

Alteration of plasma membrane H⁺-ATPase in cucumber roots under different iron nutrition

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Abstract The strategy I developed by dicotyledonous plants to efficiently acquire iron involve the action of proton-extruding H⁺-ATPases. These proteins are responsible for the solubilization of iron through rhizosphere acidification. Previously, it has been documented that acidification of the rhizosphere involve specific isoforms of plasma membrane proton pumps in response to Fe deficiency in dicot plants such as *Arabidopsis*, *Cucumis sativus*, *Lycopersicon* and *Pisum sativum*. In this work, we have investigated the effect of Fe nutritional status on the activity of plasma membrane H⁺-ATPases at the level of transcription, protein accumulation and post-translational modification. Two genes (*CsHA2* and *CsHA3*) were isolated from different parts of cucumber and their expression analyzed under different Fe treatments (0, 25 and 80 μM Fe). Transcript level of *CsHA2* was detected in both vegetative organs (roots and cotyledons), while *CsHA3* expression was limited only to the roots in immature plants. These two genes were up-regulated under Fe deficiency in cucumber roots and their expression was decreased in the presence of 80 μM Fe. We have shown that the post-translational modification of protein via phosphorylation and its accumulation as a target of activation in Fe-deficient plants might not be responsible for the increase in the H⁺-ATPase activity.

Keywords Cucumber · Iron nutrition · Proton plasma membrane pump · Roots

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Introduction

Iron is an essential micronutrient for all living organisms. It is required for life-sustaining processes such as photosynthesis, respiration, nitrogen metabolism, hormone biosynthesis and pathogen defense. Due to easy transition of oxidation state (Fe³⁺/Fe²⁺), iron has the ability to gain and lose an electron, and serves as a cofactor of redox reactions in many fundamental metabolic pathways (Marschner 1995). Iron deficiency, beside nitrogen and phosphorus, is a limiting factor for yield in agricultural soils. The content of iron in plants is relatively high in comparison to other microelements. Low iron uptake results in leaf chlorosis and reduced plant growth. On the other hand, iron excess is toxic for plants, because Fe participates in the Fenton reaction and promotes the formation of reactive hydroxyl radicals, which may cause damage to DNA, lipids and proteins, leading to oxidative stress and even to cell death (Brumbarova and Bauer 2008). Plants exposed to excessive levels of Fe show leaf bronzing and extensive tissue necrosis (Jeong and Guerinot 2009).

Despite 5 % Fe abundance at the Earth's crust, the concentration of soluble iron is relatively low in soil. In oxygenated environments, iron is mainly present as oxyhydrates with low bioavailability (Brumbarova and Bauer 2008).

Roots, especially the rhizodermis, play an important role in the uptake of nutrients from soil as well as in translocation of these nutrients to other parts of the plant (Palmgren 2001). It has been evidenced that in response to nutrient starvation some plant species radically modify the root system through changes in their architecture (hair length and density of lateral roots) as well as morphology (formation of specific transfer cells in the root epidermis). Such modifications distinctly increase the absorptive

surface area of the root system (Schikora and Schmidt 2002). Since one of the main enzymes regulating growth is plasma membrane H^+ -ATPases (“acid” growth theory), it could be postulated that they play a role in root adaptation strategies, such as increase of primary root length, initiation and elongation of lateral roots and formation of root hairs, which allow effective intake of mineral nutrients in response to fluctuation of their availability (Hodge 2004). It was found that under low concentration of Fe, the increase of root hair length and number of transfer cells are positively correlated with the amount of immunologically detectable H^+ -ATPases. These morphological and physiological responses in roots under Fe starvation might be regulated by ethylene and/or auxin signaling (Schmidt et al. 2003). Activation of plasma membrane proton pumps in the root cells is known as one of the mechanisms by which plants employing strategy I increase their capacity to acquire iron from poor soil (Kim and Guerinot 2007; Zocchi 2006). Acidification of the rhizosphere mediated by H^+ -ATPases is important for iron nutrition, because it leads to increasing solubility of ferric Fe (Walker and Connolly 2008; Ling et al. 2009). Moreover at low pH, the ferric chelate reductase reducing Fe^{3+} to Fe^{2+} is activated and an electrochemical gradient, which can be used to for iron uptake from soil, is generated. Nongraminaceous plants, such as model *Arabidopsis thaliana* and *Cucumis sativus*, use strategy I in which upon iron deficiency three proteins localized at the plasma membrane (PM) of roots are induced: proton pump, ferric chelate reductase and high-affinity transporters (Walker and Connolly 2008). It has been demonstrated that under Fe deficiency, dicotyledonous plants pump protons outside into the rhizosphere, lowering pH and increasing the solubility of iron. In this process H^+ -ATPases are involved, catalyzing the transport of protons from the cytosol across the plasma membrane (Dell’Orto et al. 2000). Furthermore, it has been shown that in Fe-limiting conditions, the activity of H^+ -ATPases and the steady-state level of the enzyme at the whole root are strongly increased. In this context Santi et al. (2005) reported that the activation of the plasma membrane proton pump of cucumber roots occurs at the transcriptional level in response to Fe deficiency. Expression analysis of *CsHA1* and *CsHA2* genes in cucumber plants showed that these two isoforms respond in a different way to Fe deprivation. Namely, in Fe-deficient roots enhanced accumulation of *CsHA1* transcript was observed, while *CsHA2* appeared to be unaffected by iron status. Enhanced transcription of *CsHA1* corresponded to an increase in the ATP-hydrolyzing activity and in a higher amount of H^+ -ATPase protein.

In addition, plasma membrane proton pumps are encoded by a multigene family and their protein products are regulated by post-translational modification. It is strongly

established that H^+ -ATPases might be regulated by reverse phosphorylation, resulting in enzyme activation or inhibition (Morsomme and Boutry 2000; Duby and Boutry 2008). This mechanism involves phosphorylation of the penultimate residue threonine (Thr) on the C-terminus region of H^+ -ATPase and is essential for the binding of 14-3-3 proteins, which modulate the activity of that enzyme (Arango et al. 2003).

The next response after solubilisation of Fe is enhanced Fe^{3+} reduction to Fe^{2+} , which is crucial for its absorption. Ferric reduction is catalyzed by the membrane-bound ferric oxidoreductase (FRO) and the plasma membrane proton pump ensures an optimal pH for the activity of this enzyme (Schikora and Schmidt 2002).

Finally Fe is transported across the plasma membrane into the root cells by iron-regulated transporters (IRT) belonging to the ZIP (ZRT, IRT-like) transporter family. Putative *FRO* genes and IRT orthologs have been identified in a number of plants (tomato, cucumber, pea, tobacco) (Jeong and Connolly 2009; Waters et al. 2007; Enomoto et al. 2007). Expression of these genes is up-regulated under Fe deficiency and is controlled by a transcription factor named FER in tomato and FIT1 in *Arabidopsis* (Fe-induced transcription factor 1) (Bauer et al. 2007). FIT1 has a characteristic motif bHLH (basic helix-loop-helix) and as a heterodimer together with the transcription factor bHLH 38 or bHLH 39 induces expression of IRT and FRO in the iron deficiency response (Walker and Connolly 2008).

Limited information is also available about the regulatory aspects of activity of plasma membrane proton pumps in contribution to iron status. Recently, one isoform of H^+ -ATPase (*CsHA1*) responsible for rhizosphere acidification under iron deficiency was identified in cucumber (Santi et al. 2005; Santi and Schmidt 2008). We identified two other genes (*CsHA2* and *CsHA3*) encoding plasma membrane proton pump in cucumber (Młodzińska et al. 2010). The *CsHA2* and *CsHA3* cDNAs encode 954 and 955 amino acid polypeptides, respectively, with calculated molecular masses of about 105 kDa. Both proteins show high similarity (about 95 %) to each other. In addition, the identification of full-length cDNA of the *CsHA2* and *CsHA3* genes allowed us to test whether both genes have a similar response to iron. There are only a few examples of the direct effect of mineral nutrition on the regulation of H^+ -ATPases activity. The aim of this work was to explain mechanisms of the alteration in the H^+ -ATPase activity at the transcriptional and posttranscriptional levels in cucumber plants under different iron regimes. Cucumber is often used as a model plant in studies on mineral nutrition, especially in iron-deficiency responses, because it is one of the most active acidifying species (Zocchi 2006). Moreover, the genome of cucumber has been recently sequenced and published (Huang et al. 2009).

Materials and methods

Plant growth

Cucumber seeds (*Cucumis sativus* L., var. Krak F1) germinated 48 h in darkness were transferred to the nutrient solution with following composition (mM): 1.7 KNO₃; 1.7 Ca(NO₃)₂; 0.33 KH₂PO₄; 0.33 MgSO₄, and microelements (μM) 10 MnSO₄ × 5H₂O; 5 H₃BO₄; 1 CuSO₄ × 5 H₂O; 0.01 ZnSO₄ × 7H₂O; 0.05 Na₂MoO₄ × 2H₂O (pH 6.5). Fe(III) EDTA was added at different concentration (0 μM—Fe deficiency, 25 and 80 μM) depending on the experiments. The pH was adjusted to 6.5 with 1 N NaOH and maintained during the entire cultivation time. Plants were grown hydroponically in the growth chamber for 5 days under 16 h photoperiod (180 μmol m⁻² s⁻¹) at 25 °C during the day and 22 °C during the night.

Isolation of plasma membranes

Plasma membranes (PM) were isolated from 5-day-old cucumber roots by two-phase partitioning according to the procedure by Larsson (1985) modified by Kłobus (1995). Finally, membrane pellet was resuspended in 5 mM BTP–MES (BTP-1,3 bis[tris](hydroxymethyl)methylaminopropane; MES-morpholineethanesulfonic acid) pH 7.5 containing 5 mM KCl, 0.1 mM EDTA and 330 mM sorbitol. The plasma membranes obtained by this method were well purified and right-side out oriented. The purity of plasma membranes was estimated on the basis of the activities of marker enzymes described previously by Kłobus (1995) and Burzyński et al. (2005). The activity of the marker enzymes of other membranes after phase partitioning was significantly lower than the activity of plasma membrane H⁺-ATPase and thus confirmed the purity of plasma membrane fraction.

ATP hydrolysis

ATPase activity of the vanadate sensitive H⁺-ATPase was determined using the procedure of Gallagher and Leonard (1982). The reaction mixture contained 50 μg protein (plasma membrane), 33 mM Tris–Mes (pH 7.5), 3 mM ATP, 2.5 mM MgSO₄, 50 mM KCl, 1 mM NaN₃, 0.1 mM Na₂MoO₄, 200 μM Na₃O₄ and 0.02 % Triton X-100. H⁺-ATPase activity was determined by measuring the release of Pi according to Ames (1966). H⁺-ATPase activity was expressed as the difference between the activity measured in the absence and presence of Na₃VO₄.

H⁺ pumping measurement

Part of the vesicles of plasma membrane was turned to the inside-out oriented form by using Brij 58 according to the

method of Kłobus and Buczek (1995) and used for measurements of the ATP-dependent proton transport. H⁺ transport activity was assayed as a decrease in the absorbance of acridine orange (A₄₉₅ nm) in medium containing 50 μg plasma membrane, 25 mM BTP–MES pH 7.5, 330 mM sorbitol, 50 mM KCl, 0.1 % BSA, 10 μM acridine orange and 0.05 % Brij 58.

Protein analysis

Protein content was determined using BSA as a standard in the presence of 0.02 % Triton X-100 (Bradford 1976).

Western blot analysis

Plasma membranes (10 μg of proteins) were incubated in 2 % (w/v) SDS, 80 mM DTT, 40 % (w/v) glycerol, 5 mM PMSF, 10 mM Tris, 1 mM EDTA and 0.05 % (w/v) bromophenol blue for 30 min at room temperature and separated on 7.5 % SDS-polyacrylamide gels (Laemmli 1970). After 1 h of electrophoresis (25 mA), proteins were electrotransferred (60 V, 200 mA) for 1.5 h to nitrocellulose using a SIGMA-ALDRICH SV10-EB10 blotting apparatus. Transfer buffer contained 25 mM Tris, 150 mM Gly and 10 % (v/v) methanol.

To identify the plasma membrane H⁺-ATPase, the blots were incubated overnight (8 °C) with polyclonal antibodies against plasma membrane H⁺-ATPase (46E5B11D) kindly provided by W. Michalke (Universität Freiburg, Germany). The antiserum was diluted 2,000-fold. After repeated washing, the nitrocellulose membrane was incubated at room temperature for 1 h with 1:4,000 diluted secondary antibody (anti-mouse, conjugated to horseradish peroxidase, Sigma-Aldrich) and visualized by staining with DAB (3,3'-diaminobenzidine tetrahydrochloride hydrate).

Phosphorylation of plasma membrane H⁺-ATPase was detected with the antibody against phosphothreonine (rabbit polyclonal to phosphothreonine, Abcam) used at concentration of 2 μg/ml after overnight incubation (8 °C). The membranes were rinsed and incubated for 1 h at room temperature with 5,000-fold secondary antibody conjugated to horseradish peroxidase (goat polyclonal to rabbit IgG, Abcam). The results were visualized by staining with DAB.

Analysis of genes expression

Total RNA was isolated from 50 mg of cotyledons and roots with Tri Reagent (Sigma). The concentration and the purity of isolated RNA were determined spectrophotometrically measuring the absorbance at 260 and 280 nm.

The expression levels of *CsHA2* and *CsHA3* in different parts of cucumber were determined with semi-quantitative

RT-PCR (Titan One Tube RT-PCR System, Roche, Germany) with specific primers. As a template in RT-PCR reaction, 0.150 μg RNA was used. A 263-bp long fragment of *CsHA2* was amplified with two specific primers: 5'-ACC CGAGTCGACAAACATCT-3' (forward) and 5'-CTTGG CACAGCAAAGTGAAA-3' (reverse) (Santi et al. 2005). For amplification of 393-bp long region of the *CsHA3* 5'AAGTTTCTGGGGTTCATGTGGAAT-3' (forward) 5'GTAACAGGAAGTGACTCTCCAGTC-3' (reverse) were used as the primers. As an internal standard, 18S RNA gene was used. RT-PCR reactions were performed with T_m at 57 °C and 22 cycles for *CsHA2* and 18 cycles for *CsHA3*, which corresponded to the log-linear phase of the amplification reaction. For the cDNA of internal standard 9 cycles were chosen. The products were visualized on ethidium bromide-containing 1.5 % agarose gels and analyzed using the BioCapt version 99 software.

Measurement of iron content

Content of iron ions was determined spectrophotometrically (atomic absorption spectrophotometer Perkin-Elmer 3300) in dry root and cotyledon tissues digested with concentrated HNO_3 in microwave system (Zou et al. 2001).

Assay of lipid peroxidation

The lipid peroxidation was measured as generation of thiobarbituric acid-reactive substances (TBARS) according to method of Sairam et al. (2002). The mixture containing 0.5 % TBA solution in 20 % TCA and PM fraction was heated at 95 °C for 30 min and then cooled in ice before centrifugation. The absorbance of the supernatant was measured at 532 nm.

Results

Effect of Fe treatment on the activity of the PM H^+ -ATPase

Fe nutrition of cucumber seedlings has an apparent effect on both the hydrolytic and transporting activities of the plasma membrane H^+ -ATPase. Data were calculated as percentage of the 25 μM Fe treatment and 100 % of PM H^+ -ATPase activity was 78.52 $\mu\text{mol Pi mg}^{-1} \text{protein h}^{-1}$. In membranes isolated from Fe-deficient cucumber roots, VO_4 -inhibited ATP hydrolysis was enhanced by almost 20 % compared to plants supplied with 25 μM Fe, whereas treatment of cucumbers with 80 μM Fe has no significant effect on it (Fig. 1). Similar results were obtained for measurements of ATP-dependent proton pumping in plasma membranes isolated from roots of plants growing in

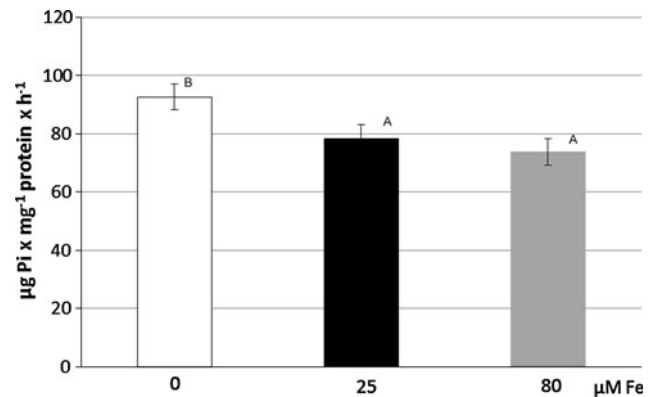


Fig. 1 Effect of Fe on the hydrolytic activity of H^+ -ATPases in plasma membranes isolated from cucumber roots treated for 5 days with different concentration of iron: Fe-free nutrient solution (white bar), 25 μM Fe (black bar) and 80 μM Fe (grey bar). The reaction mixture contained 50 μg plasma membrane protein and hydrolytic activity was measured in the absence or the presence of vanadate (a specific inhibitor of the PM H^+ -ATPases). Data were analyzed by performing ANOVA and Dunnett's test using Statistica 10 software to determine the differences between treatments and control. Means with the same letter are not significantly different ($P < 0.05$)

different Fe treatments (Fig. 2). Fe deficiency markedly increased the rate of proton extrusion (about 30 % compared with control), while the transport of H^+ in vesicles isolated from Fe-sufficient plants (80 μM Fe in nutrient solution) was only slightly reduced (94 % of control).

Effect of Fe treatment on *CsHA2* and *CsHA3* genes expression

Fe deficiency of cucumbers dramatically increased expression level of *CsHA2* (193 %) in comparison to seedlings treated with 25 μM concentration of Fe, but not in cotyledons (about 20 % increase). In contrast, the presence of 80 μM Fe in nutrient solution strongly decreased the level of specific *CsHA2* mRNA in roots and had no effect on it in cotyledons (Fig. 3a). The Fe-dependent pattern of *CsHA3* expression in roots of cucumber was similar: huge induction of gene expression under Fe deficiency and downregulation at high external Fe concentration. Moreover, it was also noted that the *CsHA3* was not expressed at all in cotyledons (Fig. 3b).

Western blotting analysis of PM H^+ -ATPase

Because iron is well known as an accelerator in the generation of reactive oxygen species (ROS), which can damage various proteins directly, we determined the amount of PM H^+ -ATPase in the absence (0 μM Fe) and in the presence of Fe (25 and 80 μM).

Western blotting analysis using antibodies raised against PM H^+ -ATPase (46E5B11D) typically showed a

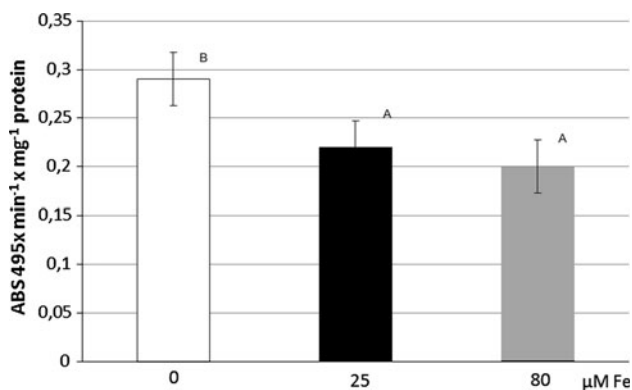


Fig. 2 Effect of Fe on the ATP-dependent proton transport activity measured in plasma membrane vesicles isolated from cucumber roots treated for 5 days with different concentration of iron: without Fe (white bar), 25 μM Fe (black bar) and 80 μM Fe (grey bar). The formation of ΔpH gradients in the vesicles were monitored as the changes in acridine orange absorbance (A₄₉₅ nm). Data were analyzed by performing ANOVA and Dunnett’s test using Statistica 10 software to determine the differences between treatments and control. Means with the same letter are not significantly different (*P* < 0.05)

H⁺-ATPase band and revealed a similar steady-state level of this protein in both Fe-sufficient and Fe-deficient plants (Fig. 4a). Protein blot analysis of plasma membranes isolated from roots exposed to different concentration of Fe with polyclonal antibodies against phosphothreonine revealed no differences between the enzyme phosphorylation under different Fe-feeding conditions (Fig. 4b).

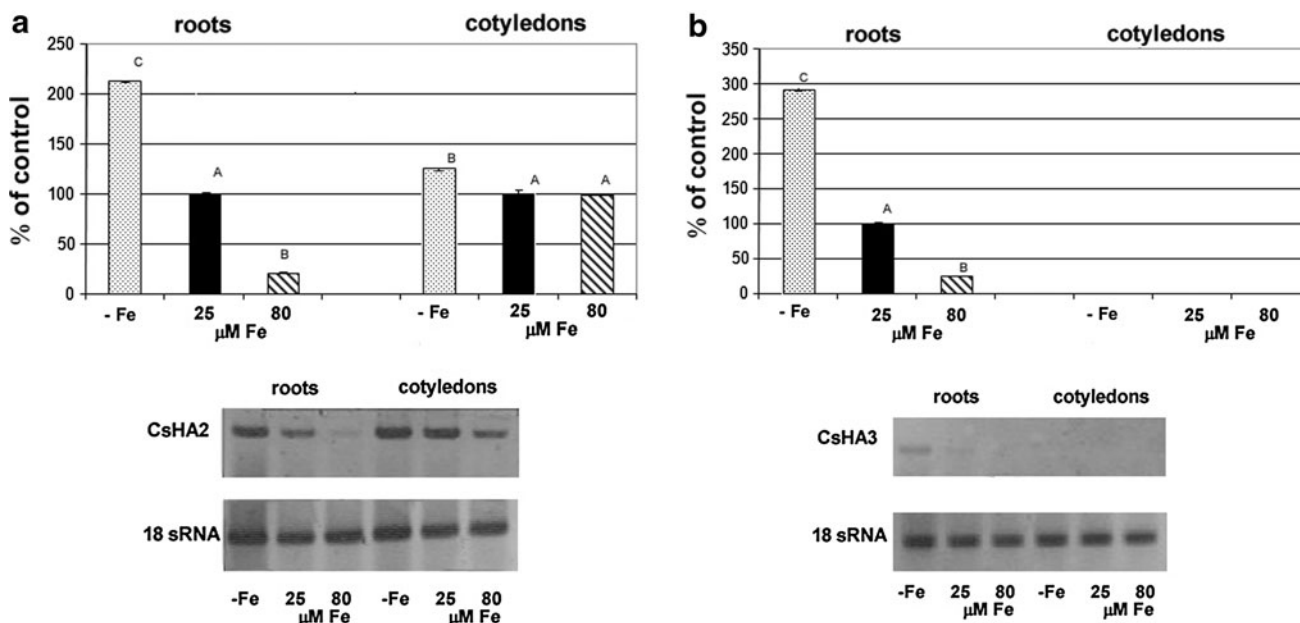


Fig. 3 Semi-quantitative RT-PCR analysis of CsHA2 (a) and CsHA3 (b) expression in cucumbers growing in nutrient solution without or with different Fe concentrations. Total RNA was isolated from cotyledons and roots untreated (–Fe) and treated for 5 days with 25 and 80 μM Fe. To evaluate the expression of PM H⁺-ATPase the 18S RNA was used as an internal standard. RT-PCR results are presented

Total iron content

The total iron content was determined in roots and cotyledons of cucumber seedlings after 5 days of growth under different iron nutritional status. The plants with 25 μM Fe in nutrient solution accumulated 0.142 mg of Fe per g of root dry weight and 0.088 mg of Fe per g of cotyledon dry weight. The iron-deficient seedlings contained 0.117 mg Fe g⁻¹ dry weight and 0.072 mg Fe g⁻¹ dry weight in roots and cotyledons, respectively. When 80 μM Fe was applied into nutrient solution the iron content increased to 0.176 or 0.114 mg per g⁻¹ dry weight in roots and cotyledons, respectively (Fig. 5).

Lipid peroxidation

The level of lipid peroxidation measured as the content of thiobarbituric acid-reactive substances (TBARS) was similar in plasma membranes isolated from cucumber roots growing in all Fe conditions (Fig. 6).

Discussion

It was previously demonstrated that the activity of H⁺-ATPase was induced by Fe deficiency in tomato, cucumber and Arabidopsis plants (Schmidt et al. 2003;

as the gel image and as a ratio of of PM H⁺-ATPase gene signal to the 18S RNA gene. All experiments were performed three times independently with comparable results. Data were analyzed by performing ANOVA and Dunnett’s test using Statistica 10 software to determine the differences between treatments and control. Means with the same letter are not significantly different (*P* < 0.05)

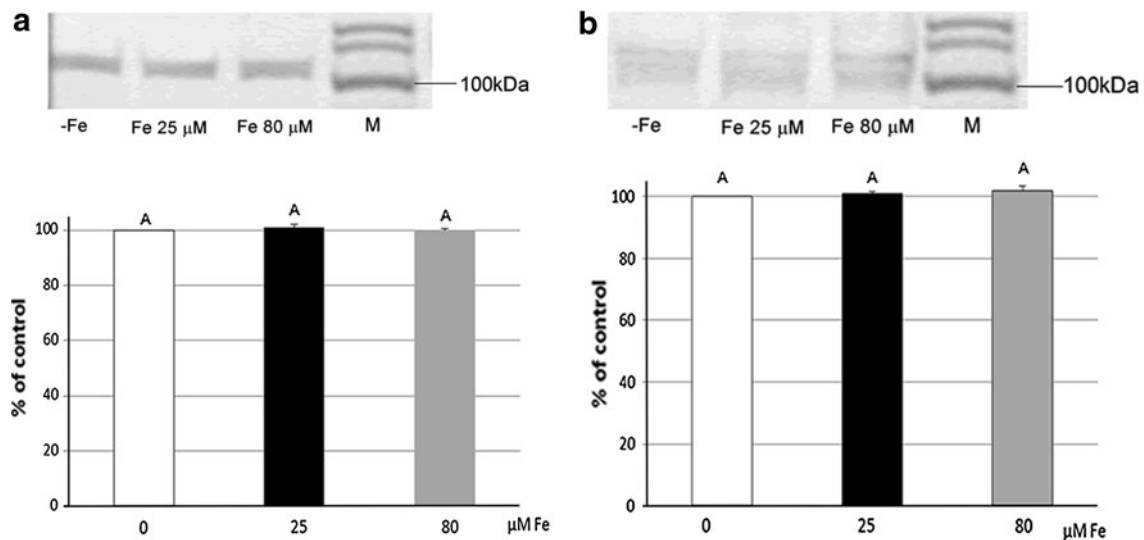


Fig. 4 Western blotting analysis of plasma membranes isolated from cucumber roots treated with different concentrations of Fe. Plasma membranes were isolated from cucumber roots exposed to 0 (–Fe), 25 or 80 μM Fe for 5 days. Immunostaining was performed using PM H^+ -ATPase antiserum 46E5B11D (a) or specific polyclonal antibodies against phosphothreonine residues (b). The H^+ -ATPases were

immunodetected with. Identical amounts (10 μg) total protein were applied in each lane. *M* protein marker. Data were analyzed by performing ANOVA and Dunnett's test using Statistica 10 software to determine the differences between treatments and control. Means with the same letter are not significantly different ($P < 0.05$)

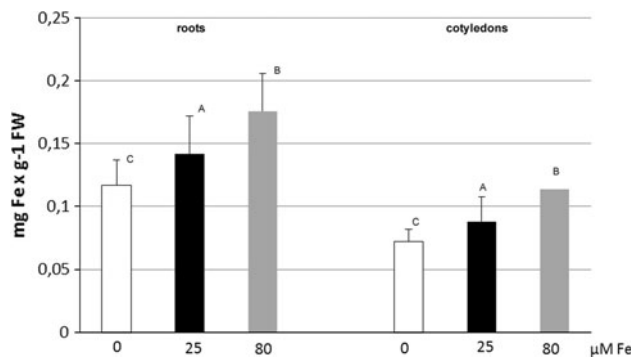


Fig. 5 Total iron content in cucumber roots and cotyledons growing in nutrient solution without or with different Fe concentrations. Data are the mean \pm SD of three replicates. Total Fe in 25 μM Fe treatment = 100 %

Rabotti and Zocchi 1994; Dell'Orto et al. 2000; Colangelo and Gueriot 2004). We also observed a slight induction in both hydrolytic and proton extrusion activity of H^+ -ATPase in membranes isolated from cucumber roots under Fe deficiency (Figs. 1, 2). This activation can result from alteration of the expression of enzyme-encoding genes. The plasma membrane proton pumps are encoded by a large family of genes differing in tissue distribution, regulation and expression level. At least 11 genes encoding H^+ -ATPases have been identified in *Arabidopsis thaliana*, 10 in *Oryza sativa*, 9 in *Nicotiana plumbaginifolia*, 8 in *Lycopersicon esculentum* and 4 in *Zea mays* (database at <http://aramemnon.uni-koeln.de/>). Modulation of activity of specific PM H^+ -ATPase isoforms by nutritional status was

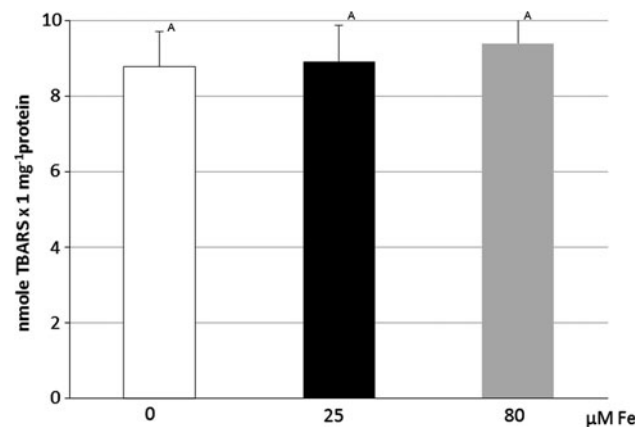


Fig. 6 Effect of iron on lipid peroxidation of plasma membranes isolated from cucumber roots treated with different concentrations of Fe. The lipid peroxidation was expressed as nmol TBARS \times 1 mg^{-1} protein using an extinction coefficient of 155 $\text{mM}^{-1} \text{cm}^{-1}$. Data were analyzed by performing ANOVA and Dunnett's test using Statistica 10 software to determine the differences between treatments and control. Means with the same letter are not significantly different ($P < 0.05$)

described earlier for maize (Santi et al. 2003), tomato (Schmidt et al. 2003) and cucumber roots (Waters et al. 2007). Several of them are suggested to be involved in lowering the pH of the soil, for example AHA2 and AHA7 in *Arabidopsis thaliana* (Fox and Gueriot 1998) or CsHA1 in *Cucumis sativus* (Santi et al. 2005). It has been well evidenced that in *Arabidopsis*, AHA2 showed high expression in roots in epidermal cells, including root hairs (Fuglsang et al. 2007; Haruta et al. 2010). Such localization

of AHA2 can suggest its potential function in nutrient uptake as well as in long-term transport in plants. Data obtained by Colangelo and Guerinot (2004) also suggest that AHA7 is involved in nutrient acquisition by *A. thaliana*. These authors found first upregulation of AHA7 under iron deficiency and then direct dependency of its expression on FIT1 (Fe deficiency induced transcription factor). On that basis, they suggested that AHA7 contributes to strategy I developed by plants to solubilize and transport iron into root cells.

In order to analyze whether externally added Fe can modify the expression of genes *CsHA2* and *CsHA3* encoding two isoforms of PM H⁺-ATPase, the level of specific transcripts was determined using the RT-PCR method. RT-PCR analysis demonstrated that at least one of them, namely the *CsHA3* gene, may also be involved in nutrient acquisition. This gene was expressed exclusively in roots in a high extent and its transcript was not detected in cotyledons. We also noted that *CsHA3* shows 88 % identity to the H⁺-ATPase AHA2 in *Arabidopsis thaliana*, which is the iron deficiency-inducible isoform. The transcript of the second gene, *CsHA2*, was found both in roots and cotyledons of young cucumber seedlings (Fig. 3a) as well as in lateral roots, shoots and leaves of mature cucumbers (Młodzińska et al. 2010). Such a strong signal of the *CsHA2* and *CsHA3* transcript in roots suggests a possible role for these isoforms of plasma membrane ATPases in response to iron limitation. In this context we suppose that under Fe deficiency protons are exuded into the rhizosphere, most likely by the *CsHA2* and *CsHA3* H⁺-ATPases. Moreover at low pH, the ferric chelate reductase reducing Fe³⁺ to Fe²⁺ is activated and electrochemical gradient, which can be used for iron uptake from soil, is generated. Some authors correlated the increase in the acidification with the development of lateral roots and transfer cells which enhances the absorptive surface of the roots (Schmidt et al. 2003). It is likely that the formation of new organs such as extra root hairs and lateral roots need to be supported by a higher proton pumps activity. However, these morphological changes in root architecture as well as physiological responses in iron-limited conditions could be different among species or genotypes. For instance, H⁺-ATPase activity in soybean roots was not correspondingly affected by Fe treatments (Zocchi et al. 2007).

In contrast, the expression of *CsHA2* and *CsHA3* genes encoding the plasma membrane proton pumps in roots dramatically decreased at 80 μM Fe (Fig. 3a, b). When iron accumulates in excess within plant cells, it can interact with hydrogen peroxide to form hydroxyl radicals and they may cause damage to DNA and proteins. Therefore, it could be possible that we observed diminished expression of *CsHA2* and *CsHA3* in roots by treatment with higher concentration of Fe. These results suggest that the

regulation of these two genes is related to the iron status. The transcripts' abundance was enhanced greatly under Fe starvation in cucumber roots, but decreased when plants were grown in a high concentration of iron. Such a broad tissue expression profile of *CsHA2* might suggest its involvement in Fe distribution through the plant rather than in its uptake from soil solution. Despite tremendous activation of *CsHA2* and *CsHA3* expression in roots of Fe-starved plants, the amount of enzyme protein was unchanged under different Fe treatments (Fig. 4a). There are at least two obvious explanations of this discrepancy: (1) usage of polyclonal antibodies instead of monoclonal antibodies raised against a specific isoform, or (2) post-translational degradation of transcripts. Higher activity of H⁺-ATPase in plasma membranes isolated from Fe-starved roots (Figs. 1, 2) indicates that the first explanation is the most likely.

It has been known for a long time that activation of H⁺-ATPase can be achieved by reverse protein phosphorylation. Our results of an immunoblotting analysis using polyclonal antiserum against phosphothreonine revealed the presence of phosphorylated residues in 100 kDa bands. It was previously documented that the plant plasma membrane H⁺-ATPase is an unusual target in that a unique phosphothreonine motif interacts with the binding of 14-3-3 protein. Whereas the most plasma membrane proteins are phosphorylated in serine residues, the marker of plasma membrane H⁺-ATPases and other few plasma membrane targets depend mainly on phosphorylation of threonine residues (Borch et al. 2002). The size of the band after western blot for phosphothreonine antibodies correspond to the band with monoclonal antibodies against H⁺-ATPases (100 kDa). It might indicate that the phosphothreonine antibodies recognized phosphorylated plasma membrane proton pumps. However, no significant differences in plasma membrane protein phosphorylation due to Fe nutrition were observed (Fig. 4b). Therefore, the post-translational modification of protein via phosphorylation as a target of activation by Fe deficiency stress cannot be responsible for the increase of H⁺-ATPase activity. Nevertheless, regulation of the H⁺ pumps by the phosphorylation was also found in relation to the transport of other ions across the plasma membrane in roots (Shen et al. 2006; Janicka-Russak and Klobus 2007).

Our results indicated that Fe content was remarkably greater in cucumber roots and cotyledons treated with 25 and 80 μM Fe than in Fe-deficient plants (Fig. 5). The increase was the highest in these two organs when plants were exposed to 80 μM Fe, reaching almost 140 % in comparison with seedlings without Fe. If iron is taken up in excess, toxic oxygen radicals can form and damage of the cell membrane system, especially the plasma membrane, can occur (Jeong and Connolly 2009). Therefore, we

examined the possible involvement of lipid peroxidation in the iron-mediated alteration of H^+ -ATPase activity when plants are grown in Fe sufficiency. However, the obtained results suggested that inhibition of hydrolytic activity and proton transport in cucumber roots treated with 80 μ M Fe might not be a consequence of lipid peroxidation (Fig. 6). These data leave open the possibility that other factors at the post-transcriptional stage might regulate the activity of H^+ -ATPase in cucumber roots to avoid excessive Fe absorption.

Taken together, our data provide evidence that the modulation of activity of plasma membrane proton pumps by the Fe signal occurs at the gene expression level. We have shown that the *CsHA3* gene exclusively expressed in roots of cucumber seedlings was upregulated by Fe deficiency, whereas *CsHA2* was expressed in both cotyledons and roots. However, a strong signal of the *CsHA2* transcript was also observed in response to Fe shortage. It will also be worth carrying out further work to elucidate whether other isoforms of ATPases in cucumber play a potential role in response to iron deficiency.

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