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Two plasma membrane H^+-ATPase genes are differentially expressed in iron-deficient cucumber plants

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Abstract

Aim of the present work was to investigate the involvement of plasma membrane (PM) H^+-ATPase (E.C. 3.6.3.6) isoforms of cucumber (*Cucumis sativus* L.) in the response to Fe deficiency. Two PM H^+-ATPase cDNAs (*CsHA1* and *CsHA2*) were isolated from cucumber and their expression analysed as a function of Fe nutritional status. Semi-quantitative reverse transcriptase (RT)-PCR and quantitative real-time RT-PCR revealed in Fe-deficient roots an enhanced accumulation of *CsHA1* gene transcripts, which were hardly detectable in leaves. Supply of iron to deficient plants caused a decrease in the transcript level of *CsHA1*. In contrast, *CsHA2* transcripts, detected both in roots and leaves, appeared to be unaffected by Fe. This work shows for the first time that a transcriptional regulation of PM H^+-ATPase involving a specific isoform occurs in the response to Fe deficiency.
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Keywords: Gene expression; H^+-ATPase; Fe deficiency; Plasma membrane; Real-time PCR; Reverse transcriptase (RT)-PCR; Rhizosphere acidification

1. Introduction

One of the mechanisms through which non-graminaceous plants (Strategy I plants) respond to Fe deprivation is a localised decrease of the root external pH. Acidification of the rhizosphere helps to mobilise sparingly soluble Fe that is otherwise not available to the plant. Once Fe solubility is increased, Fe(III)-chelates are reduced by a specific root reductase, and then Fe^{2+} is transported across the root plasma membrane (PM) via Fe^{2+} transporters [6]. Induction of root Fe(III)-reductase activity is necessary for Fe uptake under Fe deficiency, and can be uncoupled from acidifying capacity [31]. Iron uptake is enhanced during Fe deficiency through the expression of a Fe^{2+} transporter in the epidermis of roots [28]. Both reduction and uptake activities are regulated in *Arabidopsis* at the level of transcription and protein accumulation, indicating a close coupling [5].

Fe deficiency-induced lowering of the pH has been attributed to an activation of the root PM H^+-ATPase, as evidenced by increased activity and steady-state level of the enzyme at the whole root level [7], and by co-localisation of intense immunolabelling of the H^+-ATPase protein and proton extrusion at the sub-apical root zones of Fe-deficient plants [8,24]. The proton pump is responsible for the trans-membrane electrochemical gradient [18] and is abundant in transfer cells, which are the most specialised cell type for membrane transport [24]. Beside solubilisation of Fe hydroxide species, PM H^+-ATPase activity ensures an optimal pH for the activity of the Fe(III)-chelate reductase [21].

Limited information is available regarding the regulatory aspects of Fe stress-induced H^+-ATPase activity. PM H^+-ATPase is encoded by a multigene family and subjected to a multilevel regulation [18]. Evidence has been presented for a role of specific PM H^+-ATPase genes (*HA* genes) in response to various environmental stimuli, such as salt stress [29], mechanical stress [17], immersion [13], and changes in nitrate supply [22]. Whether Fe shortage induces expression of a specific PM H^+-ATPase isoform has not been elucidated until now. Here we show that Fe deficiency-induced responses involve the modulation at the transcriptional level of a specific H^+-ATPase gene in cucumber roots.

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2. Results

2.1. Isolation of CsHA partial cDNAs

To investigate the expression of PM H^+-ATPase isoforms in response to Fe deficiency, two partial cDNAs corresponding to two novel *HA* genes were isolated from cucumber roots. A forward degenerate oligonucleotide was used in 3′RACE experiments to amplify a partial cDNA of this gene. Two cDNAs were thus obtained: one 1207 bp-long and a second 1074 bp in size, which were named *CsHA1* and *CsHA2*, respectively, and submitted to EMBL database under accession No. AJ703810 (*HA1* gene) and AJ703811 (*HA2* gene). Fig. 1 shows the alignment between the deduced amino acid sequences of *CsHA1*, *CsHA2* and the corresponding regions of bean *BHA1* [4], *Arabidopsis AHA2* [12], tobacco *PMA4* [14], and two maize genes, *MHA2* and *MHA4* [10,22]. All these isoforms have also been shown to be localised at the

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AHA2      640  QRMKNYTIYAVSITIRIVFGFMLIALIWEFDFSAFMVLIIAILNDGTIMTISKDRVKPSP
PMA4      642  QRMKNYTIYAVSITIRIVFGFMFIALIWKYDFSAFMVLIIAILNDGTIMTISKDRVKPSP
BHA-1     641  QRMKNYTIYAVSITIRIVFGFLFIALIWKFDFAPFMVLIIAILNDGTIMTISKDRVKPSP
CsHA1       1  QRMKNYTIYAVSITIRIVFGFLLIALIWKFDFSPFMVLIIAILNDGTIMTISKDRVKPSP
CsHA2       1  QRMKNYTIYAVSITIRIVFGFMLIALIWKFDFSPFMVLIIAILNDGTIMTISKDRVKPSP
MHA4        1  QRMKNYTIYAVSITIRIVLGFMLIALIWQYDFSPFMVLIIAILNDGTIMTISKDRVKPSP
MHA2      639  QRMKNYTIYAVSITIRIVLGFMLIALIWQYDFSPFMVLIIAILNDGTIMTISKDRVKPSP
               ******************:**::*****::**:.*************************

AHA2      700  TPDSWKLKEIFATGVVLGGYQAIMTVIFFWAAHKTDFFSDTFGVRSIRDNNHELMGAVYL
PMA4      702  MPDSWKLKEIFATGVVLGGYQALMTVVFFWAMHDTDFFSDKFGVKSLRNSDEEMMSALYL
BHA-1     701  LPDSWKLREIFATGVVLGSYMALMTVIFFWAMKDTNFFSNKFGVRSLRLSPEEMMAALYL
CsHA1      61  LPDSWKLKEIFATGIMLGGYLALMTVIFFWIMRETDFFPEKFNVRPIKDSPKEMMAALYL
CsHA2      61  LPDSWKLKEIFATGIVLGGYLALMTVIFFWLMDGTNFFSDKFGVRSIRHNEDEMMAALYL
MHA4       61  LPDSWKLKEIFATGIVLGSYLALMTVIFFWAMHKTDFFSDKFGVRSIRDSEHEMMSALYL
MHA2      699  LPDSWKLKEIFATGIVLGGYLALMTVIFFWAMHKTDFFSDKFGVRSIRDSEHEMMSALYL
                ******:******::**.* *:***:***    *:**.:.*.*:.:: . .*:*.*:**

AHA2      760  QVSIISQALIFVTRSRSWSFVERPGALLMIAFLIAQLIATLIAVYANWEFAKIRGIGWGW
PMA4      762  QVSIISQALIFVTRSRSWSFLERPGMLLVIAFMIAQLVATLIAVYANWAFARVKGCGWGW
BHA-1     761  QVSIISQALIFVTRSRSWSFAERPGLLLLGAFLIAQLVATFIAVYANWGFARIKGMGWGW
CsHA1     121  QVSIVSQALIFVTRSRSWSYFERPGLLLMGAFVIAQLVATLIAVYANWEFAKIKGAGWGW
CsHA2     121  QVSIVSQALIFVTRSRGRSYAERPGLLLVGAFFIAQLVATLIAVYANWGFAKIKGIGWGW
MHA4      121  QVSIVSQALIFVTRSRSWSFVERPGLLLVTAFLLAQLVATFLAVYANWGFARIKGIGWGW
MHA2      759  QVSIVSQALIFVTRSRSWSFVERPGLLLVTAF-VAQLVATLIAVYANWRFARIKGIGWGW
               ****:***********. *: **** **: ** :***:**::****** **:::* ****

AHA2      820  AGVIWLYSIVTYFPLDVFKFAIRYILSGKAWLNLFENKTAFTMKKDYGKEEREAQWALAQ
PMA4      822  AGVIWLYSIIFYLPLDIMKFAIRYILSGKAWNNLLDNKTAFTTKKDYGKEEREAQWALAQ
BHA-1     821  AGVIWLYSVVTYIPLDILKFAIRYILSGKAWDNLLENKTAFTTKKDYGKEEREAQWAAAQ
CsHA1     181  AGVVWIYSVIFYIPLDFIKFAIRYILSGKAWLNLLENKTAFTTKKDYGREEREAQWAATQ
CsHA2     181  AGVIWLYSIVFYIPLDVMKFAIRYILSGKAWLNLLENKTAFTTKKDYGKEEREAQWALAQ
MHA4      181  AGVVWLYSIVFYFPLDLIKFFIRFVLSGRAWDNLLENKTAFTTKKDYGREEREAQWATAQ
MHA2      818  AGVVWLYSIVFYFPLDLLKFFIRFVLSGRAWDNLLDTRIAFTRKKDLRKGEREAQWATAQ
               ***:*:**:: *:***.:** **::***:** **::.: *** ***  : ******* :*

AHA2      880  RTLHGLQP-KE--AVNIFPEKGSYRELSEIAEQAKRRAEIARLRELHTLKGHVESVVKLK
PMA4      882  RTLHGLQP-PE--ATNLFNEKNSYRELSEIAEQAKRRAEMARLRELHTLKGHVESVVKLK
BHA-1     881  RTLHGLQP-PE--TSNLFNDKNSYRELSEIAEQAKRRAEVARLRELHTLKGHVESVVKLK
CsHA1     241  RTLHGLQPAPE--RASLFLEKNSYRELSEIAEQAKRRAEIARLRELNTLKGHVESVVKLK
CsHA2     241  RTLHGLQP-PE--STNIFSEKSSYRELSEIAEQAKRRAEIARLRELNTLKGHVESVVKLK
MHA4      241  RTLHGLQP-PEAATSTLFHDKNSYRELSEIAEQAKRRAEIARLRELNTLKGHVESVVKLK
MHA2      878  RTLHGLQP-PE--SNTLFNDKSSYRELSEIAEQAKRRAEIARLRELNTLKGHVESVAKLK
               ********  *    .:* :*.*****************:******:*********.***

AHA2      937  GLDIETP-SHYTV
PMA4      939  GLDIETIQQHYTV
BHA-1     938  GLDIDTIQQHYTV
CsHA1     299  GLDIDTIQQHYTV
CsHA2     298  GLDIDTIQQHYTV
MHA4      300  GLDIDTIQQNYTV
MHA2      935  GLDIDTIQQNYTV
               ****:*  .:***
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Fig. 1. Predicted partial amino acid sequence of *CsHA1* and *CsHA2* and alignment with the corresponding regions of *BHA1* (accession no. S52728), *AHA2* (accession no. P19456), *PMA4* (accession no. S52728), *MHA2* (accession no. X85805) and *MHA4* (accession no. AJ539534). The darkened regions correspond to the peptide against which antibodies were directed for Western analyses (see Fig. 4). * indicates identical amino acids, whereas: indicates similar ones.

PM of root cells and have been suggested to play a role in active transport of nutrients. *CsHA1* shares an 88% identity (93% similarity) with *CsHA2* and showed an 81%, 83%, 86%, 83% and 84% identity (89%, 93%, 94%, 90% and 91% similarity) with the corresponding region of *AHA2*, *PMA4*, *BHA1*, *MHA2* and *MHA4*, respectively. With these latter genes, *CsHA2* shared an 85%, 86%, 87%, 85% and 86% identity (90%, 95%, 95%, 92% and 93% similarity). Both genes could be attributed on the basis of identity percentage to the PM H^+-ATPase gene subfamily II [15].

2.2. Analysis of gene expression

The expression pattern of H^+-ATPase genes in response to Fe deficiency and Fe re-supply to Fe-deficient plants was investigated by semi-quantitative reverse transcriptase (RT)-PCR. Expression analysis of the two *CsHA* genes in roots and leaves of cucumber plants is shown in Fig. 2. *CsHA1* transcripts were detected only in roots (Fig. 2A); furthermore, Fe deficiency strongly increased their steady-state levels (+160% as compared to Fe-sufficient control roots). Supply of Fe to deficient plants for 24 h as 40 μM Fe(III)-EDTA caused a decrease in *CsHA1* transcripts to a level similar to that of control roots. *CsHA2* transcripts were detected both in roots and leaves and appeared to be unaffected by the Fe nutritional status (Fig. 2B).

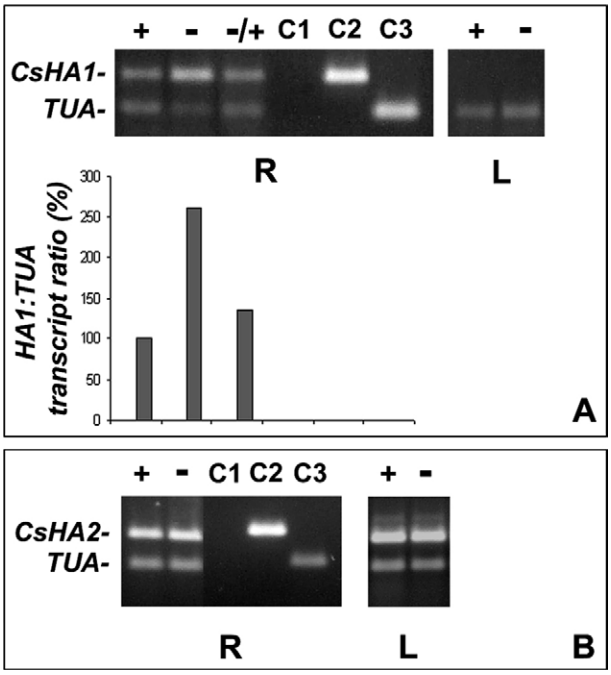

Fig. 2. Semi-quantitative RT-PCR analysis of *CsHA1* (A) and *CsHA2* (B) expression in cucumber roots (R) and leaves (L) grown with (+) or without (–) Fe. –/+ indicates Fe-deficient plants supplied for 24 h with 40 μM Fe(III)-EDTA.
C1: negative control for *CsHA1* transcripts carried out by using *CsHA2* cDNA clone as template (A) and vice versa for *CsHA2* transcripts (B). C2: positive control carried out by using *CsHA1* (A) or *CsHA2* (B) cDNA clone as template. C3: positive control for α-tubulin transcripts carried out by using *TUA* cDNA clone as template. Ethidium bromide-stained bands for *CsHA1* transcripts were quantified relatively to the band of α-tubulin. Data were calculated as percentage of the +Fe treatment.

The mRNA accumulation pattern of the two *HA* genes together with their transcript abundance were analysed by real-time RT-PCR (Fig. 3), as this technology allows to quantify mRNAs and to detect genes expressed below the limit of detection provided by RNA gel-blot analysis. Quantification revealed a 28-fold higher *CsHA1* transcript level in Fe-deficient as compared to Fe-sufficient control roots. Re-supply of Fe to deficient plants down-regulated *CsHA1* expression (Fig. 3A). *CsHA2* appeared to be only slightly up-regulated in Fe-deficient roots (1.9-fold), and was not significantly affected by Fe re-supply. *CsHA1* transcripts were detected at a very low level in leaves by real-time PCR and appeared unaffected by Fe nutritional status; *CsHA2* transcripts were similarly unchanged in the treatments (data not shown). Absolute transcript abundances of the two genes were compared

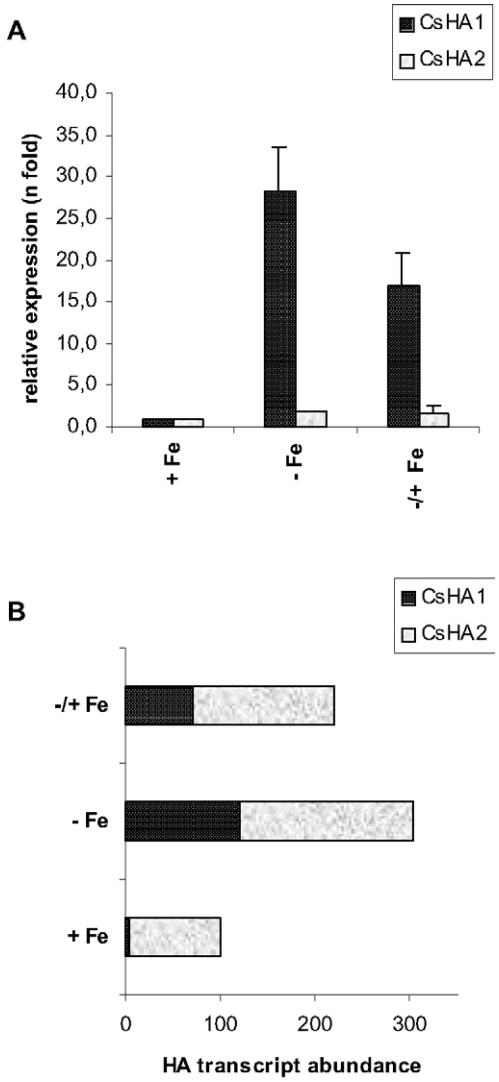

Fig. 3. Real-time RT-PCR analysis of *CsHA1* and *CsHA2* expression in cucumber roots grown with (+), without (–) Fe, or without Fe and then supplied with Fe(III)-EDTA for 24 h (–/+). A: *CsHA* mRNA levels were normalised with respect to the internal control *TUA*; changes in expression of *CsHA1* and *CsHA2* were calculated on the basis of their expression levels in Fe-sufficient control plants. Data bars represent mean ± S.E. of transcript levels from two independent experiments, run in triplicate. B: *CsHA* mRNA abundance. Total *CsHA* mRNA in +Fe plants = 100.

Treatment	+Fe	-Fe	-/+Fe
ATP hydrolysis (μmol Pi mg^{-1} prot. h^{-1})	83.8 ± 3.3	112.5 ± 4.2	97.7 ± 6.8
H^+-ATPase (Western blot)			
(%)	100	136 ± 6	113 ± 9

Fig. 4. PM H^+-ATPase activity and protein amount in PM-enriched vesicles isolated from cucumber roots. All activity data are the means of three independent experiments run in triplicate ± S.E. Total membrane proteins (ca 5 µg) were separated by denaturing 4–12% gradient SDS-PAGE and stained by Coomassie Brilliant Blue or immuno-detected with serum directed against the C-terminal domain of CsHA1 and CsHA2 as indicated in Fig. 1. Quantification of Western blot signals is expressed as percentage of the +Fe treatment (three independent experiments in triplicate ± S.E.).

in mRNA preparations from roots. In control roots *CsHA1* transcripts represented only about 4% of the total (*CsHA1* and *CsHA2*) mRNAs, whereas in roots of Fe-deficient plants the relative level of *CsHA1* messengers was increased up to 40% (Fig. 3B).

2.3. H^+-ATPase activity and Western analysis

Enhanced transcription of the *CsHA* genes was reflected by higher ATP-hydrolysing activity of the enzyme and abundance of ATPase protein (Fig. 4). Phospho-hydrolysing activity was increased by 30% in Fe-deficient roots, which was paralleled by a similar change in the amount of PM H^+-ATPase protein.

3. Discussion

The results of the present work show that a specific PM H^+-ATPase gene (*CsHA1*) strongly responds to Fe deprivation in cucumber roots. Increased PM H^+-ATPase activity and protein accumulation were previously observed in roots of Fe-deficient cucumber [7]. A transcriptional up-regulation of HA genes was also shown by northern blot analysis and related to an intense immunolabelling of the PM H^+-ATPase at the sub-apical root zones of Fe-deficient cucumber plants [8]. However, in both those studies the involvement in the Fe-stress response of a specific PM H^+-ATPase isoform was not analysed. In our work two novel *HA* cDNAs were identified in cucumber, and by both semi-quantitative RT-PCR and quantitative real-time RT-PCR the expression of each of the two genes was determined independently. By using real-time RT-PCR analysis, that is a highly sensitive and specific method to accurately measure the transcript level of relatively low-expressed members of a homogeneous gene family, it was possible to show that *CsHA1* is strongly expressed (about 30-fold more than control) almost exclusively in Fe-deficient roots, as its transcripts were not detected in leaves, and at a very low level in Fe-supplied roots. Moreover, by analysing the behaviour of the two *HA* genes after supplying Fe to Fe-deficient plants, we could show that regulation of *CsHA1* gene expression is related to the plant's iron nutritional status; in fact, transcript abundance could be greatly enhanced by Fe starvation in cucumber roots, while being decreased when Fe was supplied to the deficient plants.

The up-regulation of a specific PM H^+-ATPase occurring during the development of the response to Fe deficiency in cucumber roots is similar to what has been observed for other two PM components of the physiological adjustment induced by iron shortage in non-graminaceous plants, namely the PM Fe(III)-chelate reductase and the Fe^{2+} transporters [6], and suggests a major role of the enzyme in the molecular mechanism controlling iron uptake from the environment.

Supplying iron to Fe-deficient cucumber plants did not cause a transient increase in transcripts amount, as was observed for the Fe(III)-chelate reductase (FRO2) and the Fe^{2+} transporter (IRT1) genes in *Arabidopsis* [27]. This behaviour might be due to the duration of the treatment and/or to the amount of Fe supplied to the cucumber plants; alternatively, different regulatory mechanisms for the biochemical components of the Fe-deficiency response should be hypothesised. Coupling of PM H^+-ATPase-mediated proton extrusion and Fe(III) reduction as suggested by Rabotti et al. [20] has not yet been demonstrated.

Lowering rhizosphere pH is an important component of the adaptive response to Fe-deficiency of Strategy I plants such as cucumber. Changes in the level of CsHA1 might contribute to the acidification of the nutrient solution observed for Fe-deficient cucumber plants [2]. In tomato, density of PM H^+-ATPase was shown to be twice in Fe deficiency-induced transfer cells, frequency of which was considerably higher in acidifying root areas [24]. In addition, an increase in PM H^+-ATPase may also be required for the maintenance of optimal conditions for the activity of the PM Fe(III)-chelate reductase (i.e. low apoplastic pH and trans-PM potential homeostasis) and/or might be related to the morphological modifications occurring in Fe-deficient root tissues [24] such as the formation of extra root hairs. Interestingly, an increase in PM H^+-ATPase activity has been found also in plants that do not acidify the external solution [23,26]. Moreover, PM H^+-ATPase could be involved in the enhanced root exudation of organic acids observed during Fe deficiency [1]. Citrate increases about five folds in Fe-deficient cucumber roots [20] and, recently, turning off of a PM H^+-ATPase gene by antisense RNA has been shown to decrease citrate efflux in a carrot mutant cell line [16].

Real-time PCR is a very sensitive technology, which allows to detect and quantify variation in transcripts of genes expressed at a very low level, like *CsHA1*. In spite of an about 30-fold increase in transcript levels of this gene in Fe-deficient plants, total PM H^+-ATPase activity and protein amount increased to a lesser extent (Fig. 4). This result might be conceivably due to several factors, like the 'dilution' of active membrane vesicles by non-active cells and tissues, the loss of regulatory factors during membrane isolation, and the detection of several PM H^+-ATPase isoforms, differently affected by the nutritional status of plants, that could not be distinguished on the basis of the enzymatic assay and immunoblotting. Challenge of future work will be to localise

CsHA1 by means of tools more specific than those presently available for cucumber. Our data leave open the possibility that other factors (e.g. post-transcriptional regulation) might contribute to the observed results; a complex regulation might be needed, especially in Fe-deficient tissues, in order to avoid excessive ATP consumption. The Fe(III)-chelate reductase (FRO2) and the Fe^{2+} transporter (IRT1) genes in *Arabidopsis* are coordinately regulated at both the transcriptional and post-transcriptional levels, possibly involving ubiquitination and protein turnover in this latter case; iron uptake and storage are carefully regulated processes, because of its potential toxicity for the cell [5].

Interestingly, the C terminus of both cucumber H^+-ATPase isoforms contains the threonine (penultimate residue), phosphorylation of which is essential for binding of the regulatory 14-3-3 proteins [11]. A putative 14-3-3 protein have been shown to strongly respond to Fe deprivation in tomato [30].

In conclusion our data provide for the first time evidence that in cucumber plants a specific PM H^+-ATPase gene responds to changes in Fe availability. Modulations of expression of specific PM H^+-ATPase isoforms by nutritional signals were recently described for maize roots, in which the *MHA4* gene was found to be induced by the presence of nitrate in the nutrient solution [22]. Regulation by environmental cues may help to explain the presence of multiple genes encoding proteins highly similar in terms of sequence and function.

4. Methods

4.1. Plant growth

Three-day-old cucumber seedlings (*Cucumis sativus* L., cv. Serpente cinese) were grown under controlled conditions [7] for 5 days in nutrient solution without (–) or with (+) 80 μM Fe(III)-EDTA. Part of the Fe-deficient plants were supplied with 40 μM Fe(III)-EDTA (–/+) during the last 24 h before harvesting. Plant growth conditions were as described previously [7].

4.2. Cloning of CsHA cDNAs

Two partial cDNAs for cucumber PM H^+-ATPases were obtained by 3′-RACE (Rapid Amplification of cDNA Ends), using the 3′RACE System Kit manufactured by Invitrogen (Invitrogen Co., Carlsbad, CA). In the 3′-RACE experiments two μg of total RNA from roots were reverse-transcribed using a modified oligo-d(T), then cDNA amplified using a forward degenerate primer (5′-CAG/AAGG/GATGAAGAAC-TACAC-3′) following the manufacturer's instructions. All the PCR products, purified from a 1.2% (w/v) agarose gel, were cloned into pCR4-TOPO plasmids (TOPO TA Cloning Kit, Invitrogen Co.) and sequenced. Homology searches were carried out using the BLASTN and BLASTP algorithm [3]; identity percentages were calculated using GAP (Wisconsin GCG package, Accelrys, [9]) and sequence alignment was performed using the ClustalX (1.5b) programme [25].

4.3. RNA analysis

For semi-quantitative RT-PCR analysis, one μg of total DNase-treated RNA was reverse-transcribed using an oligo-dT primer and the Superscript™ II RT (Invitrogen Co.) in 20 μl, then 1 μl of the first strand cDNA reaction mixture was PCR-amplified with Accuprime™ Taq DNA Polymerase (Invitrogen Co.) using either *CsHA1*- or *CsHA2*-specific primers, and, in the same reaction, specific primers for the cucumber α-tubulin (*TUA* gene, accession No. AJ715498). The primers used to amplify *CsHA1* were 113F2 (5′-CTCCAACCAGCACCAGAAA-3′) and 113R1 (5′-TCCTTCATCTCTTTCTGCAACA-3′); the product was 240 bp in length. The gene-specific primers for *CsHA2* were 127F1 (5′-ACCCGAGTCGACAAACATCT-3′) and 127R1 (5′-CTTGGCACAGCAAAGTGAAA-3′), and amplified a 263 bp cDNA region. Reactions were run for twenty-seven cycles, corresponding to the log-linear phase of the amplification reaction.

Real-time RT-PCR was performed in a DNA Engine Opticon 2 (MJ Research Inc., Waltham, MA) system using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). Primer pairs were the same as described above. Amplification efficiencies were: 2.10 (*TUA*), 1.82 (*HA1*) and 1.94 (*HA2*). Target gene expression was normalised to *TUA* expression. Relative expression (*n*-fold) of the normalised target gene in the treatment was calculated by comparison to a control (+Fe) experiment considering efficiency values according to the mathematical model proposed by Pfaffl [19].

4.4. Isolation of membrane vesicles and protein analysis

Membrane vesicles were prepared using a sucrose-gradient technique as previously reported [7], except for the addition of protectants to homogenisation (20 μg ml^{-1} chymostatin, 1 μM leupeptin) and to resuspending (50 μg ml^{-1} chymostatin, 1 μM leupeptin) medium; this procedure yielded a membrane preparation with a PM H^+-ATPase activity 93% inhibited by vanadate when assayed at pH 6.5 in the presence of the detergent Brij 58 (polyoxyethylene 20 cetyl ether). PM H^+-ATPase assay was performed at 38 °C as described elsewhere [7].

For Western blot analysis antibodies raised against the C-terminal domain of both HAs were used. The 18 aa peptide AKRRAEIARLRELHTLKG (boxed in Fig. 1) was synthesised and conjugated to keyhole limpet haemocyanine by Sigma-Genosys (Sigma-Genosys Ltd., Cambridge, UK), and the same manufacturer produced the antisera. SDS-PAGE and Western blot were carried out as described elsewhere [22].

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