

# Phosphorylation of Thr-948 at the C Terminus of the Plasma Membrane H<sup>+</sup>-ATPase Creates a Binding Site for the Regulatory 14-3-3 Protein

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The plant plasma membrane H<sup>+</sup>-ATPase is activated by the binding of 14-3-3 protein to the C-terminal region of the enzyme, thus forming an H<sup>+</sup>-ATPase–14-3-3 complex that can be stabilized by the fungal toxin fusicoccin. A novel 14-3-3 binding motif, QQXYpT<sub>948</sub>V, at the C terminus of the H<sup>+</sup>-ATPase is identified and characterized, and the protein kinase activity in the plasma membrane fraction that phosphorylates this threonine residue in the H<sup>+</sup>-ATPase is identified. A synthetic peptide that corresponds to the C-terminal 16 amino acids of the H<sup>+</sup>-ATPase and that is phosphorylated on Thr-948 prevents the *in vitro* activation of the H<sup>+</sup>-ATPase that is obtained in the presence of recombinant 14-3-3 and fusicoccin. Furthermore, binding of 14-3-3 to the H<sup>+</sup>-ATPase in the absence of fusicoccin is absolutely dependent on the phosphorylation of Thr-948, whereas binding of 14-3-3 in the presence of fusicoccin occurs independently of phosphorylation but still involves the C-terminal motif YTV. Finally, by complementing yeast that lacks its endogenous H<sup>+</sup>-ATPase with wild-type and mutant forms of the *Nicotiana plumbaginifolia* H<sup>+</sup>-ATPase isoform PMA2, we provide physiological evidence for the importance of the phosphothreonine motif in 14-3-3 binding and, hence, in the activation of the H<sup>+</sup>-ATPase *in vivo*. Indeed, replacing Thr-948 in the plant H<sup>+</sup>-ATPase with alanine is lethal because this mutant fails to functionally replace the yeast H<sup>+</sup>-ATPase. Considering the importance of the motif QQXYpTV for 14-3-3 binding and yeast growth, this motif should be of vital importance for regulating H<sup>+</sup>-ATPase activity in the plant and thus for plant growth.

## INTRODUCTION

The highly conserved family of 14-3-3 proteins is expressed in all eukaryotic organisms analyzed thus far, and members of the family are involved in the regulation of many activities (reviewed in Chung et al., 1999). The 14-3-3 proteins have been shown to bind to their target proteins in a sequence-specific and phosphorylation-dependent manner. Approximately half of the 14-3-3-interacting proteins contain the motif RSXpSXP (where X stands for any amino acid and p denotes a phosphorylated amino acid; Muslin et al., 1996), and others contain a similar motif, RXY/FXpSP (Yaffe et al., 1997). However, several 14-3-3 binding proteins do not have any of these conserved motifs, and some have non-phosphorylated motifs (Du et al., 1996; Petosa et al., 1998).

In plants, enzymes shown to bind 14-3-3 proteins include nitrate reductase (Bachmann et al., 1996; Moorhead et al.,

1996), sucrose–phosphate synthase (Toroser et al., 1998), and the plasma membrane H<sup>+</sup>-ATPase (Jahn et al., 1997; Oecking et al., 1997). With nitrate reductase and sucrose–phosphate synthase, binding of 14-3-3 protein involves phosphorylated motifs similar to that described by Muslin et al. (1996); 14-3-3 binding to these enzymes results in inhibition of activity (Bachmann et al., 1996; Moorhead et al., 1996; Toroser et al., 1998). In contrast, 14-3-3 binding to the plasma membrane H<sup>+</sup>-ATPase activates that enzyme (Jahn et al., 1997; Oecking et al., 1997; Baunsgaard et al., 1998).

Binding of 14-3-3 protein to the H<sup>+</sup>-ATPase can be demonstrated after treating intact tissue (Jahn et al., 1997; Oecking et al., 1997) or isolated plasma membranes (Baunsgaard et al., 1998) with the fungal toxin fusicoccin, which stabilizes the H<sup>+</sup>-ATPase–14-3-3 complex. The 14-3-3 protein binds to the C-terminal region of the H<sup>+</sup>-ATPase (Jahn et al., 1997; Oecking et al., 1997), which contains an autoinhibitory domain (Palmgren et al., 1991). Removal of this domain by proteolysis or by deletion at the gene level results in an activated form of the enzyme with a higher  $V_{max}$  and a lower  $K_m$  for ATP, a more alkaline pH optimum, and a more efficient coupling between ATP hydrolysis and H<sup>+</sup>

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pumping (Palmgren et al., 1990, 1991; Baunsgaard et al., 1996). These effects on the H<sup>+</sup>-ATPase are identical to those observed after treatment of intact tissue with fusicoccin (Johansson et al., 1993).

Because the C-terminal region of the H<sup>+</sup>-ATPase does not contain any of the 14-3-3 binding motifs described previously, we have looked for a novel motif. Recently, we found that fusicoccin-induced 14-3-3 binding to the H<sup>+</sup>-ATPase protects a phosphorylated amino acid at the C terminus (Olsson et al., 1998). This amino acid residue, identified as Thr-948, is the next to last amino acid from the C terminus of the H<sup>+</sup>-ATPase, and we proposed that this phosphorylated residue is part of a binding motif for 14-3-3 (Olsson et al., 1998).

In this study, we identify and characterize the novel 14-3-3 binding motif, QQXYpTV, at the C terminus of the H<sup>+</sup>-ATPase. We show that binding of 14-3-3 to this motif in the absence of fusicoccin depends absolutely on the phosphorylation of Thr-948; however, 14-3-3 binding in the presence of fusicoccin occurs independently of phosphorylation but still involves the C-terminal YTV motif. Moreover, by expressing wild-type and mutant forms of the *Nicotiana plumbaginifolia* H<sup>+</sup>-ATPase isoform PMA2 (for plasma membrane H<sup>+</sup>-ATPase isoform 2) in yeast, we provide physiological evidence for the importance of the phosphothreonine motif in 14-3-3 binding and, hence, in the *in vivo* activation of the H<sup>+</sup>-ATPase.

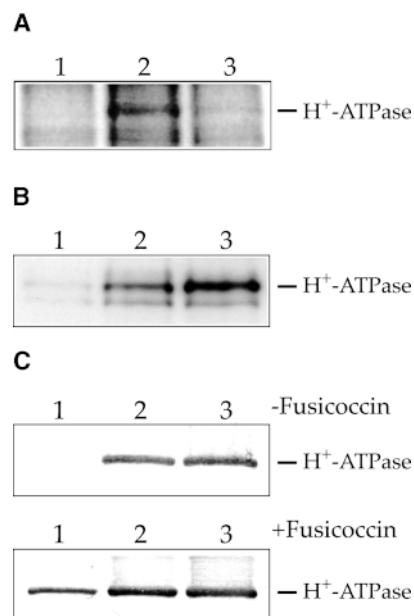
## RESULTS

### In Vivo Phosphorylation of the H<sup>+</sup>-ATPase Creates a Binding Site for 14-3-3

As was shown recently, the plant plasma membrane H<sup>+</sup>-ATPase can be isolated in a phosphorylated form from spinach leaf tissue infiltrated with fusicoccin and <sup>32</sup>P-orthophosphate (Olsson et al., 1998). The <sup>32</sup>P-labeled amino acid was identified as Thr-948 (numbering according to the *Arabidopsis* H<sup>+</sup>-ATPase isoform 1 [AHA1]). Furthermore, because the phosphate of the phosphorylated Thr-948 was protected from turnover by fusicoccin-dependent 14-3-3 binding to the H<sup>+</sup>-ATPase, the phosphorylated Thr-948 was suggested to be part of a binding site for 14-3-3.

That the phosphorylated Thr-948 is protected by 14-3-3 binding was indicated by the fact that the H<sup>+</sup>-ATPase was labeled with phosphorus-32 only when the ATP pool of the plant material was equilibrated with <sup>32</sup>P-orthophosphate before the addition of fusicoccin, as shown in Figure 1A (lane 2). Fusicoccin induces irreversible binding of 14-3-3 protein to the H<sup>+</sup>-ATPase (Jahn et al., 1997; Oecking et al., 1997), and the H<sup>+</sup>-ATPase was not labeled with phosphorus-32 when the fusicoccin was added before the <sup>32</sup>P-orthophosphate (lane 3), that is, when formation of the H<sup>+</sup>-ATPase-14-3-3 complex preceded phosphorus-32 labeling of the

ATP pool used for phosphorylation of the H<sup>+</sup>-ATPase. Furthermore, labeling with phosphorus-32 was not observed in the absence of fusicoccin (lane 1), that is, when phosphorylated Thr-948 was not protected from endogenous phosphatases by the formation of a stable H<sup>+</sup>-ATPase-14-3-3 complex. This interpretation is now confirmed by data obtained with polyclonal antibodies directed against phosphothreonine. Thus, in Figure 1B, we show that phosphothreonine-specific antibodies recognize a phosphorylated H<sup>+</sup>-ATPase in lanes 2 and 3. The extent of phosphorylation is even greater in lane 3 than in lane 2, most probably be-



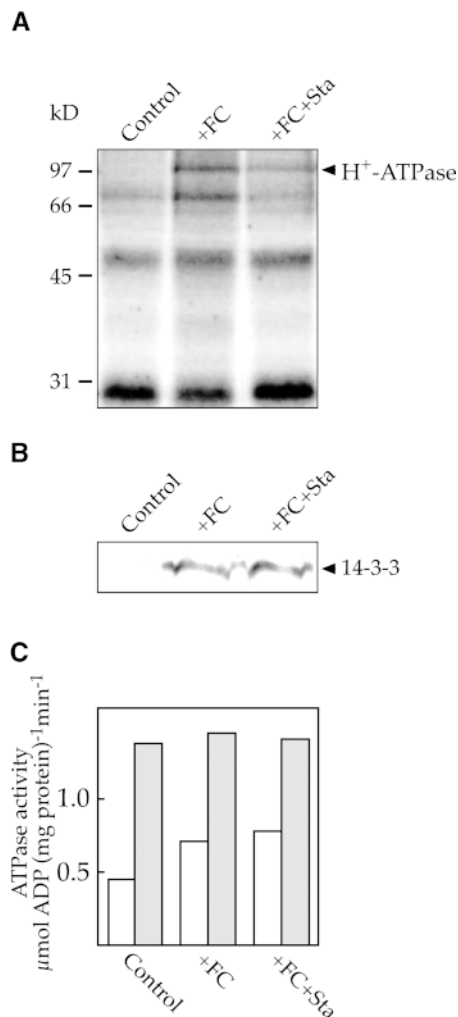
**Figure 1.** In Vivo Phosphorylation and 14-3-3 Binding of the Plasma Membrane H<sup>+</sup>-ATPase.

(A) Phosphorus-32 labeling of the H<sup>+</sup>-ATPase as visualized by using a PhosphorImager.

(B) Immunostaining of the H<sup>+</sup>-ATPase with an antibody directed against phosphothreonine.

(C) 14-3-3 protein overlays demonstrating the binding of 14-3-3 to the H<sup>+</sup>-ATPase in the absence (–) and presence (+) of fusicoccin.

Pieces of spinach leaves were infiltrated with medium containing <sup>32</sup>P-orthophosphate in the absence (lanes 1) or presence (lanes 2 and 3) of fusicoccin and incubated for a total of 90 min. In lanes 2, fusicoccin was added after 60 min of incubation with <sup>32</sup>P-orthophosphate, whereas in lanes 3, fusicoccin was added 15 min before the addition of <sup>32</sup>P-orthophosphate. Plasma membranes were isolated, and polypeptides were separated by SDS-PAGE (20 μg of protein per lane) and transferred to a polyvinylidene difluoride (PVDF) membrane. A Coomassie blue-stained and dried SDS gel was used in (A); PVDF membranes were used in (B) and (C). To detect 14-3-3 binding, we incubated PVDF membranes with 0.7 μM digoxigenin (DIG)-labeled GF14ω in the absence or presence of 5 μM fusicoccin. GF14ω was visualized with an antibody directed against DIG-labeled proteins. The position of the H<sup>+</sup>-ATPase is indicated.



**Figure 2.** Effects of the Protein Kinase Inhibitor Staurosporine.

**(A)** Effect on *in vivo* phosphorylation of the H<sup>+</sup>-ATPase.

**(B)** Effect on plasma membrane-bound 14-3-3.

**(C)** Effect on H<sup>+</sup>-ATPase activity.

Pieces of spinach leaves were infiltrated with media containing <sup>32</sup>P-orthophosphate and incubated in the absence (Control) or presence (+FC) of fusicoccin or of fusicoccin and staurosporine (+FC+Sta) for a total of 90 min. Staurosporine (1 μM) was present for all 90 min, whereas fusicoccin (5 μM) was present only during the last 30 min. In **(A)**, plasma membranes were separated by SDS-PAGE (20 μg of protein per lane), and <sup>32</sup>P-labeled polypeptides were visualized by using a PhosphorImager. The position of the H<sup>+</sup>-ATPase is indicated. In **(A)**, numbers at left refer to molecular mass standards. In **(B)**, isolated plasma membranes were treated with Brij 58 (detergent/protein ratio of 10:1) and resuspended. This treatment turns all vesicles cytoplasmic-side out (Johansson et al., 1995) and serves to remove proteins loosely attached to the cytosolic surface of the plasma membrane and soluble proteins enclosed in cytoplasmic-side-in vesicles. Thus, only firmly bound 14-3-3 should remain. Polypeptides were separated by SDS-PAGE (10 μg of protein per lane), transferred to a blotting membrane, and probed with an antiserum directed against a barley 14-3-3 protein. The position of the 14-3-3

cause fusicoccin in lane 3 already was present in the infiltration medium, whereas in lane 2, the leaf pieces had been incubated for 1 hr with <sup>32</sup>P-orthophosphate before being treated with fusicoccin. In control experiments with antibodies directed against phosphoserine and phosphotyrosine, there was no staining of the H<sup>+</sup>-ATPase (data not shown), confirming the specificity of the antibodies used. Thus, phosphothreonine-specific antibodies, rather than labeling with phosphorus-32, may be used conveniently to detect phosphorylation of Thr-948 in the H<sup>+</sup>-ATPase.

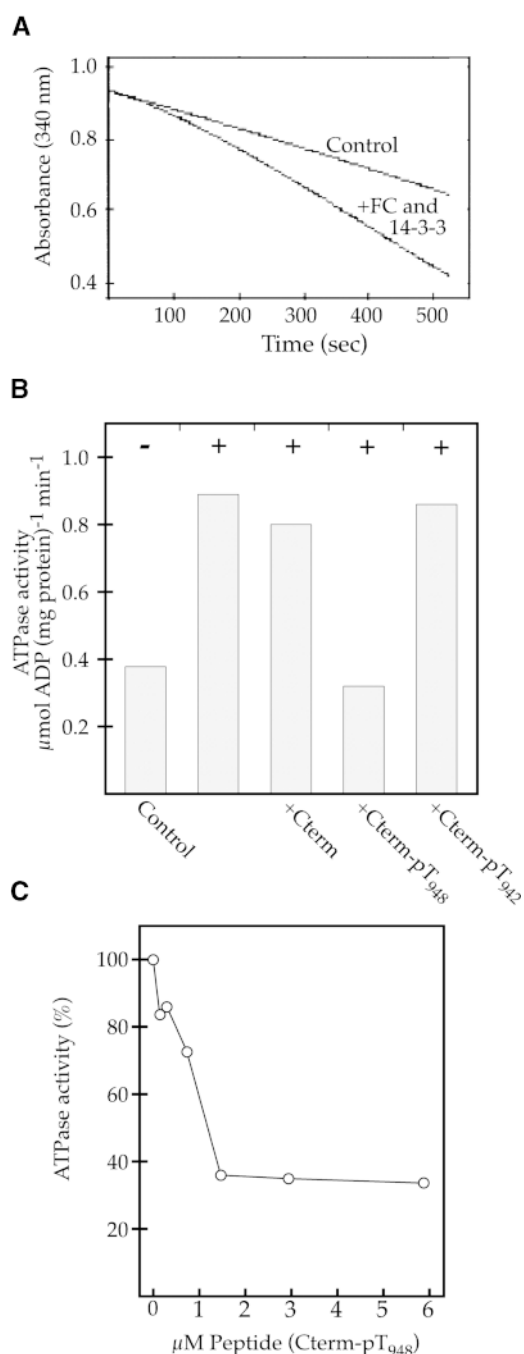
The 14-3-3 overlays in Figure 1C show that the phosphorylated H<sup>+</sup>-ATPase binds 14-3-3 *in vitro* also in the absence of fusicoccin (top, lanes 2 and 3), whereas the nonphosphorylated H<sup>+</sup>-ATPase binds 14-3-3 only in the presence of fusicoccin (lanes 1). This means that phosphorylation of Thr-948 creates a binding site for 14-3-3 and that fusicoccin somehow replaces the need for this phosphorylation.

We were able to show that fusicoccin-dependent 14-3-3 binding *in vivo* is not dependent on a phosphorylated H<sup>+</sup>-ATPase. As shown in Figure 2A, staurosporine, a Ser/Thr protein kinase inhibitor with some specificity for protein kinase C in animals (Rüegg and Burgess, 1989), markedly reduced the phosphorylation of the H<sup>+</sup>-ATPase. The reduced phosphorylation of the H<sup>+</sup>-ATPase caused by staurosporine (Figure 2A) did not diminish the amount of 14-3-3 bound to the plasma membrane (Figure 2B) and had no effect on the fusicoccin-dependent activation of the H<sup>+</sup>-ATPase (Figure 2C). Binding of 14-3-3 protein to the plasma membrane in the presence of fusicoccin is mainly accomplished through the H<sup>+</sup>-ATPase, as can be observed in 14-3-3 protein overlays (data not shown) and during purification of the H<sup>+</sup>-ATPase-14-3-3 complex (Oecking et al., 1997).

#### A Peptide Mimicking the 14-3-3 Binding Site Inhibits 14-3-3 Binding to and Activation of the H<sup>+</sup>-ATPase

The spinach leaf plasma membrane H<sup>+</sup>-ATPase can be activated *in vitro* by recombinant 14-3-3 protein (*Arabidopsis* GF14ω) and fusicoccin (Figure 3A), as was demonstrated earlier for the *Arabidopsis* isoform AHA1 expressed in yeast (Baunsgaard et al., 1998). As shown in Figure 3A, the spinach leaf H<sup>+</sup>-ATPase was gradually activated by GF14ω and fusicoccin; after ~10 min, the rate of ATP hydrolysis was

protein is indicated. In **(C)**, H<sup>+</sup>-ATPase activity was measured with plasma membranes isolated from control tissue, tissue incubated with fusicoccin (+FC), and tissue incubated with fusicoccin and staurosporine (+FC+Sta). Open bars indicate activity in the presence of 0.05% (w/v) Brij 58 to obtain 100% cytoplasmic-side-out vesicles and thus assay all H<sup>+</sup>-ATPase molecules. Hatched bars represent activity in the presence of 0.02% (w/v) lysophosphatidylcholine to fully activate all H<sup>+</sup>-ATPase (Johansson et al., 1993).



**Figure 3.** Effects of 14-3-3 Protein, Fusicoccin, and Peptides Mimicking the C Terminus of the H<sup>+</sup>-ATPase on the ATP Hydrolytic Activity of the Plasma Membrane H<sup>+</sup>-ATPase.

**(A)** In vitro activation of the H<sup>+</sup>-ATPase by adding fusicoccin and 14-3-3 protein (+FC and 14-3-3) to plasma membranes isolated in the absence of fusicoccin and thus containing nonactivated H<sup>+</sup>-ATPase. The rate of ATP hydrolysis was enzymatically coupled to the hydrolysis of NADH and was monitored through the decrease in absorbance at 340 nm. The rate of ATP hydrolysis in the control was 0.54

doubled. To corroborate that phosphorylated Thr-948 is part of a binding site for 14-3-3, we synthesized synthetic peptides that mimicked the C terminus of the H<sup>+</sup>-ATPase (Figure 4) and tested their ability to prevent the activation of the H<sup>+</sup>-ATPase by GF14 $\omega$  and fusicoccin. The peptides corresponded to the C-terminal 16 amino acids of the H<sup>+</sup>-ATPase (KLKGLDIDT<sub>942</sub>LQQAYT<sub>948</sub>V) and either were not phosphorylated (Cterm) or were phosphorylated on Thr-942 (Cterm-pT<sub>942</sub>) or Thr-948 (Cterm-pT<sub>948</sub>). Figure 3B shows that Cterm-pT<sub>948</sub> completely prevented the activation observed in the presence of GF14 $\omega$  and fusicoccin, whereas neither the nonphosphorylated peptide nor Cterm-pT<sub>942</sub> had an effect on the in vitro activation of the H<sup>+</sup>-ATPase. Inhibition was half-maximal at  $\sim 0.6$   $\mu$ M of Cterm-pT<sub>948</sub> and reached a plateau at  $\sim 1$   $\mu$ M (Figure 3C).

These data were confirmed when the interaction between the H<sup>+</sup>-ATPase and digoxigenin (DIG)-labeled GF14 $\omega$  was investigated by using overlay assays. As demonstrated in Figure 5A, the presence of 1  $\mu$ M of the Cterm-pT<sub>948</sub> peptide completely prevented fusicoccin-dependent 14-3-3 binding to the nonphosphorylated H<sup>+</sup>-ATPase. This peptide also inhibited 14-3-3 binding to the phosphorylated H<sup>+</sup>-ATPase in the absence of fusicoccin, but this binding was not completely prevented even with 10  $\mu$ M of peptides (Figure 5B). The addition of the peptides Cterm or Cterm-pT<sub>942</sub> to the incubation medium did not affect the H<sup>+</sup>-ATPase/14-3-3 interaction.

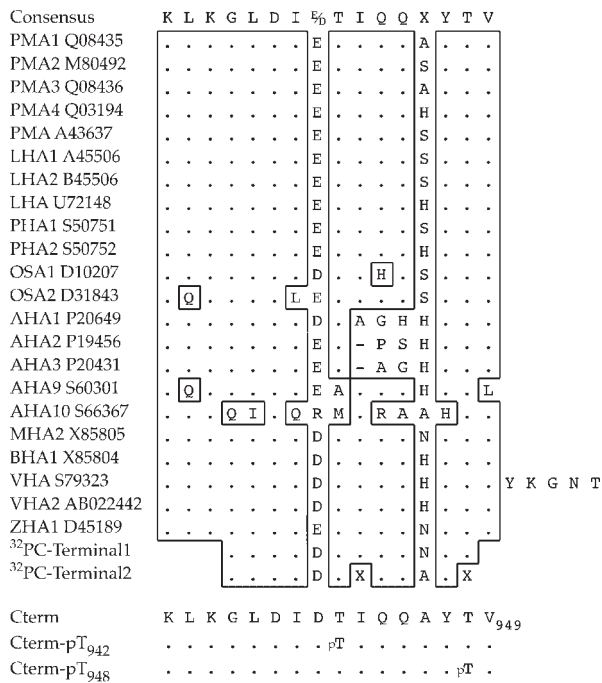
### In Vitro Phosphorylation of the H<sup>+</sup>-ATPase

As shown in Figure 1, the H<sup>+</sup>-ATPase cannot be isolated in phosphorylated form from spinach leaf tissue unless the tis-

$\mu$ mol ADP (mg protein)<sup>-1</sup> min<sup>-1</sup>. In the presence of 7  $\mu$ M fusicoccin and 0.7  $\mu$ M 14-3-3 protein (GF14 $\omega$ ), the final rate of ATP hydrolysis was 1.10  $\mu$ mol ADP (mg protein)<sup>-1</sup> min<sup>-1</sup>.

**(B)** Effects of peptides mimicking the C terminus of the H<sup>+</sup>-ATPase on the activation of the H<sup>+</sup>-ATPase by 14-3-3 and fusicoccin. Plasma membranes were preincubated with different synthetic peptides and GF14 $\omega$  for 15 min before the addition of fusicoccin (+). The control did not receive GF14 $\omega$  and fusicoccin (-). The twofold activation observed in the presence of GF14 $\omega$  and fusicoccin (+) was abolished in the presence of the peptide phosphorylated on Thr-948 (Cterm-pT<sub>948</sub>). The nonphosphorylated peptide (Cterm) or the peptide phosphorylated on Thr-942 (Cterm-pT<sub>942</sub>) had no effect on activity. The synthetic peptides correspond to the last 16 amino acids at the C terminus of the H<sup>+</sup>-ATPase (see Figure 4).

**(C)** Concentration dependence of the inhibition by the peptide Cterm-pT<sub>948</sub>. Plasma membranes were preincubated with 0.7  $\mu$ M GF14 $\omega$  and various amounts of the synthetic peptide Cterm-pT<sub>948</sub> in the presence of 7  $\mu$ M fusicoccin. One hundred percent activity was equal to 0.70  $\mu$ mol ADP (mg protein)<sup>-1</sup> min<sup>-1</sup>.



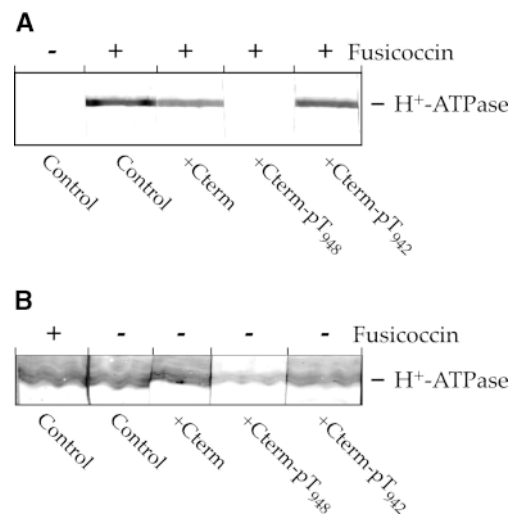
**Figure 4.** Alignment of C-Terminal Amino Acid Sequences of Plant Plasma Membrane H<sup>+</sup>-ATPases.

The C-terminal amino acid sequences (the last 15 to 21 amino acids, depending on the isoform) of plant plasma membrane H<sup>+</sup>-ATPases found in the database and the two sequences found in spinach leaves with Thr-948 phosphorylated in vivo, <sup>32</sup>PC-Terminal1 and <sup>32</sup>PC-Terminal2 (Olsson et al., 1998), were aligned. MacVector (Intelligenetics, Oxford Molecular Group, Oxford, UK) was used for the multiple alignment. Sequence identity is indicated with boxes and dots. The peptides mimicking the C terminus of the H<sup>+</sup>-ATPase and used in activity measurements, and 14-3-3 protein overlays are shown. Numbering in the peptides is according to the Arabidopsis isoform AHA1. The aligned C-terminal sequences were obtained from the following H<sup>+</sup>-ATPases: PMA isoforms from *N. plumbaginifolia* (tobacco); LHA isoforms from *Lycopersicon esculentum* (tomato); PHA isoforms from *Solanum tuberosum* (potato); OSA isoforms from *Oryza sativa* (rice); AHA isoforms from Arabidopsis (thale cress); MHA2 from *Zea mays* (maize); BHA1 from *Phaseolus vulgaris* (kidney bean); VHA isoforms from *Vicia faba* (fava bean); and ZHA1 from *Zostera marina* (sea grass).

is first incubated with fusicoccin. In vitro, however, the H<sup>+</sup>-ATPase is slowly phosphorylated when spinach leaf plasma membrane vesicles are incubated with ATP and Mg<sup>2+</sup>, as was detected by using phosphothreonine-specific antibodies (Figures 6A and 6B). This phosphorylation also was observed in the absence of added 14-3-3 and fusicoccin (Figure 6B), although the rate of phosphorylation was greater in the presence of 14-3-3 and fusicoccin (Figure 6A). Probably, most of the soluble phosphatases that are active in vivo are removed during plasma membrane isolation,

making protection of the phosphothreonine by 14-3-3 binding less important. As shown in Figure 6C, binding of GF14 $\omega$  to the in vitro-phosphorylated H<sup>+</sup>-ATPase in the absence of fusicoccin was proportional to the extent of phosphorylation, as observed above for the in vivo-phosphorylated H<sup>+</sup>-ATPase (Figure 1C).

As demonstrated in Figure 7, the interaction between the in vitro-phosphorylated H<sup>+</sup>-ATPase and GF14 $\omega$  was completely prevented by 10  $\mu$ M (but not by 1  $\mu$ M; data not shown) of the Cterm-pT<sub>948</sub> peptide. The nonphosphorylated peptide (Cterm) as well as Cterm-pT<sub>942</sub> had no effect. These results are similar to those observed with the in vivo-phosphorylated H<sup>+</sup>-ATPase (Figure 5B).

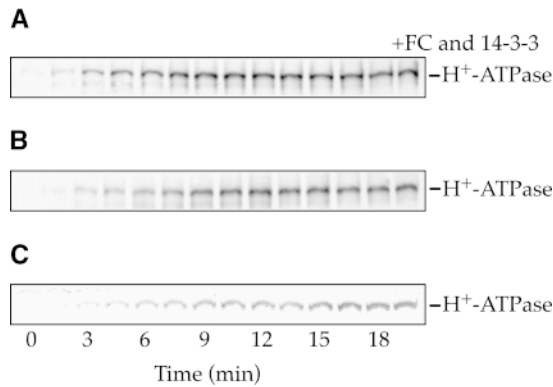


**Figure 5.** Effects of Peptides Mimicking the C Terminus of the H<sup>+</sup>-ATPase on the Binding of 14-3-3 to the H<sup>+</sup>-ATPase.

(A) 14-3-3 protein overlay with plasma membranes isolated from leaves in the absence of fusicoccin and thus containing nonphosphorylated H<sup>+</sup>-ATPase.

(B) 14-3-3 protein overlay with plasma membranes isolated from leaves incubated with fusicoccin and thus containing phosphorylated H<sup>+</sup>-ATPase.

Plasma membranes (260  $\mu$ g of protein) were subjected to SDS-PAGE, and the polypeptides were transferred to a PVDF membrane. The membrane was cut into strips and incubated with 0.7  $\mu$ M DIG-labeled 14-3-3 (GF14 $\omega$ ) in the absence (-) or presence (+) of 5  $\mu$ M fusicoccin (Control), and (A) in the presence of 5  $\mu$ M fusicoccin (+) and 1  $\mu$ M peptide (different ones as indicated) for 2 hr, or (B) in the absence of fusicoccin (-) but the presence of 10  $\mu$ M peptide (different ones as indicated) for 2 hr. GF14 $\omega$  was detected with an antibody directed against DIG-labeled proteins. The position of the H<sup>+</sup>-ATPase is indicated. The synthetic peptides correspond to the last 16 amino acids at the C terminus of the H<sup>+</sup>-ATPase (see Figure 4). One peptide was not phosphorylated (+Cterm), the second was phosphorylated at Thr-948 (Cterm-pT<sub>948</sub>), and the third was phosphorylated at Thr-942 (Cterm-pT<sub>942</sub>).



**Figure 6.** In Vitro Phosphorylation and 14-3-3 Binding of the H<sup>+</sup>-ATPase.

(A) and (B) Immunostaining of the H<sup>+</sup>-ATPase with phosphothreonine-specific antibodies.

(C) 14-3-3 protein overlay of a protein gel blot corresponding to the one in (B).

Plasma membranes isolated from leaves in the absence of fusicocin and thus containing nonphosphorylated H<sup>+</sup>-ATPase were incubated with ATP and Mg<sup>2+</sup> in the absence (B) or presence (A) of fusicocin and 14-3-3 (+FC and 14-3-3). Samples were withdrawn every 90 sec, and polypeptides were separated by SDS-PAGE and transferred to a PVDF membrane. In (A) and (B), the membrane was probed with phosphothreonine-specific antibodies, whereas in (C) the membrane was incubated with DIG-labeled 14-3-3 (GF14 $\omega$ ) and subsequently with DIG-specific antibodies. The position of the H<sup>+</sup>-ATPase is indicated.

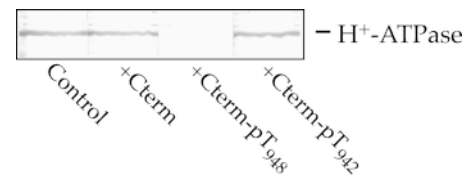
### Physiological Importance of Thr-948

To demonstrate the physiological relevance of 14-3-3 binding to the phosphorylated Thr-948 site, we investigated the effects of various mutations in the *N. plumbaginifolia* H<sup>+</sup>-ATPase gene *pma2* when it was expressed in the *Saccharomyces cerevisiae* strain YAK2. The two wild-type H<sup>+</sup>-ATPase genes (*PMA1* and *PMA2*) are deleted from this yeast strain, and its survival on galactose medium is made possible by the presence of the yeast H<sup>+</sup>-ATPase gene *PMA1* under the control of the galactose-dependent *GAL1* promoter. The *N. plumbaginifolia* *pma2* gene is under the control of the yeast *PMA1* promoter and is expressed on both galactose and glucose medium (de Kerchove d'Exaerde et al., 1995). Hence, shifting the yeast YAK2 from galactose to glucose medium results in the yeast becoming completely dependent on the plant H<sup>+</sup>-ATPase PMA2 for survival (de Kerchove d'Exaerde et al., 1995). We used the *N. plumbaginifolia* *pma2* gene for three reasons. First, when *pma2* is expressed in yeast, its product PMA2 can functionally replace the yeast plasma membrane H<sup>+</sup>-ATPase (de Kerchove d'Exaerde et al., 1995). Second, fusicocin binding can be detected in plasma membranes isolated from yeast expressing plant PMA2, and plant PMA2/yeast 14-3-3

complexes are present in these plasma membranes (Piotrowski et al., 1998). Third, the amino acid sequence at the C terminus of the *N. plumbaginifolia* H<sup>+</sup>-ATPase isoform PMA2 (QQSYT<sub>955</sub>V) is similar to the two C-terminal peptide sequences found in spinach leaf H<sup>+</sup>-ATPase (QQNYT and QQAY; Figure 4), with the Thr-955 in PMA2 thus corresponding to the Thr-948 discussed above.

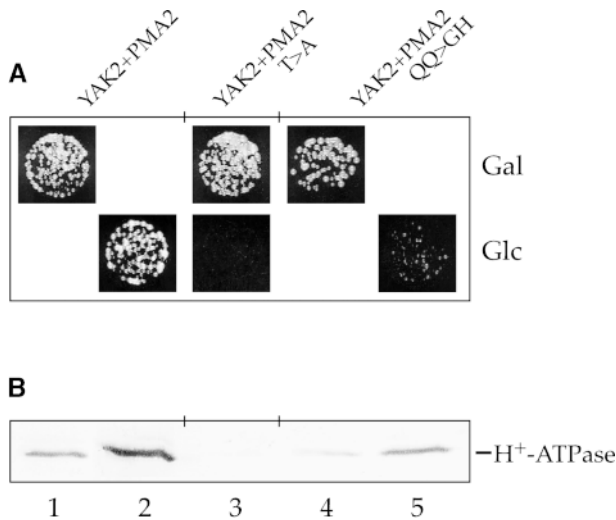
To clarify the importance of the motif QQXYpTV for 14-3-3 binding, we generated two mutants of the *pma2* gene. In one mutant, the threonine residue was changed to an alanine residue (T955A), and in the other mutant, the relatively conserved QQ (see Figure 4) was changed to GH (Q951G/Q952H). In this way, the second mutant obtained a C-terminal sequence (GHSYTV) similar to that of the Arabidopsis isoform AHA1 (GHHYTV). Notably, the three Arabidopsis isoforms AHA1, AHA2, and AHA3 all lack the QQ motif and do not (or only very poorly) complement yeast mutants that lack their endogenous H<sup>+</sup>-ATPase (Villalba et al., 1992; Palmgren and Christensen, 1993, 1994).

As shown in Figure 8A (left), YAK2 cells expressing the wild-type plant PMA2 grew well on both galactose and glucose media. Moreover, as demonstrated by the 14-3-3 overlay in Figure 8B (lanes 1 and 2), the wild-type plant PMA2 expressed in these cells bound 14-3-3. More 14-3-3 was bound when the yeast cells were grown on glucose medium (lane 2), in which they were dependent on the plant H<sup>+</sup>-ATPase for survival. When the PMA2 T955A mutant was expressed in YAK2 cells, growth was detected only on galactose medium (Figure 8A, center); that is, the PMA2 T955A mutant could not functionally replace the yeast H<sup>+</sup>-ATPase, which suggests that Thr-955 is necessary for growth. As shown in Figure 8B (lane 3), plasma membranes obtained from yeast expressing the PMA2 T955A mutant (grown on galactose medium) showed no 14-3-3 binding to the mutated H<sup>+</sup>-ATPase. The plant PMA2 mutant Q951G/Q952H could functionally replace the yeast H<sup>+</sup>-ATPase, although



**Figure 7.** Effects of Peptides Mimicking the C Terminus of the H<sup>+</sup>-ATPase on the Binding of 14-3-3 to in Vitro-Phosphorylated H<sup>+</sup>-ATPase.

Plasma membranes were incubated with ATP and Mg<sup>2+</sup> for 30 min to phosphorylate the H<sup>+</sup>-ATPase; polypeptides were separated by SDS-PAGE (260  $\mu$ g of protein) and transferred to a PVDF membrane. The membrane was cut into strips and incubated with 0.7  $\mu$ M DIG-labeled 14-3-3 (GF14 $\omega$ ) and 10  $\mu$ M peptide (various ones as indicated) for 2 hr. GF14 $\omega$  was detected with an antibody directed against DIG-labeled proteins. The position of the H<sup>+</sup>-ATPase is indicated.



**Figure 8.** Growth of Yeast Cells Expressing Wild-Type or Mutant Plant H<sup>+</sup>-ATPase and Analysis of 14-3-3 Binding.

(A) Growth of the yeast (*S. cerevisiae*) strain YAK2 expressing the yeast H<sup>+</sup>-ATPase under the control of the galactose-dependent *GAL1* promoter transformed with the wild-type plant (*N. plumbaginifolia*) H<sup>+</sup>-ATPase PMA2 (YAK2+PMA2; left), mutant PMA2 T955A (YAK2+PMA2 T>A; middle), or the mutant PMA2 Q951G/Q952H (YAK2+PMA2 QQ>GH; right). The ability of the plant wild-type and mutant H<sup>+</sup>-ATPases to functionally replace the yeast H<sup>+</sup>-ATPase was analyzed by shifting from galactose (Gal; top row) to glucose (Glc; bottom row) medium. No growth was detected on glucose medium with yeast expressing the PMA2 T955A mutant.

(B) 14-3-3 protein overlay showing the binding of 14-3-3 to the wild-type and mutant forms of the plant H<sup>+</sup>-ATPase PMA2 in the absence of fusicoccin: lane 1, wild-type PMA2 from yeast grown on galactose; lane 2, wild-type PMA2 from yeast grown on glucose; lane 3, mutant PMA2 T955A from yeast grown on galactose; lane 4, mutant PMA2 Q951G/Q952H from yeast grown on galactose; and lane 5, mutant PMA2 Q951G/Q952H from yeast grown on glucose. Plasma membranes (5  $\mu$ g of protein) derived from the individual yeast strains in (A) were subjected to SDS-PAGE, and the polypeptides were transferred to a nitrocellulose membrane and incubated with 0.7  $\mu$ M His-tagged 14-3-3 (*N. tabacum* isoform T14-3c).

the yeast grew very slowly on glucose medium (Figure 8A, right). The slow growth on glucose correlated to less binding of 14-3-3 protein when compared with wild-type PMA2 (cf. lanes 2 and 5 in Figure 8B).

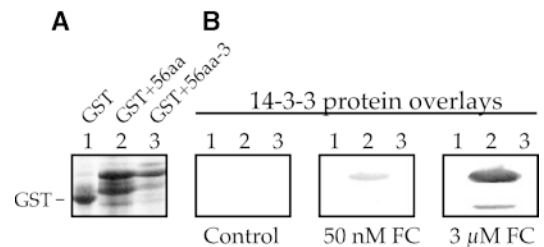
To investigate whether the C-terminal amino acid residues also are involved in fusicoccin-dependent but phosphorylation-independent 14-3-3 binding, we constructed two glutathione *S*-transferase (GST) fusion proteins containing parts of the C terminus of *N. plumbaginifolia* PMA2 and expressed them in *Escherichia coli* to avoid phosphorylation of Thr-955. As seen in the 14-3-3 protein overlays in Figure 9B, a GST fusion protein containing the C-terminal 56 amino ac-

ids bound 14-3-3 in a fusicoccin-dependent manner (lanes 2). However, removal of the last three amino acids (YT<sub>955</sub>V) completely abolished 14-3-3 binding (lanes 3), demonstrating the involvement of the C terminus not only in phosphorylation-dependent 14-3-3 binding but also directly in fusicoccin-dependent 14-3-3 binding.

## DISCUSSION

The plasma membrane H<sup>+</sup>-ATPase is a key enzyme required for nutrient uptake and plant growth, and its activity is regulated by several physiological stimuli (reviewed in Michelet and Boutry, 1995). The mechanisms for regulation are, however, not fully understood. The fungal toxin fusicoccin has long been used as a tool for studying H<sup>+</sup>-ATPase activation. Fusicoccin activates the H<sup>+</sup>-ATPase by inducing an irreversible bond between the C-terminal region of the H<sup>+</sup>-ATPase and 14-3-3 protein, thereby displacing the C-terminal auto-inhibitory domain (Jahn et al., 1997; Oecking et al., 1997). 14-3-3 proteins usually bind to phosphorylated motifs in their target proteins, for which two optimal motifs have been identified: RSXpSXP (Muslin et al., 1996) and RXY/FXpSP (Yaffe et al., 1997). However, the C-terminal region of the plant H<sup>+</sup>-ATPase contains no amino acid sequence with obvious similarity to either of these motifs. Other target proteins for 14-3-3 binding also lack these motifs, and non-phosphorylated motifs have been identified (Du et al., 1996; Petosa et al., 1998).

Several reports indicate that the H<sup>+</sup>-ATPase of higher plants can be phosphorylated both *in vivo* and *in vitro*. Schaller and Sussman (1988) showed that the H<sup>+</sup>-ATPase is phosphorylated *in vivo* and *in vitro* on serine and threonine



**Figure 9.** 14-3-3 Binding to GST Fusion Proteins Containing C-Terminal Parts of the *N. plumbaginifolia* H<sup>+</sup>-ATPase PMA2.

(A) Protein-stained SDS gel with GST and GST fusion proteins expressed in *E. coli*. Lane 1 shows the GST protein (GST); lane 2, a GST fusion protein containing the C-terminal 56 amino acids of the H<sup>+</sup>-ATPase (GST+56aa); and lane 3, a GST fusion protein containing the last 56 amino acids at the C terminus, except for the very last three amino acids (YTV) (GST+56aa-3).

(B) 14-3-3 protein overlays corresponding to the gel in (A) in the presence of increasing concentrations of fusicoccin (FC).

residues by  $\text{Ca}^{2+}$ -stimulated protein kinases, but no effect on activity was shown. Vera-Estrella et al. (1994) and Xing et al. (1996) reported that in vivo dephosphorylation of the  $\text{H}^+$ -ATPase in tomato cells activated the enzyme. Recently, Camoni et al. (1998) demonstrated that the  $\text{H}^+$ -ATPase from maize roots is phosphorylated in the C-terminal region by a  $\text{Ca}^{2+}$ -dependent protein kinase in vitro. More recently, Thr-948 at the  $\text{H}^+$ -ATPase C terminus was identified as an in vivo phosphorylation site (Olsson et al., 1998). Because the phosphate of phosphorylated Thr-948 was found to be protected from turnover by fusicoccin-dependent 14-3-3 binding, this phosphorylated residue was suggested to be part of a binding site for 14-3-3.

Using phosphothreonine-specific antibodies, we have now confirmed that the phosphate of phosphorylated Thr-948 is protected from turnover in vivo by fusicoccin-dependent 14-3-3 binding to the  $\text{H}^+$ -ATPase (Figure 1B). Moreover, by using 14-3-3 protein overlays, we demonstrate that phosphorylation of Thr-948 creates a binding site for 14-3-3 (Figure 1C). Notably, binding of 14-3-3 to the phosphorylated  $\text{H}^+$ -ATPase is not dependent on fusicoccin, whereas 14-3-3 binding to the nonphosphorylated enzyme is dependent on fusicoccin both in vitro (Figure 1C) and in vivo (Figure 2B). In  $\text{H}^+$ -ATPase assays, we found that a synthetic peptide corresponding to the C-terminal 16 amino acids of the  $\text{H}^+$ -ATPase and phosphorylated on Thr-948 (Cterm-pT<sub>948</sub>; Figure 4) completely prevents the activation of the  $\text{H}^+$ -ATPase that is observed in the presence of recombinant 14-3-3 (GF14 $\omega$ ) and fusicoccin (Figure 3). Furthermore, this peptide prevents both fusicoccin-dependent 14-3-3 binding to nonphosphorylated  $\text{H}^+$ -ATPase and fusicoccin-independent 14-3-3 binding to phosphorylated  $\text{H}^+$ -ATPase (Figure 5). The fusicoccin-independent 14-3-3 binding to the in vivo phosphorylated  $\text{H}^+$ -ATPase was not completely inhibited even at a peptide concentration of 10  $\mu\text{M}$ , whereas the fusicoccin-dependent 14-3-3 binding to nonphosphorylated  $\text{H}^+$ -ATPase was completely inhibited at a peptide concentration of only 1  $\mu\text{M}$ . Possibly, the presence of fusicoccin increases the affinity between the peptide and the 14-3-3 protein, hence making the peptide a more potent inhibitor of  $\text{H}^+$ -ATPase–14-3-3 complex formation. Taken together, our data show that phosphorylation of Thr-948 creates a specific binding site for 14-3-3. However, our data also show that fusicoccin-dependent 14-3-3 binding to the  $\text{H}^+$ -ATPase occurs even when Thr-948 is not phosphorylated.

A protein kinase phosphorylating the  $\text{H}^+$ -ATPase on a threonine residue is associated with the plasma membrane (Figure 6). Similar to the in vivo phosphorylation reaction, in vitro phosphorylation creates a binding site for 14-3-3, the 14-3-3 binding being proportional to the extent of phosphorylation of the  $\text{H}^+$ -ATPase (Figure 6C). 14-3-3 binding in this case also was inhibited by the peptide phosphorylated on Thr-948 (Figure 7). These data suggest that the in vitro–phosphorylated threonine is identical to Thr-948. Given the very low degree of phosphorylation in vitro, we have failed so far to positively identify the in vitro–phosphorylated thre-

onine residue. Thus, we estimate that only a small percentage of the  $\text{H}^+$ -ATPase molecules are phosphorylated in vitro compared with >50% being phosphorylated in vivo (data not shown). The  $\text{H}^+$ -ATPase phosphorylated in vivo bound much more 14-3-3 protein than did the  $\text{H}^+$ -ATPase phosphorylated in vitro, reflecting the difference in the extent of phosphorylation (cf. Figures 5B and 7). The difference in extent of phosphorylation also may explain the observed difference in inhibition by the peptide Cterm-pT<sub>948</sub> of 14-3-3 protein binding to the in vivo– and in vitro–phosphorylated  $\text{H}^+$ -ATPase (Figures 5B and 7).

To demonstrate the importance of phosphorylated Thr-948 in vivo, we expressed the  $\text{H}^+$ -ATPase isoform PMA2 from *N. plumbaginifolia* in the YAK2 strain of *S. cerevisiae*. When this yeast strain is grown on glucose medium, the yeast  $\text{H}^+$ -ATPase PMA1 is no longer expressed, and yeast growth becomes completely dependent on the ability of the plant  $\text{H}^+$ -ATPase to functionally replace the yeast  $\text{H}^+$ -ATPase. Indeed, yeast growth under these conditions is directly proportional to the activity of the plant  $\text{H}^+$ -ATPase (Regenberg et al., 1995). The *N. plumbaginifolia* isoforms PMA2 and PMA4 are the only plant  $\text{H}^+$ -ATPase isoforms tested to date that can support yeast growth when the yeast  $\text{H}^+$ -ATPase gene *PMA1* is turned off (de Kerchove d'Exaerde et al., 1995; Luo et al., 1999). In contrast, the three Arabidopsis  $\text{H}^+$ -ATPase isoforms AHA1, AHA2, and AHA3 are not (or only poorly) able to functionally replace the yeast  $\text{H}^+$ -ATPase (Villalba et al., 1992; Palmgren and Christensen, 1993, 1994), unless, as shown for AHA2, the plant enzyme is activated either by deletion of the C-terminal region or by addition of fusicoccin to the growth medium (Palmgren and Christensen, 1993; Regenberg et al., 1995; Baunsgaard et al., 1998). The C-terminal amino acid sequences of the *N. plumbaginifolia* isoforms PMA2 (QQSYT<sub>955</sub>V) and PMA4 (QQHYT<sub>956</sub>V) differ from those of the Arabidopsis isoforms AHA1 (GHHYT<sub>948</sub>V), AHA2 (PSHYT<sub>947</sub>V), and AHA3 (AGHYT<sub>948</sub>V) with respect to the QQ motif, which is conserved in most plant  $\text{H}^+$ -ATPases (Figure 4).

To test the importance of Thr-948 and the QQ motif, we expressed two mutants of the plant PMA2 in yeast. In one mutant, PMA2 T955A, the threonine (Thr-955 in *N. plumbaginifolia* PMA2) was exchanged for an alanine. In the other mutant, Q951G/Q952H, the QQ motif was exchanged for GH, making the C-terminal sequence (GHSYTV) of this mutant similar to that of the Arabidopsis isoform AHA1 (GHHYTV). The PMA2 T955A mutant could not functionally replace the yeast  $\text{H}^+$ -ATPase PMA1 (Figure 8A, center), and the yeast expressing the Q951G/Q952H mutant grew only slowly on glucose medium. Based on the facts that *N. plumbaginifolia* PMA2 allows yeast growth in the absence of yeast  $\text{H}^+$ -ATPase and forms a stable complex with yeast 14-3-3, it was suggested that the plant PMA2–yeast 14-3-3 complex represents an activated state of the plant  $\text{H}^+$ -ATPase and that the ability to form such a complex explains why this plant isoform can functionally replace the yeast  $\text{H}^+$ -



ATPase (Piotrowski et al., 1998). This interpretation is supported by our data on yeast growth and 14-3-3 binding to wild-type PMA2 and to the T955A and Q951G/Q952H mutants, which show a good correlation between growth on glucose and the ability of the plant wild-type and mutant H<sup>+</sup>-ATPases to bind 14-3-3 (Figure 8). Thus, the T955A mutant, which grew only on galactose medium, did not bind 14-3-3 at all (Figure 8B, lane 3). No binding was expected, because phosphorylation of the missing residue is necessary to obtain 14-3-3 binding in the absence of fusicoccin (Figure 1).

Why do the yeast cells expressing the Q951G/Q952H mutant grow poorly on glucose medium? The 14-3-3 binding motifs identified by Muslin et al. (1996) and Yaffe et al. (1997) have a basic amino acid residue at position -3 or -4. This basic residue probably constitutes a phosphorylation signal for animal protein kinases A and C (Yaffe et al., 1997). The C-terminal sequences of plant H<sup>+</sup>-ATPases do not contain any basic residues at position -3 or -4 relative to Thr-948. However, positions -2 to -4 are enriched in glutamine (Q), histidine (H), and asparagine (N) (Figure 4), amino acids with bulky hydrophilic side chains that may constitute phosphorylation signals for plant protein kinases. If the QQ motif, in contrast to the GH, PS, and AG motifs of the Arabidopsis isoforms AHA1, AHA2, and AHA3, is a phosphorylation signal well recognized by a yeast protein kinase, this would explain why the PMA2 and PMA4 isoforms of *N. plumbaginifolia*, but not the Arabidopsis isoforms, can functionally replace the yeast H<sup>+</sup>-ATPase. The crucial point would be the extent of phosphorylation of Thr-948 and concomitant 14-3-3 binding and activation of the plant H<sup>+</sup>-ATPase. This interpretation is supported by the data in Figure 8B, which show much stronger 14-3-3 binding to the wild-type H<sup>+</sup>-ATPase (lane 2) than to the Q951G/Q952H mutant (lane 5). Because 14-3-3 binding in the absence of fusicoccin is a direct measure of the amount of phosphorylated H<sup>+</sup>-ATPase (Figures 1 and 6), this suggests that much less phosphorylated (and thus activated) H<sup>+</sup>-ATPase is present in the plasma membrane of yeast expressing the Q951G/Q952H mutant than in that of yeast expressing the wild-type plant H<sup>+</sup>-ATPase.

Both fusicoccin-dependent and fusicoccin-independent 14-3-3 binding are inhibited by the peptide phosphorylated on Thr-948 (Figures 5 and 7), suggesting that both types of binding involve the same binding site on the 14-3-3 protein. Similarly, the C terminus of the H<sup>+</sup>-ATPase is involved not only in phosphorylation-dependent 14-3-3 binding but also in phosphorylation-independent but fusicoccin-dependent 14-3-3 binding. Thus, a GST fusion protein containing the C-terminal 56 amino acids of *N. plumbaginifolia* PMA2 binds 14-3-3 in the presence of fusicoccin, whereas a similar fusion protein lacking only the last three amino acids (YTV; Figure 4) does not bind 14-3-3 (Figure 9). We used GST fusion proteins expressed in *E. coli* to avoid phosphorylation of Thr-955; the lack of phosphorylation was confirmed by the lack of binding in the absence of fusicoccin. The data in Figure 9 clearly indicate that the C-terminal three amino acids are necessary for formation of a fusicoccin-stabilized

H<sup>+</sup>-ATPase-14-3-3 complex in the absence of phosphorylation.

Comparing the C termini of all H<sup>+</sup>-ATPase isoforms in the database, the minimal motif for phosphorylation-dependent 14-3-3 binding seems to be YpTV, including an enrichment of Q, H, and N at positions -2 to -4 (Figure 4). This motif is found in all of the H<sup>+</sup>-ATPase isoforms except AHA 10. The C-terminal H<sup>+</sup>-ATPase motif does not match any of the sequence motifs involved in the binding of 14-3-3 that have been described to date (Palmgren et al., 1998) and thus represents a novel motif. Striking differences are the phosphorylation of a threonine rather than a serine residue and the absence of a proline at position +2. According to the structural data, this proline produces a change in chain direction, which allows the remainder of the binding polypeptide to exit the binding cleft of the 14-3-3 protein (Yaffe et al., 1997). This structural problem is avoided with the H<sup>+</sup>-ATPase, in which phosphorylated Thr-948 is the next to last amino acid in the C terminus of most H<sup>+</sup>-ATPase isoforms (Figure 4).

Considering the importance of the motif QQXYpTV for 14-3-3 binding and yeast growth, as demonstrated above, this motif is expected to be of vital importance for regulating H<sup>+</sup>-ATPase activity in the plant and hence for plant growth.

## METHODS

### Plant Material

Spinach (*Spinacia oleracea*) was grown in a greenhouse with supplementary light (210  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ , 350 to 800 nm, G/86/2 HPLR 400 W; Philips, Eindhoven, The Netherlands). Expanding leaves of 4- to 5-week-old plants were used.

### In Vivo Phosphorylation

Spinach leaves (5 g) were cut into small pieces and infiltrated under vacuum with 30 mL of 0.33 M sucrose, 10 mM Mes-1,3-bis(tris[hydroxymethyl]methylamino)propane (BTP), pH 6.0, 0.1 mM EGTA, 0.1 mM EDTA, and 1 mCi of <sup>32</sup>P-labeled orthophosphate. After a 1-hr incubation at room temperature, 5  $\mu\text{M}$  fusicoccin was added, and incubation was continued for an additional 30 min. Alternatively, fusicoccin was present in the infiltration medium at the beginning of the experiment, and the <sup>32</sup>P-labeled orthophosphate was added 15 min later. The total incubation time in this case was also 90 min. Controls did not receive fusicoccin. After removal from the incubation buffer, plasma membranes were isolated as described below.

### Isolation of Plant Plasma Membranes

Plasma membranes were purified from a microsomal fraction by two-phase partitioning (Larsson et al., 1994). The final plasma membrane pellet was resuspended in 10 mM Mops-BTP, pH 7.0, 20%

(v/v) glycerol, 5 mM EDTA, and 1 mM DTT and was either used immediately or stored at  $-80^{\circ}\text{C}$  until required.

### H<sup>+</sup>-ATPase Assay

ATPase activity was measured as described by Palmgren (1990). In this assay, ATP hydrolysis is coupled enzymatically to oxidation of NADH by use of pyruvate kinase and lactate dehydrogenase. The rate of ATP hydrolysis is measured as the absorbance decrease of NADH at 340 nm. The assay medium contained 10 mM Mops-BTP, pH 7.0, 2 mM ATP, 4 mM  $\text{MgCl}_2$ , 140 mM KCl, 1 mM DTT, 0.25 mM NADH, 1 mM phosphoenol pyruvate, 50  $\mu\text{g}$  of lactate dehydrogenase (in glycerol; Boehringer Mannheim, Mannheim, Germany), 50  $\mu\text{g}$  of pyruvate kinase (in glycerol; Boehringer Mannheim), 0.05% (w/v) Brij 58 (Sigma), and 10 to 50  $\mu\text{g}$  of plasma membrane protein in a total volume of 1 mL. To determine the effect of synthetic peptides, we preincubated the plasma membranes with the purified Arabidopsis recombinant 14-3-3 protein isoform GF14 $\omega$  and the different peptides for 15 min at room temperature, followed by an additional 10-min incubation in the presence of 7  $\mu\text{M}$  fusicochin before measuring H<sup>+</sup>-ATPase activity.

### SDS-PAGE and Immunoblotting

Samples were solubilized at room temperature in SDS sample buffer, and polypeptides were separated by SDS-PAGE according to Laemmli (1970). Either gels were stained with Coomassie Brilliant Blue R 250 or polypeptides were electrophoretically transferred to an Immobilon polyvinylidene difluoride (PVDF) transfer membrane (Millipore, Bedford, MA) for immunostaining. Affinity-purified phosphothreonine-specific antibodies (Zymed, San Francisco, CA) were used as primary antibody. The secondary antibody was conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (Amersham, Little Chalfont, UK). <sup>32</sup>P-labeled proteins were visualized by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

### 14-3-3 Protein Overlays

Plasma membranes isolated from spinach leaves were subjected to SDS-PAGE, blotted to an Immobilon PVDF transfer membrane, and blocked in 2% (w/v) BSA. A digoxigenin (DIG)-3-*O*-methylcarbonyl- $\epsilon$ -aminocaproic acid-*N*-hydroxysuccinimide protein labeling kit (Boehringer Mannheim) was used to label recombinant 14-3-3 GF14 $\omega$ , and the PVDF membranes were incubated with 1.0 or 10  $\mu\text{M}$  (monomer concentration) DIG-labeled 14-3-3 GF14 $\omega$  in a buffer containing 20 mM Hepes-KOH, pH 7.7, 5 mM  $\text{MgCl}_2$ , 75 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2% (w/v) milk powder, and 0.04% (w/v) Tween 20 (Fullone et al., 1998) in the presence of 5  $\mu\text{M}$  fusicochin when specified. After incubation at room temperature for 2 hr, the membranes were washed three times in 200 mM  $\text{K}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$ , pH 7.5, 150 mM NaCl, and 0.1% (w/v) Tween 20 (PBS-T) and treated with an alkaline phosphatase-conjugated antibody directed against DIG-labeled proteins for 30 min. The membranes were washed three times in PBS-T, and the proteins interacting with 14-3-3 were visualized with nitro blue tetrazolium and 5-chromo-4-chloro-3-indolyl phosphate.

With yeast plasma membranes and glutathione *S*-transferase (GST) fusion proteins, the following protocol was used. Yeast plasma

membranes or lysates of *Escherichia coli* expressing GST-fused C-terminal PMA2 peptides were separated by SDS-PAGE and transferred to nitrocellulose. Nonspecific sites were blocked by incubation for 1 hr at room temperature with 2% (w/v) milk powder in 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, and 1 mM  $\text{MgCl}_2$  (TBS). Subsequently, the membrane was incubated overnight at  $4^{\circ}\text{C}$  with the purified 6-His-tagged 14-3-3 isoform T14-3c of *Nicotiana tabacum* diluted to 20  $\mu\text{g mL}^{-1}$  (0.7  $\mu\text{M}$  14-3-3 monomer) in 20 mM Tris-HCl, pH 7.5, 20% (w/v) glycerol, 5 mM  $\text{MgSO}_4$ , and 2 mM DTT. After washing with TBS, immunodetection of His-tagged 14-3-3 was performed by applying the anti-His antibody (Qiagen, Hilden, Germany; monoclonal antibody raised against the 6-His epitope of the pQE vectors) in combination with goat anti-mouse immunoglobulin-conjugated alkaline phosphatase and the enzyme substrates nitro blue tetrazolium and 5-chromo-4-chloro-3-indolyl phosphate.

### In Vitro Phosphorylation

Plasma membranes (13 mg mL<sup>-1</sup>) isolated from spinach leaves were incubated with 0.4% (w/v) Brij 58, 20 mM ATP, and 40 mM  $\text{MgCl}_2$ . During the time course of the incubation, samples were withdrawn every 90 sec and solubilized in SDS sample buffer (Laemmli, 1970). For the 14-3-3 protein overlay in Figure 7, plasma membranes were incubated as described above for 30 min.

### Growth Media

*Saccharomyces cerevisiae* cells were grown at  $30^{\circ}\text{C}$  in minimal medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco, Detroit, MI), 2% (w/v) glucose or galactose, and 20 mM  $\text{KH}_2\text{PO}_4$  supplemented with all amino acids and nucleotides required for growth. The pH was adjusted with KOH to pH 6.5.

*E. coli* was grown at  $37^{\circ}\text{C}$  in  $2 \times$  YT medium (1% [w/v] yeast extract, 1.6% [w/v] tryptone, and 0.5% [w/v] NaCl) containing 100  $\mu\text{g mL}^{-1}$  ampicillin.

### Strains and Plasmids

The YAK2 strain of *S. cerevisiae* and the construction of the plasmid  $2\mu\text{p}(PMA1)pma2$  are described in detail in de Kerchove d'Exaerde et al. (1995). In brief, the H<sup>+</sup>-ATPase genes *PMA1* and *PMA2* were deleted from the parental strain, and the resulting strain was complemented with yeast *PMA1* under the control of the galactose-dependent *GAL1* promoter in the centromeric plasmid pRS-316 (Sikorski and Hieter, 1989). The multicopy plasmid  $2\mu\text{p}(PMA1)pma2$  harbors the *N. plumbaginifolia* H<sup>+</sup>-ATPase PMA2 cDNA under the control of the yeast *PMA1* promoter.

A DNA fragment encoding 98 amino acids at the C terminus of the *N. plumbaginifolia* H<sup>+</sup>-ATPase PMA2 was amplified by polymerase chain reaction (PCR) and cloned into pBluescript SK+ (Stratagene, La Jolla, CA). Site-directed mutagenesis then was performed by using the GeneEditor Kit (Promega, Mannheim, Germany). Subsequently, the mutated DNA fragment was exchanged for the wild-type DNA fragment of  $2\mu\text{p}(PMA1)pma2$ , yielding  $2\mu\text{p}(PMA1)pma2$  T955A and  $2\mu\text{p}(PMA1)pma2$  Q951G/Q952H.

YAK2 was transformed with  $2\mu\text{p}(PMA1)pma2$ ,  $2\mu\text{p}(PMA1)pma2$  T955A, or  $2\mu\text{p}(PMA1)pma2$  Q951G/Q952H. The resulting yeast strains (YAK2+PMA2, YAK2+PMA2 T955A, and YAK2+PMA2 Q951G/Q952H) were used to test the ability of wild-type and mutant

PMA2 to functionally replace the yeast H<sup>+</sup>-ATPase on minimal medium containing glucose as the sole carbon source.

### Expression and Purification of Proteins

The recombinant 14-3-3 protein GF14 $\omega$  from Arabidopsis was expressed and purified according to the manufacturer's directions (PET System Manual; Novagen, Madison, WI). *E. coli* BL21(DE3) was transformed by heat shock with use of the pET15b vector (Novagen) containing a GF14 $\omega$  cDNA insert (Lu et al., 1994), kindly provided by R.J. Ferl (Biotechnology and Horticultural Sciences, University of Florida, Gainesville). Purification was conducted according to the manufacturer's protocol by using a Ni<sup>2+</sup> HiTrap chelating column (Pharmacia Biotech, Uppsala, Sweden) under nondenaturing conditions.

For expression of the *N. tabacum* 14-3-3 isoform T14-3c as a 6-His-tagged protein in *E. coli*, the corresponding cDNA (Piotrowski and Oecking, 1998) was amplified by PCR and cloned into the His-tagged expression vector pQE-30 (Qiagen, Chatsworth, CA). The construct was expressed in *E. coli* M15, and 10 mg of native protein was purified from 100 mL of culture by using Ni-NTA agarose (Qiagen), according to the manufacturer's protocol.

For expression of the *N. plumbaginifolia* H<sup>+</sup>-ATPase PMA2 C-terminal region fused to GST in *E. coli*, DNA fragments encoding PMA2 peptides 901-956 and 901-953 were amplified by PCR. The resulting fragments were cloned into the GST expression vector pGEX-4T-1 (Pharmacia Biotech).

PCR products and products of mutagenesis were verified by sequencing with a Sequenase 2.0 kit (Amersham).

### Preparation of Yeast Plasma Membranes

Yeast cells first were grown in 600 mL of minimal medium containing 2% (w/v) galactose until their OD<sub>600</sub> reached ~0.6. After sedimentation and washing, the cells were used to inoculate 2 × 1 L of minimal medium, one containing 2% (w/v) galactose and the other containing 2% (w/v) glucose (to avoid expression of yeast PMA1). When the OD<sub>600</sub> reached ~1.0, the yeast cells were collected by centrifugation and washed twice with cold water.

For membrane preparation, yeast cells were homogenized on a vortex-type mixer with glass beads (0.5 mm in diameter) in 20% (w/v) glycerol, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 2 mM phenylmethylsulfonyl fluoride (GTED 20). After filtration through a glass filter, the homogenate was centrifuged at 3000g for 10 min. The supernatant was diluted with one volume of GTED 20 and ultracentrifuged for 45 min at 100,000g to collect the membrane fraction, which was resuspended in ~2 mL of GTED 20. For preparation of plasma membranes, the pH was adjusted to pH 5.2 by the dropwise addition of 2 M acetic acid. The supernatant obtained after a short centrifugation (45 sec at 10,000g) contained the plasma membranes and was neutralized immediately with KOH followed by dilution with 1 volume of GTED 20. Plasma membranes were collected by centrifugation; resuspended in 20% (w/v) glycerol, 10 mM Tris-HCl, pH 7.5, 1 mM DTT, and 1 mM MgSO<sub>4</sub>; frozen in liquid nitrogen; and stored at -80°C.

### Protein Determination

Protein was measured essentially as described by Bearden (1978), with BSA as the standard.

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