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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 activities (i.e. Fe(III)-chelate reductase [FC-R], H^+-ATPase and Iron Regulated Transporters [IRT]), under such condition. In this study, cDNA amplified fragment length polymorphism (cDNA-AFLP) analysis was employed to identify genes differentially expressed at the root apex level following Fe deficiency treatment application. The expression patterns of the most interesting transcript derived fragments (TDFs) were validated through semiquantitative RT-PCR analysis. This study allowed us to identify a set of new cucumber genes overexpressed under Fe deficiency, such as those coding for calmodulin, SNAP, TIM23 and V-PPase. Furthermore, we also observed that calmodulin protein accumulated in Fe-deficient root apices. This last result allows us to hypothesise that a signal transduction cascade might be involved in the transduction of the Fe deficiency signal or, more likely, it may be involved in the induction and regulation of the metabolic changes induced by Fe deficiency

Additional Key words: calmodulin, *Cucumis sativus* L., iron deficiency, semiquantitative RT-PCR (sqRT-PCR), Strategy I, transcript-derived fragments (TDFs), transcriptomic analysis.

Abbreviations: AFLP, amplified fragment length polymorphism; BPDS, bathophenantrolinedisulfonate; PVDF, Polyvinylidene Difluoride; TDF, transcript derived fragments

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Introduction

Iron exists, in well aerated soils, mainly as scarcely soluble oxides and oxi-hydroxides and therefore is not freely available to plants uptake, notwithstanding its 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 cell. Iron deficiency induces various responses at the root level, aimed to increase the availability of the ion 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 i) the Fe reduction capacity of root tissues, ii) the acidification of the rhizosphere to increase Fe solubility and iii) the uptake activities in rhizodermal root cells (i.e. Fe(III)-chelate reductase [FC-R], H^+-ATPase and Iron Regulated Transporters [IRT], respectively) (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 iron; in fact, the energetic metabolism of carbohydrates has been shown to be 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 soybean (*Glycine max* (L.) Merr.) plants (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 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 labor-intensive 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.

1 However, it should be noted that molecular components that are controlled by post-transcriptional regulation or
2 by post-translational modification cannot be identified.
3 The AFLP technology has been used predominantly for assessing the degree of variability among plant cultivars,
4 establishing linkage groups in crosses and saturating genomic regions with markers for gene landing efforts. The
5 AFLP fragments may also be used as physical markers to determine the overlap and positions of genomic clones
6 and to integrate genetic and physical maps (Vuylsteke *et al*. 2007 and references therein, Beharav *et al*. 2010,
7 Kang *et al*. 2010, Sikdar *et al*. 2010, Cuesta *et al,* 2010).
8 Keeping the above in view, the objective of our study was to identify genes that were differentially expressed in
9 response to Fe deficiency in cucumber (*Cucumis sativus* L.). Cucumber can be considered a model Strategy I plant
10 since it exhibits intense responses to Fe deficiency compared to other species (e.g. strong rhizosphere
11 acidification). While the responses of cucumber plants to Fe deficiency have been largely characterized only at
12 the biochemical level, the studies of Fe-deficient *Arabidopsis thaliana* plants are mainly performed at molecular
13 level, lacking a satisfactory biochemical characterization (Curie and Briat, 2003). In this study, cDNA amplified
14 fragment length polymorphism (cDNA-AFLP) analysis was employed to identify genes which showed differential
15 expression under Fe deficiency treatment at the root apex level in cucumber roots. The expression patterns of
16 the some TDFs were validated through semiquantitative RT-PCR analysis. Several differentially expressed cDNA
17 fragments were isolated and sequenced, and their possible functions are discussed.
18
19 **Materials and methods**
20 **Plant material and growth conditions**: Cucumber plants (*Cucumis sativus* L. cv. Marketmore 76) were grown
21 in a nutrient solution as reported by Vigani *et al*. (2009). Seven-day-old plants grown in a complete nutrient
22 solution were divided, one half was transferred to the same nutrient solution and the other half to a nutrient
23 solution without Fe. Sampling was performed 7 days after the transfer, and 4 hours after light onset.
24 **In vivo localisation of the reduction capacities**: Visualization and localization of Fe(III) reduction was
25 performed by embedding the roots in a agar medium as described in Marschner *et al.* (1982) in the presence of
26 the bathophenantrolinedisulfonate (BPDS) reagent which forms a stable, water soluble, red complex with Fe^{2+}
27 and only a weak complex with Fe^{3+}.
28 **cDNA Preparation and AFLP Analysis**: Total RNA was isolated from 100-200 mg of root apexes (0.5-1 cm from
29 the tip) using TRIzol reagent (Sigma Aldrich, Milano Italy) according to the manufacturer's instructions. Poly A^+
30 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 (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/info/msg1.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, Texas) according to the manufacturer's instructions. The recombinant plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen, Milano Italy) following the manufacturer's protocol. Resulting sequences were then analysed by Primm (Primm s.r.l., 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.

Semiquantitative RT-PCR analysis: Root tissues were pulverised in liquid nitrogen using a mortar and pestle, total RNA was extracted using Trizol® reagent (Invitrogen, Milano Italy) 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 analysis of Strategy I genes (Fig 1C) and of TDFs was performed according to Donnini *et al* (2010). The relative expression level of the TDFs (Supplemental Figure 1) was determined es reported in supplental analytical procedures. The primers used for semiquantitave RT-PCR were designed from the selected sequences of the TDFs using the FAST-PCR Version (Supplemental Table 2).

Protein gel blot analysis: 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

1 peroxidase Staining Kit Mouse (Sigma) according to the manufacturer's instructions. Protein concentration was
2 determined according to Bradford (1976).
3

4 **Results and Discussion**

5 We focused our analysis on the root apex only, since it is the main site displaying ion uptake in which Strategy I
6 activities are strongly induced (Landsberg 1986, 1994). Cucumber plant displayed notable morphological
7 changes at the root apex level (Fig.1A) and showed pronounced *in vivo* Fe reduction (Fig.1B) compared to the
8 control. Furthermore, the genes encoding the enzymes typically induced under Fe-deficiency induced (FC-R, H^+-
9 ATPase and IRT) were over-expressed in –Fe root apex compared with the control (Fig. 1C).
10 Surprisingly, TDFs homologous to FC-R, H^+-ATPase and IRT were not detected in Fe-deficient roots, nor were
11 other typical metabolic markers related to Strategy I response (e.g. phospho*enol*pyruvate carboxylase). The lack
12 of detection of these TDFs could be explained by taking into account the possible limitations inherent in the
13 cDNA-AFLP methodology: i) the primers used may have been unable to amplify the transcripts relative to
14 Strategy I genes; ii) the under-representation of transcripts lacking a recognition site for the restriction enzymes
15 used; iii) the cDNA-AFLP technology is based on a double digest, short fragments may be generated and
16 subsequently lost in the analysis; iv) the missing TDFs could have a sequence length under 100 bp or above 500
17 bp, which was the range considered in cDNA-AFLP analysis. About 2000 fragments ranging from 100 to 500 bp
18 in length were typically observed on the gels examined. We identified 26 differentially expressed TDFs (Table
19 1). These TDFs were eluted from the gel, re-amplified, and cloned. Twenty-two (85%) out of 26 TDFs were up
20 regulated and 4 (15%) were down regulated under Fe deficiency.
21 An investigation in the Cucurbit genome database revealed a relationship between the selected 26 clones and the
22 genes involved in environmental stress. The biological role of some of these cloned genes can be inferred from
23 sequence similarity to previously studied plant proteins. Among the 22 up-regulated TDFs, the comparison
24 between the homologues of these sequences and those in the database suggested that most of them are involved
25 in processes such as protein synthesis (e.g. TDFs # 5, 6 and 19), in the respiration pathway (e.g. TDFs # 7, 8 and
26 9), ion transport, compartmentalization and cellular homeostasis (e.g. TDFs # 10 and 12), cell signalling (e.g. TDF
27 # 14) and cell division (e.g. TDFs # 15, 16 and 20). The remaining TDFs were not found in the cucumber database
28 (e.g.TDFs # 2 and 17, 18, 21, 22, 25). In Table 1 the TDFs homologies are reported. Out of the 26 clones, we
29 selected some of those more interesting for the Fe deficiency topic (TDFs marked by an asterisk in the Table 1),

1 to validate our cDNA-AFLP data through semiquantitative RT-PCR analysis according to methods suggested by
2 several authors (Bachem *et al*. 1996, Ditt *et al*. 2001, Sojikula *et al*. 2010).
3
4 **TDFs with putative function in mitochondrial respiration**
5 The TDF #7 was homologous to TIM23 gene (AT1G7530.1) from *Arabidopsis thaliana* (Table 1) encoding for a
6 translocase which represents the main entry site for proteins addressed to the matrix and the inner
7 mitochondrial membrane (Bauer *et al*. 2000; Koehler 2000; Jensen *et al*. 2002; Endo *et al*. 2003; Rehling *et al*.
8 2003).
9 The TDF identified by cDNA-AFLP (Fig. 2) and confirmed by sqRT-PCR (Fig. 3A, supplemental Fig.1) showed an
10 overexpression in Fe-deficient root apices which suggests an increase in the mitochondrial protein import under
11 Fe deficiency. Despite the recent observation that the content of many proteins decreases in mitochondria of Fe-
12 deficient cucumber (Vigani *et al*. 2009), it has been hypothesized that mitochondria still play a pivotal role in
13 metabolic changes occurring under Fe deficiency through the activation of alternative metabolic pathways (i.e.
14 alternative NAD(P)H dehydrogenase and metabolite shuttles) (Vigani and Zocchi 2009, 2010). In agreement with
15 our results, Lister *et al*. (2004) suggest that the transcription of mitochondrial import component genes is
16 induced when mitochondrial function is limited. In fact, *Arabidopsis thaliana* cells treated with rotenone and
17 antimycin A showed a strongly induced expression of mitochondrial import component genes, TIM23 among
18 them. Since TIM23 is specifically involved in the protein transport into the matrix, its induction suggests an
19 enhancement of protein request in the matrix. In fact, it is well documented that some activities belonging to the
20 Krebs cycle (López-Millan *et al.* 2000) and the demand for related proteins (Li *et al*. 2008) increase under such
21 stress condition.
22 TDF #8 was homologous to AT4G02580 gene from *Arabidopsis thaliana* (Tab. 1) encoding for a 24 kDa subunit of
23 mitochondrial complex I [NADH dehydrogenase (ubiquinone)]. The TDF identified by cDNA-AFLP (Fig. 2) and
24 confirmed by sqRT-PCR (Fig. 3A, supplemental Fig.1) showed an overexpression in Fe-deficient root apices.
25 Thimm *et al*. (2001) observed a transcript overexpression of a NADH oxidoreductase, suggesting a
26 transcriptional induction of a gene encoding for a complex I subunit. However we previously observed an almost
27 undetectable protein band of NAD9 (a Complex I subunit) while complex I activity was strongly decreased
28 (Vigani *et al*. 2009). The contrasting data between transcriptional induction and activity inhibition of complex I
29 could be explained in two ways: i) the presence of different transcriptional and translational regulations, ii)
30 transcript induction observed in plants grown first in the presence of Fe and then subjected to Fe deprivation,

1 while the protein content and complex I activity determined on 10-day-old cucumber plants grown directly, after
2 germination, in the absence of Fe.
3
4 **TDFs with putative function in the proton and vesicle transport**
5 TDF #10 was homologous to the AVP2 gene (AT1G78920) from *Arabidopsis thaliana,* encoding for a vacuolar-
6 PPase (V-PPase) (Table 1). The TDF identified by cDNA-AFLP (Fig. 2) and confirmed by sqRT-PCR (Fig. 3A,
7 supplemental Fig.1), showed an overexpression in Fe-deficient root apices. Among the proton pumps, the plant
8 cells possess a vacuolar H^+-PPase (V-PPase), which is able to acidify the vacuolar lumen by hydrolysing PPi
9 (Maeshima 2000). The overexpression of the TDF #10 in cucumber root apices suggests an induction of vacuolar
10 lumen acidification under Fe deficiency. On the contrary, Espen *et al*. (2000) showed a vacuolar pH increase in
11 Fe-deficient cucumber roots. However, it has been shown that the V-PPase is not restricted only to the tonoplast
12 but it is also present and active in the plasmalemma (PM), in the trans-Golgi network (TGN) and in
13 multivesicular bodies (Ratajczak et al. 1999). Thus, the transcript induction of a V-PPase in cucumber root apices
14 could be also related to the vesicular traffic in the cell and not only to its tonoplast activity. In fact, we have also
15 identified a gene over-expressed in this condition (TDF #26, Fig. 2, Fig. 3 and supplemental Fig. 1), homologous
16 to AT3G56190 gene from *Arabidopsis thaliana* coding for α-SNAP (Soluble NSF Attachment Protein) (Table 1).
17 SNAP proteins belong to the SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptors)
18 protein family, which are involved in a specific vesicles fusion process during vesicular transport (Uemura *et al.*
19 2004). Vesicles are used to transport molecules, for instance proteins, to specific locations. The docking of the
20 vesicle to the target membrane, causes the recruitment of two general soluble trafficking factors, NSF (N-
21 ethylmaleimide-Sensitive Factor) and α-SNAP leading to membrane fusion and delivery of the contents of the
22 transport vesicle (Fairman and Wagner 2003). This finding sheds new light on the link between exocytosis
23 processes and Fe deficiency responses occurring in roots, mainly for two reasons. The first one could be related
24 to the formation of transfer cells - a process well documented in the Fe-deficient root tissues (Landsberg 1994),
25 but not yet completely understood. We hypothesize that an enhancement of vesicle trafficking towards to the PM
26 increases the apposition of organic compounds for cell wall synthesis. The second reason could be related to the
27 over-expression of the V-PPase gene observed in this study. We hypothesize that Fe deficiency can promote
28 exocytosis processes in the cell, enhancing vesicular traffic from the cytosol to the PM. The vesicles formed could
29 be characterized by the presence of V-PPase which could acidify the vacuolar lumen. Once the vesicles containing
30 protons reach the PM the fusion of these membranes could lead at the same time to a) the apoplastic

acidification assisting the PM H^+-ATPase activity and b) 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 putatively encode calmodulin

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 (Ikura and Ames 2006; Bouche *et al*. 2005). The over-expression of the transcript encoding for calmodulin in Fe-deficient root apexes has been observed as a polymorphic band in cDNA-AFLP (Fig. 2) and confirmed by sqRT-PCR analysis (Fig. 3A, supplemental Fig.1). Moreover, there are no papers about CaM induction under the condition of 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, the Fig. 3B showed that the transcript strongly increased about 1 day after the induction of the Fe deficiency condition, while the protein weakly accumulated after 1 and 3 days and just after 5 days of the removal of the Fe from the nutrient solution 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).

We aware that the identification of the precise role of these genes and their specific involvement under Fe deficiency needs further investigation.

References

Bachem, C.W.B., van der Hoeven, R.S., de Brujin, S.M., Vreugdenhil, D., Zabeau, M., Visser, R.G.F.: Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. - Plant J. **9:** 745-753, 1996.

Bauer, M.F., Hofmann, S., Neupert, W., Brunner, M.: Protein translocation into mitochondria: the role of TIM complexes. - Trends Cell. Biol. **10**: 25–31, 2000.

Beharav, A., Maras, M., Kitner, M., Šuštar-Vozlič, J., Sun, G.L., Doležalová, I., Lebeda, A., Meglič, V.: Comparison of three genetic similarity coefficients based on dominant markers from predominantly self-pollinating species. - Biol. Plant. **54**: 54-60, 2010.

Bouche, N., Yellin, A., Snedden, W.A., Fromm, H.: Plant-specific calmodulin-binding proteins. - Annu. Rev. Plant Biol. **56**, 435-466, 2005.

Bradford, M.M.: A rapid and sensitive method for the quantization of micrograms quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Breyne, P., Dreesen, R., Cannoot, B., Rombaut, D., Vandepoele, K., Rombauts, S., Vanderhaeghen, R., Inzé, D., Zabeau, M.: Quantitative cDNA-AFLP analysis for genome-wide expression studies. - Mol. Gen. Genom. **269**: 173–179, 2003.

Breyne, P., Zabeau, M.: Genome-wide expression analysis of plant cell cycle modulated genes. - Curr. Opin. Plant Biol. **4**: 136–142, 2001.

Buckhout, T.J., Yang, T.J., Schmidt, W.: Early iron-deficiency-induced transcriptional changes in Arabidopsis roots as revealed by microarray analyses. - BMC Genomics **10**: 147, 2009.

Colangelo, E.P., Guerinot, M.L.: The essential basic helix-loop-helix protein FIT1 is required for Fe deficiency response.- Plant Cell **16**: 3400-3412, 2004.

Cuesta, C., Ordás, R.J., Rodríguez, A. Fernández, B.: PCR-based molecular markers for assessment of somaclonal variation in *Pinus pinea* clones micropropagated *in vitro*. - Biol. Plant. **54**: 435-442, 2010.

Curie, C., Briat, J.F.: Iron transport and signalling in plants. Annu. Rev. Plant Biol. **54**: 183-206, 2003.

De Nisi, P., Zocchi, G.: Phosphoenolpyruvate carboxylase in cucumber (*Cucumis sativus* L.) roots under iron deficiency: activity and kinetic charaterisation. - J. Exp. Bot. **51**: 1903-1909, 2000.

Ditt, R.F., Nester, E.W., Comai, L.: Plant gene expression response to *Agrobacterium tumefacien*s. - Proc. Natl. Acad. Sci. USA. **98**: 10954-10959, 2001.

Donnini, S., Prinsi, B., Negri, A.S., Vigani, G., Espen, L., Zocchi, G.: Proteomic characterization of iron deficiency response in Cucumis sativus L. roots. - BMC Plant Biol. **10**: 268, 2010.

Endo, T., Yamamoto, H., Esaki, M.: Functional cooperation and separation of translocators in protein import into mitochondria, the double-membrane bounded organelles. - J. Cell. Sci. **116:** 3259-3267, 2003.

Espen, L., Dell'Orto, M., De Nisi, P., Zocchi, G.: Metabolic responses in cucumber (*Cucumis sativus* L.) root under Fe-deficiency: a 31NMR in vivo study. - Planta. **210**: 985-992, 2000.

Fairman, R., Wagner, J.: Structural studies of α-SNAP. - Biology 300ª Quarter 1 Biochemistry of Proteins & Nucleic Acids 2003.

Fukumura, R., Takahashi, H., Saito, T., Tsutsumi, T., Fujimori, A., Sato, S., Tatsumi, K., Araki, R., Abe, M.: A sensitive transcriptome analysis method that can detect unknown transcripts. - Nucl. Ac. Res. **31**: 94, 2003.

Hartings, H.: High resolution fingerprinting of transcribed genes by means of a modified cDNA-AFLP method. Short Communications. - Maydica*.* **44**: 179-181, 1999.

Ikura, M., Ames, J.B.: Genetic polymorphism and protein conformational plasticity in the calmodulin superfamily: two ways to promote multifunctionality. - Proc. Natl. Acad. Sci. USA. **103:**1159-1164, 2006.

Jensen, R., Dunn, C.: Protein import into and across the mitochondrial inner membrane: role of the TIM23 and TIM22 translocation. - Biochim. Biophys. Acta. **1592**: 25-34, 2002.

Kang, S.-Y., Lee, K.J., Lee, G.-J., Kim, J.-B., Chung, S.-J., Song, J.Y., Lee, B.-M., Kim, D.S.: Development of AFLP and STS markers linked to a waterlogging tolerance in Korean soybean landraces. - Biol. Plant. **54**: 61-68, 2010.

Koehler, C.M.: Protein translocation pathways of the mitochondrion. - FEBS Lett. **476**: 27-31, 2000.

Landsberg, E.C.: Function of rhizodermal transfer cells in the Fe stress response mechanism of *Capsicum annuum* L. - Plant Physiol. **82:** 511-517, 1986.

Landsberg, E.C.: Transfer cell formation in sugar beet roots induced by latent Fe deficiency. - Plant Soil. **165**: 197–205, 1994.

Li, J., Wu, X.D., Hao, S.T., Wang, X.J., Ling, H.Q.: Proteomic response to iron deficiency in tomato root. -Proteomics. **8**: 2299-311, 2008.

Lister, R., Chew, O., Lee, M.N., Heazlewood, J.L., Clifton, R., Parker, K., Millar, A.H., Whelan, J.: A transcriptomic and proteomic characterization of the arabidopsis mitochondrial protein import apparatus and its response to mitochondrial dysfunction. - Plant Physiol. **134:** 777-789, 2004.

López-Millán, A.F., Morales, F., Andaluz, S., Gogorcena, Y., Abadía, A., De Las Rivas, J., Abadía, J.: Responses of sugar beet roots to iron deficiency. Changes in carbon assimilation and oxygen use. - Plant Physiol. **124:** 885-897, 2000.

Maeshima, M.: Vacuolar H^+-pyrophosphatase. - Biochim. Biophys. Acta. **1465**: 37-51, 2000.

Marschner, H., Römheld, V., Ossenberg-Neuhaus, H.: Rapid method for measuring changes in pH and reducing process along roots of intact plants. - Zeit. Pflanz. Boden. **105**: 407-416, 1982.

O'Rourke, J.A., Charlson, D.V., Gonzalez, D.O., Vodkin, L.O., Graham, M.A., Cianzio, S.R., Grusak, M.A., Shoemaker, R.C.: Microarray analysis of iron deficiency chlorosis in near-isogenic soybean lines. - BMC Genomics. **8**: 476, 2007.

Ratajczak, R., Hinz, G., Robinson, D.G.: Localization of pyrophosphatase in membranes of cauliflower inflorescence cells. - Planta. **208:** 205-211, 1999.

Rehling, P., Pfanner, N., Meisinger, C.: Insertion of hydrophobic membrane proteins into the inner mitochondrial membrane-a guided tour. - J.Mol.Biol. **326**: 639-657, 2003.

1 Sikdar, B., Bhattacharya, M., Mukherjee, A., Banerjee, A., Ghosh, E., Ghosh, B., Roy, S.C.: Genetic diversity in
2 important members of *Cucurbitaceae* using isozyme, RAPD and ISSR markers. - Biol. Plant. **54:** 135-140, 2010.
3 Sojikula, P., Kongsawadworakula, P., Viboonjuna, U., Thaiprasitc, J., Intawong, B., Narangajavanaa, J., Svastid,
4 M.R.J.: AFLP-based transcript profiling for cassava genome-wide expression analysis in the onset of storage
5 root formation. - Physiol. Plant. **140**: 198-198, 2010
6 Thimm, O., Essigmann, B., Kloska, S., Altmann, T., Buckhout, T.J.: Response of arabidopsis to iron deficiency stress
7 as revealed by microarray analysis. - Plant Physiol. **127:** 1030–1043, 2001.
8 Uemura, T., Ueda, T., Ohniwa, R.L., Nakano, A., Takeyasu, K., Sato, M.H.: Systematic analysis of SNARE molecules in
9 Arabidopsis: dissection of the post-Golgi network in plant cells. - Cell Struct. Funct. **29**: 49-65, 2004.
10 Vigani, G., and Zocchi, G.: Effect of Fe deficiency on mitochondrial alternative NAD(P)H dehydrogenases in
11 cucumber roots. - J. P. Physiol. **167**: 666-669.
12 Vigani, G., Maffi, D., Zocchi, G.: Iron availability affects the function of mitochondria in cucumber roots. - New
13 Phytol. **182**: 127-136, 2009.
14 Vigani, G., Zocchi, G.: The fate and the role of mitochondria in Fe deficient roots of Strategy I plants. - Plant Signal.
15 Behav. **4**: 375-379, 2009.
16 Vuylsteke, M., Peleman, J.D., van Eijk, M.J.T.: AFLP-based transcript profiling (cDNA-AFLP) for genome-wide
17 expression analysis. - Nat. Prot. **2:** 1399-1413, 2007.
18 Waters, B.M., Lucena, C., Romera, F.J., Jester, G.G., Wynn, A.N., Rojas, C.L., Alcantara, E., Perez-Vicente, R.: Ethylene
19 involvement in the regulation of the H^+-ATPase CsHA1 gene and of the new isolated feric reductase CsFRO1
20 and iron transporter CsIRT1 genes in cucumber plants. - Plant Physiol. Biochem. **45**: 293-301, 2007.
21 Zocchi, G.: Metabolic changes in iron-stressed dicotyledonous plants. In: Barton, L.L., Abadía, J., (ed.): Iron
22 Nutrition in Plants and Rhizospheric Microorganisms. Pp 359-370. Dordrecht - The Netherlands - Springer
23 2006.
24