culture medium and reduction of Fe(III) within 7 d (Fig. 1A and 1B). Gene expression analysis carried out by semi-quantitative RT-PCR revealed that *CsFRO1*, *CsIRT1*, *CsHA1* and *Cspepc1* transcripts (Fig. 3A) are increased during this period accordingly with the iron deficiency induction of the iron uptake system. This induction is well correlated with the increase in the relative enzymatic activities (Table 1). This work for the first time put in relation the induction of specific genes for iron uptake as *CsFRO1* and *CsIRT1* and that of the *CsHA1* and *Cspepc1*. From these experiments it is clear that the whole

iron deficiency response is under the same gross control and that supply of iron rapidly de-induces the expression of these transcripts altogether (Fig. 3B), greater for the *CsFRO1* and *CsIRT1* (they serve specifically for iron uptake), which reach the level seen in the control roots within 48 h, than for the *CsHA1* and *Cspepc1*, that are less specific and also serve for other important cellular functions (Chollet et al., 1996; Palmgren, 2001). A further possible explanation of this different response could relay on the fact that for *Cs*FRO1 and *Cs*IRT1 we can assume a primary coordinate regulation, both local and systemic in response to a direct event (presence or absence of iron). For what concern the *Cs*HA1 and the *Cs*pepc1 a kind of secondary regulation can be supposed in view of a less direct involvement of these two activities in the iron-deficiency responses. It seems possible to hypothesise a sequential coupled regulation which involves these four genes: direct or primary for the response of *Cs*FRO1 and *Cs*IRT1, to promote iron uptake, and secondary or metabolic for *Cs*HA1 and *Cs*pepc1. What is intended for metabolic is the necessity to increase the production of NAD(P)H and ATP for the FC-R and the H^+-ATPase activities, respectively, that brings to an increase in the rate of glycolysis and perhaps of the pentose phosphate pathway (Rabotti et al. 1995; Espen et al. 2000), along with the necessity to extrude protons which tend to accumulate as the glycolysis rate increases in a sort of pH-stat mechanism (Sakano, 1998). Thus, the activation of *Cs*HA1 and *Cs*pepc1 transcripts should seem to be stimulated as a metabolic consequence of iron starvation rather than by a direct system. This seems to be in agreement with the microarray analysis shown by Colangelo and Guerinot (2004) where there is no evidence that these two last genes are targets of FIT1 regulation. Of course, we can not ruled out the possibility that other regulatory mechanisms operate, such as posttranscriptional modifications, as suggested for FRO2

and IRT1 in Arabidopsis (Connolly et al., 2003), that could involve also the H^+-ATPase and the PEPC. Furthermore, even the possibility of posttranslational modifications, that might finely regulate the activities of the enzymes correlated with the iron deficiency responses, can not also be ruled out. This might explain why the H^+-ATPase and the PEPC activities can be reduced though their transcripts are still consistently presents (Table 1 and Fig. 3B).

In addition to the activity of *Cs*FRO1 and *Cs*IRT1, we show that also other activities considered to be linked to the iron deficiency response, the *Cs*HA1 (in particular) and the *Cs*pepc1, may be regulated through the same signal. A transient increase in both gene expression and enzymatic activities could be seen when iron starved roots are supplied with iron, showing a local control by substrate. In fact, when starved roots are supplied with iron which acts as a local inducer, all the enzyme activities assayed are increased in the first 24 h (Table 1); concerning the gene expression we could not really appreciate any substantial increase, but in any case their expression is not diminished within this period. A possible explanation of this apparent lack of induction, that has been shown at the moment mainly in Arabidopsis, could be ascribed to a different regulation in cucumber or to the fact that the expression, already high when iron is supplied, might mask a possible further increase.

What kind of signals are involved in the response to iron deficiency is not yet known. We can assume that iron itself, through its movements in the xylematic and phloematic saps, may signal the iron status of the plant. It can act, according to the dual regulation model proposed by Vert *et al.* (2003), either as a local inducer signal and as a repressive systemic signal once its concentration inside the plant reaches a satisfactory level. Whether iron acts directly or in association with other molecules is not yet

known. On the other hand, iron deficiency itself cannot be considered a promotive systemic signal, but other molecules or mechanisms could act to induce such responses. In a study on iron deficiency responses in Arabidopsis by using microarray analysis (Thimm *et al.*, 2001) it was found that in the shoot, several genes involved in the metabolism and export of carbohydrate, are strongly up-regulated in this condition, 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 roots under iron stress deficiency is very high since they necessitate an increased amount of reducing equivalents, energy and tricarboxylic cycle intermediates to sustain all the processes induced by this condition (Zocchi, 2006 and reference therein). Indeed, an increase in the sugar concentration has been demonstrated in the phloem of iron deficient bean plants (De Vos *et al.*, 1986). Whether sugars, or other molecules transported along with them in the phloem, are responsible for a systemic signal in iron deficiency response is still unknown. The promotive signal has been assigned to several molecules until now, such as IAA (Landsberg, 1984; Römheld & Marschner, 1986), ethylene (Lucena et al., 2006), sugar (Bienfait *et al.*, 1987), iron complexed by a ligand (Kruger *et al.*, 2002) and recently to nitric oxide (Graziano *et al.*, 2002). Regulation of the transcription factors that control the expression of genes involved in iron uptake and metabolism are characterized in bacteria (Escolar *et al.*, 1999), in yeast (*Saccharomyces cerevisiae* (Yamaguchi-Iwai *et al.*, 2002) and in vertebrate (Papanikolaou & Pantopoulos, 2005). Recently, also in tomato and Arabidopsis transcription factors controlling the iron deficiency responses and the iron uptake has been described (Colangelo & Guerinot, 2004; Jacoby et al., 2004;

Brumbarova & Bauer, 2005; Yuan et al., 2005) suggesting that an analogous system might operate at the plant level.

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Table I. *Effect of iron nutritional status on plasma membrane and PEPC root activities of plant grown in the presence or absence of iron and after iron resupply.*

H^+-ATPase, Fe(III)-chelate reductase (FC-R) and PEPC activities were determined in the root apical segments of 8-day-old plants grown in iron sufficient or iron deficient nutrient solution and after Fe resupply to the -Fe roots. Data are the mean of three independent experiments. SE never exceeds 8%. Data are expressed as nmol NADH mg $prot^{-1}$ min^{-1}

	Time	H^+-ATPase		FC-R		PEPC	
treatment		+ Fe	- Fe	+ Fe	- Fe	+ Fe	- Fe
	7 d	103	157	40	142	82	235
Iron resupply	24 h		160		196		310
	48 h		140		151		220
	72 h		116		96		103

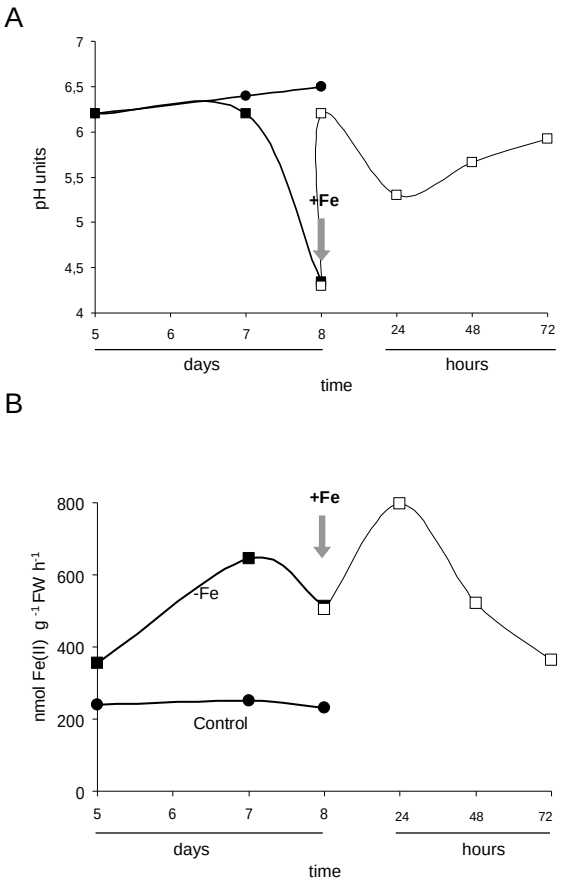

Figure 1.

Time courses of acidification (A) and reduction (B) capacity of cucumber roots grown in the presence (100 µM Fe) (closed circle) or in the absence of Fe (closed square). Arrow indicates the Fe resupply (open square) (100 µM Fe-EDTA). A representative experiment is shown.

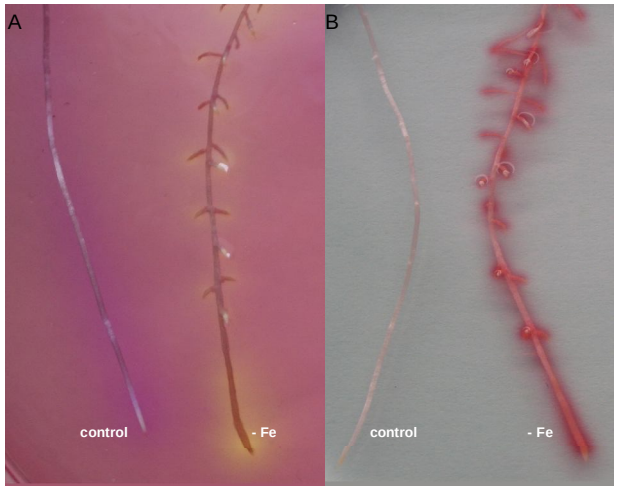

Figure 2.

Visualization of medium acidification (A) and iron reduction (B) capacity along cucumber primary roots. Excised primary roots were incubated in 0.1% agar medium and the acidification was detected as pH change of the indicator Bromocresole Purple (yellow); the reduction was determined as the Fe^{2+}-$(BPDS)_3$ complex formation (red).

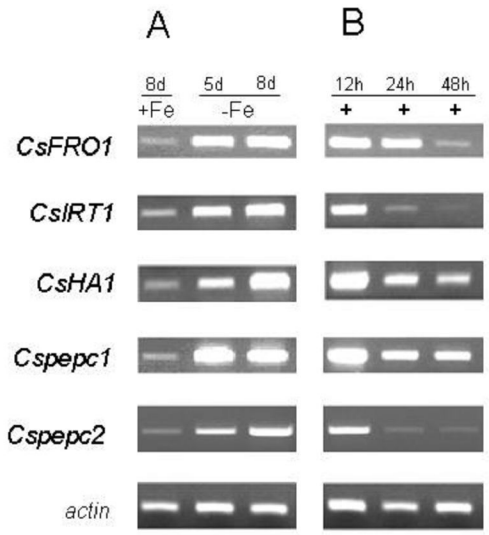

Figure 3.

Iron deficiency-dependent expression of Strategy I responsive and PEPC genes. Total RNA was extracted from roots grown in the presence (+ Fe 8d) or in the absence of iron (-Fe 5d, 8d) and 12h, 24h and 48h after iron resupply. The transcript levels of *CsFRO1*, *CsIRT1*, *CsHa1*, *Cspepc1* and *actin* of roots grown under different iron nutritional status were monitored by semi-quantitative RT-PCR. In A, expression pattern of transcripts after 5 and 8 days of iron deficiency respect to the control. In B, expression pattern after 12h, 24h and 48h of iron resupply to Fe-deficient 8-day-old plants.