A plant plasma membrane H\(^+\)-ATPase expressed in yeast is activated by phosphorylation at its penultimate residue and binding of 14-3-3 regulatory proteins in the absence of fusicoecn

Olivier Maudoux*,§, Henri Batoko*§¶, Claudia Oecking†, Kris Gevaert‡, Joel Vandekerckhove‡, Marc Boutry*|| and Pierre Morsomme*#

* Unité de Biochimie Physiologique, Université Catholique de Louvain, Croix du Sud 2-20, B-1348 Louvain-la-Neuve, Belgium
† Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität, D-44780 Bochum, Germany
‡ Flanders Interuniversity Institute for Biotechnology, Department of Biochemistry, University of Ghent, Belgium.
§ These authors contributed equally to this work.
¶ Present address: Department of Plant Sciences, University of Oxford, Oxford, OX1 3RB, UK
# Present address: Biozentrum, University of Basel, 4056, Switzerland

|| Corresponding author:
Marc Boutry
Tel: 32-10-473621, Fax: 32-10-473872, E-mail: boutry@fysa.ucl.ac.be
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Summary

The *Nicotiana plumbaginifolia* plasma membrane H\(^+\)-ATPase isoform PMA2, equipped with a 6-His tag, was expressed in *Saccharomyces cerevisiae* and purified. Unexpectedly, a fraction of the purified tagged PMA2 associated with the two yeast 14-3-3 regulatory proteins, BMH1 and BMH2. This complex was formed *in vivo* without treatment with fusicoccin, a fungal toxin known to stabilize the equivalent complex in plants. When gel filtration chromatography was used to separate the free ATPase from the 14-3-3/H\(^+\)-ATPase complex, the complexed ATPase was twice as active as the free form. Trypsin treatment of the complex released a smaller complex, composed of a 14-3-3 dimer and a fragment from the PMA2 C-terminal region. The latter was identified by Edman degradation and mass spectrometry as the PMA2 C-terminal 57 residues, whose penultimate residue (Thr 955) was phosphorylated. *In vitro* dephosphorylation of this C-terminal fragment prevented binding of 14-3-3 proteins, even in the presence of fusicoccin. Mutation of Thr 955 to alanine, aspartate or a stop codon prevented PMA2 from complementing the yeast H\(^+\)-ATPase. These mutations were also introduced in an activated PMA2 mutant (Gln14 → Asp) characterized by a higher H\(^+\) pumping activity. Each mutation directly modifying Thr 955 prevented 14-3-3 binding, decreased ATPase specific activity and reduced yeast growth. We conclude that the phosphorylation of Thr 955 is required for 14-3-3 binding and that formation of the complex activates the enzyme.
Introduction

The plant plasma membrane proton-pump ATPase (H⁺-ATPase) mediates ATP-dependent H⁺ extrusion from the cell, creating the driving force for secondary transport of many solutes into and out of the cell. The H⁺-ATPase belongs to the P-type ATPase family characterized by a catalytic phosphorylated intermediate (for reviews, see (1-3)).

Plant H⁺-ATPases belong to a multigene family, with individual members expressed in particular cell types at specific developmental stages (4-8). The existence of several H⁺-ATPase genes suggest the gene products exhibit diverse catalytic or regulatory properties. Heterologous expression in the yeast *Saccharomyces cerevisiae* has notably allowed this premise to be addressed but also demonstrated that different *Arabidopsis thaliana* or *Nicotiana plumbaginifolia* H⁺-ATPase isoforms have distinct kinetics (9-11). In vivo functional differences have also been found for two *N. plumbaginifolia* H⁺-ATPase isoforms which confer distinct growth properties on yeast, depending on the external pH (11).

The plant H⁺-ATPase contains an auto-inhibitory domain located in the C-terminal region downstream of the last transmembrane span (12). Removal of this regulatory region by tryptic digestion (12) or by mutagenesis (13,14) gave enhanced H⁺-ATPase activity. The plant H⁺-ATPase is activated by direct, reversible interaction between its C-terminal region and regulatory 14-3-3. (15-17). The 14-3-3 proteins are present in all eukaryotes and act as regulators in various signal transduction pathways (18,19). The H⁺-ATPase/14-3-3 complex formed in plants is labile and can only be studied in the presence of fusicoccin. This fungal toxin binds to, and stabilizes, a complex of H⁺-ATPase and 14-3-3 proteins (15-17).
Another mode of plant H⁺-ATPase regulation is kinase-mediated phosphorylations, which has been shown by both *in vitro* (20-23) and *in vivo* (24-26) approaches. However, the effect of H⁺-ATPase phosphorylation on the enzyme is much debated, with separate reports associating increased H⁺-ATPase activity with either dephosphorylation (22,23,25,27) or phosphorylation (21,26,28). This controversy could reflect different phosphorylation sites within H⁺-ATPase, including one involving 14-3-3 binding. The 14-3-3 proteins mediate signal transduction by binding to consensus motifs containing a phosphoserine residue (29,30). Putative phosphorylation motifs are present in the H⁺-ATPase C-terminal region but none is a known 14-3-3 binding motif (31). The penultimate residue (Thr) of a spinach H⁺-ATPase engaged in a complex with 14-3-3’s upon fusicoccin treatment is phosphorylated and protected from turnover (26). However, it is not known if phosphorylation is a prerequisite for 14-3-3 protein binding and whether phosphorylation would have occurred in the absence of fusicoccin.

The PMA2 (plasma membrane H⁺-ATPase) H⁺-ATPase from *N. plumbaginifolia* has several characteristics that make it a good model for regulatory studies in yeast. It can functionally replace the *S. cerevisiae* plasma membrane H⁺-ATPase, allowing plasma membranes containing only this isoform to be obtained (10). Although functional replacement of yeast H⁺-ATPase by wild-type PMA2 requires an external pH higher than 5.0, several activated pma2 mutants that confer grow at pH 4, or even pH 3 have been selected. These mutations, found in various regions of the enzyme, all give structural modifications that cause displacement of the regulatory C-terminal region and enhanced proton pumping activity (13,32). Finally, the expression of PMA2 in yeast gives strong fusicoccin-binding activity that involves a complex with yeast 14-3-3 regulatory proteins (33).
By equipping both wild type PMA2 and an activated PMA2 mutant with an N-terminal His-tag to facilitate their purification, we report the surprising result that PMA2 phosphorylated at its penultimate residue forms a stable complex with 14-3-3s in the absence of fusicoccin. This binding gives both increased H^+-ATPase activity and more effective complementation of yeast growth.
Experimental procedures

Construction of strains

The plasmid 2µp(PMA1)pma2, containing either the wild type \textit{N. plumbaginifolia} pma2 cDNA or the mutant E14D (32), was modified by PCR to introduce a sequence coding for 6 histidine residues at either the C-terminus (before the STOP codon) or the N-terminus (between codons 3 and 4).

For C-terminal tagging, the primers Pth1 (5' GTCACAAGATCTCGAAGT 3') and Pth2 (5' TGGGTCTCCATGGTTCAATGGTGATGGTGATGGTGAAACAGTGTAT GATTGCTG3') were used to generate a first PCR product containing a unique NcoI restriction site downstream of the stop codon. The primers Pth3 (5' CATTGAACCATGGAGACCCAAAGTCAGAGTGTGTTCGGCCA 3') and Pth4 (5' GCAGGTCGACTCTTAGAGGA 3') were used to amplify the 3' untranslated region, giving a second PCR product. The two partly overlapping PCR products were combined and amplified as a single fragment, using primers Pth1 and Pth4. The new fragment was digested with BglII/XbaI and used to replace the corresponding fragment in plasmid 2µp(PMA1)pma2.

For N-terminal tagging, an identical strategy was used, except that the primers were Pth5 (5' GAATTCGAGCTCGGTACC 3'), Pth6 (5' GTGATGGTGATGGTGATGCTCCCCCATACTTGCTTCAATTTCCTCAGTACCATCACCATCACAAACCTGAGGTGTTAGATGC 3'), Pth7 (5' ATGGGGGAGCATCACCATCAC 3'), and Pth8 (5' GCTTTAGAGCTCGAGCTCGGAGCC 3'). The full length PCR product, digested by SacI/NheI, was used to replace the corresponding fragment in plasmid 2µp(PMA1)pma2.
Plasmids with N-tagged wild-type or E14D PMA2 were modified by PCR to exchange the penultimate residue, Thr 955, for Ala (T955A), Asp (T955D) or a stop codon (T955STOP).

**Solubilization and purification of the histidine-tagged H⁺-ATPase**

Plasma membranes were prepared according to Morsomme *et al.* (13). All buffers contained 1 mM PMSF and 2μg/ml of leupeptin, aprotinin, antipain, pepstatin and chymostatin (protease inhibitor cocktail) and all manipulations were at 4°C.

The plasma membranes were resuspended (5 mg/ml) in 20 mM imidazole pH 7.5, 1 mM MgCl₂, 500 mM KCl, 10 % glycerol. A 10 % (w/v) solution of polyoxyethylene 8 lauryl ether (C12E8) in the same buffer was added to give a final detergent:protein ratio of 1:2 (w/w), the sample was thoroughly mixed for 10 min and centrifuged at 100,000 g for 1 h. The pellet was resuspended (5 mg/ml) in buffer TK20 (20 mM imidazole pH 7.5, 1 mM MgCl₂, 150 mM KCl, 20% (v/v) glycerol). A 10 % (w/v) solution of dodecylmaltoside (DDM) in buffer TK20 was added to give a detergent:protein ratio of 1:1 (w/w), the sample was thoroughly mixed and centrifuged as above. The supernatant was added to a Ni-NTA agarose matrix (Quiagen) (0.5 ml of matrix, 2.5 mg of proteins), the mixture incubated for 1 h on a rotary wheel at 4 °C, loaded into a column and then washed 4 times with 500 μl of buffer TK20 containing 0.05 % (w/v) DDM. Bound proteins were eluted with 250 mM imidazole pH 7.5, 1 mM MgCl₂, 150 mM KCl, 20% (v/v) glycerol. Separation of the free H⁺-ATPase and the H⁺-ATPase/14-3-3 complex was achieved using a Bio-Prep SE 100/17 column (BioRad) equilibrated with 0.01% (w/v) DDM, 10 mM imidazole pH 7.0, 50 mM KCl, 1 mM MgCl₂. Coomassie blue-stained proteins were quantified using ImageMaster1D software (Pharmacia), with phosphorylase b (97 kDa) as the standard.
Western blot analysis was performed using specific antibodies directed against soluble regions of PMA2 (13,32) and the enhanced biochemiluminescence method.

**Tryptic digestion**

For the analysis of 14-3-3 binding to PMA2, the tryptic digestion was performed at room temperature directly on Nickel resin-bound purified H\textsuperscript+-ATPase (solubilized from ten mg plasma membrane proteins) at a trypsin/protein ratio of 1/166. The digestion was performed in 100 mM Tris-HCl (pH 8.5) in a final volume of 185 µl. After 1 h at 20°C, the reaction was stopped by addition of 1 mM phenylmethylsulfonyl fluoride. The digestion medium and two 125 µl washes with 100 mM Tris-HCl (pH 8.5) were collected by centrifugation from a Micro Bio-Spin Chromatography Column (Bio-Rad) and pooled. The peptide mixture was fractionated using a BioLogic Duo-Flow System (Bio-Rad) equipped with a UNO anion-exchange column (UNO Q-1 column, Bio-Rad). Elution was performed with a linear gradient between buffer A (20 mM Bis-Tris (pH 6.5), 1 mM MgSO\textsubscript{4}) and buffer B (20 mM Bis-Tris (pH 6.5), 1 mM MgSO\textsubscript{4}, 1 M NaCl).

**H\textsuperscript+-ATPase kinetics**

The standard ATPase assay was described previously (32). When solubilized ATPases were used, 0.1% (w/v) sonicated asolectin was added.

**Liposome preparation and enzyme reconstitution**

Liposome preparation, reconstitution of membrane-bound enzyme, and the measurement of ATP-dependent proton pumping were performed as described previously (13), except that reconstitution was performed by mixing 10 µg of the
purified protein with 0.7 mg of asolectin in a total volume of 150 µl of 50 mM potassium acetate, 10 mM Mes buffer pH 7.0. The mixture was frozen in liquid nitrogen and ATP-dependent proton pumping was measured after thawing the mixture on ice. The reaction was initiated with MgATP and stopped by addition of the ionophore, nigericin, or the protonophore, FCCP.

**Fusicoccin binding assay**

Fusicoccin-binding activity was measured as described previously (33) using 9’-nor-8’-hydroxy[3H]fusicoccin (1.06 Mbq nmol⁻¹, 10 nM) as the radioligand. All tests were performed in duplicate.

**Analysis of 14-3-3 binding in yeast**

Blue native electrophoresis (34) was performed with a polyacrylamide gradient gel (5-18%). The tryptic digests were prepared in 15 % glycerol, 50 mM Bis-Tris/HCl, pH 7.0 and applied without Coomassie blue. The dye which induced the protein charge shift was provided during electrophoresis by including 0.02 % Coomassie blue (SERVA Bleu G) in cathode buffer.

The second dimension (SDS-PAGE, (35)) was performed using a 16.5% T, 3% C gel overlaid with a 10% T, 3% T spacer gel and a 4% T, 3% C stacking gel. Western blot analysis was performed using specific antibodies directed against the C-terminal region of PMA2 (13) and the enhanced biochemiluminescence method.

**Peptide purification**

Anion-exchange chromatography fractions were precipitated with 10 % trichloroacetic acid. After 30 min on ice, the proteins were pelleted (20,000 g for 30 min) and
resuspended in 50 µl of 2% SDS (w/v) in water. The sample was diluted 20 fold and fractionated using a reverse-phase column (2.1 x 250 mm, ProSphere C18, Alltech) connected to an ABI 140B HPLC. Solvent A was 0.1 % TFA in water and solvent B was 0.1 % TFA in 85/15 acetonitrile/water (v/v). A linear gradient was developed over 100 min from 5% B to 100% B.

**Peptide binding experiments**

The peptide isolated by RP-HPLC (40 pmol or 0.265 µg) was dried under vacuum, resuspended in 10 mM Tris-HCl pH 8.5 and dephosphorylated at 37°C for 60 min using one unit of calf intestine alkaline phosphatase (Boehringer Mannheim). For 14-3-3 binding experiments, the dephosphorylated peptide was resolved by SDS-PAGE (35), and transferred to a PVDF membrane. The membrane was saturated for 30 min with 3% (w/v) milk powder, 0.5 % Tween-80 in TBS (50 mM Tris-HCl, pH 7.8, 150 mM NaCl) and then washed three times for 10 min in 0.1% Tween-80 in TBS. The membrane was incubated overnight at 4°C with purified ^35S- labeled His-tagged 14-3-3 (20 µg/ml in 20 mM Tris/HCl, pH 7.8, 20 % glycerol, 5 mM MgSO₄, 2 mM DTT). His-tagged 14-3-3 (N. tabacum, isoform T14-3c) expressed in E. coli was labeled in a minimal medium supplemented with PRO-MIX (~70 % L-[^35S] methionine and 30 % L-[^35S] cysteine)(Amersham Pharmacia Biotech) and purified on Ni²⁺-NTA (Quiagen). After three washes for 5 min with 0.1 % Tween in TBS, the membrane was dried and autoradiographed.

**MALDI-TOF analysis**

Protein bands excised from a Coomassie blue-stained gel or the RP-HPLC purified C-terminal peptide of PMA2 were digested overnight using 0.5 µg trypsin in a total
volume of 50 µl 100 mM Tris-HCl pH 8.7/ H$_2^{18}$O (1/1). The peptides generated were partially mass-tagged at their C-terminal carboxyl moiety by incorporating $^{18}$O-isotope (36). Following digestion, a fraction of the peptide mixture (about 10% of the total) was concentrated on Poros R2 beads and analyzed by MALDI-MS peptide mass fingerprinting (37). The remaining peptide mixture was separated by reverse-phase HPLC on a 1 mm i.d. C-18 column (1 x 50 mm, Vyda Separations Group, Hesperia, CA, USA) (37). Eluting peptides were automatically collected in 50 µl aliquots, to which 5 µg Poros R2 beads suspended in 15 µl 0.1% TFA were added. Fractions were completely dried and the peptides desorbed from the beads using 0.7 µl of MALDI-matrix solution (a 5-fold dilution of 20 mg $\alpha$-cyano-4-hydroxycinnamic acid and 4 mg 2,5-dihydroxybenzoic acid dissolved in 500 µl 0.1% TFA in water/acetonitrile (1/1)) (37). The matrix-peptide solution was then transferred onto the MALDI-target, air-dried and analyzed. All MALDI mass spectra were obtained using a Bruker Reflex III Instrument (Bruker Instruments Inc., Bremen, Germany), with the delayed extraction option. RP-HPLC fractions from the purified C-terminal peptide of PMA2 were first analyzed in reflectron mode and then subjected to PSD-analysis to be verified. For the identification of protein bands excised from the polyacrylamide gel, the Sequest program (38) used PSD-spectral data to search a 290,000-entries non-redundant protein database for matches.
Results

A histidine-tag at the C-terminus of PMA2 affects yeast growth

In order to purify the *N. plumbaginifolia* PMA2 expressed in yeast, a His-tag was added to either the C-terminal residue or to the N-terminal region (between residues 3 and 4) of the wild-type PMA2 and a mutated PMA2 (E14D). The mutant enzyme, which contains a glutamate to aspartate replacement at position 14, was more active and allowed yeast to grow at a lower pH (13). After transformation, the plasmid encoding the essential yeast H⁺-ATPase was eliminated by selection on a suicide substrate (10).

The yeast strain expressing the N-tagged wild-type PMA2 grew at pH 6.0, but not at lower pHs, like the untagged wild-type PMA2 (Fig. 1). However, the C-tagged wild-type PMA2 failed to replace the yeast H⁺-ATPase. In the case of the activated mutant (E14D), the N-tagged protein allowed growth down to pH 3.6, just like the untagged protein. However, C-tagging of the mutant reduced growth at both pH 6.0 and pH 4.0 and abolished growth at pH 3.6 (Fig. 1). These results indicate that C-terminal tagging of either the wild-type or mutant PMA2 interferes with enzyme function. Conversely, N-terminal tagging had no obvious deletions effects on enzyme function.

The purified N-tagged H⁺-ATPase retains the enzymatic properties of the membrane-bound form

Purified plasma membranes from yeast strains expressing the N-tagged wild-type or E14D PMA2 were stripped with C12E8, the H⁺-ATPases solubilized using DDM (39) and purified on a Ni column (Fig. 2).
The purified wild-type and mutated PMA2 were analyzed to determine whether the effects of the activating mutation were unaffected by purification. The N-tagged versions were chosen for analysis, since yeast growth was not affected by this modification. The E14D PMA2 had a lower Km and a higher Vmax than the wild-type PMA2 (Table 1) and its pH optimum was shifted towards alkaline values (pH 7.0-7.2, compared with pH 6.4-6.8 for the wild-type). Moreover, the ATPase activity of the E14D PMA2 was not stimulated by lysophosphatidylcholine (LPC), while that of the wild-type PMA2 was enhanced 3-fold (Table 1). These data concur with the differences previously obtained with the membrane-bound non-tagged wild-type and mutant enzymes (13,32).

The purified enzymes successfully reconstituted into liposomes, allowing an estimate of the coupling between proton-pumping and ATP hydrolysis. The mutant PMA2 had greater proton-pumping and ATPase activity and a higher coupling ratio than the wild-type (Table 1). The activated state of the E14D mutant, previously described for the membrane-bound form, was conserved after purification and reconstitution, and is therefore an intrinsic property of the plant H⁺-ATPase. Trypsin digestion and western blotting with region specific antibodies (32) confirmed that the C-terminal region is more accessible for the mutant enzyme than for the wild-type (data not shown).

**PMA2 forms a stable complex in vivo with yeast 14-3-3 homologs**

Electrophoretic analysis and Coomassie blue-staining of the fractions obtained after Ni affinity chromatography confirmed the enrichment of the plant H⁺-ATPase (Fig. 2). Antibody-reactive minor bands with molecular masses of about 60 and 50 kDa were identified as proteolytic products of PMA2 (not shown). Two additional proteins with apparent molecular masses of 32 and 35 kDa copurified with both the N-tagged wild-
type and mutant PMA2, but did not react with any of the anti-PMA2 antibodies. When these protein bands were excised from the gel, digested with trypsin, and the digest analyzed by MALDI-MS peptide mass fingerprinting, they were identified, using a non-redundant protein database, as the yeast BMH1 (P29311) and BMH2 (P34730). These initial findings were verified by separation of the peptide mixture by RP-HPLC and linear mode MALDI-MS analysis. Several peptide ions were then selected for MALDI-PSD analysis and identified as fragments from BMH proteins (Table 2). These identifications were confirmed using antibodies specific for yeast 14-3-3 proteins (40) (not shown).

A fraction of the N-tagged wild-type and mutant plant PMA2 therefore formed, in vivo, a stable complex with yeast 14-3-3 proteins. Complex formation did not require fusicoccin, as in plants (15-17) or in yeast expressing an Arabidopsis H⁺-ATPase isoform (41). Conversely, 14-3-3 protein was not detected in association with the C-tagged PMA2 (Fig. 2), indicating that tagging at this position interfered with 14-3-3 binding.

Since only a fraction of the H⁺-ATPases forms a complex with 14-3-3 proteins, we separated the two forms by size-exclusion chromatography. Both the wild-type and mutant H⁺-ATPases segregated as a free form and a 14-3-3 complex, the latter being relatively more abundant in the case of the mutant (Fig. 3A). Comparison of the specific ATPase activities of the two forms (with protein measured as the amount of the 100 kDa band on SDS gels) showed that the ATPase/14-3-3 complex was approximately twice as active as the free enzyme (Fig. 3B). This applied to both the wild-type and mutant forms (Fig. 3B). In the complex, the ATPase/14-3-3 molar ratio was 0.76 ± 0.09.
The PMA2-14-3-3 complex binds fusicoccin very efficiently in vitro

The action of fusicoccin at the molecular level is not well understood. In plants, fusicoccin stabilizes, or even induces, the formation of the H⁺-ATPase-14-3-3 complex and increases ATPase activity (15-17, 26, 41-43). In contrast, pretreatment of yeast cells expressing PMA2 does not affect the ATPase activity (33). Although, the present study showed that a very stable PMA2-14-3-3 complex was formed in vivo in the absence of fusicoccin, it was of interest to determine whether it could bind fusicoccin. Fusicoccin binding activity (Fig. 2) was enhanced 15- to 18-fold by the Ni affinity column purification of either the N-tagged wild-type or E14D PMA2, showing that the fusicoccin-binding activity co-purified with the H⁺-ATPase. The Kd for both the membrane-bound and affinity-purified enzymes was less than 1 nM (Fig. 2), were unchanged by affinity purification and were similar to values estimated using plant membranes (44). After gel filtration of the purified fraction, the fusicoccin-binding activity co-purified with the complex (1378 and 1717 pmol/mg for the wild-type and mutant forms, respectively) while the free H⁺-ATPase did not show binding. The C-terminal tagged PMA2 mutant, which did not form a complex with 14-3-3 proteins (Fig. 2), also did not bind fusicoccin.

Homo- and heterodimers of 14-3-3 proteins interact with the PMA2 C-terminal region

The part of PMA2 which participates in the complex was identified by tryptic digestion of the more readily obtained purified E14D PMA2/14-3-3 complex. SDS-PAGE showed that the H⁺-ATPase was completely degraded into smaller products while the two 14-3-3 isoforms seemed intact (Fig. 4A).
Blue native electrophoresis was used to test whether the intact 14-3-3 proteins formed a dimer with a fragment of PMA2. Preliminary experiments indicated that the H\(^+-\)ATPase/14-3-3 complex, although stable in low or high pH, in high salt or upon urea treatment, dissociated upon tryptic digestion. We therefore stabilized the purified complex by fusicoxin treatment. Trypsin digestion was performed in the absence and presence of 5 µM fusicoxin. In the absence of the toxin, three clear bands were observed (Fig. 4B). These cross-reacted with anti-14-3-3 antibodies, but not with anti-H\(^+-\)ATPase C-terminus antibodies (see below). In the presence of fusicoxin, the three bands had a lower electrophoretic mobility (Fig. 4B) and cross-reacted with both anti-14-3-3 and anti-H\(^+-\)ATPase C-terminus antibodies (not shown), suggesting that a PMA2 C-terminus/14-3-3 complex was preserved.

The blue native electrophoresis separated bands were resolved by second dimension electrophoresis under denaturing conditions (SDS-PAGE, Fig. 4C-E). In the absence of fusicoxin, the complex was resolved into a set of spots identified by western blot as BMH1 or BMH2 (not shown), which suggests that the complex was originally organized as a homo- or heterodimer (Fig. 4, inset). In the presence of fusicoxin (Fig. 4D), an additional spot of ~6,500 Da was observed. A minor spot of ~7,800 Da was also present. Western blotting identified both polypeptides as fragments from the H\(^+-\)ATPase C-terminal region (Fig. 4E).

**14-3-3 proteins interact with the PMA\(_2\) C-terminal region**

The H\(^+-\)ATPase region involved in the complex was characterized in more detail. The complex obtained after trypsin treatment was purified by anion exchange chromatography. The last major peak (Fig. 5, fractions 5-6) was identified by western blotting as the 14-3-3/C-terminal fragment complex (not shown). This was solubilized
in 2 % SDS, and the suitably diluted proteins were separated by reversed phase chromatography. The PMA2 C-terminal fragment, again identified by western blotting and its sequence determined by Edman degradation revealed the presence of a major peptide corresponding to the last 57 residues of PMA2 (150 pmol).

Identification of the penultimate residue of the PMA2 C-terminal region by MALDI-TOF mass spectrometry as a phospho-threonine

The total mass of the RP-HPLC purified major tryptic peptide of PMA2 binding to the 14-3-3 proteins was obtained by MALDI-MS operating in linear mode. The peptide had an average mass of 6635.93 Da. This corresponded to the mass of the last 57 amino acids of PMA2 ((M+H)+av=6554.39 Da) plus 80 Da, characteristic of a single phosphorylated moiety. In order to identify which amino acid was phosphorylated, the purified 57-residue peptide was digested with trypsin and the resulting peptide mixture was separated by RP-HPLC. MALDI-PSD was used to determine the sequences of the observed tryptic peptides. As shown in Table 3, except for the last 13 amino acids, the complete sequence of the 57 amino acid long C-terminal part of PMA2 could be obtained using MALDI-PSD spectra. One of the RP-HPLC fractions contained a peptide with a mass of 1546.54 Da. This corresponded to the theoretical mass of the last 13 amino acids (NH2-GLDIETIQQSYTV-COOH, (M+H)+mono=1466.73 Da) with one phosphorylated residue. Furthermore, the loss of 80 and 98 Da from the intact precursor ion, observed in the reflectron mode (Fig. 6A), typifies the presence of a phosphorylated residue (45). Since the proposed peptide has at least three putative phosphorylation sites (Thr 949, Ser 953 and Thr 955), MALDI-PSD analysis was performed in order to identify the phosphorylated residue. Comparison of the MALDI-PSD spectra of the ‘native’ (phosphorylated) peptide and
a dephosphorylated peptide, confirmed that Thr 955, the penultimate residue, was phosphorylated (Fig. 6B).

**Dephosphorylation of Thr955 prevents in vitro interaction of 14-3-3 and the C-terminus of PMA2**

The involvement of phospho-threonine in the formation of the 14-3-3/PMA2 complex was assessed using the dephosphorylated PMA2 57-residue C-terminal peptide. An overlay assay was used to determine the effect of alkaline phosphatase treatment on 14-3-3 binding (43). In the absence of fusicoccin, the 14-3-3 proteins bound to the PMA2 peptide (Fig. 7, lane 1). The binding depended a phosphorylated Thr 955 since alkaline phosphatase pretreatment abolished binding (Fig. 7, lane 2). In the presence of fusicoccin, a much stronger signal (lane 3), that was absent for the dephosphorylated peptide (lanes 4), was observed. The toxin therefore increased formation of the complex, provided the peptide was phosphorylated.

**Mutation of Thr955 prevents 14-3-3 binding, decreases H⁺-ATPase activity and alters yeast growth**

To show that in vivo 14-3-3 binding depends on PMA2 phosphorylation at Thr 955, this residue in both wild-type and E14D mutant pma2 cDNA was mutated to alanine, to aspartate or to a termination codon.

With the wild-type PMA2, none of the mutations supported yeast growth when the plasmid with the yeast H⁺-ATPase gene was eliminated. This demonstrated that Thr 955 was essential for PMA2 to confer yeast growth. Even the negative charge of Asp could not mimic a phosphorylated Thr.
The set of Thr 955 mutations introduced in E14D PMA2 were more informative because they allowed yeast growth when the plasmid expressing the yeast H\(^+\)-ATPase gene was removed. However, all Thr 955 mutations (Ala, Asp or stop) gave reduced yeast growth at pH 5.0, and prevented growth at pH 4.0. In contrast, the strain expressing the unmodified E14D PMA2 grew normally at both pHs (Fig. 8A). Although all mutant membranes showed levels of PMA2 comparable to the E14D membranes, none of mutant membranes displayed 14-3-3 proteins (Fig. 8B). In addition, ATPase activity for the three mutants was reduced by 43-56 %. This indicated that the lack of phosphorylation and 14-3-3 binding gave a less active H\(^+\)-ATPase (Fig. 8B).
Discussion

Expression in yeast has allowed the purification and analysis of His-tagged wild-type and activated mutant (E14D) H⁺-ATPase PMA2 from N. plumbaginifolia. Because inclusion of an N-terminal His tag affected neither the ATPase activity nor yeast growth, these constructs allowed a detailed comparison of the purified enzymes. As previously shown for the membrane-bound enzyme (32), the activated state of the purified mutant correlated with a structural modification that made the C-terminal region more accessible to trypsic degradation. The kinetic and structural differences seen between the wild-type and E14D PMA2 are therefore inherent to the enzyme.

Purification of the N-tagged wild-type and mutant PMA2 unexpectedly revealed that both yeast 14-3-3 proteins (BMH1 and BMH2) co-purified with the H⁺-ATPase. Regulatory 14-3-3 proteins have been found to interact with plant plasma membrane H⁺-ATPases, but only after treatment with fusicoccin. The complex formed between 14-3-3 and plasma membrane H⁺-ATPase is thought to act as the fusicoccin receptor, and treatment with the fungal toxin stabilizes this complex (15-17,26,41-43). A C-terminal domain, which is specific to plant H⁺-ATPases, is involved in complex formation with 14-3-3s (15,16,43). This suggests 14-3-3 proteins may be potential modulators of H⁺-ATPase activity. In plants, complex formation is only observed in the presence of fusicoccin. This has precluded determination of whether the complex can be formed in the absence of fusicoccin and, if so, whether H⁺-ATPase activity is enhanced. The present study of PMA2 expression in yeast therefore provides the final evidence of a stable in vivo H⁺-ATPase/14-3-3 complex formed in the absence of fusicoccin. This complex is sufficiently stable to resist drastic treatments with 4 M urea and either low (4.0) or high (10.5) pH.
Separation of the free H\textsuperscript{+}-ATPase from the H\textsuperscript{+}-ATPase/14-3-3 complex by gel filtration chromatography showed that the specific activity of the complex was significantly (2-fold) higher than that of the free form of both the wild-type and E14D mutant. The C-tagged H\textsuperscript{+}-ATPase and isoforms mutated in the penultimate residue served as useful negative controls. They failed to form a complex with 14-3-3 proteins. These modifications reduced ATPase activity and either abolished (wild-type PMA2) or diminished (E14D PMA2) complementation of yeast PMA1.

On the basis of Coomassie blue staining, the molar ratio of H\textsuperscript{+}-ATPase and 14-3-3s in the complex is close to 0.8; a higher ratio was found using silver staining, suggesting that the actual ratio is close to 1. The regulatory 14-3-3 proteins occur only as a dimer (46), but, since one dimer can interact with two partners at the same time (47), the minimal complex may consist of a H\textsuperscript{+}-ATPase dimer linked through a 14-3-3 dimer.

The PMA2 region interacting with the 14-3-3 proteins appears within the last 57 amino acid residues of PMA2. Fusicoccin is not required for complex formation because it was absent during yeast growth, membrane isolation, PMA2 solubilization and purification. However, in vitro fusicoccin treatment was required during trypsin digestion to stabilize the complex between the 14-3-3’s and the tryptic-generated PMA2 C-terminal region. This implies that fusicoccin masks the last four trypsin sites or modifies the structure of the complex, preventing access of the protease to the PMA2 C-terminal region engaged in the 14-3-3 dimer. Furthermore, although the 57-residue peptide was able to bind 14-3-3’s in the absence of fusicoccin (overlay assay), fusicoccin addition caused formation of a higher complex amount. This suggests that fusicoccin stabilizes the interactions between the PMA2 C-terminal region and the 14-3-3’s.
Our data support the hypothesis that the PMA2 C-terminal region contains two domains. The N-terminal one was proposed to be the inhibitory domain interacting with the rest of the enzyme because 18 point mutations identified in this domain displace the C-terminal region and render the enzyme more active (32). In this work, the C-terminal half of the C-terminal region is shown to interact with 14-3-3 proteins. This interaction specifically involves phosphorylation of the penultimate residue (Thr 955) of PMA2. Olsson et al. (26) had identified a phosphorylated Thr in spinach H⁺-ATPase. In this case, in vivo treatment with fusicoccin was necessary and it was not clear whether phosphorylation and 14-3-3 binding were independent of fusicoccin treatment.

The inability to detect non-phosphorylated peptide during MALDI-TOF analysis suggests that all PMA2 molecules engaged in the complex are phosphorylated at Thr 955. Furthermore, this phosphorylation is indispensable for the complex formation. This was shown by overlay assay with the purified peptide obtained after in vitro dephosphorylation and by the analysis of the mutants obtained after site-directed mutagenesis of Thr 955.

Site-directed mutagenesis of Thr 955 in the wild-type PMA2 did not allow yeast growth when the yeast H⁺-ATPase gene was removed. This provides direct evidence of the important physiological consequence of interfering with Thr 955 phosphorylation. To rule out artifacts such as protein instability, mutagenesis of Thr 955 was performed in the E14D mutant of PMA2, which confers sufficient ATPase activity to replace yeast PMA1. In the E14D background, prevention of Thr 955 phosphorylation still allowed replacement of yeast H⁺-ATPase, but gave a lower growth rate. The mutated E14D PMA2 was synthesized to the same extent and targeted to the plasma membrane. However, no 14-3-3 binding was observed and
ATPase activity was reduced. *In vivo* treatment of yeast cell expressing PMA2 with fusicoccin did not increase the amount of yeast 14-3-3 associated with the plasma membrane, did not activate PMA2 and did not improve growth (33). Thus fusicoccin treatment has no effect on the phosphorylation of Thr 955.

The proportion of the H⁺-ATPase occurring as a complex with 14-3-3 is higher in cells expressing the mutant E14D than in those expressing the wild-type PMA2. This observation can be correlated with the higher fusicoccin-binding activity of both the membrane and purified fractions. The higher proportion of complex may be related to the better accessibility of the mutant C-terminal as revealed by trypsin treatment of the membrane (32) or soluble enzyme (data not shown). The enhanced ATPase activity and yeast growth seen with the E14D mutant cannot be fully explained by increased 14-3-3 binding activity. Indeed, the C-tagged E14D mutant, and the N-tagged E14D mutant modified in the penultimate residue did not bind 14-3-3 proteins, but these enzymes had higher ATPase activity and conferred better growth than did the wild-type PMA2. In addition to allowing better 14-3-3 binding, the E14D mutation has an inherent positive effect on enzyme activity. This fact is also supported by the existence of a mutant PMA2 lacking the 74 C-terminal residues (32). This mutant does not recruit 14-3-3 proteins to the plasma membrane (not shown) and does not bind fusicoccin (33), but has a high ATPase activity and fully supports yeast growth (32).

What is true for PMA2 may not apply to plant H⁺-ATPase isoforms expressed in yeast. Indeed, the *A. thaliana* AHA2 H⁺-ATPase isoform, purified using a His-tag at the N-terminus, does not seem to form a 14-3-3 complex (48) and its fusicoccin-binding activity in membrane fractions is very low in the absence of *in vivo* fusicoccin treatment (0.04 pmol mg⁻¹ protein) (41) compared with the N-tagged PMA2 (29.6 pmol mg⁻¹ protein) (41).
pmol mg\(^{-1}\) protein). This low fusicoccin-binding activity can be correlated with the observation that aha2 did not complement yeast PMA1. This suggests that, for full activity of plant H\(^{+}\)-ATPases, 14-3-3 proteins are required. Plants express several H\(^{+}\)-ATPases, sometimes in the same cell type and at the same developmental stage. This is the case of PMA2 and PMA4, the two most highly expressed isoforms in *N. plumbaginifolia* (7). Expression of these isoforms in yeast has shown them to have different kinetic behaviors and to confer different sensitivities to external pH (11). It will therefore be important to characterize the phosphorylation status and the 14-3-3 complex formation of PMA4 to see whether the two H\(^{+}\)-ATPase isoforms are subject to a different C-terminus phosphorylation and/or 14-3-3 binding capacity.

While this paper was under review, two reports addressed 14-3-3 binding to plant H\(^{+}\)-ATPase (49,50). They also concluded that 14-3-3 binding to plant H\(^{+}\)-ATPases involved the C-terminal region, including a phosphorylated threonine as the penultimate residue.
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Footnotes

The abbreviations are: AHA: Arabidopsis H+-ATPase; PMA: plasma membrane H+-ATPase; DDM: dodecylmaltoside; LPC: lysophosphatidylcholine, TFA: trifluoroacetic acid.
Figure legends

Fig. 1: Yeast growth conferred by tagged and untagged wild-type and E14D PMA2. Yeast cells expressing either the wild-type (YAKpma2) or the mutated PMA2 (E14D) as their untagged (none) or N-terminal (N-ter) or C-terminal (C-ter) tagged form were replicated on glucose medium at different pHs (pH 6.0, 4.0, and 3.6) and grown at 30°C for five days. For the wild-type PMA2 tagged at the C-ter, the strain still contained the plasmid bearing the yeast PMA1 under the GAL1 promoter.

Fig. 2: Plasma membrane-bound and affinity-purified fractions of the tagged wild-type and E14D PMA2. A. Plasma membrane-bound (20 µg) and Ni-affinity purified fractions (5 µg) of the N-tagged wild-type PMA2 and the N- or C-tagged E14D PMA2 were analyzed by SDS-PAGE and stained with Coomassie blue. MW corresponds to molecular weight markers. The corresponding specific ATPase activity and fusicoccin (FC)-binding activity (Bmax and Kd) for each fraction are shown below the gel. ND: not determined.

Fig. 3: Separation of free H⁺-ATPase and the H⁺-ATPase/14-3-3 complex by size-exclusion chromatography. A. Ni-affinity purified wild-type PMA2 (50 µg) was separated by size-exclusion chromatography (see experimental procedures) and the fractions (1/25) analyzed by silver-stained SDS-PAGE gel (shown for fractions 23 to 35). B. 500 µl of fractions 25 and 33 of wild-type and E14D PMA2 were precipitated by 10% TCA and analyzed by
Coomassie blue-stained SDS-PAGE and the amount of protein in the 100 kD band estimated by densitometry. This value was used to calculate the specific activity of each form of the ATPase from ATPase assays of the separated soluble forms. An arbitrary value of 1 was assigned to fraction 25.

**Fig. 4.** Analysis of the PMA2 H⁺-ATPase/14-3-3 complex obtained after tryptic treatment.

(A) Purified PMA2 was digested (+) or not digested (-) with trypsin as described in “Experimental procedures”, samples were electrophoresed on a Tris-Tricine polyacrylamide gel and stained with Coomassie Blue. (B) Purified PMA2 incubated in the absence (-) or presence (+) of 5 µM fusicoccin was digested by trypsin as described under “Experimental procedures”. The resulting products were electrophoresed by Blue native-PAGE. (C-E) Gel strips obtained after Blue native-PAGE as in B were subjected to SDS-PAGE. The gels were stained with Coomassie Blue (C, D) or immunoblotted with anti-H⁺-ATPase C-terminus antibodies (E). Inset: detail of 14-3-3 homo- and heterodimers.

**Fig. 5.** Ion exchange chromatography of the PMA2 C-terminus/14-3-3 complex obtained after tryptic treatment.

Tryptic fragments of PMA2/14-3-3 complex, obtained as in Fig. 4, were subjected to UNOQ chromatography using a 0-500 mM NaCl gradient. One-ml fractions were collected. Fractions 5-6 correspond to the 14-3-3/PMA2 C-ter complex were detected by western blot analysis (not shown). Absorbance (AU), the gradient progression (%B) are displayed.
Fig. 6. (A) MALDI-MS spectrum of the C-terminal fragment of PMA2.

After RP-HPLC of fractions 5-6 resulting from UNOQ chromatography (Fig.5), the fraction containing the PMA2 C-terminal fragment was digested with trypsin and analyzed by MALDI-MS. The peptide with a mass of 1546.54 Da displays two satellite peaks 80 and 98 Da smaller than the intact peptide, indicating that the peptide contains a phosphorylated residue. (B) MALDI-PSD spectrum of the phosphorylated (right) or dephosphorylated (left) peptide present in the spectrum shown in (A). The observed $b_n$-ions verifying the sequence NH$_2$GLDIETIQQSYT$^*$V-COOH ($T^*$=phosphothreonine) are indicated.

Fig. 7. Dephosphorylation of Thr 955 prevents 14-3-3 binding.

The 57-residue C-terminal peptide (0.25 µg) obtained after ion exchange and RP-HPLC chromatography was incubated for 1 h at 37°C in 20 µl dephosphorylation buffer (50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5) in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of one unit of calf intestine alkaline phosphatase. The peptide was separated by SDS-PAGE, blotted onto a PVDF membrane (lanes 1-4) and lanes 5 and 6 stained with Coomassie blue. After saturation, the rest of the membrane was incubated with $^{35}$S- labeled 14-3-3 as indicated in “Experimental procedures” in the absence (lanes 1 and 2) or presence of 5 µM fusicoccin (lanes 3 and 4).

Fig. 8. Mutation of Thr 955 reduces growth of the yeast expressing the E14D PMA2 H$^+$-ATPase.

(A) The strain expressing E14D PMA2 and the three Thr 955 mutants (E14D-Ala, E14D-Asp, and E14D-stop) were spread on a solid rich glucose medium at pH 4.0 or 5.0 and grown for five days. (B) Purified plasma membranes (2 µg proteins) of these
strains were electrophoresed on a 12% Tris-Glycine polyacrylamide gel and blotted onto a PVDF membrane. PMA2 H⁺-ATPase and yeast 14-3-3 proteins were immunodetected with the corresponding antibodies. ATPase activity is indicated below.
Table 1. Kinetic properties of the purified tagged wild-type and E14D PMA2

| N-tagged H^+-ATPase | Purified H^+-ATPase | Reconstituted vesicles |
|---------------------|---------------------|-----------------------|
|                     | Km¹ (µM)            | Vmax¹ (µmol Pi/min/mg) | pH optimum² | LPC activation¹,³ | ACMA quenching⁴ (% quenching/min/mg) | ATPase activity⁴ (µmol Pi/min/mg) | quenching/ATPase ratio |
| Wild-type PMA2      | 144 ± 9             | 22.4 ± 1.6             | 6.4 - 6.8   | 332 ± 16          | 5.6 ± 0.7                              | 17.3 ± 1.2                         | 323                   |
| E14D PMA2           | 46 ± 3              | 36.8 ± 1.8             | 7.0 - 7.2   | 101 ± 5           | 19.3 ± 0.7                             | 36.4 ± 1.3                         | 530                   |

¹ Data are the mean ± SD of two independent plasma membrane preparations
² Range of optimal pH within which no significant difference was found
³ Specific ATPase activity without LPC was taken as 100%
⁴ Data are the mean ± SD of three independent reconstitutions
Table 2. Identification of 30 kDa bands co-purifying with $H^+$-ATPase by mass spectrometry

| Protein band$^1$ | Matched sequence | Identified protein          |
|------------------|------------------|----------------------------|
| P32              | NH$_2$-TASEIATTELPPTHPIR-COOH | BMH1 and BMH2 (yeast) |
|                  | NH$_2$-TVASSGQELSVEER-COOH       | BMH1 (yeast)               |
| P35              | NH$_2$-YLAEFSSGDAR-COOH         | BMH1 and BMH2 (yeast) |
|                  | NH$_2$-VFYYK-COOH               | BMH1 and BMH2 (yeast) |
|                  | NH$_2$-AVASSGQELSVEER-COOH      | BMH2 (yeast)              |

$^1$ P32 and P35 refer to the bands of the corresponding size (kDa) identified in figure 2.
Table 3. MALDI-PSD based identification of tryptic peptides from the C-terminal region of PMA2

| Position | (M+H)\textsuperscript{+} \textit{measured} | Sequence |
|----------|--------------------------------|----------|
| 900-918  | 2167.97 Da                     | (-)LFSEATNFNELNQLAEEAK\textit{(R)} |
| 920-925  | 715.51 Da                      | \textit{(R)}RAEIAER\textit{(QR)}   |
| 928-933  | 740.52 Da                      | \textit{(QR)}ELHTLK\textit{(G)}    |
| 934-941  | 854.52 Da                      | \textit{(K)}GHIVESVK\textit{(LK)}  |
| pH  | Wild-type PMA2 | E14D PMA2 |
|-----|----------------|-----------|
| 6.0 | ![Image](image1) | ![Image](image2) |
| 4.0 | ![Image](image3) | ![Image](image4) |
| 3.6 | ![Image](image5) | ![Image](image6) |

**His-tag:**

- None
- N-ter
- C-ter
| MW (kDa) | Plasma membrane fraction | Purified fraction |
|----------|--------------------------|------------------|
| 94       | N-tag PMA2               | N-tag PMA2       |
| 67       | N-tag E14D               | N-tag E14D       |
| 43       | C-tag E14D               | C-tag E14D       |
| 30       | PMA2                     | BMH2             |
|          |                          | BMH1             |

### ATPase activity (µmolPi/min/mg)

| N-tag PMA2 | N-tag E14D | C-tag E14D | BMH2 | BMH1 |
|------------|------------|------------|------|------|
| 0.69       | 1.03       | 0.8        | 22.4 | 36.8 |
| 27.2       |            |            |      |      |

### FC-binding activity:

| Bmax (pmol/mg) | Kd (nM) |
|----------------|---------|
| N-tag PMA2 | N-tag E14D | C-tag E14D | BMH2 | BMH1 |
| 29.6 | 50.8 | <0.2 | 482 | 923 |
| 0.82 | 0.85 | nd  | 0.79 | 0.81 |
| nd  | nd  | nd  | nd  | nd  |
A

B

WT | E14D
---|---
1 0.59 ± 0.10 | 1 0.55 ± 0.16

ATPase activity (arbitrary units)
A. Tryptic treatment

|                | - | + |
|----------------|---|---|
| Plant H⁺-ATPase|   |   |
| Yeast 14-3-3   |   |   |

B. FC pre-treatment

|                | - | + |
|----------------|---|---|
| 14-3-3 dimers  |   |   |
| PMA2 C-terminal region |   |

C. Native-PAGE

D. SDS-PAGE

E. BMH2 BMH1

|                | - | + |
|----------------|---|---|
| BMH2 BMH1      |   |   |

|                | - | + |
|----------------|---|---|
| Yeast 14-3-3   |   |   |
| PMA2 C-terminal region |   |

|                | - | + |
|----------------|---|---|
| FC pre-treatment |   |   |
14-3-3 dimer + PMA2
C-terminal region
A.  

Amino acid Ion Observed Mass  Amino acid Ion Observed Mass  
Gb b1 / G b1 /  
Lb b2 / L b2 170.90  
Db b3 286.29 D b1 286.41  
Ib b4 399.51 I b4 399.65  
Eb b5 528.74 E b5 528.78  
Tb b6 629.77 T b6 629.56  
Ib b7 742.75 I b7 742.78  
Qb b8 / Q b8 871.28  
Qb b9 999.12 Q b9 999.24  
Sb b10 1086.16 S b10 1085.70  
Yb b11 1249.12 Y b11 1249.29  
phosphoT b12 1430.27 T b12 1350.14  
Vb b13 / V b13 /  

B.  

1470 1480 1490 1500 1510 1520 1530 1540 1550 1560 1570 m/z 

(MH-HPO₄)⁺  

(MH-H₂PO₄)⁺
|                      | 14-3-3 overlay | Coomassie blue |
|----------------------|----------------|----------------|
| FC during overlay    | -              | -              |
| Alkaline phosphatase | -              | +              |

![Image of gel analysis](http://www.jbc.org/Downloaded from)
A.

pH 4.0

pH 5.0

B.

|          | E14D | E14D-Ala | E14D-Asp | E14D-stop |
|----------|------|----------|----------|-----------|
| ATPase activity (μmolPi/min/mg) | 1.33 ± 0.18 | 0.59 ± 0.10 | 0.61 ± 0.04 | 0.76 ± 0.06 |

PMA2

Yeast 14-3-3
References

1. Michelet, B., and Boutry, M. (1995) *Plant Physiol.* **108**, 1-6
2. Palmgren, M. G. (1998) *Adv. Bot. Res.* **28**, 1-70
3. Sussman, M. R. (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45**, 211-234
4. Harper, J. F., Manney, L., and Sussman, M. R. (1994) *Mol. Gen. Genet.* **244**, 572-87
5. Ewing, N. N., and Bennett, A. B. (1994) *Plant Physiol.* **106**, 547-57
6. Michelet, B., Lukaszewicz, M., Dupriez, V., and Boutry, M. (1994) *Plant Cell* **6**, 1375-89
7. Moriau, L., Michelet, B., Bogaerts, P., Lambert, L., Michel, A., Oufattole, M., and Boutry, M. (1999) *Plant J.* **19**, 31-41
8. DeWitt, N. D., and Sussman, M. R. (1995) *Plant Cell* **7**, 2053-67
9. Palmgren, M. G., and Christensen, G. (1994) *J. Biol. Chem.* **269**, 3027-33
10. de Kerchove d'Exaerde, A., Supply, P., Dufour, J. P., Bogaerts, P., Thines, D., Goffeau, A., and Boutry, M. (1995) *J. Biol. Chem.* **270**, 23828-37
11. Luo, H., Morsomme, P., and Boutry, M. (1999) *Plant Physiol.* **119**, 627-34
12. Palmgren, M. G., Sommarin, M., Serrano, R., and Larsson, C. (1991) *J. Biol. Chem.* **266**, 20470-5
13. Morsomme, P., de Kerchove d'Exaerde, A., De Meester, S., Thines, D., Goffeau, A., and Boutry, M. (1996) *EMBO J.* **15**, 5513-26
14. Palmgren, M. G., and Christensen, G. (1993) *FEBS Lett.* **317**, 216-22
15. Jahn, T., Fuglsang, A. T., Olsson, A., Bruntrup, I. M., Collinge, D. B., Volkmann, D., Sommarin, M., Palmgren, M. G., and Larsson, C. (1997) *Plant Cell* 9, 1805-1814

16. Oecking, C., Piotrowski, M., Hagemeier, J., and Hagemann, K. (1997) *Plant J.* 12, 441-453

17. Olivari, C., Meanti, C., De Michelis, M. I., and Rasi-Caldogno, F. (1998) *Plant Physiol.* 116, 529-537

18. Aitken, A. (1996) *Trends Cell Biol.* 6, 341-347

19. Chung, H. J., Sehnke, P. C., and Ferl, R. J. (1999) *Trends Plant Sci.* 4, 367-371

20. Schaller, G. E., and Sussman, M. R. (1988) *Planta* 173, 508-518

21. Suzuki, Y. S., Wang, Y. L., and Takemoto, J. Y. (1992) *Plant Physiol.* 99, 1314-1320

22. Vera-Estrella, R., Barkla, B. J., Higgins, V. J., and Blumwald, E. (1994) *Plant Physiol.* 104, 209-215

23. Desbrosses, G., Stelling, J., and Renaudin, J. P. (1998) *Eur. J. Biochem.* 251, 496-503

24. Sekler, I., Weiss, M., and Pick, U. (1994) *Plant Physiol.* 105, 1125-32

25. Xing, T., Higgins, V. J., and Blumwald, E. (1996) *Plant Cell* 8, 555-564

26. Olsson, A., Svennelid, F., Ek, B., Sommarin, M., and Larsson, C. (1998) *Plant Physiol.* 118, 551-5

27. Lino, B., Baizabal-Aguirre, V. M., and Gonzalez de la Vara, L. E. (1998) *Planta* 204, 352-9

28. van der Hoeven, P. C., Siderius, M., Korthout, H. A., Drabkin, A. V., and de Boer, A. H. (1996) *Plant Physiol.* 111, 857-65
29. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) *Cell* 84, 889-97
30. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) *Cell* 91, 961-71
31. De Boer, B. (1997) *Trends Plant Sci.* 2, 60-66
32. Morsomme, P., Dambly, S., Maudoux, O., and Boutry, M. (1998) *J. Biol. Chem.* 273, 34837-42
33. Piotrowski, M., Morsomme, P., Boutry, M., and Oecking, C. (1998) *J. Biol. Chem.* 273, 30018-23
34. Schägger, H., Cramer, W. A., and von Jagow, G. (1994) *Anal. Biochem.* 217, 220-230
35. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 368-379
36. Gevaert, K., Demol, H., Verschelde, J.-L., Van Damme, J., De Boeck, S., and Vandekerckhove, J. (1997) *J. Prot. Chem.* 16, 335-42
37. Gevaert, K., Demol, H., Sklyarova, T., Vandekerckhove, J., and Houthaeve, T. (1998) *Electrophoresis* 19, 909-17
38. Griffin, P. R., MacCoss, M. J., Eng, J. K., Blevins, R. A., Aaronson, J. S., and Yates, J. R., 3rd. (1995) *Rapid Commun. Mass Spectrom.* 9, 1546-51
39. Johansson, F., Sommarin, M., and Larsson, C. (1994) *Physiol. Plant.* 92, 389-396
40. van Heusden, G. P., Griffiths, D. J., Ford, J. C., Chin, A. W. T. F., Schrader, P. A., Carr, A. M., and Steensma, H. Y. (1995) *Eur. J. Biochem.* 229, 45-53
41. Baunsgaard, L., Fuglsang, A. T., Jahn, T., Korthout, H. A., de Boer, A. H., and Palmgren, M. G. (1998) *Plant J.* 13, 661-71
42. DeMichelis, M. I., RasiCaldogno, F., Pugliarello, M. C., and Olivari, C. (1996) *Plant Physiol.* **110**, 957-964

43. Fullone, M. R., Visconti, S., Marra, M., Fogliano, V., and Aducci, P. (1998) *J. Biol. Chem.* **273**, 7698-702

44. Aducci, P., Marra, M., Fogliano, V., and Fullone, M. R. (1995) *J. Exp. Bot.* **46**, 1463-1478

45. Annan, R. S., and Carr, S. A. (1997) *J. Prot. Chem.* **16**, 391-402

46. Tzivion, G., Luo, Z., and Avruch, J. (1998) *Nature* **394**, 88-92

47. Schultz, T. F., Medina, J., Hill, A., and Quatrano, R. S. (1998) *Plant Cell* **10**, 837-47

48. Lanfermeijer, F. C., Venema, K., and Palmgren, M. G. (1998) *Protein Expr. Purif.* **12**, 29-37

49. Svennelid, F., Olsson, A., Piotrowski, M., Rosenquist, M., Ottman, C., Larsson, C., Oecking, C., and Sommarin, M. (1999) *Plant Cell* **11**, 2379-2392

50. Fuglsang, A. T., Visconti, S., Drumm, K., Jahn, T., Stensballe, A., Mattei, B., Jensen, O. N., Aducci, P., and Palmgren, M. G. (1999) *J. Biol. Chem.* **274**, 36774-80
A plant plasma membrane H+-ATPase expressed in yeast is activated by phosphorylation at its penultimate residue and binding of 14-3-3 regulatory proteins in the absence of fusicoccin
Olivier Maudoux, Henri Batoko, Claudia Oecking, Kris Gevaert, Joel Vandekerckhove, Marc Boutry and Pierre Morsomme

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