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cDNA-AFLP analysis reveals a set of new genes differentially expressed in cucumber root apexes in response to iron deficiency

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Abstract

Cucumber (*Cucumis sativus* L.) is considered a model plant for the investigation of Fe deficiency responses, since it strongly exhibits typical strategy I, *i.e.* increased activities of Fe(III)-chelate reductase, H^+-ATPase and iron regulated transporters. In this study, cDNA amplified fragment length polymorphism analysis was employed to identify genes differentially expressed in the root apex following Fe deficiency. The expression patterns of the most interesting transcript derived fragments were validated by semiquantitative reverse transcriptase - polymerase chain reaction. A set of new genes overexpressed under Fe deficiency, such as those coding for calmodulin, SNAP, TIM23 and V-PPase were identified. Furthermore, we also observed that calmodulin protein accumulated in Fe-deficient root apexes.

Additional key words: calmodulin, *Cucumis sativus*, semiquantitative RT-PCR, transcript-derived fragments, transcriptomic analysis.

Introduction

In well aerated soils, iron exists mainly as scarcely soluble oxides and oxi-hydroxides and therefore is not freely available for plant uptake, despite its high abundance. In plants, Fe uptake and homeostasis are tightly regulated to ensure both a sufficient supply of Fe from the soil and the avoidance of a toxic excess in the cells. Iron deficiency induces various responses aimed to increase the availability of the Fe in the rhizosphere for plant uptake. Strategy I plants (dicotyledonous and non-graminaceous plants) are able to respond to a lack of Fe in the soil by increasing *1*) the Fe reduction capacity of root tissues, *2*) the acidification of the rhizosphere to increase Fe solubility and *3*) the increased uptake by rhizodermal root cells by increaseng activities of Fe(III)-chelate reductase (FC-R), H^+-ATPase and iron regulated transporters (IRT) (Curie and Briat 2003). The increase in FC-R and H^+-ATPase activities needs a continuous supply of energetic substrates to keep the system working. This effort needs a reorganization of metabolic pathways to efficiently sustain activities linked to the acquisition of Fe; in fact, the metabolism of sugars is involved in these responses (Zocchi 2006, Vigani and Zocchi 2009). Thus, it is evident that the effects of Fe deficiency on plant metabolism are multifaceted, and that more information is required to increase our comprehension of the regulatory mechanisms activated by plants under this condition.

Proteome and transcriptome analyses are the most useful tools to obtain a wide collection of information about the metabolic status of a particular tissue and under particular conditions. For Strategy I plants, some studies on proteomic investigations under Fe deficiency (Li *et al*. 2008, Donnini *et al*. 2010), as well as some studies showing a microarray analysis of differentially expressed transcript profiles in Fe-deficient *Arabidopsis thaliana* (Thimm *et al*. 2001, Buckhout *et al*. 2009) and *Glycine max* (O'Rourke *et al*. 2007) have recently been published. Transcript profiling techniques allow the simultaneous examination of gene expression under different experimental conditions. The PCR-based technique of cDNA-amplified fragment length polymorphism (cDNA-AFLP) is widely available at a low cost, even if for various

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Abbreviations: AFLP - amplified fragment length polymorphism; BPDS - bathophenantrolinedisulfonate; FC-R - Fe(III)-chelate reductase; IRT - iron regulated transporters; PVDF - polyvinylidene difluoride; RT-PCR - reverse trascriptase-polymerase chain reaction; TDF - transcript derived fragments.

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plant species there is little information at the molecular level (Breyne and Zabeau 2001). The sensitivity and specificity of the method allows the detection of even poorly expressed genes and permits us to distinguish between homologous sequences (Breyne *et al*. 2003, Fukumura *et al*. 2003). This is an extremely efficient mRNA fingerprinting method for the isolation of those genes which show differential expression in stressed conditions. Moreover this technique enables the discovery of genes that have not been previously identified or predicted from sequence analysis. However, it should be noted that molecular components that are controlled by post-transcriptional regulation or by post-translational modification cannot be identified.

The AFLP technology has been used predominantly for assessing the degree of variability among plant cultivars, establishing linkage groups in crosses and saturating genomic regions with markers for gene landing efforts. The AFLP fragments may also be used as physical markers to determine the overlap and positions of genomic clones and to integrate genetic and physical maps (Vuylsteke *et al*. 2007 and references therein, Beharav *et al*. 2010, Cuesta *et al.* 2010, Kang *et al*. 2010, Sikdar *et al*. 2010).

Keeping the above in view, the objective of our study was to identify genes that were differentially expressed in response to Fe deficiency in cucumber. Cucumber can be considered a model Strategy I plant since it exhibits more intense responses to Fe deficiency compared to other species (*e.g*. strong rhizosphere acidification). While the responses of cucumber plants to Fe deficiency have been largely characterized only at the biochemical level, the studies of Fe-deficient *Arabidopsis thaliana* plants are mainly performed at molecular level, lacking a satisfactory biochemical characterization (Curie and Briat 2003). In this study, cDNA amplified fragment length polymorphism (cDNA-AFLP) analysis was employed to identify genes which showed differential expression under Fe deficiency treatment at the root apex. The expression patterns of some transcript derived fragments (TDFs) were validated through semiquantitative RT-PCR analysis. Several differentially expressed cDNA fragments were isolated and sequenced, and their possible functions are discussed.

Materials and methods

Cucumber (*Cucumis sativus* L. cv. Marketmore 76) plants were grown in a nutrient solution as reported by Vigani *et al*. (2009). One half of 7-d-old plants grown in a complete nutrient solution was transferred to the same nutrient solution and the other half to a nutrient solution without Fe. Sampling was performed 7 d after the transfer, and 4 h after light onset.

Visualization and localization of 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 bathophenantrolinedisulfonate (BPDS) reagent which forms a stable, water soluble, red complex with Fe^{2+} and only a weak complex with Fe^{3+}.

Total RNA was isolated from 100 - 200 mg of root apexes (0.5 - 1 cm from the tip) using *TRIzol* reagent (*Sigma-Aldrich*, Milano Italy) according to the manufacturer's instructions. Poly A^+ RNA was isolated following the *Oligotex* mRNA spin-column protocol (*Qiagen*, Milano Italy). The concentration of Poly A^+ RNA was checked spectrophotometrically at 260 nm and about 500 ng of Poly A^+ RNA from each sample was used for cDNA preparation. First strand cDNA was synthesized following the reverse transcriptase *SuperScript* protocol (*Invitrogen*, Milano Italy). Contaminating genome DNA was removed by DNaseI treatment (*Amersham Biosciences*, Uppsala, Sweden). The cDNA-AFLP analysis was performed according to Bachem *et al.* (1996) as modified by Hartings (1999) (see Table I in the supplementary analytical procedures; for more detail refer to the website http://www.diprove.unimi.it/groups/phys_rg2.htm or contact the corresponding author). The validation of all the steps of the experiment have been done with three independent biological replicates and each of them has been technically repeated twice.

The eluted TDFs were cloned into the *pBlueScript II* plasmid (*Stratagene*, Cedar Creek, TX, USA) according to the manufacturer's instructions. The recombinant plasmids were purified using *QIAprep Spin Miniprep* kit (*Qiagen*) following the manufacturer's protocol. Resulting sequences were then analysed by *Primm* (Milano, Italy). After removal of vector sequences, database search was performed. The nucleotide as well as translated protein sequences were analyzed for their homology using the publicly available Nucleotide collection of the *Cucurbit genome database* (http://www.icugi.org/cgi-bin/ICuGI/tool/blast.cgi) using *Cucumber database* (*v2.0*) and *BLASTN* program. For further information about the AFLP procedure and used primers contact the corresponding author or visit the http://www.diprove.unimi.it/info/msg1.htm.

For semiquantitative RT-PCR analysis root tissues were pulverised in liquid nitrogen using a mortar and pestle, total RNA was extracted using *Trizol*® reagent (*Invitrogen*) and first strand cDNA synthesis was carried out using the *iScript*™ cDNA synthesis kit (*Bio-Rad*, Milano, Italy) according to the manufacturer's instructions. Actin, which is a gene constitutively expressed, was used as an internal control. Semiquantitative RT-PCR (sqRT-PCR) analysis of Strategy I genes (Fig 1*C*) and of TDFs was performed according to Donnini *et al* (2010). The relative expression level of the TDFs was determined as reported in supplemental analytical procedures. The primers used for sqRT-PCR were designed from the selected sequences of the TDFs using the *FAST-PCR version* (Suppl. Table 2, see above

mentioned www.pages).

Soluble proteins extracted from roots of plants grown in the presence and in the absence of Fe were loaded onto a discontinuous SDS-polyacrylamide gel according to De Nisi and Zocchi (2000). After SDS-PAGE, proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane filters (*Sigma*) and the immunoreaction with monoclonal anti-calmodulin antiserum (*Sigma*) was performed according to the manufacturer's instructions. The detection procedure was performed by *ExtraAvidin* peroxidase staining kit (*Sigma*) according to the manufacturer's instructions. Protein content was determined according to Bradford (1976).

Results and discussion

We focused our analysis on the root apex only, since it is the main site displaying Fe uptake in which Strategy I activities are strongly induced (Landsberg 1986, 1994). Cucumber plant grown under Fe-deficiency displayed notable morphological changes at the root apex (Fig. 1*A*) and showed more pronounced *in vivo* Fe reduction (Fig.1*B*) compared to the control. Furthermore, the genes encoding the enzymes typical for Fe-deficiency (FC-R, H^+-ATPase and IRT) were over-expressed (Fig. 1*C*).

Surprisingly, TDFs homologous to FC-R, H^+-ATPase and IRT were not detected in Fe-deficient roots, as well as other typical metabolic markers related to Strategy I response (*e.g*. phosphoenolpyruvate carboxylase). The lack of detection of these TDFs could be explained by taking into account the possible limitations inherent in the cDNA-AFLP methodology: *1*) the primers used may have been unable to amplify the transcripts relative to Strategy I genes; *2*) the under-representation of transcripts lacking a recognition site for the restriction enzymes used; *3*) the cDNA-AFLP technology is based on a double digest, short fragments may be generated and subsequently lost in the analysis; *4*) the missing TDFs could have a sequence length under 100 bp or above 500 bp, which was the range considered in cDNA-AFLP analysis. About 2000 fragments ranging from 100 to 500 bp in length were typically observed on the gels examined. We identified 26 differentially expressed TDFs (Table 1). These TDFs were eluted from the gel, re-amplified, and cloned. Twenty-two (85 %) out of 26 TDFs were up regulated and 4 (15 %) were down regulated under Fe deficiency.

An investigation in the *Cucurbit genome database* revealed a relationship between the selected 26 clones and the genes involved in environmental stress. The biological role of some of these cloned genes can be inferred from sequence similarity to previously studied plant proteins. Among the 22 up-regulated TDFs, the comparison between the homologues of these sequences and those in the database suggested that most of them are involved in processes such as protein synthesis (*e.g*. TDFs # 5, 6 and 19), in the respiration pathway (*e.g*. TDFs # 7, 8 and 9), ion transport, compartmentalization and cellular homeostasis (*e.g*. TDFs # 10 and 12), cell signalling (*e.g*. TDF # 14) and cell division (*e.g*. TDFs # 15, 16 and 20). The remaining TDFs were not found in the cucumber database (*e.g*. TDFs # 2 and 17, 18, 21, 22, 25). Out of the 26 clones, we selected some of those more interesting for the Fe deficiency (TDFs marked by an asterisk in the Table 1), to validate our cDNA-AFLP data through semiquantitative RT-PCR analysis according to methods suggested by several authors (Bachem *et al*. 1996, Ditt *et al*. 2001, Sojikula *et al*. 2010).

The TDF #7 was homologous to TIM23 gene (AT1G7530.1) from *Arabidopsis thaliana* (Table 1) encoding for a translocase which represents the main entry site for proteins addressed to the matrix and the inner mitochondrial membrane (Bauer *et al*. 2000, Koehler 2000, Jensen *et al*. 2002, Endo *et al*. 2003, Rehling *et al*. 2003).

The TDF identified by cDNA-AFLP (Fig. 2) and confirmed by sqRT-PCR (Fig. 3*A*) showed an over-expression in Fe-deficient root apexes which suggests an increase in the mitochondrial protein import under Fe deficiency. Despite the recent observation that the content of many proteins decreases in mitochondria of Fe-deficient cucumber (Vigani *et al*. 2009), it has been hypothesized that mitochondria still play a pivotal role in

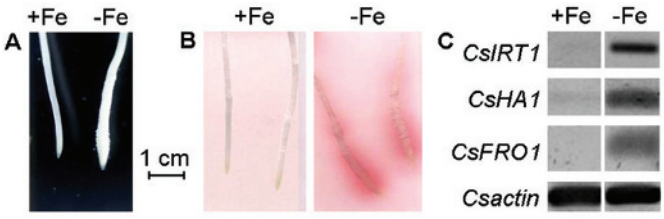

Fig. 1. *A* - Morphological changes of root apex of cucumber plants growing in the presence (+Fe) and in absence (-Fe). *B* - Visualization of Fe reduction capacity along cucumber primary roots. Excised primary roots were incubated in 0.1 % agar medium and the reduction was determined as the Fe^{2+}-$(BPDS)^3$ complex formation (red). *C* - sqRT-PCR analysis of Strategy I (*CsIRT1*, *CsHA1*, *CsFRO1*) genes in root apexes of +Fe and -Fe cucumber plants.

Table 1. Similarity of transcript-derived fragments (TDFs) expressed during Fe deficiency in cucumber root apex with known gene sequences ([a] - cDNA-AFLP fragments generated by different primer combinations showing sequence similarity *Cucurbit genome database* using *BLASTN* program; [e] - E-value was used to indicate the significance of sequence similarity. The TDF up-regulated under Fe deficiency were indicated with plus (+), while the TDFs down-regulated were indicated with minus (-). *TDFs selected for the validation performed by semiquantitative RT-PCR.

TDF #	Primer combination	TDF length [bp]	UniGene[a] ID	E-value[e]	accession No.	Homology in the *Arabidopsis thaliana* genome (*TAIR* database) accession No.	annotation	E-value[e]	Expression
1	B11/M36	230	CU11427	e-103	Csa000816	-			-
2	B12/M42	310	CU83639	4e-48	-				-
3	B12/M45	230	-						-
4	B13/M33	180	CU27578	2e-78		AT5G49720	glycosyl hydrolase 9A1	1e-054	-
5	B12/M39	230	CU47898	4e-98	Csa013450	AT1G31830	aminoacids permease family protein	3e-21	+
6	B12/M40	120	CU9563	1e-40	Csa006172	AT1G07920	elongation factor EF1α	4e-121	+
7*	B11/M56	200	CU14835	2e-47	Csa009905	AT1G7530	ATTIM23	8e-011	+
8*	B11/M42	320	CU8941	e-117	Csa003028	AT4G02580	NADH-ubiquinone oxidoreductase 24 kDa subunits	8e-059	+
9	B13/M40	100	CU72418	3e-29	Csa010829	AT3G27890	NQR(NADPH:quinone oxidoreductase FMN reductase)	6e-019	+
10*	B10/M45	170	CU31994	2e-12	Csa009847	AT1G78920	AVP2	5e-018	+
11	B10/M49	250	-						+
12	B11/M40	170	CU10438	1e-66	Csa001755	AT2G19900	ATNADP-ME1 (malic enzyme)	6e-080	+
13	B13/M54	100	CU11470	3e-13	Csa020702	AT5G20060	phospholipase/carboxylase family protein	7e-023	+
14*	B10/M59	140	CU10012	3e-54	Csa012897	AT3G438101	calmodulin (CAM7)	2e-016	+
15	B12/M31	240	CU11377	e-110	Csa009074	AT5G19770	tubulin (TUA3)	9e-078	+
16	B11/M33	250	CU11377	e-107	Csa69627	AT5G19770	tubulin (TUA3)	9e-078	+
17	B12/M35	230	-						+
18	B11/M41	160	-						+
19	B11/M42	200	CU10384	3e-89	Csa009906	AT1G54270	eukaryotic translation initiation factor (EIF4A-2)	1e-172	+
20	B11/M52	310	CU4051	e-156	Csa014935	AT3G02550	LBD41 (LOB domain-containig protein 41)	4e-070	+
21	B11/M60	150	-						+
22	B12/M42	160	-						+
23	B13/M33	240	CU7554	6e-94	Csa018980	AT5G19590	unknown protein	5e-038	+
24	B13/M55	280	CU29575	e-129	Csa015840	AT4G27480	glycosyltrasferase family 14 protein	2e-015	+
25	B13/M57	100	-						+
26*	B12/M41	125	CU23961	2e-49	Csa005687	AT3G56190	α-SNAP	7e-036	+

metabolic changes occurring under Fe deficiency through the activation of alternative metabolic pathways, *i.e*. alternative NAD(P)H dehydrogenase and metabolite shuttles (Vigani and Zocchi 2009, 2010). In agreement with our results, Lister *et al*. (2004) suggested that the transcription of mitochondrial import component genes was induced when mitochondrial function was limited. In fact, *Arabidopsis thaliana* cells treated with rotenone and antimycin A showed a strongly induced expression of mitochondrial import component genes, TIM23 among them. Since TIM23 is specifically involved in the protein transport into the matrix, its induction suggests an enhancement of protein request in the matrix. In fact, it is well documented that some activities belonging to the Krebs cycle (López-Millan *et al.* 2000) and the demand for related proteins (Li *et al*. 2008) increase under such stress conditions.

TDF #8 was homologous to AT4G02580 gene from *Arabidopsis thaliana* (Table 1) encoding for a 24 kDa subunit of mitochondrial complex I [NADH dehydrogenase (ubiquinone)]. The TDF identified by cDNA-AFLP (Fig. 2) and confirmed by sqRT-PCR (Fig. 3*A*) showed an overexpression in Fe-deficient root apexes. Thimm *et al*. (2001) observed a transcript overexpression of a NADH oxidoreductase, suggesting a transcriptional induction of a gene encoding for a complex I subunit. However we previously observed an almost undetectable protein band of NAD9 (complex I subunit) while

complex I activity was strongly decreased (Vigani *et al*. 2009). The contrasting data between transcriptional induction and activity inhibition of complex I could be explained in two ways: *1*) the presence of different transcriptional and translational regulations, *2*) transcript induction observed in plants grown first in the presence of Fe and then subjected to Fe deprivation, while in Vigani *et al*. (2009) the protein content and complex I activity were determined on 10-d-old cucumber plants grown directly, after germination, in the absence of Fe.

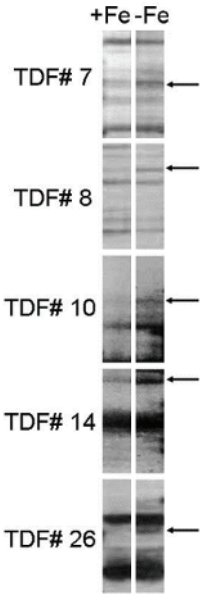

Fig. 2. Portions of cDNA-AFLP gel autoradiogram showing selected TDFs induced in root apexes of Fe-deficient cucumber plants. These TDFs have been chosen for the successive semi-quantitative RT-PCR analysis. The TDF # 7 was obtained using B11 primer in combination with M56 primer; the TDF # 8 was obtained using B11 primer in combination with M42 primer; the TDF # 10 was obtained using B10 primer in combination with M45 primer; the TDF # 14 was obtained using B10 primer in combination with M56 primer; the TDF # 26 was obtained using B12 primer in combination with M41 primer.

TDF #10 was homologous to the *AVP2* gene (AT1G78920) from *Arabidopsis thaliana,* encoding for a vacuolar-PPase (V-PPase) (Table 1). The TDF identified by cDNA-AFLP (Fig. 2) and confirmed by sqRT-PCR (Fig. 3*A*, Fig. 1*C*), showed an overexpression in Fe-deficient root apexes. Among the proton pumps, the plant cells possess a vacuolar H^+-PPase (V-PPase), which is able to acidify the vacuolar lumen by hydrolysing PPi (Maeshima 2000). The overexpression of the TDF #10 in cucumber root apexes suggests an induction of vacuolar lumen acidification under Fe deficiency. On the contrary, Espen *et al*. (2000) showed a vacuolar pH increase in Fe-deficient cucumber roots. However, it has been shown that the V-PPase is not restricted only to the tonoplast but it is also present and active in the plasmalemma (PM), in the trans-Golgi network (TGN) and in multivesicular bodies (Ratajczak *et al*. 1999). Thus, the transcript induction of a V-PPase in cucumber root apexes could be also related to the vesicular traffic in the cell and not only to its tonoplast activity. In fact, we have also identified a gene over-expressed in this condition (TDF #26, Figs. 2, 3), homologous to AT3G56190 gene from *Arabidopsis thaliana* coding for α-SNAP (Soluble NSF attachment protein) (Table 1). SNAP proteins belong to the soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNARE) protein family, which are involved in a specific vesicle fusion process during

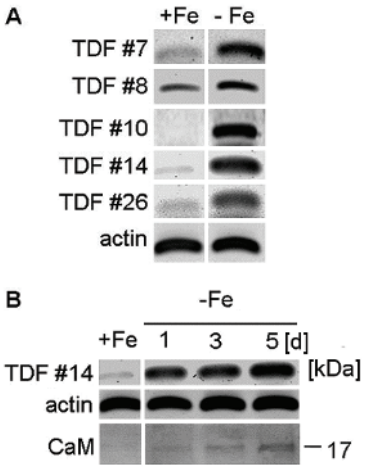

Fig. 3. Semi-quantitative RT-PCR analysis of the expression of the different TDFs in response to Fe deficiency in cucumber root apexes. *A* - agarose gels of the selected TDFs clones amplified by sqRT-PCR. *B* - Semi-quantitative RT-PCR analysis of the expressione of the TDF# 14 (*top*) and Western blot analysis of calmodulin (CaM) (*bottom*) in cucumber root apexes after 1, 3 and 5 d of Fe deficiency.

vesicular transport (Uemura *et al.* 2004). Vesicles are used to transport molecules, for instance proteins, to specific locations. The docking of the vesicle to the target membrane causes the recruitment of two general soluble trafficking factors N-ethylmaleimide-sensitive factor (NSF) and α-SNAP leading to membrane fusion and delivery of the contents of the transport vesicle (Stenbeck, 1998). This finding sheds new light on the link between exocytosis processes and Fe deficiency responses occurring in roots, mainly for two reasons. The first one could be related to the formation of transfer cells - a process well documented in the Fe-deficient root tissues (Landsberg 1994), but not completely understood yet. We hypothesize that an enhancement of vesicle trafficking towards to the PM increases the apposition of organic compounds for cell wall synthesis. The second reason could be related to the over-expression of the V-PPase gene observed in this study. We hypothesize that Fe deficiency can promote exocytosis in the cells, enhancing vesicular traffic from the cytosol to the PM. The vesicles formed could be characterized by the presence of V-PPase which could acidify the vacuolar lumen. Once the vesicles containing protons reach the PM, the fusion of these membranes could lead at the

same time to the apoplastic acidification assisting the PM H^+-ATPase activity and/or the proton extrusion into the apoplast using the energy from PPi hydrolysis avoiding the consumption of ATP, which in this condition is a metabolic limiting factor.

TDF #14 was homologous to *CAM7* gene (AT3G438101) from *Arabidopsis thaliana* encoding for calmodulin (Table 1). Calmodulin (CaM) is a conserved multifunctional calcium sensor that mediates intracellular Ca^{2+} signalling and regulates diverse cellular processes by interacting with calmodulin-binding proteins (Bouche *et al*. 2005, Ikura and Ames 2006). The over-expression of the transcript encoding for CaM in Fe-deficient root apexes has been observed as a polymorphic band in cDNA-AFLP (Fig. 2) and confirmed by sqRT-PCR analysis (Fig. 3*A*). Moreover, there are no papers about CaM induction under Fe deficiency. This finding is not unexpected since new stimuli were provided by changing the growing conditions. This finding might suggest a direct involvement of CaM in the transduction of the Fe deficiency signal or, more likely, its involvement in the induction and regulation of the metabolic changes which accompany the responses induced by Fe deficiency. Of course we are aware that more results are required to finally corroborate this hypothesis. Interestingly, Fig. 3*B* showed that the transcript abundance strongly increased about 1 d after the induction of the Fe deficiency condition, while the protein weakly accumulated after 1 and 3 d and just after 5 d of the induction of the Fe deficiency the CaM protein accumulated strongly. Moreover, some authors observed a transcript induction of genes encoding for 14-3-3 proteins and some protein kinases in Fe-deficient *Arabidopsis thaliana* roots which should be target proteins of the CaM-transduced signal (Colangelo and Guerinot. 2004, Buckhout *et al.* 2009).

Conclusion

This study allowed to identify, for the first time, genes differentially expressed induced by Fe deficiency in cucumber plant, such as those coding calmodulin, SNAP, TIM23 and V-PPase. Thus, at the transcript level, several processes appear to be induced by Fe deficiency, which might be common components of the responses to different perturbations, with integration from other stress-specific signalling cascades. However, the identification of the precise role of these genes and their specific involvement under Fe deficiency needs further investigation.

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