Mutational Analysis of the Interaction between 14-3-3 Proteins and Plant Plasma Membrane H⁺-ATPase*

Received for publication, October 29, 2002, and in revised form, December 10, 2002
Published, JBC Papers in Press, December 30, 2002, DOI 10.1074/jbc.M211039200

Sabina Visconti, Lorenzo Camoni, Maria Rosaria Fullone, Marco Lalle, Mauro Marra, and Patrizia Aducci

From the Department of Biology, University of Rome “Tor Vergata,” via della Ricerca Scientifica, I-00133, Rome, Italy, and Department of Biochemical Sciences “A. Rossi-Fanelli,” University of Rome “La Sapienza,” piazzale Aldo Moro 5, I-00185 Rome, Italy, and Department of Biological and Environmental Studies, University of Sannio, via Port’Arsa 11, I-82100, Benevento, Italy.

In this study, we report on mutational studies performed to investigate the mechanism of binding of 14-3-3 proteins to the plasma membrane H⁺-ATPase of plant cells. In fact, although the molecular basis of the interaction between 14-3-3 and the known mode-1 and mode-2 consensus sequences are well characterized, no information is available regarding the association with the H⁺-ATPase, which contains the novel binding site YTV totally unrelated to the 14-3-3 canonical motifs. To this purpose, different mutants of the maize 14-3-3 GF14-6 isoform were produced and used in interaction studies with the plasma membrane H⁺-ATPase and with a peptide reproducing the 14-3-3 binding site of the enzyme. The ability of 14-3-3 mutants to stimulate H⁺-ATPase activity was also tested. To investigate the mechanism of fusccocin-dependent interaction, binding experiments between 14-3-3 proteins and mutants of the extreme portion of the H⁺-ATPase C terminus were also carried out. The results demonstrate that mutations of Lys⁵⁶ and Val¹⁸⁵ within the amphipathic groove disrupt the ability of GF14-6 to interact with H⁺-ATPase and to stimulate its activity. Moreover, substitution of Asp³⁰⁸ and Asp³¹⁰ in the MHA2 H⁺-ATPase C terminus greatly decreased association with GF14-6, thereby demonstrating a crucial role of negatively charged residues in the fusccocin-dependent interaction.

14-3-3 are a family of highly conserved proteins widespread in eukaryotic organisms. A number of biological activities have been attributed to 14-3-3, including the control of cell cycle, differentiation, apoptosis, targeting of proteins to different cellular locations, and the coordination of multiple signal-transduction pathways (1–7). The common feature of 14-3-3 proteins is the ability to bind target proteins, and this accounts for their diverse regulatory functions (2, 3, 6). Most 14-3-3-interacting proteins bind their targets in a phosphorylation-dependent manner. Muslin et al. (8) suggested a consensus motif RXS\[L/E/A/M\]SpXP (Sp indicates a phosphoserine) found in several proteins known to associate with 14-3-3 proteins. This sequence has been defined in more detail by peptide library screening: two different phosphoserine motifs, R[S/Ar]+[S/Ar]Sp[L/E/A/M]P and R[X[S/Ar]]+Sp[L/E/A/M]P (where Ar indicates an aromatic residue and + a basic residue), defined as mode-1 and mode-2 sequences respectively, were proposed (9). However these motifs cannot account for all 14-3-3 interactions and, even though phosphorylation seems to be a general prerequisite for 14-3-3 binding, proteins interacting in a phosphorylation-independent manner have also been identified (10–12).

The 14-3-3 dimers have a characteristic cup-like shape with a highly conserved inner surface and a more variable outer surface (13, 14). In the concave surface, each monomer presents an amphipathic groove formed by a cluster of basic residues (Lys⁴⁹, Arg⁵⁶, Lys¹²⁰, Arg¹²⁷) on one side, and hydrophobic residues (Val¹⁷⁶, Leu¹⁸¹, Leu¹⁸⁶, Leu²⁰⁷) on the other side. Crystallization studies of 14-3-3 with phosphorylated peptides reproducing mode-1 consensus sequence demonstrated that phosphoserine interacts by salt bridges with the side chains of basic residues of the 14-3-3 groove (9, 15). Accordingly, mutations of Lys⁴⁹, Arg⁵⁶, and Arg¹²⁷ hamper the association of 14-3-3 with Raf-1 and exoenzyme S (16). Substitution of residues in the hydrophobic cluster also affects binding properties (17).

In plants, much less information about the mechanism of interaction between 14-3-3 proteins and their targets is available. Most of the binding sequences so far identified are ascribable to the mode-1 motif (2, 7). A remarkable exception is represented by the plasma membrane H⁺-ATPase. In fact, it contains, at the extreme end of its C-terminal domain, the 14-3-3 binding site YTV (18), which is totally unrelated to those previously identified. Phosphorylation of the threonine residue in the YTV sequence, which has been demonstrated to occur in vivo (19), is required for the interaction with 14-3-3 proteins (18, 20). Association of 14-3-3 proteins with the H⁺-ATPase is stabilized by the toxin fusccocin (FC)¹, known to strongly activate the H⁺-ATPase (21). Moreover, FC is able to trigger the interaction of 14-3-3 with the H⁺-ATPase also in the absence of phosphorylation (22).

Here we report on mutational studies carried out to elucidate the molecular basis of the interaction between 14-3-3 proteins and the plasma membrane H⁺-ATPase of plant cells. Different mutants of the maize 14-3-3 GF14-6 isoform (K56E, K56Q, K56R, and V185E) were produced and used in interaction studies with the plasma membrane H⁺-ATPase and with a peptide reproducing the 14-3-3 binding site of the enzyme. The ability of 14-3-3 mutants to stimulate the phosphohydrolytic activity

¹ The abbreviations used are: FC, fusccocin; GST, glutathione-S-transferase; ER, endoplasmic reticulum; MHA2, Maize H⁺-ATPase isoform 2; AHA1, Arabidopsis thaliana H⁺-ATPase isoform 1.
of the H\(^{-}\)-ATPase was also tested and correlated to the interaction data. Finally, to investigate the mechanism of the FC-dependent interaction, binding experiments between 14-3-3 proteins and mutants of the extreme portion of the H\(^{-}\)-ATPase C terminus were carried out.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**FC was prepared according to Ballio et al. (23); tritiated dihydroFC ([\(^{3}H\)]FC) with specific activity 0.77 TBq/mmol was obtained according to Ballio et al. (24).

[(\(^{32}P\)]ATP (specific activity 110 TBq/mmol) and thrombin were from Amersham Biosciences (Uppsala, Sweden). Protein kinase A, catalytic subunit, and regulatory subunit (bl15Vp peptide biotinylated-LKDLIDITOKQNY-TpV (Tp, phosphothreonine) and MHA2–776p peptide biotinylated-ALIFVTRSRSpWSFVE (Sp, phosphoserine) were synthesized by Neo-Strasbourg (Strasbourg, France). Chemicals for gel electrophoresis were from Bio-Rad ( Hercules, California, USA). All other reagents were of analytical grade.

**Plant Material—** Maize seeds (Zea mays L. cv. Santos) from Dekalb (Mestre, Italy) were germinated and grown in the dark for 6 days, as already described (25).

**Escherichia Coli Strains—** E. coli strains were grown at 37 °C in LB liquid medium or agar plates (15 g/l). Ampicillin was used at concentration of 100 μg/ml. Strain DH5α was used for plasmid propagation and strain BL21 (DE3) was used for protein expression.

**Site-directed Mutagenesis of 14-3-3 Protein—** pGEX-2TK carrying the GF14-6 cDNA clone was used as a template for mutagenesis of GF14-6. Site-directed mutagenesis of Lys60 was obtained by PCR amplification of the 108–787 fragment using a 5′ oligonucleotide primer containing the desired mutations and SacI restriction site complementary to the 3′/H11032-TTAACTTCTCAGAGTTCTACTATGAGAT-3′ (coding primer 5′-CTCTTGTCTGTTGCATACAGGAAC-3′). The 3′ end of the GF14-6 cDNA was directly cloned in pGEM-T vector (Promega, Madison, Wisconsin, USA) and then subcloned in SacI-EcoRI fragment. After 18 cycles of PCR (30 s at 95 °C and 1 min at 53 °C, 11 min at 72 °C), 10 units of DpnI were added to the mixture to digest the pX18 DNA template, and reaction was carried out at 37 °C for 2 h. 20 μl of the mixture were used to transform E. coli DH5α competent cells. Incorporation of mutation was controlled by DNA sequencing.

**Site-directed Mutagenesis of H\(^{-}\)-ATPase C-terminal Domain—** PCR-Ext2 carrying the cDNA fragment encoding the last 103 amino acid residues of the H\(^{-}\)-ATPase MHA2 isoform (Maize H\(^{-}\)-ATPase isoform 2) (pGEX-E DNA clone) (22) was used as a template. Mutagenesis was obtained by PCR using mutagenic oligonucleotide primers complementary to the 3′ end of the cDNA fragment containing the desired mutations and EcoRI restriction site, and a primer based on the 5′ end of the cDNA fragment corresponding to 2788–2792 region of the MHA2 gene (26) containing BamHI restriction site.

**Isolation of Maize Plasma Membranes—** Two-phase partitioned plasma membranes from maize roots were obtained as previously described (26).

**Purification of ER from Yeast Expressing AHA1—** Plasma membrane H\(^{-}\)-ATPase AHA1 isoform (Arabidopsis thaliana H\(^{-}\)-ATPase isoform 1) was expressed in Saccharomyces cerevisiae as previously described (27).

**SDS-PAGE and Overlay Assay—** SDS-PAGE was performed as described by Laemmli (29), in a Mini Protein apparatus (Bio-Rad).

**RESULTS**

**K56E and V185E Mutations Do Not Result in Significant Structural Changes of GF14-6—**In the 14-3-3 isoform, mutations of Lys49 and Val176 residues in 14-3-3 proteins and the peculiar YTpV binding sequence of the FC-binding site, did not result in significant structural changes of GF14-6 proteins (25).

**Expression in E. coli of Recombinant Proteins—** Wild-type and mutated GF14-6 proteins were expressed in E. coli as fusion proteins with the glutathione S-transferase (GST) using pGEX-2TK vector, whereas wild-type and mutant C-terminal domain of MHA2 H\(^{-}\)-ATPase were expressed as GST-fusion proteins using pGEX-2T vector, following the procedure described by Fullone et al. (22).

**ACKNOWLEDGMENTS**

Fuller, S. A.; et al. The mechanism of the FC-dependent interaction, binding experiments between 14-3-3 proteins and mutants of the extreme portion of the H\(^{-}\)-ATPase C terminus were carried out.
lar dichroism analysis and by testing their ability to dimerize. CD spectra of mutants overlapped that of wild-type protein (data not shown). To check the dimerization properties of mutants, overlay assay experiments were carried out. In this system, $^{32}$P-labeled GF14-6 wild-type, K56E, and V185E proteins were used as probes and the same unlabeled proteins, immobilized on nitrocellulose membrane, as baits. Autoradiography, reported in Fig. 2, shows that both $^{32}$P-labeled mutated proteins were able to interact each other and with the wild-type, demonstrating that the ability of mutants to form dimers was unaffected.

K56E and V185E Mutations hamper the interaction of GF14-6 to the $H^+$/H$_{11001}$-ATPase—The effect of K56E and V185E mutations on the association of 14-3-3 proteins with the $H^+$/H$_{11001}$-ATPase was investigated by means of the overlay assay. $^{32}$P-labeled K56E, V185E, and wild-type GF14-6 proteins were used as probes and the $H^+$/H$_{11001}$-ATPase from maize roots as a bait. The results of autoradiography are reported in Fig. 3a. K56E (2) and V185E (3) mutations severely hampered the association of 14-3-3 protein with the $H^+$/H$_{11001}$-ATPase. Binding of mutants, compared with wild-type, is reduced also in the presence of FC (Fig. 3a), which is known to strongly increase association of 14-3-3 with the $H^+$/H$_{11001}$-ATPase (22).

It is known that 14-3-3 proteins can interact with the $H^+$/H$_{11001}$-ATPase also in a phosphorylation-independent manner. In these conditions, the association is completely dependent on FC (18, 20, 22). To verify whether K56E and V185E mutations affect also this interaction, the ability of mutated proteins to bind the recombinant C-terminal domain of $H^+$/H$_{11001}$-ATPase, expressed in E. coli, as a GST-fusion protein, was tested. As shown in Fig. 3b, for both mutants, the interaction with the C-terminal domain of the $H^+$/H$_{11001}$-ATPase was almost completely abolished.

Lys56 Mutations Suggest a Role for a Positive Charge in the Interaction with the $H^+$/H$_{11001}$-ATPase—The relevance of the contribution of Lys$^{56}$ to the $H^+$/H$_{11001}$-ATPase binding was further studied by mutating it in Glu and Arg and expressing the mutated GST-fusion proteins in E. coli.

The effect of mutations on the interaction was tested by overlay experiments. As reported in Fig. 4a, substitution of

![Fig. 1. Alignment between the Zea mays 14-3-3 GF14-6 isoform and the mammalian 14-3-3 isoform. 14-3-3 residues Lys$^{56}$ and Val$^{176}$, corresponding to Lys$^{56}$ and Val$^{185}$ in GF14-6, are indicated in bold.](image)

![Fig. 2. Dimerization properties of GF14-6 mutants. Overlay assay was carried out with wild-type and mutant proteins treated with thrombin to remove GST. 0.5 μg of K56E, V185E, and wild-type GF14-6 were subjected to SDS-PAGE, and, after blotting, nitrocellulose membranes were incubated with $^{32}$P-labeled wild-type GF14-6 (a), $^{32}$P-labeled K56E mutant (b) or $^{32}$P-labeled V185E mutant (c), in the conditions indicated under “Experimental Procedures.”](image)
Lys56 with the polar residue Gln only partially restored the binding activity (3). On the other hand, the interaction with the H+/ATPase was completely rescued by the conservative mutation K56R (4). The results demonstrate that the positive charge is also required in the FC-dependent interaction; in fact, K56Q mutant showed a very weak interaction with both H+/ATPase (Fig. 4a) and its C-terminal domain (Fig. 4b).

Fig. 3. Effect of mutations K56E and V185E of GF14-6 on its interaction with the H+/ATPase. The overlay assay was performed subjecting 10 µg of plasma membrane proteins (a) or 1 µg of recombinant MHA2 GST-C terminus (b) to SDS-PAGE. After blotting, nitrocellulose membranes were incubated with 32P-labeled wild-type GF14-6 (lane 1), 32P-labeled K56E (lane 2), or 32P-labeled V185E (lane 3) in the absence or in the presence of 10 µM FC.

Lys56 and Val185 Mutations Affect the Interaction of GF14-6 with Peptides Reproducing 14-3-3 Binding Sites of H+/ATPase—A phosphorylated biotinyl-peptide (bL15Vp) reproducing the last 15 amino acid residues of the MHA2 H+/ATPase and containing the 14-3-3-YTpV binding sequence was used in interaction studies with GF14-6 mutants. The peptide was immobilized onto a streptavidin-agarose resin and incubated with 32P-labeled wild-type and mutant proteins. Interestingly, the results, reported in Fig. 5, were only partially in accordance with those obtained by overlay assays. In fact, although, as expected, K56E and V185E mutants did not interact with bL15Vp peptide and K56Q interacted very weakly, the conservative mutant K56R bound bL15Vp more efficiently than the wild-type GF14-6 did.

The same binding experiments were carried out with a phosphorylated peptide (MHA2–776p) reproducing a putative 14-3-3-binding site located in the cytosolic stretch between the 8 and 9 helices of the MHA2 H+/ATPase. This peptide contains the mode-1 related sequence RSRSpWS, and it has been reported to interact with GF14-6 protein (28); however, the physiological relevance of this interaction has not yet been demonstrated.
The results were substantially in accordance with those obtained with bL15Vp peptide. Yet a remarkable difference was observed when the experiments were performed in the presence of FC; in fact, whereas FC strongly stabilized the bL15Vp/14-3-3 complexes, it was completely ineffective on the MHA2-776p/14-3-3 interaction (Fig. 5). These data were confirmed by assaying the ability of peptides/14-3-3 complexes to bind a tritiated derivative of FC. In fact, as reported in Fig. 6, only the complexes between bL15Vp peptide and wild-type GF14-6, or K56R mutant, were able to bind [3H]FC.

Lys<sup>56</sup> and Val<sup>185</sup> Mutations Affect 14-3-3-induced Stimulation of H<sup>+</sup>-ATPase—The effect of Lys<sup>56</sup> and Val<sup>185</sup> mutations on the in vitro activation of the H<sup>+</sup>-ATPase induced by 14-3-3 proteins was tested by using purified ER vesicles of S. cerevisiae expressing the H<sup>+</sup>-ATPase AHA1 isoform of Arabidopsis thaliana. In this system, a significant and reproducible stimulation of H<sup>+</sup>-ATPase can be obtained by exogenous 14-3-3 proteins, provided that FC is also added.

ER membranes were incubated with different amounts of wild-type or mutated GF14-6 proteins in the presence of 10 μM FC. The results, reported in Fig. 7, demonstrate that the ability to stimulate the H<sup>+</sup>-ATPase activity depends on the ability to associate with it. In fact, only the wild-type and K56R GF14-6, which efficiently bind H<sup>+</sup>-ATPase in the interaction experiments (Fig. 4), stimulated AHA1 phosphohydrolytic activity.

Effect of the D938A/D940A Double Mutation and the T947E Mutation, Both in the H<sup>+</sup>-ATPase C Terminus, on FC-dependent Interaction with GF14-6—R18 is a synthetic peptide selected from a phage display library for its ability to bind 14-3-3 proteins in a phosphorylation-independent manner (32). It has been proposed that binding property is due to the motif WLDLE. Interestingly, in the H<sup>+</sup>-ATPase C terminus a very similar sequence, namely 936GLDID (numbered according to MHA2 isoform), located close to the C-terminal end is present. To verify whether this sequence could be involved in the phosphorylation-independent, FC-dependent interaction, single and double mutation of the two acidic residues, Asp<sup>938</sup> and Asp<sup>940</sup>, were obtained.

The wild-type and mutated C-terminal domains were expressed in E. coli as GST-fusion proteins and assayed for their ability to interact with 32P-labeled GF14-6 in overlay experiments, in the presence of 10 μM FC.

As shown in Fig. 8A, mutation D940A (lane 3) did not affect the binding of recombinant H<sup>+</sup>-ATPase C terminus, mutation D938A (lane 2) reduced the binding, and double mutation (lane 4) completely abolished it. These results demonstrated the relevance of the two negatively charged residues for the FC-dependent interaction with GF14-6.

A further indication of the importance of negative charges in the FC-dependent interaction was obtained by means of the T947E mutant. It contains a negatively charged residue in place of the phosphorylatable threonine residue, within the YTV 14-3-3 binding sequence. This mutant had been produced to verify whether the negative charge of the glutamic acid could mimic the phosphate group. Autoradiography reported in Fig. 8B showed that no interaction occurred between GF14-6 and the mutated C terminus. Surprisingly, however, in the presence of FC, the T947E mutant (lane 2) interacted more efficiently than did the wild-type C-terminal domain.

DISCUSSION

The plasma membrane H<sup>+</sup>-ATPase of plant cell is regulated by 14-3-3 proteins. It contains the 14-3-3 binding motif YTV, located at the extreme end of the C-terminal autoinhibitory region of the enzyme. This is a novel sequence completely unrelated to the well characterized mode-1 and mode-2 motifs, which accounts for most of 14-3-3 interactions with target proteins. Hence, we were interested in identifying residues important for binding activity of a plant 14-3-3 protein and ascertaining whether the interaction with the H<sup>+</sup>-ATPase underlies the same mechanism reported for the interaction with canonical motifs (9, 15). To this purpose, single amino acid mutations known to affect the binding properties of animal 14-3-3 proteins were reproduced in the plant 14-3-3 isoform GF14-6, and their effects on the ability to interact with and to activate the H<sup>+</sup>-ATPase were investigated.

In this study, we show that K56E and V185E mutations drastically hamper the interaction of GF14-6 with the H<sup>+</sup>-ATPase. These results indicate that Lys<sup>56</sup> and Val<sup>185</sup> are both crucial for binding to the YTV sequence, thereby suggesting that basic and hydrophobic clusters in the conserved amphipathic groove of 14-3-3 are involved also in this interaction.
This finding is worth noting, because the H\textsuperscript{+}-ATPase binding site lacks the main features of classical consensus sequences, namely a basic residue at the -3/-4 position as well as a proline at the +2 position. It has been suggested that the proline residue is important to fold the binding site, allowing the entrance of the C-terminal portion back toward to the binding groove (33); probably in the H\textsuperscript{+}-ATPase the localization of the phosphoamino acid residue at the extreme C-terminal end of the protein renders the role of the proline residue unnecessary. Interestingly, the 14-3-3 binding sequence GHSL of the glycoprotein Ib-IX-V complex protein (34), which also lacks the proline at +2 Sp position, is likewise located at the extreme C-terminal end of the protein.

The importance of a positively charged residue at position 56 of GF14-6 has been further probed by substituting the Lys residue with a Gln or Arg residue. In comparison with the mutation K56E, the polar K56Q partially, and the conservative K56R completely, restored the binding activity. Interestingly, efficiencies of H\textsuperscript{+}-ATPase stimulation by mutants directly paralleled binding affinities.

The interaction mechanism with H\textsuperscript{+}-ATPase was further investigated by studying the binding of wild-type and mutant GF14-6 to the BL15Vp peptide, reproducing the last 15 residues of MHA2 H\textsuperscript{+}-ATPase and containing the YTP\textsuperscript{V} sequence. The results were in good agreement with those observed with the whole enzyme in the overlay experiments. However, the K56R mutant, compared with the wild-type GF14-6, displays a much higher affinity toward the BL15Vp peptide than to the H\textsuperscript{+}-ATPase. A possible explanation is that the BL15Vp peptide does not exactly match the conformational features occurring in the whole enzyme, which can be important in the physiological interaction of the enzyme.

It is worth noting that no FC effect was detected when the interaction studies were performed with the MHA2-776p peptide, which reproduces a putative 14-3-3 binding site in the H\textsuperscript{+}-ATPase (28) related to a mode-1 binding motif. This strongly suggests that the ability to bind FC is a unique feature of the complex between 14-3-3 protein and the extreme portion of the C-terminal domain of the proton pump.

FC strongly stabilizes the interaction between 14-3-3 and the H\textsuperscript{+}-ATPase and brings about 14-3-3 binding also in the absence of enzyme phosphorylation. The results of the overlay assay with the recombinant unphosphorylated C-terminal domain of the H\textsuperscript{+}-ATPase demonstrated that Lys\textsuperscript{56} and Val\textsuperscript{185} are both essential for FC-induced binding. This indicates that the same amphipathic 14-3-3 binding groove is involved also in the FC-dependent interaction, occurring in the absence of phosphorylation. This result appears particularly relevant because it has been observed that binding with unphosphorylated 14-3-3 targets does not require the same hydrophobic residues involved in the interaction with phosphorylated proteins (35).

It has been proposed (9) that the function of the basic cluster in 14-3-3 proteins is to interact with the negatively charges of the phosphoamino acid within the binding site; in this respect, the question is open about the effect of Lys\textsuperscript{56} mutation, which abolishes the binding also in the absence of phosphorylation of the C-terminal domain of the H\textsuperscript{+}-ATPase. An explanation for this result was obtained by studying the effect of point mutation of the H\textsuperscript{+}-ATPase C terminus. In fact, substitution of the two Asp with Ala residues, within the sequence 938GLDID (numeration according to MHA2 isoform) located close to the YTV sequence, completely abolished the ability of recombinant C-terminal domain of H\textsuperscript{+}-ATPase to bind GF14-6 in the presence of FC. This demonstrates that negative charges play a role also in the FC-dependent interaction, probably mimicking the phosphate group function in the 14-3-3/target complex formation. This hypothesis has also been proposed for the unphosphorylated R18 peptide, which contains the very similar sequence WLDLE; in fact, in the 14-3-3/R18 complex, the two
Asp and Glu acidic residues of peptide are located next to the basic cluster of the 14-3-3 protein (15). Intriguingly exoxenzyme S, which interacts with 14-3-3 proteins in a phosphorylation-independent manner, possesses a homologous motif, 24FGA-
DAE, in its C-terminal region (12). The role of negative charges in the FC-dependent interaction can also be inferred from data obtained with the T947E mutant. In fact, the introduction of a further negatively charged residue brought about a marked increase in the ability to bind 14-3-3 protein.

In conclusion, our data show that positively charged residues in GF14-6 binding groove and negatively charged residues in the C-terminal domain of H⁺-ATPase play a crucial role in binding between these proteins, even when, as in the FC-dependent interaction, it occurs in the absence of H⁺-ATPase phosphorylation.

Acknowledgments—We thank Prof. M.G. Palmgren for the gift of the yeast strain expressing the AHA1 H⁺-ATPase. We are grateful to Banca di Roma for funding the purchase of the plant growth chamber.

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*J. Biol. Chem.* 2003, 278:8172-8178.
doi: 10.1074/jbc.M211039200 originally published online December 30, 2002

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