Activation of Plant Plasma Membrane H\(^{+}\)-ATPase by 14-3-3 Proteins Is Negatively Controlled by Two Phosphorylation Sites within the H\(^{+}\)-ATPase C-terminal Region\(^*\)\

Received for publication, September 22, 2008, and in revised form, November 20, 2008. Published, JBC Papers in Press, December 15, 2008, DOI 10.1074/jbc.M807311200

Geoffrey Duby, Wojciech Poreba, Dominik Piotrowiak, Krzysztof Bobik, Rita Derua, Etienne Waekens, and Marc Boutry

From the Institut des Sciences de la Vie, Université Catholique de Louvain, Croix du Sud, 5-15, 1348 Louvain-la-Neuve, Belgium and Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit Leuven, 3000 Leuven, Belgium

The proton pump ATPase (H\(^{+}\)-ATPase) of the plant plasma membrane is regulated by an autoinhibitory C-terminal domain, which can be displaced by phosphorylation of the penultimate Thr residue and the subsequent binding of 14-3-3 proteins. We performed a mass spectrometric analysis of PMA2 (plasma membrane H\(^{+}\)-ATPase isoform 2) isolated from Nicotiana tabacum suspension cells and identified two new phosphorylated residues in the enzyme 14-3-3 protein binding site: Thr\(^{931}\) and Ser\(^{938}\). When PMA2 was expressed in Saccharomyces cerevisiae, mutagenesis of each of these two residues into Asp prevented growth of a yeast strain devoid of its own H\(^{+}\)-ATPases. When the Asp mutations were individually introduced in a constitutively activated mutant of PMA2 (E14D), they still allowed yeast growth but at a reduced rate. Purification of His-tagged PMA2 showed that the T931D or S938D mutation prevented 14-3-3 protein binding, although the penultimate Thr\(^{935}\) was still phosphorylated, indicating that Thr\(^{935}\) phosphorylation is not sufficient for full enzyme activation. Expression of PMA2 in an N. tabacum cell line also showed an absence of 14-3-3 protein binding resulting from the T931D or S938D mutation. Together, the data show that activation of H\(^{+}\)-ATPase by the binding of 14-3-3 proteins is negatively controlled by phosphorylation of two residues in the H\(^{+}\)-ATPase 14-3-3 protein binding site. The data also show that phosphorylation of the penultimate Thr and 14-3-3 binding each contribute in part to H\(^{+}\)-ATPase activation.

Plasma membrane H\(^{+}\)-ATPases play a key role in nutrient transport by creating pH and potential gradients across the cell membrane, which are used by a whole set of secondary transporters (e.g. the use of protons in symport or antiport). Besides being involved in nutrition, H\(^{+}\)-ATPases are also implicated in various physiological roles, such as control of the stomatal aperture, cell elongation, plant development, organ movement, and intracellular pH homeostasis, although evidence for the direct involvement of H\(^{+}\)-ATPases in some of these roles is scarce (for reviews, see Refs. 1 and 2). Considering the high levels of H\(^{+}\)-ATPases in the plasma membrane and the large variety of physiological roles, one would expect this enzyme to be tightly regulated. These proteins are encoded by a gene family of about 10 members in Arabidopsis thaliana, Oryza sativa, and Nicotiana plumbaginifolia (3, 4). Depending on the gene, expression is either restricted to particular cell types or widespread in the plant, with the possibility of more than one gene being expressed in a given cell type at the same developmental stage, thus precluding the characterization of a single isoform from plant material (3).

One case of H\(^{+}\)-ATPase post-translational regulation that has been extensively described at the molecular level involves the C-terminal autoinhibitory domain (5). Phosphorylation of the penultimate residue of H\(^{+}\)-ATPase, a Thr, triggers the binding of regulatory 14-3-3 proteins, resulting in the formation of an activated complex (6–10). This complex was recently shown to be a dodecamer of six H\(^{+}\)-ATPases and six 14-3-3 proteins (11, 12). A still unresolved question concerns the respective roles of Thr phosphorylation and 14-3-3 binding. Are they both required before the enzyme is activated, or does 14-3-3 binding further activate an enzyme already partly activated by Thr phosphorylation? Another possibility would be to consider that Thr phosphorylation fully activates H\(^{+}\)-ATPase and that 14-3-3 binding stabilizes the activated form.

H\(^{+}\)-ATPase activation by Thr phosphorylation and 14-3-3 binding has been observed in guard cells upon blue light activation (8, 13); in various organs treated with fusicoccin, a fungal toxin, which stabilizes the H\(^{+}\)-ATPase-14-3-3 complex (7, 14, 15); and in plant cells upon metabolic activation (11, 16, 17). However, H\(^{+}\)-ATPase regulation by phosphorylation is not limited to the penultimate Thr residue. Evidence has been accumulating that phosphorylation of other unidentified residues is linked to H\(^{+}\)-ATPase inhibition in beet and oat root cells (18–20) and cultured tobacco and tomato cells (21–24). Proteomic analysis of Arabidopsis plasma membranes identified three phosphorylated H\(^{+}\)-ATPase residues corresponding to Ser\(^{899}\), Ser\(^{940}\), (25), and Thr\(^{881}\) (26) of AHA2 (Arabidopsis H\(^{+}\)-ATPase isoform 2) in addition to the penultimate Thr\(^{247}\) (25, 26). Treatment with the bacterial elicitor flagellin results in
Plant H⁺-ATPase Phosphorylation

decreased phosphorylation of AHA2 Thr⁸⁸¹ and Thr⁹⁴⁷ and increased phosphorylation of Ser⁹⁳⁰ (26). Recently, a Ser/Thr protein kinase, PKS5, was shown to phosphorylate Ser⁹³¹ of AHA2 both in vitro and in a yeast expression system, and this resulted in prevention of 14-3-3 protein binding and lower enzyme activity; however, attempts to confirm the phosphorylation of this residue in vivo by mass spectrometry (MS³) failed (27). Proteomics analysis of plasma membrane proteins from Arabidopsis seedlings supplied with sucrose showed that phosphorylation of Thr⁸⁸¹ in the AHA2 C-terminal region resulted in enzyme activation by a phosphorylation event outside the 14-3-3 binding site (16).

In this study, we took advantage of a transgenic N. tabacum BY2 cell line expressing His-tagged PMA2 (plasma membrane H⁺-ATPase isoform 2) (28), one of the two most widely expressed H⁺-ATPase isoforms in N. plumbaginifolia (3), and performed MS analysis on the purified His-tagged PMA2. Two phosphorylated sites, Thr⁹³¹ and Ser⁹³⁸, were identified. Expression of PMA2 mutated at these sites in yeast and tobacco cells strongly suggested that their phosphorylation interferes with the binding of 14-3-3 proteins and therefore H⁺-ATPase activation.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Yeast strains were grown on rich medium containing 2% glucose, 2% yeast extract (YD medium) or on minimal medium containing 2% galactose, 0.7% yeast nitrogen base without amino acids (Difco), 0.115% drop mix composed of all amino acids required for growth (DOGal medium) (29). The pH was adjusted to 4.0 or 6.5 with HCl or KOH, and 2% agar was added to obtain solid medium. 5'-Fluoroorotic acid (5'-FOA) medium was prepared as described in Ref. 29.

Genetic Constructions—The yeast plasmid 2µ(pMA1)6hispm2a contains the pma2 gene, with six His codons between residues 3 and 4, under the control of the S. cerevisiae PM-A1 promoter, the LEU2 gene for selection, and the 2µ plasmid-derived sequence for high copy number replication of the plasmid (30). The generation of the PMA2-E14D, PMA2-P154R, and PMA2-N510L activating mutants has been described previously (31). PMA2-P154R and PMA2-N510K were tagged with 6 His residues by replacing the NheI/BglII DNA fragment from 2µ(pMA1)6hispm2a by that from 2µ(pMA1)pma2P154R or 2µ(pMA1)pma2N510K, respectively. PMA2-E14D was His-tagged by PCR as described in Ref. 32. The mutation of Thr⁹³¹ and Ser⁹³⁸ to Ala and Asp residues was achieved by amplifying a modified fragment between the BglII and Xbal restriction sites of 2µ(pMA1)6hispm2a plasmid by triple PCR and inserting it into 2µ(pMA1)6hispm2a opened by BglII and Xbal restriction. The Thr⁹³¹, Ser⁹³⁸ double mutants were obtained using the same PCR strategy, except that the starting plasmids were 2µ(pMA1)6hispm2aS938D and 2µ(pMA1)6hispm2aS938A. Insertion of the Ser⁹³⁸ and Thr⁹³¹ mutations into 2µ(pMA1)6hispm2aE14D was performed by exchanging the BglII/XbaI DNA fragments. The PMA2-P154R and PMA2-N510L mutants carrying the Ser⁹³⁸ mutations were obtained by replacing the NheI/BglII DNA fragment from 2µ(pMA1)6hispm2aS938D with that from 2µ(pMA1)pma2P154R or 2µ(pMA1)pma2N510K, respectively. The YAK2 yeast strain has disrupted yeast MAI and PMA2 genes and contains a centromeric plasmid carrying the yeast MAI gene under the control of the GAL1–10 promoter and the URA3 gene for selection (30).

The plant binary vectors used for the N. tabacum BY2 cell transformation were obtained by first inserting a SmaI and XbaI fragment from the yeast 2µ(pMA1)6hispm2a, -S938A, S938D, T931A, and T931D plasmid, corresponding, respectively, to the PMA2-S938A, PMA2-S938D, PMA2-T931A, and T931D coding sequence into the pAUX3131 vector (33) between the N. plumbaginifolia PMA4 promoter reinforced with two copies of the cauliflower mosaic virus 35S enhancer (34) and the nos terminator. In the second step, PMA2 expression cassettes were transferred as an I-SceI fragment into the plant binary vector pPZP-RCS2 (33) containing the nptII marker gene.

Membrane Isolation—All buffers were supplemented with 1 mm phenylmethanesulfonyl fluoride and the protease inhibitors leupeptin, aprotinin, antipain, pepstatin, and chymostatin (each 2 μg/ml).

Yeast microsomal and plasma membrane fractions were obtained as described previously (31), except that the homogenization buffer was 250 mm sorbitol, 10 mm EDTA, 5 mm EGTA, 20% ethylene glycol, 30 mm β-glycerophosphate, 10 mm NaF, 5 mm NaMoO₄, 5 mm dithiothreitol, 60 mm Tris, pH 8.0 (HCl), and the suspension buffer was 250 mm sorbitol, 20% ethylene glycol, 30 mm β-glycerophosphate, 10 mm NaF, 60 mm Tris, pH 8.0 (HCl).

Microsomal membranes were prepared from BY2 cells as in Ref. 11. The homogenization and suspension buffers were supplemented with phosphatase inhibitors and had the same composition as those used for yeast microsomal membrane preparation, except that 0.6% polyvinylpolypyrrolidone was added to the homogenization buffer.

N. tabacum BY2 Cell Culture and Transformation—BY2 cells were maintained on MS medium (catalog number 2610024; MP Biomedicals, LLC) supplemented with 3% sucrose, 0.2 mg/liter 2,4-dichlorophenoxyacetic acid, 0.2 g/liter KH₂PO₄, pH 5.8, 50 mg/liter myo-inositol, 5 mg/liter thiamine-HCl. BY2 cells were diluted 20-fold each week in fresh medium and grown as described previously (35). Transient expression of the mutated PMA2 isoforms was performed by co-cultivating for 68 h 24 ml of 4-day-old BY2 cell culture with 7 ml of Agrobacterium tumefaciens strain LBA4404virG (36) transformed with the different pPZP-PMA2 and grown to an A₆₀₀ of 1.4. A. tumefaciens and BY2 cells were washed and co-cultured in BY2 cell medium at pH 5.3 supplemented with 10 mm glucose and 50 μm acetylsyngone.

PMA2 Purification—PMA2 isoforms expressed in yeast or in N. tabacum BY2 cells were purified on Ni²⁺-NTA-agarose (Qiagen). The yeast microsomal fraction (2 mg/ml) was stripped for 30 min on 4 °C in TK20 buffer (20 mm imidazole, 100 mm KCl, 10% glycerol, 1 mm MgCl₂, pH 7.5) supplemented with 0.25% octaoxyethylene tetradecyl. After centrifugation for 30 min at 100,000 × g at 4 °C, the pellet was resuspended in

³ The abbreviations used are: MS, mass spectrometry; Mes, 2-morpholinoethanesulfonic acid; 5'-FOA, 5-fluoroorotic acid; NTA, nitrilotriacetic acid.
Identification of phosphorylated PMA2 peptides by neutral loss

| Neutral loss scanning | m/z         | Sequence                  | Number of phosphorylations |
|-----------------------|-------------|---------------------------|----------------------------|
| Neutral loss 32.7     | 579.5       | 9238ELHTLKGHVESVVK        | 2                          |
| Neutral loss 49       | 797.6       | 475ESTGPPWQFGILLPLDPDRPR  | 2                          |
| Neutral loss 901      | 920.0       | 920RAEIAQRLEHTLK or 425VSNGAPEQILNLHNNK | 1  |
| Neutral loss 922.3    | 923.0       | 515MoxITGDLAIGKETGR       | 1                          |
| Neutral loss 954      | 924.0       | 515MITGDLAIGKETGR         | 2                          |
| Neutral loss 1011     | 924.0       | 924QRELEHTLKGHVESVVK or 315MTAEEMAGMDVLCSDK | 2  |

* The position of the first residue in the PMA2 sequence is indicated.

**RESULTS**

Identification of New PMA2 Phosphorylated Sites—Our aim was to identify phosphorylation sites in PMA2, one of the two most widely expressed H⁺-ATPases in *N. plumbaginifolia*, using an *N. tabacum* cell line (BY2) expressing His₆-tagged PMA2 (28). PMA2 was solubilized from the microsomal fraction, purified by Ni²⁺-NTA chromatography, and electrophoresed on SDS gels. The band corresponding to PMA2 was excised from the gel and digested with trypsin and phosphorylated tryptic peptides enriched by PhosTrapp titanium beads (PerkinElmer Life Sciences) or by immobilized metal (Fe³⁺) affinity chromatography (POROS MC20; Applied Biosystems). Peptide analysis by neutral loss of 32.4 and 49 Da, corresponding to the loss of a phosphate residue (98 Da) in peptides bearing three (98/3) or two (98/2) positive charges, respectively, indicated the presence of several phosphorylated PMA2 peptides located in the C-terminal region or in the large loop of the enzyme (see supplemental Fig. 1 and Table 1). Among these peptides, we sought to confirm the identification of the phosphorylated residues in the C-terminal region, because they belong to the 14-3-3 binding site. Using multiple reaction monitoring, we confirmed the presence of one phosphopeptide containing phosphorylated Ser⁹³⁸ and another containing phosphorylated Thr⁹³¹ and Ser⁹³⁸ (Fig. 1). Both residues are located within the C-terminal region interacting with 14-3-3 proteins (12) and are well conserved in the H⁺-ATPase family.
His6-tagged PMA2 mutants were expressed from a yeast plasmid in the S. cerevisiae YAK2 strain in which the cell’s own two H^+-ATPase expression. To confirm the results, we shifted the expressing wild-type PMA2 and PMA2-S938A, the latter growing better yeast growth, probably by decreasing the interaction of the C-terminal autoinhibitory domain with the rest of the protein and making Thr^{935} more accessible to phosphorylation and 14-3-3 binding. This in turn might reduce access of phosphatases to phosphorylated Thr^{935} (31). As shown in Fig. 3, in this PMA2-activated form, the T931A, T931D, or S938D mutation reduces H^+-ATPase activity to a level that does not allow yeast growth (data not shown).

To further investigate the role of these phosphorylated residues, we introduced the Thr^{931} and Ser^{938} single mutations into the constitutively activated PMA2-E14D mutant, in which the activating mutation results in higher H^+-ATPase activity and better yeast growth, probably by decreasing the interaction of the C-terminal autoinhibitory domain with the rest of the protein and making Thr^{935} more accessible to phosphorylation and 14-3-3 binding. This in turn might reduce access of phosphatases to phosphorylated Thr^{935} (31). As shown in Fig. 3, in this PMA2-activated form, the T931A, T931D, or S938D mutation did not prevent the appearance of colonies after 5'-FOA selection and did not affect yeast growth at pH 6.5 on solid medium (left) and in liquid culture (duplication time between 2.6 and 2.9 h for PMA2-E14D and E14D-mutated strains). However, when grown under more demanding conditions (i.e. at pH 4.0 (right)), PMA2-E14D/T931D and PMA2-E14D/T931A cells did not grow (more than 20 h duplication time in liquid culture), whereas PMA2-E14D/S938D (6.5 h duplication time) grew more slowly than those expressing PMA2-E14D (5 h) or PMA2-E14D/S938A (4.8 h), showing that the activating E14D mutation only partly overruled the inhibitory effect of the other mutations.

Since both Thr^{931} and Ser^{938} are in the 14-3-3 protein binding domain, we wondered whether the growth reduction was chemical that is converted into a toxic compound by the URA3 gene product. Since this gene is present on the plasmid bearing the yeast PMA1, growth in the presence of 5'-FOA selects for strains that have lost this plasmid and thus the yeast PMA1 but contain a functional plant H^+-ATPase gene present on the other plasmid. Colonies were obtained for the strains expressing wild-type PMA2 and PMA2-S938A, the latter growing faster (Fig. 2B). No colony was ever obtained with the other constructs, confirming that the T931A, T931D, or S938D mutation reduces H^+-ATPase activity to a level that does not allow yeast growth (data not shown).

Characterization of PMA2 Thr^{931} and Ser^{938} Mutants Expressed in Yeast—For a more detailed characterization, Thr^{931} and Ser^{938} were mutagenized singly or in combination to Ala (to prevent phosphorylation) or Asp (to mimic the negative charge of a phosphorylated residue), and the resulting Thr931 to dehydroamino-2-butyric acid.

Western blotting confirmed the presence of the PMA2 mutants (data not shown), ruling out an indirect effect of the mutations on H^+-ATPase expression. To confirm the results, we shifted the transformed strains to glucose medium containing 5'-FOA, a chemical that is converted into a toxic compound by the URA3 gene product. Since this gene is present on the plasmid bearing the yeast PMA1, growth in the presence of 5'-FOA selects for strains that have lost this plasmid and thus the yeast PMA1 but contain a functional plant H^+-ATPase gene present on the other plasmid. Colonies were obtained for the strains expressing wild-type PMA2 and PMA2-S938A, the latter growing faster (Fig. 2B). No colony was ever obtained with the other constructs, confirming that the T931A, T931D, or S938D mutation reduces H^+-ATPase activity to a level that does not allow yeast growth (data not shown).

To further investigate the role of these phosphorylated residues, we introduced the Thr^{931} and Ser^{938} single mutations into the constitutively activated PMA2-E14D mutant, in which the activating mutation results in higher H^+-ATPase activity and better yeast growth, probably by decreasing the interaction of the C-terminal autoinhibitory domain with the rest of the protein and making Thr^{935} more accessible to phosphorylation and 14-3-3 binding. This in turn might reduce access of phosphatases to phosphorylated Thr^{935} (31). As shown in Fig. 3, in this PMA2-activated form, the T931A, T931D, or S938D mutation did not prevent the appearance of colonies after 5'-FOA selection and did not affect yeast growth at pH 6.5 on solid medium (left) and in liquid culture (duplication time between 2.6 and 2.9 h for PMA2-E14D and E14D-mutated strains). However, when grown under more demanding conditions (i.e. at pH 4.0 (right)), PMA2-E14D/T931D and PMA2-E14D/T931A cells did not grow (more than 20 h duplication time in liquid culture), whereas PMA2-E14D/S938D (6.5 h duplication time) grew more slowly than those expressing PMA2-E14D (5 h) or PMA2-E14D/S938A (4.8 h), showing that the activating E14D mutation only partly overruled the inhibitory effect of the other mutations.

Since both Thr^{931} and Ser^{938} are in the 14-3-3 protein binding domain, we wondered whether the growth reduction was
related to a lower activation of the enzyme due to reduced phosphorylation of the penultimate Thr\textsuperscript{955} and/or reduced 14-3-3 protein binding. We and others previously measured phosphorylation of the Thr\textsuperscript{955} using commercial anti-phospho-Thr antibodies (27, 32), but the identification of other phosphorylated Thr residues (e.g. PMA2 Thr\textsuperscript{931}) calls into question the specificity of the signal observed with these antibodies. In the present study, we therefore used a monoclonal antibody, PMA2pT, raised against a synthetic peptide mimicking the phosphorylated PMA2 C-terminal peptide\textsuperscript{949TIQQSTpTV} (where pT represents phosphothreonine), which has been demonstrated to be specific for Thr\textsuperscript{955}-phosphorylated PMA2.4

His\textsubscript{6}-tagged wild type or mutant PMA2 was purified from the different yeast strains and analyzed by Western blotting (see Fig. 4, A and B, for quantification). As expected, no Thr\textsuperscript{955} phosphorylation or co-purified 14-3-3 proteins were seen with PMA2-E14D/T955A.

In agreement with the reduction of yeast growth at low pH, S938D mutation of PMA2-E14D resulted in a large reduction in Thr\textsuperscript{955} phosphorylation levels (down to 27%) and almost no co-purified 14-3-3 proteins (down to 2.2%), showing that this mutation has a dramatic effect on 14-3-3 protein binding. This was also supported by the fact that, at the similar level of Thr\textsuperscript{955} phosphorylation seen with wild-type PMA2 and PMA2-E14D/S938D, almost no 14-3-3 proteins were bound to the latter compared with the amount bound to the wild-type PMA2.

In contrast, introduction of the S938A mutation into PMA2 did not influence the level of Thr\textsuperscript{955} phosphorylation compared with wild-type PMA2 but significantly increased the binding of 14-3-3 proteins by 44%, in agreement with the greater capacity of this mutant to sustain yeast growth at pH 6.5 compared with the wild-type PMA2. Introduction of the S938A mutation into

---

4 K. Bobik, Y. Nizet, C. Vandermeeren, J. Kanczewska, G. Duby, and M. Boutry, submitted for publication.

---

**FIGURE 3.** Comparative growth at pH 6.5 and 4.0 of yeast strains expressing PMA2 mutants. After selection on \( ^{5}-\)FOA-containing medium, YAK2 yeast cells expressing the indicated PMA2 mutant were grown overnight in YD medium, diluted to an \( A_{600} \) of 1, 0.1, 0.01, or 0.001; plated on solid YD medium; and allowed to grow for 2 days on YD medium at pH 6.5 or for 4 days on YD medium at pH 4.0. The untransformed YAK2 strain was plated as a negative control.

**FIGURE 4.** Immunodetection of Thr\textsuperscript{955} phosphorylation and bound 14-3-3 proteins for the different mutants on the PMA2 wild-type or E14D activating mutant background. A, a microsomal fraction (500 \( \mu \)g of protein) isolated from a 36-h culture (YD, pH 6.5) of yeast strains expressing wild-type or mutant PMA2 was solubilized, and the His\textsubscript{6}-tagged PMA2 was purified by Ni\textsubscript{2+}-NTA chromatography and analyzed by SDS-PAGE and Western blotting using antibodies against PMA2 (top), PMA2 phospho-Thr\textsuperscript{955} (pPMA2; middle), or 14-3-3 proteins (bottom). Note that the double band identified by anti-14-3-3 protein antibodies corresponds to the two \( S. \) cerevisiae 14-3-3 proteins. B, PMA2 phospho-Thr\textsuperscript{955}/PMA2 signal ratio (dark gray bars) and 14-3-3 proteins/PMA2 signal ratio (light gray bars) from A. The signal for each band was normalized to that for PMA2-E14D (set at 100%), and then the signal ratios were calculated. Quantification was performed using Image Station 4000R and Molecular Imaging Software from Eastman Kodak Co. The data are the mean and the S.E. for the results from three independent experiments.
the PMA2-E14D strain did not result in significant modification of phosphorylation or binding of 14-3-3 proteins, agreeing with the lack of growth modification resulting from this additional mutation. These results indicate that Ser938 phosphorylation has a much stronger impact on 14-3-3 protein binding than on Thr955 phosphorylation.

Modifying Thr931 to Ala and Asp reduced Thr955 phosphorylation to 56 and 32%, respectively, compared with the control PMA2-E14D, whereas the 14-3-3 protein binding was abolished in both cases, indicating that Thr931 phosphorylation affects the 14-3-3 protein binding more than Thr955 phosphorylation and that a high level of Thr955 phosphorylation together with 14-3-3 protein binding are required to allow yeast growth at low pH.

Since many other activating mutants of PMA2 have been identified (38), we extended our analysis by combining the Ser938 mutations with two other activating mutations, P154R and N510K, which are localized in the small and large loop, respectively, unlike E14D, which is in the N-terminal region. These three activated mutants result in reduced constraint of the C-terminal inhibitory domain and have been proposed to take part into the enzyme domain interacting with the C-terminal inhibitory domain (38, 39). The single P154R and N510K activating mutations resulted in yeast growth at pH 4.0 similar to that obtained with E14D (Fig. 3). Combining these mutations with either S938A or S938D had the same effects as the same combination with the E14D mutation (i.e. no major modifications with S938A but slower growth at pH 4.0 (Fig. 3), reduced Thr955 phosphorylation, and absence of 14-3-3 protein binding (Fig. 5) with S938D). We conclude that the activation process of each of these three activating mutations, localized in three different regions, was partly reduced by mutation of Ser938 into Asp, indicating that full activation depends on 14-3-3 protein binding.

To link the above data to H+ -ATPase activity, ATPase assays were performed on the plasma membrane fraction from the Ser938 mutants, which were chosen because Ser938 mutations into Ala and Asp residue resulted in different phenotypes. As shown in Fig. 6A, PMA2-S938A had a higher activity than PMA2, in agreement with the better growth and increased 14-3-3 protein binding. As expected, all of the mutants on the PMA2-E14D background displayed higher ATPase activity than the wild-type PMA2. No statistically significant difference was observed between PMA2-E14D and PMA2-E14D combined with the T955A or S938A mutation. Unexpectedly, the PMA2-E14D/S938D mutant displayed slightly higher activity compared with the PMA2-E14D isofrom instead of lower activity, as expected from the slower growth at pH 4.0. Since the
H^+-ATPase expression levels of the different mutants were similar, we wondered whether the in vitro ATPase activity reflected the in vivo proton pumping activity of these enzymes. Acidification of the external medium by intact cells was monitored as previously (38, 40) to assess the in vivo H^+-ATPase activity. As shown in Fig. 6B, at external pH 6.5, no significant difference was observed between the strains expressing PMA2-E14D or PMA2-E14D/S938D, whereas, at external pH 4.0, the latter had a 28% reduced acidification rate compared with the former (p < 0.03), in agreement with the slower growth.

Characterization of PMA2 Thr^{931} and Ser^{938} Mutants Expressed in Tobacco Cells—All of the above data were obtained using yeast. Although 14-3-3 proteins are well conserved, we cannot rule out the possibility that, in some cases, yeast 14-3-3 proteins might behave differently from plant 14-3-3 proteins. To examine whether the marked differences in 14-3-3 protein binding of the S938D and T931D mutations seen in yeast were recapitulated in plants, we performed a transient expression of wild-type PMA2 and the S938A, S388D, T931A, and T931D PMA2 mutants in N. tabacum BY2 suspension cells by co-cultivation with transformed A. tumefaciens.

In a previous study (11), we showed that, under standard culture conditions, little phosphorylation of PMA2 Thr^{955} and little 14-3-3 protein binding are seen in N. tabacum BY2 cells, but the addition of fusicoccin, a fungal toxin known to stabilize the H^+-ATPase-14-3-3 complex, results in a dramatic increase in the amount of this complex and of Thr^{955} phosphorylation. In the present study, 10 µM fusicoccin was added to the BY2 cells for 30 min before ectopic wild-type or mutant His<sub>6</sub>-tagged PMA2 was purified from the microsomal fraction of BY2 cells. Western blotting (Fig. 7) showed that PMA2-S938D as well as PMA2-T931A and PMA2-T931D had a lower level of phosphorylated Thr^{955} and of bound 14-3-3 proteins than wild-type PMA2 or PMA2-S938A, confirming the data obtained in yeast.

**DISCUSSION**

A well-characterized activation mechanism of H^+-ATPase consists of the phosphorylation of its penultimate residue and the binding of 14-3-3 protein dimers. Eight additional phosphorylation sites have been identified in the C-terminal region of A. thaliana or O. sativa H^+-ATPases through proteomics analysis of plasma membranes or through more detailed analysis (16, 25–27, 41, 42). In the present work, we focused on the phosphorylation sites of PMA2 from N. plumbaginifolia, which belongs to a different H^+-ATPase subfamily than AHA2, an Arabidopsis isoform in which the phosphorylation sites have been well studied. Two phosphorylated residues, Thr<sup>931</sup> and Ser<sup>938</sup>, have been identified by mass spectrometry in the C-terminal region of PMA2 in N. tabacum culture cells.

These two residues were further studied in a yeast expression system by site-directed mutagenesis. Table 2 summarizes the most important data on yeast growth, Thr<sup>955</sup> phosphorylation, and 14-3-3 protein binding for the wild-type PMA2 and its different mutants. His-tagging of the mutant forms allowed their purification together with bound 14-3-3 proteins. This avoided performing Western blotting with a membrane fraction, which might have given a 14-3-3 protein signal not totally due to H^+-ATPase, since 14-3-3 proteins can bind to many different proteins. H^+-ATPase purification also made it possible to measure 14-3-3 proteins bound to the native H^+-ATPase instead of to a partly denatured enzyme, as in far Western blotting. Another important feature of our study was the use of monoclonal antibodies directed specifically against the Thr<sup>955</sup>-phosphorylated form of PMA2, thus avoiding the use of general phosphothreonine antibodies that cannot distinguish between the penultimate Thr and other Thr residues that might be phosphorylated, such as PMA2 Thr<sup>931</sup> (this work) or PMA2 Thr<sup>889</sup>, which is homologous to AHA2 Thr<sup>881</sup> (16).

Phosphorylation of Thr<sup>931</sup> or a homologous residue had not been previously identified in a plant H^+-ATPase. This residue is located in the C-terminal region that binds a 14-3-3 protein...
Plant $H^+\text{-ATPase}$ Phosphorylation

dimer. Its mutagenesis to Ala and Asp resulted in abolition of yeast growth when introduced into wild-type PMA2 or its reduction when associated with the activating mutation E14D (Table 2). Analysis of the purified mutants showed that the penultimate Thr$^{955}$ was still phosphorylated to some extent, but no 14-3-3 proteins were bound. How can we explain that a mutation (Asp) expected to mimic a phosphorylated residue and another one (Ala) expected to prevent phosphorylation resulted in the same effect? A three-dimensional structure of the H$^+\text{-ATPase}$ C-terminal region crystallized with 14-3-3 proteins has been obtained (12). A 14-3-3 protein dimer simultaneously binds two interacting H$^+\text{-ATPase}$ peptides, each of which terminates as a loop within the typical 14-3-3 protein binding groove. Thr$^{931}$ lies in a small loop between two helices embedded in this groove. The fact that an Ala mutation has the same effect as the Asp mutation of a phosphorylated residue is not surprising in this case, since an Ala residue, which is more favorable to the formation of a helical structure, is expected to disturb the loop as much as an Asp residue. Actually, the T931A mutation in a construct consisting of the last 52 residues of PMA2 fused to intein also impairs 14-3-3 protein binding in vitro (12). The homologous residue in Arabidopsis AHA2 has also been pointed out as an important residue for 14-3-3 protein binding (43).

As with Thr$^{931}$, mimicking Ser$^{938}$ phosphorylation by mutating it to Asp had a greater effect on 14-3-3 protein binding than on Thr$^{955}$ phosphorylation. Ser$^{938}$ is located in a helical structure embedded in the complex, and its phosphorylation is predicted to disturb the association of the two H$^+\text{-ATPase}$ C-terminal regions within the 14-3-3 protein dimer. In particular, phosphorylation at this position introduces a negative charge close to that of Glu$^{938}$, and the charge repulsion between the two residues could destabilize the enzyme C-terminal secondary structure (12). Characterization of a total protein extract from trichloroacetic acid-treated cells to inactivate any phospha-tase activity showed that the Thr$^{955}$ phosphorylation was also affected in vivo by the S938D mutation (data not shown), suggesting that in the absence of 14-3-3 protein binding, phosphoryl-Thr$^{955}$ is not protected and thus more susceptible to phosphatases in vivo. Mutation of PMA2-Ser$^{938}$ into Ala did not significantly modify Thr$^{955}$ phosphorylation but increased 14-3-3 protein binding and resulted in better yeast growth. The affinity of 14-3-3 proteins for the C-terminal region of PMA2 S938A has already been analyzed in vitro and was about 3 times higher with Ala than with Ser at position 938 (12). It therefore seems that a Ser instead of an Ala residue at position 938 is the “price to pay” to allow regulation by phosphorylation.

Similar effects of Asp or Ala mutation of Ser$^{938}$ on 14-3-3 protein binding have been recently reported for the homologous residue in AHA2 (Ser$^{931}$) (27). These authors identified a protein kinase, PKS5, which is able to phosphorylate this residue in vitro or in vivo in a yeast expression system. However, phosphorylated AHA2-Ser$^{931}$ has still to be identified in Arabidopsis. In the plant cells, both PMA2 T931D and S938D mutants were shown to be affected at the level of their Thr$^{955}$ phosphorylation and capacity to bind 14-3-3 proteins as it was observed in the yeast system. Phosphorylation of PMA2 Thr$^{955}$ was even more affected in the plant context expression. This could probably result from the fact that experiments performed in yeast were made using the PMA2-E14D activated form or that plant phosphatases are more efficient in dephosphorylating the unphosphorylated Thr$^{955}$. These effects of Ser$^{938}$ and Thr$^{931}$ mutagenesis observed in a plant cell context as well as the fact that both residues were found to be phosphorylated in BY2 cells support the relevance of their role in H$^+\text{-ATPase}$ regulation in the plant. In addition, phosphorylation of the AHA2 residue homologous to PMA2 Ser$^{938}$ has been proposed to down-regulate the enzyme activity under high pH growth conditions, suggesting a role in vivo of this regulation (27).

The strain combining the activating mutant PMA2-E14D and the mutation T955A, which prevented phosphorylation of this residue and subsequent 14-3-3 protein binding, grew at the same rate as the strain expressing PMA2-E14D and hence faster than wild-type PMA2 at pH 6.5 but, unlike the strain expressing PMA2-E14D, did not grow at pH 4.0 (Table 2). This suggests that the enzyme activation by the E14D mutation depends only in part on Thr$^{955}$ phosphorylation and 14-3-3 protein binding. This conclusion is corroborated by the observation that both of the Thr$^{931}$ mutants and the S938D mutant of PMA2-E14D also showed a growth intermediate between that with PMA2-E14D or wild-type PMA2.

An interesting observation was made with the S938D mutation on the PMA2-E14D background. It reduced growth and proton pumping at pH 4.0, but its in vitro ATPase activity at pH 6.5 was slightly increased. This discrepancy suggests that the in vitro ATPase activity might not reflect the actual in vivo performance of the enzyme. This might result from a partial uncoupling of ATPase hydrolysis and proton pumping as observed for AHA2-D684N (44). Determination of whether this is the case will require a detailed analysis of this mutant, combining in vivo and in vitro approaches.

A still unresolved question regarding H$^+\text{-ATPase}$ activation through its C-terminal region is whether phosphorylation of the penultimate Thr (Thr$^{955}$ in PMA2) is sufficient for activation (in which case, 14-3-3 protein binding might play a role in stabilization), whether both phosphorylation and 14-3-3 protein binding are required before the enzyme is activated, or whether each process contributes partly to activation. This question can now be answered by comparing the growth of the different mutants. Yeast expressing PMA2 or PMA2-S938A showed the same level of Thr$^{955}$ phosphorylation, but the S938A form had more 14-3-3 protein bound, and the yeast strain grew faster at pH 6.5 than the wild type (Table 2). This suggests that the binding of 14-3-3 proteins contributes to enzyme activation. What about Thr$^{955}$ phosphorylation? Neither PMA2-E14D/T955A nor PMA2-E14D/S938D mutant bound 14-3-3 proteins, whereas the latter, unlike the former, still contained phosphorylated Thr$^{955}$, and the yeast strain still grew at pH 4.0. These data suggest that Thr$^{955}$ phosphorylation and the binding of 14-3-3 proteins each contribute in part to the activation process.

In conclusion, we have identified two new phosphorylation sites, Thr$^{931}$ and Ser$^{938}$, in N. plumbaginifolia PMA2. Their study by directed mutagenesis suggests that phosphorylation at each position interferes with the binding of 14-3-3 proteins to
the C-terminal region and thus prevents full activation of the enzyme.

Acknowledgments—We thank Sebastien Lievyns and Bertrand Magy for technical assistance.

REFERENCES

1. Sondergaard, T. E., Schulz, A., and Palmgren, M. G. (2004) Plant Physiol. 136, 2475–2482
2. Duby, G., and Boutry, M. (2008) Pflug. Arch. Eur. J. Physiol., in press
3. Arango, M., Gevaudant, F., Oufattole, M., and Boutry, M. (2003) Planta 216, 355–365
4. Baxter, I., Tchieu, J., Sussman, M. R., Boutry, M., Palmgren, M. G., Gribkov, M., Harper, J. F., and Axelsen, K. B. (2003) Plant Physiol. 132, 618–628
5. Palmgren, M. G., Sommarin, M., Serrano, R., and Larsson, C. (1999) J. Biol. Chem. 266, 20470–20475
6. Camoni, L., Iori, V., Marra, M., and Aducci, P. (2000) J. Biol. Chem. 275, 9919–9923
7. Fuglsang, A. T., Visconti, S., Drumm, K., Jahn, T., Stensballe, A., Mattei, B., Jensen, O. N., Aducci, P., and Palmgren, M. G. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9311–9316
8. Kinoshita, T., and Shimazaki, K. (1999) EMBO J. 18, 5548–5558
9. Maudoux, O., Batoko, H., Oecking, C., Gevaert, K., Vandekerckhove, J., Boutry, M., and Morsomme, P. (2000) J. Biol. Chem. 275, 17762–17770
10. Svennelid, F., Olsson, A., Piotrowski, M., Rosenquist, M., Ottmann, C., Larsson, C., Oecking, C., and Sommarin, M. (1999) Plant Cell 11, 2379–2391
11. Kanczewska, J., Marco, S., Vandermeeren, C., Maudoux, O., Rigaud, J. L., and Boutry, M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 11675–11680
12. Ottmann, C., Marco, S., Jasper, N., Marcon, C., Schauer, N., Weyand, M., Vandermeeren, C., Duby, G., Boutry, M., Wittinghofer, A., Rigaud, J. L., and Oecking, C. (2007) Mol. Cell 25, 427–440
13. Emi, T., Kinoshita, T., and Shimazaki, K. (2001) Plant Physiol. 125, 1115–1125
14. Wurtele, M., Jelich-Ottmann, C., Wittinghofer, A., and Oecking, C. (2003) EMBO J. 22, 987–994
15. Fullone, M. R., Visconti, S., Marra, M., Fogliano, V., and Aducci, P. (1998) J. Biol. Chem. 273, 7698–7702
16. Niittyla, T., Fuglsang, A. T., Palmgren, M. G., Frommer, W. B., and Schulze, W. X. (2007) Mol. Cell Proteomics 6, 1711–1726
17. Camoni, L., Marra, M., Garufi, A., Visconti, S., and Aducci, P. (2006) Plant Cell Physiol. 47, 743–747
18. Lino, B., Baizabal-Aguirre, V. M., and Gonzalez de la Vara, L. E. (1998) Planta 204, 352–359
19. Schaller, G. E., and Sussman, M. R. (1988) Plant Physiol. 86, 512–516
20. Suzuki, Y. S., Wang, Y., and Takemoto, J. Y. (1992) Plant Physiol. 99, 1314–1320
21. Desbrosses, G., Stelling, J., and Renaudin, J. P. (1998) Eur. J. Biochem. 251, 496–503
22. Schaller, A., and Oecking, C. (1999) Plant Cell 11, 263–272
23. Vera-Estrella, R., Barkla, B. J., Higgins, V. J., and Blumwald, E. (1994) Plant Physiol. 104, 209–215
24. Xing, T., Higgins, V. J., and Blumwald, E. (1996) Plant Cell 8, 555–564
25. Nuhse, T. S., Stensballe, A., Jensen, O. N., and Peck, S. C. (2003) Mol. Cell Proteomics 2, 1234–1243
26. Nuhse, T. S., Bottrill, A. R., Jones, A. M., and Peck, S. C. (2007) Plant J. 51, 931–940
27. Fuglsang, A. T., Guo, Y., Cuin, T. A., Qiu, Q., Song, C., Kristiansen, K. A., Bych, K., Schulz, A., Shabala, S., Schumaker, K. S., Palmgren, M. G., and Zhu, J. K. (2007) Plant Cell 19, 1617–1634
28. Woloszynska, M., Kanczewska, J., Drabkin, A., Maudoux, O., Dambly, S., and Boutry, M. (2003) Ann. N. Y. Acad. Sci. 986, 198–203
29. Treco, D. A., and Lundblad, V. (2001) Current Protocols in Molecular Biology, pp. 13.1.1–13.1.7, John Wiley & Sons, Inc., New York
30. de Kerchove d’Exaerde, A., Supply, P., Dufour, J. P., Bogaerts, P., Thines, D., Golfeau, A., and Boutry, M. (1995) J. Biol. Chem. 270, 23828–23837
31. Morsomme, P., Dambly, S., Maudoux, O., and Boutry, M. (1998) J. Biol. Chem. 273, 34837–34842
32. Dambly, S., and Boutry, M. (2001) J. Biol. Chem. 276, 7017–7022
33. Goderis, I. J., De Bolle, M. F., Francois, I. E., Wouters, P. F., Broekaert, W. F., and Cammue, B. P. (2002) Plant Mol. Biol. 50, 17–27
34. Zhao, R. M., Moriau, L., and Boutry, M. (1999) Plant Sci. 149, 157–165
35. Nagata, T., Nemoto, Y., and Hasezawa, S. (1992) Int. Rev. Cytol. 132, 1–30
36. van der Fits, L., Deakin, E. A., Hoge, J. H. C., and Memelink, J. (2000) Plant Mol. Biol. 43, 495–502
37. Wach, A., Ahlers, J., and Graber, P. (1990) Eur. J. Biochem. 189, 675–682
38. Morsomme, P., de Kerchove d’Exaerde, A., De Meester, S., Thines, D., Golfeau, A., and Boutry, M. (1996) EMBO J. 15, 5513–5526
39. Pedersen, B. P., Buch-Pedersen, M. J., Morth, J. P., Palmgren, M. G., and Nissen, P. (2007) Nature 450, 1111–1114
40. Martinez-Munoz, G. A., and Kane, P. (2008) J. Biol. Chem. 283, 20309–20319
41. Ookura, T., Komatsu, S., Kawamura, Y., and Kasamo, K. (2005) Jap. Jpn Agr. Res. Q. 39, 99–104
42. Sugiyama, N., Nakagami, H., Mochida, K., Daudi, A., Tomita, M., Shirasu, K., and Ishihama, Y. (2008) Mol. Syst. Biol. 4, 193
43. Fuglsang, A. T., Borch, J., Bych, K., Jahn, T. P., Roepstorff, P., and Palmgren, M. G. (2003) J. Biol. Chem. 278, 42266–42272
44. Buch-Pedersen, M. J., Rudashevskaya, E. L., Berner, T. S., Venema, K., and Palmgren, M. G. (2006) J. Biol. Chem. 281, 38285–38292