Blue light activates the plasma membrane H^+ -ATPase by phosphorylation of the C-terminus in stomatal guard cells

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The opening of stomata, which is driven by the accumulation of K^+ salt in guard cells, is induced by blue light (BL). The BL activates the H^+ pump; however, the mechanism by which the perception of BL is transduced into the pump activation remains unknown. We present evidence that the pump is the plasma membrane H^+ -ATPase and that BL activates the H^+ -ATPase via phosphorylation. A pulse of BL (30 s, 100 $\mu\text{mol}/\text{m}^2/\text{s}$) increased ATP hydrolysis by the plasma membrane H^+ -ATPase and H^+ pumping in *Vicia* guard cell protoplasts with a similar time course. The H^+ -ATPase was phosphorylated reversibly by BL, and the phosphorylation levels paralleled the ATP hydrolytic activity. The phosphorylation occurred exclusively in the C-termini of H^+ -ATPases on both serine and threonine residues in two isoproteins of H^+ -ATPase in guard cells. An endogenous 14-3-3 protein was co-precipitated with H^+ -ATPase, and the recombinant 14-3-3 protein bound to the phosphorylated C-termini of H^+ -ATPases. These findings demonstrate that BL activates the plasma membrane H^+ -ATPase via phosphorylation of the C-terminus by a serine/threonine protein kinase, and that the 14-3-3 protein has a key role in the activation.

Keywords: blue light/gas exchange/ H^+ pump/protein kinase/stomata

Introduction

Blue light (BL)-specific responses are ubiquitous in plant cells, and include phototropism, inhibition of stem elongation and stomatal opening (Kaufman, 1993; Short and Briggs, 1994; Ahmad and Cashmore, 1996). Stomatal pores surrounded by a pair of guard cells in the epidermis regulate gas exchange between leaves and the atmosphere, and thus allow CO_2 entry for photosynthesis and transpirational stream in higher plants (Zeiger, 1983; Assmann, 1993; Pockman *et al.*, 1995).

The opening of stomata is mediated by an accumulation of K^+ salt in guard cells, and K^+ accumulation through the voltage-gated K^+ channel is driven by an inside-negative, electrical potential across the plasma membrane (Hedrich and Schroeder, 1989; Assmann, 1993). This electrical potential is created by a BL-activated H^+ pump which has been suggested to be H^+ -ATPase in the plasma

membrane (Assmann *et al.*, 1985; Shimazaki *et al.*, 1986; Schroeder, 1988; Schwartz *et al.*, 1991; Amodeo *et al.*, 1992). However, the mechanism by which the perception of BL is transduced into H^+ pump activation is largely unknown. Previous studies have revealed that inhibitors of serine/threonine protein kinases inhibit BL-dependent H^+ pumping in *Vicia* guard cell protoplasts (GCPs) and light-induced stomatal opening in *Commelina* epidermis (Shimazaki *et al.*, 1992, 1993; Assmann and Shimazaki, 1999), suggesting that protein phosphorylation is involved in the BL signalling pathway of stomatal guard cells; however, there is no direct evidence for this.

The plasma membrane H^+ -ATPase has been shown to be encoded in multigene families in many plants (Sussman, 1994), and tissue-specific distribution of one type of isoform has been reported (Dewitt and Sussman, 1995; Michelet and Boutry, 1995). Two mRNAs encoding the H^+ -ATPase isoproteins (VHA1 and VHA2, *Vicia* H^+ -ATPase isoforms 1 and 2) are expressed in *Vicia* guard cells, and cDNA clones for these have been isolated (Nakajima *et al.*, 1995; Hentzen *et al.*, 1996). Since the plasma membrane H^+ -ATPase generates a H^+ electrochemical gradient, and provides a driving force for the uptake of various nutrients such as K^+ , nitrate, sulfate, sucrose and amino acids across the plasma membrane in many cell types and tissues of plant, the regulatory mechanism of this enzyme has been studied extensively (Serrano, 1989; Palmgren, 1991; Sussman, 1994; Michelet and Boutry, 1995; Sze *et al.*, 1999). The H^+ -ATPase activity is thought to be regulated by an autoinhibitory domain in the C-terminal region of the enzyme. Removal of a 7–10 kDa fragment from the C-terminal end of H^+ -ATPase generates a high-activity state of H^+ -ATPase with a higher V_{max} , a lower K_m for ATP, a changed pH dependence with higher activity at physiological pH and an increased coupling of H^+ transport with ATP hydrolysis (Palmgren *et al.*, 1990; Morsomme *et al.*, 1996; Sze *et al.*, 1999). Very similar results were obtained by *in vivo* treatment of intact tissue with the fungal toxin fusaric acid (FC), an activator of the H^+ -ATPase, and it is suggested that FC induces a displacement of the C-terminal autoinhibitory domain of H^+ -ATPase from its catalytic site (Palmgren, 1991; Johansson *et al.*, 1993; Rasi-Caldogno *et al.*, 1993). Such displacement of the C-terminus may be achieved by phosphorylation and dephosphorylation with physiological stimuli, and the C-terminus can be a substrate for protein kinase and protein phosphatase *in vivo* and *in vitro* (Schaller and Sussman, 1988; Serrano, 1989; Palmgren, 1991; Suzuki *et al.*, 1992; Sekler *et al.*, 1994; Vera-Estrella *et al.*, 1994; Xing *et al.*, 1996; Camoni *et al.*, 1998; Sze *et al.*, 1999).

In plants, the 14-3-3 protein was identified initially as an FC-binding protein (Korthout and de Boer, 1994; Marra *et al.*, 1994; Oecking *et al.*, 1994). Recent studies have

demonstrated that the 14-3-3 protein interacts directly with the C-terminal autoinhibitory domain of H⁺-ATPase as a positive modulator and that the complex of H⁺-ATPase and 14-3-3 protein is stabilized by FC (Jahn *et al.*, 1997; Oecking *et al.*, 1997; Fullone *et al.*, 1998). The 14-3-3 protein may act as a natural ligand of H⁺-ATPase, and interaction between the C-terminus of H⁺-ATPase and 14-3-3 protein can be a physiological regulatory mechanism of H⁺-ATPase activity (Baunsgaard *et al.*, 1998). The 14-3-3 proteins are ubiquitous in eukaryotic cells and have a highly conserved primary sequence; they have been demonstrated to bind selectively to the phosphorylated motifs in their target proteins (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). However, the plasma membrane H⁺-ATPase in plants lacks such consensus motifs, and there is no evidence that the 14-3-3 protein binds to the phosphorylated motif of H⁺-ATPase under physiological conditions.

In this study, we demonstrate that the BL-activated H⁺ pump is the plasma membrane H⁺-ATPase, and that the H⁺-ATPase is activated by BL via exclusive phosphorylation of the C-terminus in guard cells. We also indicate that the 14-3-3 protein binds to the C-terminus *in vivo* only when the C-terminus is phosphorylated by BL.

Results

Activation of the plasma membrane H⁺-ATPase by BL

Figure 1A shows a typical BL-dependent H⁺ pumping in GCPs from *Vicia faba*. Stimulation of GCPs with a short pulse of BL (30 s, 100 $\mu\text{mol}/\text{m}^2/\text{s}$) superimposed on the background red light (RL, 600 $\mu\text{mol}/\text{m}^2/\text{s}$) induced H⁺ pumping. It began 30 s after the start of stimulation and was sustained for 15 min with the maximum rate at ~ 2.5 min. The GCPs, once stimulated by a single pulse, responded to the second pulse, but the magnitude decreased to 60% when the interval between the first and the second pulse was 20 min, consistent with the response in an intact leaf (Iino *et al.*, 1985). To show that this BL-activated H⁺ pump is the plasma membrane H⁺-ATPase, ATP hydrolysis in response to BL was determined after immediate disruption of GCPs (Figure 1B). The hydrolytic activity in guard cell extract was increased 30 s after the pulse, had reached maximum at 2.5 min, and decreased gradually to the basal level within 20 min. When the second pulse was applied to GCPs 20 min after the first one, the ATP hydrolysis was increased again. The ATP hydrolytic activity was inhibited by 100 μM vanadate, an inhibitor of the plasma membrane H⁺-ATPase (Figure 1B). The similarity of the kinetic properties between H⁺ pumping and ATP hydrolysis suggests that BL-dependent H⁺ pumping is mediated by the plasma membrane H⁺-ATPase. The maximum rates of BL-stimulated ATP hydrolysis and BL-dependent H⁺ pumping were 1.5 nmol Pi/h/ μg protein and 1.7 nmol H⁺/h/ μg protein, respectively. ATP hydrolysis is sufficient to account for the observed H⁺ pumping, since the enzyme is assumed to have a stoichiometry of 2H⁺/ATP (Spanswick, 1981) or H⁺/ATP (Briskin and Reynolds-Niesman, 1991). It is also possible that BL concomitantly improves the coupling between H⁺ pumping and ATP hydrolysis (Baunsgaard *et al.*, 1996; Morsomme *et al.*, 1996).

Fig. 1. BL-dependent H⁺ pumping in GCPs and BL-stimulated ATP hydrolysis in guard cell extract from *V. faba*. (A) BL-dependent H⁺ pumping in GCPs was measured by the decrease of pH in the medium. A second pulse of BL (2nd BL) was applied 20 min after the first one. (B) ATP hydrolytic activity was measured by determining the Pi released from 2 mM ATP for 30 min. GCPs were pre-incubated under RL for 40 min at 24°C, then illuminated with a pulse of BL. GCPs were disrupted by adding medium containing 100 mM MOPS-KOH pH 7.5, 5 mM EDTA, 200 mM NaCl, 1 mM PMSF, 20 μM leupeptin, 1 mM DTT and 0.05% Triton X-100 to an equal volume of GCP suspension at the indicated times. A second pulse of BL was applied as shown in (A), and GCPs were disrupted 2.5 min after the pulse (2nd BL). Vanadate was added at 100 μM . (C) ATP hydrolytic activity as a function of ATP concentration was determined under RL (open symbols) and 2.5 min after the pulse of BL superimposed on the RL (closed symbols). (D) Lineweaver-Burk plot from the data shown in (C). A pulse of BL was applied to the GCP suspension for 30 s at 100 $\mu\text{mol}/\text{m}^2/\text{s}$ superimposed on the background RL at 600 $\mu\text{mol}/\text{m}^2/\text{s}$. All measurements were done at 24°C.

The apparent K_m for ATP and the V_{max} extrapolated from double-reciprocal plots were 0.29 mM and 1.96 nmol Pi/h/ μg protein under the background RL, and were changed to 0.17 mM and 3.23 nmol Pi/h/ μg protein by the pulse of BL (Figure 1C and D). These changes are analogous to the mode of H⁺-ATPase activation by proteolytic removal or FC-induced displacement of the C-terminus of H⁺-ATPase (Palmgren *et al.*, 1990; Johansson *et al.*, 1993; Rasi-Caldogno *et al.*, 1993).

Reversible phosphorylation of the plasma membrane H⁺-ATPase by BL

To determine *in vivo* phosphorylation levels of plasma membrane H⁺-ATPase, GCPs were incubated with [³²P]-orthophosphate, and H⁺-ATPase was immunoprecipitated using specific antibodies. Autoradiograms reveal that the phosphorylation level of H⁺-ATPase having a molecular

Fig. 2. Changes in the phosphorylation levels of the plasma membrane H⁺-ATPase in GCPs in response to BL. (A) Autoradiogram of immunoprecipitated H⁺-ATPase. The reaction was terminated by adding an equal volume of medium containing 100 mM MOPS-KOH pH 7.5, 5 mM EDTA, 200 mM NaCl, 1 mM PMSF, 20 μ M leupeptin, 4 mM DTT, 20 mM NaF, 1 mM ammonium molybdate, 200 nM calyculin A and 2% Triton X-100 to the GCP suspension at the indicated times. The second pulse was applied to GCPs 20 min after the first one, and the reaction was terminated 2.5 min after the pulse. The resulting supernatant was immunoprecipitated immediately by adding the antiserum against the hydrophilic loop of H⁺-ATPase at 0.5% (v/v). In each lane, the immunoprecipitated H⁺-ATPase obtained from 100 μ g of guard cell proteins was subjected to SDS-PAGE in 12.5% gels. The protein on SDS-PAGE was dried, and Fuji RX-film was exposed to the dried gels for 4 days at room temperature (see Materials and methods). (B) Immunoprecipitated H⁺-ATPase was detected using the antibodies against the C-terminus of H⁺-ATPase conjugated to alkaline phosphatase (Sigma-Genosys Ltd., Cambridge, UK) at 500-fold dilution. In this experiment, the secondary antibodies were not used. The numbers on the left indicate the molecular masses. Experiments repeated three times on different occasions gave similar results.

mass of 95 kDa was increased by BL (Figure 2A), reached a maximum 2.5 min after the BL pulse, and decreased gradually to the basal level within 20 min. When the second pulse of BL was applied 20 min after the first, the H⁺-ATPase was rephosphorylated. These responses were specific to BL (data not shown). The results indicate that the plasma membrane H⁺-ATPase is phosphorylated by BL, and that the phosphorylation levels of H⁺-ATPase are closely correlated with the BL-stimulated ATP hydrolysis (Figures 1B and 2A). The antibodies recognized a band of 95 kDa protein as H⁺-ATPase in the immunoprecipitate, and there was no change in the amount due to the pulse of BL (Figure 2B), suggesting that the increase in the activity is due to post-translational activation of H⁺-ATPase.

Fluence dependency of BL-dependent ATP hydrolysis and phosphorylation of H⁺-ATPase

Stomatal response in intact leaves and H⁺ pumping in GCPs by BL are both fluence dependent (Iino *et al.*, 1985; Shimazaki *et al.*, 1986). In accord with the physiological responses, ATP hydrolysis by the plasma membrane H⁺-ATPase was also fluence dependent. ATP hydrolysis increased linearly with the increase in the fluence rate up

Fig. 3. H⁺ pumping, ATP hydrolysis and phosphorylation of H⁺-ATPase as a function of BL fluence rate. (A) Maximum rate of BL-dependent H⁺ pumping in GCPs. (B) ATP hydrolytic activity of guard cell extracts. The reaction was terminated by adding the medium (see Figure 1B) under RL and 2.5 min after the pulse of BL. (C) Autoradiograms of immunoprecipitated H⁺-ATPase. Reactions were terminated under RL or 2.5 min after the pulse of BL at various fluence rates by adding the solubilizing medium. Immunoprecipitated H⁺-ATPase was subjected to SDS-PAGE. Other conditions were the same as described for Figure 2A. The pulse duration of BL was 30 s. The fluence rate of BL was adjusted with neutral density filters. Each experiment was done using 100 μ g of guard cell proteins.

to 50 μ mol/m²/s of BL and was saturated at 100 μ mol/m²/s, with half-saturation at 7.5 μ mol/m²/s when the pulse duration was 30 s (Figure 3B). The rate of H⁺ pumping and the levels of H⁺-ATPase phosphorylation showed similar fluence dependency (Figure 3A and C). The phosphorylation of H⁺-ATPase was inhibited by K-252a, an inhibitor of serine/threonine protein kinase, in a concentration-dependent manner (Figure 4B), and this inhibitor similarly inhibited BL-dependent H⁺ pumping (Figure 4A). These results suggest that phosphorylation of plasma membrane H⁺-ATPase is required for its activation, and that the increased H⁺-ATPase activity is responsible for BL-dependent H⁺ pumping in GCPs.

Exclusive phosphorylation of the C-terminus of H⁺-ATPase

Figure 5 shows the amino acid sequences of the C-termini of two H⁺-ATPase isoforms deduced from isogenes of *VHA1* and *VHA2* expressed in guard cells (Nakajima *et al.*, 1995; Hentzen *et al.*, 1996). Treatment of these H⁺-ATPases with cyanogen bromide (CNBr), which should split the H⁺-ATPase polypeptide chains on the

Fig. 4. Inhibition of BL-dependent H⁺ pumping and phosphorylation of H⁺-ATPase by a protein kinase inhibitor. **(A)** BL-dependent H⁺ pumping in GCPs as a function of K-252a concentration. K-252a dissolved in dimethylsulfoxide (DMSO) was added to the GCP suspension 20 min before the pulse of BL at the indicated concentrations. The final concentration of DMSO was 0.5%. Other conditions were the same as described for Figure 1A. **(B)** Autoradiogram of immunoprecipitated H⁺-ATPase at various concentrations of K-252a. GCPs were solubilized under RL and 2.5 min after the start of BL pulse (BL), and H⁺-ATPase was separated by SDS-PAGE. Solid arrowhead indicates the position of H⁺-ATPase. The H⁺-ATPase in each lane was immunoprecipitated from 100 μg of guard cell proteins. Other conditions were the same as described for Figure 2A. Experiments repeated twice on different occasions gave similar results.

VHA1	YLQVSIVSQALIFVTRSRSGMLNAPGLLLGAFLIAQLI	800
VHA2	YLQVSIVSQALIFVTRSRGWSFLERPGALLVIAFLIAQLI	800
VHA1	ASLIAVYANWAFARIQIGIGWGAGVIWLYSIIFYPLDII	840
VHA2	ATLIAVYANWGFAGVQIGIGWGAGVIWLYSIVFYIPLDVM	840
VHA1	KFATRYFLSGKAWSN-LENKTAFTTKKDYGKGEREAQWAH	879
VHA2	KFAIRYILSGKAWNLLDNKTAFTTKKDYGKEEREAQWAH	880
VHA1	ALWTLHGLEPPSSGIFHEKNSYRELSEIAEQAKRRAEVA	919
VHA2	AQRTLHGLQPPSSGIFNEKSSYRELSEIAEQAKRRAEVA	920
VHA1	RLRELHTLKGHVESVVKLGKGLDIDTIQQHYTVYKGMT	956
VHA2	RLRELHTLKGHVESVVKLGKGLDIDTIQQHYTV	952

Fig. 5. Amino acid sequence alignment of the C-terminal region of VHA1 and VHA2. The amino acid sequences of VHA1 (S79323, position 761–956) and VHA2 (AB022442, position 761–952) are aligned. A dash indicates a gap introduced to allow for optimal alignment of the sequences. Asterisks show identical amino acids. Solid arrows indicate the methionine residues. The amino acid sequence underlined was used as an antigen of the C-terminus (see Materials and methods).

carboxyl side of Met782 and Met840, will generate 19.7 and 12.9 kDa of the C-terminal regions from VHA1 (positions 783–956) and VHA2 (position 841–952), respectively, and the other regions of the H⁺-ATPase will be digested below 12 kDa. We fragmented the immunoprecipitated H⁺-ATPase with CNBr and separated the peptides by SDS-PAGE. The resulting 21 and 15 kDa

peptides, both of which had slightly higher molecular masses than expected, were found to be phosphorylated by BL, with each peptide consisting of two closely spaced bands (Figure 6A). As shown in Figure 6B, polyclonal antibodies raised against the C-terminal region of VHA1 (positions 851–956, Figure 5) recognized both the 21 and 15 kDa peptides, each band having two closely spaced peptides. Radioactivities in these peptides determined using a liquid scintillation counter comprised >98% of those in unfragmented H⁺-ATPase (data not shown). These results suggest that BL induces exclusive phosphorylation of the C-terminus in the plasma membrane H⁺-ATPase in guard cells. BL-induced phosphorylation of both peptides was inhibited by K-252a at 10 μM (Figure 6A).

Phosphorylation of serine and threonine residues in H⁺-ATPase isoproteins

Interestingly, the antibodies against the C-terminus also recognized both the 19 and 13 kDa peptides (Figure 6B). These 19 and 13 kDa peptides are very likely to be the C-termini of VHA1 and VHA2, respectively, based on their deduced molecular masses (Figure 5), and the 21 and 15 kDa peptides seem to be their respective phosphorylated forms, since the apparent molecular mass is known to be increased by phosphorylation on SDS-PAGE (Short *et al.*, 1993; Morrison *et al.*, 1998). In support of this, both the 21 and 15 kDa peptides were eliminated by the inhibition of phosphorylation through the protein kinase inhibitor K-252a (Figure 6B).

To investigate whether the 13 kDa peptide is the C-terminus of VHA2, the recombinant C-terminus of VHA2 (position 836–952) was expressed in *Escherichia coli* as a GST-VHA2 fusion protein with a molecular mass of 38 kDa, and compared with the 13 kDa protein in the immunoprecipitate (Figure 7A). The fusion protein was digested with CNBr at the Met840 residue (see Figure 5), followed by Western blotting, revealing that the recombinant 13 and undigested 23 kDa peptides were recognized by the antibodies against the C-terminus (Figure 7B, lane 2). In the immunoprecipitate, both the 13 and 19 kDa peptides were found when the H⁺-ATPase was digested with CNBr (Figure 7B, lane 1), and the 13 kDa peptide showed the same mobility as that of the recombinant 13 kDa peptide on SDS-PAGE. This result suggests that the 13 kDa peptide in the immunoprecipitate is the C-terminus of VHA2, and thus the 19 kDa peptide is very likely the C-terminus of VHA1 in the guard cells of *V. faba*.

The major proportion of the C-terminal fragments existed in the forms of 19 and 13 kDa peptides, and 21 and 15 kDa peptides were produced upon stimulation with BL (Figure 6B), suggesting that the majority of the plasma membrane H⁺-ATPase molecules are in a non-phosphorylated state in guard cells under resting conditions.

To determine the phosphoamino acids, the phosphorylated 21 and 15 kDa peptides of the C-termini were analysed using cellulose thin-layer electrophoresis. The 21 and 15 kDa peptides were found to be phosphorylated on both serine and threonine residues (Figure 6D). No phosphotyrosine was found. This result suggests that a serine/threonine protein kinase phosphorylates H⁺-ATPase in response to BL. Very high radiolabelling was observed

Fig. 6. Exclusive phosphorylation of the C-terminus of the plasma membrane H^+ -ATPase in guard cells by BL and determination of phosphoamino acids. (A) Autoradiograms of immunoprecipitated H^+ -ATPase and its CNBr-treated products (+CNBr) under RL, BL and with K-252a. The H^+ -ATPase in each lane was immunoprecipitated by the antibodies from 200 μ g of proteins of GCPs as shown in Figure 2, followed by SDS-PAGE. Solubilization of GCPs and the addition of K-252a to GCPs were done in the same way as shown in Figures 2 and 4. Arrows indicate the phosphorylated C-terminal fragments. (B) Western blotting of the CNBr-treated H^+ -ATPase with polyclonal antibodies against the C-terminus. Arrows indicate the peptides recognized by the antibodies. (C) Western blotting of the CNBr-treated H^+ -ATPase using pre-serum as a control. (D) Analysis of the phosphoamino acids in 21 and 15 kDa peptides by cellulose thin-layer electrophoresis (see Materials and methods). The positions of each of the phosphoamino acids indicated by the arrows were determined from standards.

Fig. 7. Mobilities of the C-terminus of VHA2 from guard cells and of recombinant VHA2 on SDS-PAGE. (A) Expression of GST-C-terminal fusion protein of VHA2 in *E. coli*. The fusion protein with a molecular mass of 38 kDa was separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The 28 kDa protein found seems to be the degradation product of the fusion protein. (B) Mobilities of immunoprecipitated H^+ -ATPase in GCPs (lane 1) and the recombinant C-terminus of VHA2 (lane 2) which had been digested by CNBr. The digested peptides were separated by SDS-PAGE. The C-termini were detected by Western blotting using the antibodies against the C-terminus.

in the threonine residue of the 15 kDa peptide, and this may reflect the multiple threonine residues in the C-terminus which are phosphorylated by BL.

Co-precipitation of a 14-3-3 protein with H^+ -ATPase

Silver staining of the immunoprecipitate revealed that a 32 kDa protein was co-precipitated with the plasma membrane H^+ -ATPase (Figure 8A). A pulse of BL increased the amount of this protein, and the changes in the amount paralleled both the levels of phosphorylation

Fig. 8. Co-precipitation of a 32 kDa protein with H^+ -ATPase. (A) Silver staining of the immunoprecipitated product which was separated by SDS-PAGE. GCPs were incubated under background RL for 40 min, then illuminated with a pulse of BL. The reaction was terminated before (RL) and after the BL pulse at the indicated times by adding the solubilizing medium. A second pulse of BL was applied to GCPs 20 min after the first one, and the reaction was terminated 2.5 min after the pulse (2nd BL). K-252a was added at 10 μ M. The H^+ -ATPase from 100 μ g of GCP protein was immunoprecipitated in each lane. (B) Western blotting of a 14-3-3 protein using polyclonal antibodies against recombinant 14-3-3 protein from *Arabidopsis*. Experimental conditions were the same as those in (A). (C) Western blotting of a 14-3-3 protein in GCPs under RL, BL and in the presence of K-252a. Solubilized guard cell proteins (12.5 μ g) were separated by SDS-PAGE. Other conditions were the same as in (A). Western blotting using pre-serum as control gave no specific staining (data not shown). Experiments repeated three times on different occasions gave similar results. Arrows indicate the positions of the 32 kDa protein.

Fig. 9. Binding of the 14-3-3 protein to the plasma membrane H⁺-ATPase. (A) Purified recombinant GST (29 kDa, lane 1) and GST-14-3-3 fusion protein (58 kDa, lane 2) from *E. coli* on SDS-PAGE stained with Coomassie Brilliant Blue. (B) Far Western blotting was done using recombinant GST-14-3-3 protein as a probe, and the GST fusion protein and GST were immunodetected by anti-GST antibodies. GCPs were incubated under background RL for 40 min, then illuminated with a pulse of BL. GCPs were solubilized with solubilizing medium for SDS-PAGE before (RL) and after the pulse of BL (BL) at the indicated times. A second pulse of BL was applied 20 min after the first one, and GCPs were solubilized 2.5 min after the start of the pulse (2nd BL). Guard cell proteins (12.5 µg) separated by SDS-PAGE were transferred to a nitrocellulose membrane. (C) Inhibition of the 14-3-3 protein binding to H⁺-ATPase by 10 µM K-252a. Other conditions were the same as in (B). (D) Far Western blotting using recombinant GST instead of GST-14-3-3 fusion protein for the control. (E) Western blotting of H⁺-ATPase by the antibodies in GCPs. (F) Far Western blotting using recombinant GST-14-3-3 protein as a probe. Immunoprecipitated H⁺-ATPase from 200 µg of proteins of GCPs was digested by CNBr, and the digested peptides were separated by SDS-PAGE. Other conditions are the same as in Figure 6A. (G) Far Western blotting using recombinant GST as a probe for the control. Other conditions are the same as in (F).

and the hydrolytic activity of H⁺-ATPase. The increase in the amount of co-precipitated 32 kDa protein was suppressed through the inhibition of protein phosphorylation in the presence of K-252a (Figure 8A). Recent reports using FC indicate that a 14-3-3 protein associates directly with the C-terminus of H⁺-ATPase and activates the H⁺-ATPase, and that the molecular mass of this protein is ~30 kDa (Jahn *et al.*, 1997; Oecking *et al.*, 1997; Baunsgaard *et al.*, 1998; Fullone *et al.*, 1998; Sze *et al.*, 1999). Thus it is possible that the 32 kDa protein is the 14-3-3 protein. To examine this possibility, polyclonal antibodies were raised against *Arabidopsis* GF14 ϕ as a typical plant 14-3-3 protein (Lu *et al.*, 1994), and the 32 kDa protein was recognized by the antibodies (Figure 8B). These results suggest that the 32 kDa protein is a 14-3-3 protein, and that the phosphorylation of H⁺-ATPase may be responsible for the tight association of this protein. Changes in the amount of 32 kDa protein were not due to *de novo* synthesis or degradation because the total amount of the 32 kDa protein in GCPs was not changed by the pulse of BL or by K-252a (Figure 8C).

To determine the concentration of 14-3-3 protein and H⁺-ATPase in the immunoprecipitate, both proteins were analysed by Western blot. The result suggested that the concentration of 14-3-3 protein was comparable with that of H⁺-ATPase although the specificity of the antibody to endogenous 14-3-3 protein in guard cells was not considered. Since the phosphorylated H⁺-ATPase was ~50% of the total H⁺-ATPase in the immunoprecipitate (Figure 6), the ratio of 14-3-3 protein to phosphorylated H⁺-ATPase was estimated to be >1.

14-3-3 protein binds to the phosphorylated C-terminus of the H⁺-ATPase

To investigate whether the 14-3-3 protein binds to the H⁺-ATPase *in vitro*, we generated the recombinant GST-

14-3-3 fusion protein for far Western blotting (Figure 9A). The 14-3-3 fusion protein was found to bind to immunoprecipitated H⁺-ATPase, and the amount of bound 14-3-3 protein was increased by the BL pulse and decreased afterwards (Figure 9B). The amount of 14-3-3 protein was increased in response to the second pulse of BL (Figure 9B, 2nd BL). These increases were eliminated by inhibition of protein phosphorylation with K-252a (Figure 9C), indicating that the phosphorylation was essential to the tight binding of the 14-3-3 protein.

It is very likely that the 14-3-3 protein is bound to the C-terminus, because the terminus is exclusively phosphorylated. As shown in Figure 9F, the 14-3-3 protein was bound to 21 and 15 kDa peptides, but not to the 19 and 13 kDa peptides (see Figure 6B). The amount of bound 14-3-3 protein was increased by BL, and the protein bound to the 15 kDa peptide, which had not been observed under RL or with K-252a, became evident after the BL pulse. The increase in binding of the 14-3-3 protein by BL was inhibited by K-252a (Figure 9F). These results, together with evidence that the 32 kDa protein is the 14-3-3 protein, indicate that an endogenous 14-3-3 protein in guard cells binds to the C-terminus of the plasma membrane H⁺-ATPase only when it is phosphorylated and that the amount of bound 14-3-3 protein is proportional to H⁺-ATPase activity. This provides further evidence that the C-terminus is involved in the regulation of the activity of plasma membrane H⁺-ATPase under physiological conditions. The binding was specific to 14-3-3 protein because recombinant GST did not bind H⁺-ATPase (Figure 9D). Western blot analysis revealed that the amount of H⁺-ATPase in GCPs was not changed by BL (Figure 9E).

A small amount of 14-3-3 protein was bound to the H⁺-ATPase (Figure 9B and C) and to the C-terminus (Figure 9F) both under RL and in the presence of K-252a.

These results suggest that a small proportion of H^+ -ATPase exists in a phosphorylated state. Consistent with this result, a low level of phosphorylation of the H^+ -ATPase was found without BL and with K-252a (Figures 1A and 6A).

Discussion

BL activates the H^+ pump in guard cells, inducing stomatal opening, and this BL-activated H^+ pump has been suggested to be the plasma membrane H^+ -ATPase (Assmann *et al.*, 1985; Shimazaki *et al.*, 1986; Schwartz *et al.*, 1991; Amodeo *et al.*, 1992; Roelfsema *et al.*, 1998). However, operation of a BL-sensitive plasma membrane redox system containing flavoproteins rather than the H^+ -ATPase has been proposed as an entity responsible for H^+ pumping (Gautier *et al.*, 1992; Parvathi and Raghavendra, 1995; Assmann and Shimazaki, 1999). In this study, we provide unequivocal evidence indicating that the BL-activated H^+ pump is the plasma membrane H^+ -ATPase in guard cells. Exploiting the fact that the plasma membrane H^+ -ATPase in guard cells is sensitive to BL, the pulse of BL could be used as a tool for modulation of H^+ -ATPase activity to investigate its regulatory mechanism.

It is clear that the plasma membrane H^+ -ATPase in guard cells possesses low- and BL-dependent high-activity states (Figure 1). Our results show that phosphorylation is required for the high-activity state of H^+ -ATPase in guard cells. The plasma membrane H^+ -ATPase was phosphorylated reversibly by a pulse of BL, and the levels of phosphorylation were positively correlated with the rates of ATP hydrolysis and H^+ pumping under all the experimental conditions tested (Figures 1–4). Inhibition of the phosphorylation by a protein kinase inhibitor suppressed the H^+ pumping (Figure 4). Recently, Olsson *et al.* (1998) have found that the C-terminus of spinach H^+ -ATPase is phosphorylated on the threonine residue in the presence of FC, an activator of H^+ -ATPase; however, the relationship between the level of phosphorylation and H^+ -ATPase activity is unclear. The results provide the first evidence demonstrating that phosphorylation increases the activity of plasma membrane H^+ -ATPase in response to physiological stimuli in higher plants.

By contrast, several lines of evidence indicate that dephosphorylation activates the plasma membrane H^+ -ATPase and that phosphorylation inhibits the activity. Fungal pathogen-induced dephosphorylation of H^+ -ATPase *in vivo* in cultured tomato cells (Vera-Estrella *et al.*, 1994; Xing *et al.*, 1996) and alkaline phosphatase-mediated dephosphorylation of H^+ -ATPase *in vitro* in tobacco cells (Desbrosses *et al.*, 1998) result in an increase of H^+ -ATPase activity. Systemin, a primary wounding signal, is suggested to inhibit H^+ -ATPase activity via Ca^{2+} -dependent phosphorylation of H^+ -ATPase in cultured tomato cells (Schaller and Oecking, 1999). The Ca^{2+} -dependent phosphorylation of H^+ -ATPase and inhibition of H^+ pumping have been reported in root cells of oat and beet (Schaller and Sussman, 1988; Suzuki *et al.*, 1992; Lino *et al.*, 1998). These results contrast sharply with our present results. The reason for the discrepancy is not clear at present, and the opposite effect of the phosphorylation on the activity may be due to differences in the site and residue of phosphorylation in

the H^+ -ATPase molecule. Desbrosses *et al.* (1998) have suggested that the plasma membrane H^+ -ATPase in tobacco cells is activated through the dephosphorylation of a phosphothreonine residue which does not localize in the C-terminal domain.

As shown in Figures 1B and 3B, we consistently found the basal ATP hydrolysis by the plasma membrane ATPase before the application of BL, but there was no apparent H^+ pumping in GCPs under these condition. This is probably because our measurement condition is the state of equilibrium between H^+ release by the pumping and H^+ uptake by the photosynthetic CO_2 fixation, and thus we could not observe the H^+ pumping. In agreement with this interpretation, the H^+ release (acidification) occurs when the photosynthetic CO_2 fixation in GCPs is suppressed by DCMU, an inhibitor of photosystem II, under RL (Shimazaki and Zeiger, 1987). In addition to this, it is possible that the H^+ -ATPase activity might be induced by the disruption of GCPs.

Although the messages of *VHA1* and *VHA2* genes accumulate in *Vicia* guard cells (Hentzen *et al.*, 1996), there is no direct evidence for the presence of their isoproteins. Expression of H^+ -ATPases at high density in guard cells has been demonstrated by immunological methods (Villalba *et al.*, 1991; Becker *et al.*, 1993), but these methods cannot discriminate between isoproteins such as *VHA1* and *VHA2*. In our study, the treatment of guard cell H^+ -ATPases with CNBr generated different sizes of C-terminal peptides with the predicted molecular masses of *VHA1* and *VHA2*, respectively (Figures 5 and 6B). One of the peptides thought to be the *VHA2* fragment in guard cells was identified using recombinant *VHA2*, together with evidence that the peptide was recognized by antiserum against the C-terminus (Figure 7). These results suggest that the isoproteins of both *VHA1* and *VHA2* are likely to be expressed in guard cells, and that treatment of H^+ -ATPase with CNBr may make it possible to discriminate these isoproteins by their size differences on SDS-PAGE.

The plasma membrane H^+ -ATPases are encoded in multigene families, and tissue-specific expression of gene isoforms has been reported (Michelet and Boutry, 1995). In *Arabidopsis thaliana*, for example, the *AHA3* (*Arabidopsis H⁺-ATPase isoform 3*) gene and *AHA3* isoprotein have been demonstrated, using epitope tagging, to be expressed in companion cells in a cell-specific manner (Dewitt and Sussman, 1995). The isoprotein has been shown to have biochemical properties different from those of other isoproteins; *AHA3* has a higher K_m for ATP and a lower sensitivity to vanadate than *AHA1* and *AHA2* when these are expressed in yeast (Palmgren and Christensen, 1994). Since guard cells respond specifically to BL by activating the plasma membrane H^+ -ATPase, it is expected that a guard cell-specific isoprotein with a unique regulatory domain catalyses this reaction. In guard cells of *V.faba*, two mRNAs encoding the H^+ -ATPase isoproteins (*VHA1* and *VHA2*) are expressed; however, these genes are not unique to guard cells and it is suggested that they are expressed in roots, leaves, stems and flowers (Nakajima *et al.*, 1995; Hentzen *et al.*, 1996). Thus, it is most likely that the H^+ -ATPase(s) in guard cells are phosphorylated via a guard cell-specific signalling pathway which can respond to BL.

We have found that an endogenous 14-3-3 protein binds to the plasma membrane H⁺-ATPase in guard cells when the C-terminus is phosphorylated, and the amount of the bound 14-3-3 protein parallels the H⁺-ATP activity (Figures 8 and 9). Recent investigations indicate that the H⁺-ATPase-14-3-3 protein complex is stabilized by FC, and that the complex maintains the high-activity state of H⁺-ATPase (Jahn *et al.*, 1997; Baunsgaard *et al.*, 1998; Fullone *et al.*, 1998; Olsson *et al.*, 1998; Piotrowski *et al.*, 1998; Sze *et al.*, 1999). Therefore, in guard cells, it is also likely that the H⁺-ATPase-14-3-3 complex acts as a high-activity state of H⁺-ATPase. The complex in guard cells seems to dissociate upon dephosphorylation, resulting in decreased H⁺-ATPase activity (Figures 1B and 2). However, the difference may exist in the formation of the H⁺-ATPase-14-3-3 complex between BL and FC. BL-dependent complex formation obligatorily requires phosphorylation of the H⁺-ATPase. (Figure 9), but the FC-induced complex formation does not seem to require the phosphorylation (Sze *et al.*, 1999). The complex formation is facilitated markedly by FC without phosphorylation *in vitro* (Fullone *et al.*, 1998).

It has been demonstrated that 14-3-3 proteins bind to phosphorylated motifs containing phosphoserine residues of RSXpSXP and RXY/FXpSXP in their target proteins (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). The C-termini of H⁺-ATPases in guard cells were found to be phosphorylated on serine and threonine residues in response to BL (Figure 6). However, the C-termini of H⁺-ATPases in plant isoforms including VHA1 and VHA2 lack such typical consensus motifs. Thus, the unique motifs responsible for binding of the 14-3-3 protein should lie within the C-terminus of H⁺-ATPase. Very recently, Axelsen *et al.* (1999) evaluated the role of the C-terminus of AHA2 by replacing 87 amino acid residues in this region, and characterized the kinetic properties of the enzymes which were expressed in yeast. They have suggested that there are two potential 14-3-3 binding motifs, which have the sequences R⁹⁰¹ELSE and R⁹²⁰ELHTL. The same sequence motifs are also found in the C-termini of both VHA1 and VHA2 in similar locations.

In recent years, much progress has been made in identifying BL photoreceptors responsible for inhibition of hypocotyl elongation and phototropisms. Flavin- and pterin-binding photolyase homologues, CRY1 and CRY2, in the case of hypocotyl growth (Ahmad and Cashmore, 1993, 1996), and a flavin-containing protein kinase, NPH, in the case of phototropism (Huala *et al.*, 1997; Christie *et al.*, 1998), have been identified as BL photoreceptors. However, there is no evidence that CRY1, CRY2 and NPH1 are involved in the BL response of stomata. Accumulated evidence suggests that the carotenoid zeaxanthin, a component of the xanthophyll cycle in chloroplasts, mediates the perception of BL in stomatal guard cells (Zeiger and Zhu, 1998; Frechila *et al.*, 1999). It would be very interesting to know how zeaxanthin in chloroplasts affects the activities of protein kinases or phosphatases which may be localized in the cytosol or the plasma membrane.

In conclusion, we demonstrate that a pulse of BL activates the plasma membrane H⁺-ATPase via phosphorylation in the C-terminus of guard cells. The phosphorylation of H⁺-ATPase by BL is mediated by a serine/threonine

protein kinase which may be involved in BL signalling pathways unique to guard cells. A 14-3-3 protein binds to the phosphorylated C-terminus of H⁺-ATPase as a physiological ligand and may have a key role for activation of the plasma membrane H⁺-ATPase. This is the first evidence demonstrating that protein phosphorylation is involved in the BL response of stomata. We note, however, that mutational analysis will provide more direct evidence for the physiological role of phosphorylation and the 14-3-3 protein in the regulation of H⁺-ATPase.

Materials and methods

Plant materials and isolation of GCPs

Plants of *V. faba* (cv. Ryosai Issun) were cultured hydroponically in a greenhouse as described previously (Shimazaki *et al.*, 1992). GCPs were isolated enzymatically from the lower epidermis of 4- to 8-week-old leaves of *V. faba* according to previous methods with modifications (Shimazaki *et al.*, 1992; Mawson 1993). The first step digestion medium (pH 5.4) contained 1% (w/v) cellulase R-10 (Yakult Pharmaceutical Industry Co., Tokyo, Japan), 0.1% (w/v) polyvinylpyrrolidone K-30, 0.2% (w/v) bovine serum albumin (BSA), 1 mM CaCl₂, 0.25 M mannitol and 10 mM MES. The second step digestion medium (pH 5.4) contained 0.6% (w/v) cellulase RS, 0.005% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan), 0.3% (w/v) BSA, 1 mM CaCl₂, 0.25 M mannitol, 5 mM ascorbic acid, 20 µg/ml chloramphenicol and 10 mM MES. The first step digestion was carried out for 1.5 h at 12°C with shaking (50 strokes/min), and the second step digestion was for 12 h at 12°C without shaking. Isolated GCPs were stored in 0.4 M mannitol and 1 mM CaCl₂ on ice in the dark until use. Protein was determined by the method of Bradford (1976) using BSA as a standard.

Measurement of BL-dependent H⁺ pumping

BL-dependent H⁺ pumping by GCPs from *V. faba* was measured with a glass pH electrode (Beckman 39532) using a dual-beam protocol described previously (Shimazaki *et al.*, 1992). The reaction mixture (1.0 ml) consisted of 0.125 mM MES-NaOH pH 6.0, 1 mM CaCl₂, 0.4 M mannitol, 10 mM KCl and GCPs (50 µg of protein), unless otherwise stated. The light intensity was 600 µmol/m²/s for background RL and 100 µmol/m²/s for BL.

ATP hydrolysis

ATP hydrolytic activity was measured by determining the Pi released from ATP (Kinoshita *et al.*, 1995) with slight modifications. The suspension medium of GCPs (1.0 ml) consisted of 5 mM MES-KOH pH 6.0, 10 mM KCl, 1 mM CaCl₂, 0.4 M mannitol and GCPs (0.25 mg of protein). The GCPs in the medium were incubated under background RL for 40 min at 24°C, and illuminated with a pulse of BL for 30 s. An aliquot of withdrawn GCP suspension (25 µl) was mixed with an equal volume (25 µl) of the medium containing 100 mM MOPS-KOH pH 7.5, 5 mM EDTA, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 µM leupeptin, 1 mM dithiothreitol (DTT) and 0.05% Triton X-100 to disrupt the GCPs. The disrupted GCPs were mixed immediately with an equal volume of the reaction mixture for ATPase measurement. The reaction mixture consisted of 50 mM MOPS-KOH pH 7.0, 0.2 M mannitol, 100 mM NaCl, 100 mM KNO₃, 2.5 mM EDTA, 20 mM MgCl₂, 10 µg/ml oligomycin, 1 mM ammonium molybdate, 0.5 mM PMSF and 10 µM leupeptin. The reaction was then initiated by adding ATP at 2 mM and was run for 30 min at 24°C.

Determination of phosphorylation levels of the plasma membrane H⁺-ATPase in guard cells

The GCP suspensions of 0.5–1.0 ml (1.0 mg of protein/ml) were incubated with ³²P-labelled orthophosphate (³²Pi) (0.25 mCi/mg protein) under background RL for 80 min at 24°C, then illuminated with a 30 s pulse of BL. The BL-dependent reaction was terminated at the indicated times by addition of an equal volume of the medium containing 100 mM MOPS-KOH pH 7.5, 5 mM EDTA, 200 mM NaCl, 1 mM PMSF, 20 µM leupeptin, 4 mM DTT, 20 mM NaF, 1 mM ammonium molybdate, 200 nM calyculin A and 2% Triton X-100 to the GCPs, followed by centrifugation for 10 min at 10 000 g. The resulting supernatant was mixed with antiserum against the plasma membrane H⁺-ATPase at 0.5% (v/v). After incubation for 12 h with gentle mixing at 4°C, protein A–

agarose (Santa Cruz Biotech., Inc., CA) was added to the supernatant at 2.5%, and kept for 2 h at 4°C with gentle mixing. The sample was then centrifuged for 1 min at 10 000 g and the pellet was washed three times with 1 ml of ice-cold Tris-buffered saline (TBS). The pellet obtained was resuspended in the solubilizing medium (Laemmli 1970), centrifuged at 10 000 g for 1 min to remove agarose, and the supernatant was used for SDS-PAGE. Separated proteins on the gel were stained once with Coomassie Brilliant Blue for 1 h to visualize the protein profiles. The destained gel was immersed in Enlightening (New Research Products, Boston, MA) for 15 min and dried on a gel drier. Autoradiography was carried out by exposing Fuji RX film (Fuji Film, Tokyo, Japan) to the dried gel for 1–4 days at room temperature.

Light source

RL for background illumination was obtained from a tungsten lamp (Sylvania EXR 150 W) by passing the light through a red glass filter (Corning 2-61) and BL was obtained from a tungsten lamp (Sylvania EXR 300 W) through a blue glass filter (Corning 5-60). Photon flux density was determined with a quantum meter (Li-Cor, model 185A, Lincoln, NE).

Generation of polyclonal antibodies

The conserved hydrophilic loop containing the ATP-binding site and the C-terminus of H⁺-ATPase from *Vfaba* (VHA1) [S79323] (Nakajima *et al.*, 1995), and 14-3-3 protein (GF140) from *Arabidopsis* [L09111] (Lu *et al.*, 1994) were used separately as antigens. These three antigens were expressed in *E.coli* as recombinant proteins. RNA was extracted from *Vicia* GCPs (8×10^6) and from whole tissues of 3-week-old *Arabidopsis* (cv. Columbia) with acid guanidine thiocyanate-phenol-chloroform (Isogen, Nippon Gene, Japan) according to the method of Chomczynski and Sacchi (1987). The first strand cDNA was synthesized from this total RNA using oligo(dT)₁₂₋₁₈ as a primer (Takara, Japan). Three genes for the conserved hydrophilic loop and the C-terminus of VHA1, and 14-3-3 protein (GF140) were amplified from the first strand cDNA by PCR (Model PC-700, Astec, Japan) with two primers. The primers (5'-CCGGATCCATGGCGGACGTTCTTGTAGTG-3' and 5'-CCGGATCCAGCATCGGCAACAGCAATT-3') for the hydrophilic loop, (5'-CCGGATCCCGCTATTCTTGAGTGGA-3' and 5'-CCGGATCCTTATGTGTTGCCTTTGTAC-3') for the C-terminus and (5'-CCGGATCCATGGCGGACGTTCTTGTAGTG-3' and 5'-CCGGATCCATGATCTCCTTCTATTC-3') for the 14-3-3 protein were used, all of which contained the *Bam*HI site at both ends. The resulting amplified DNA fragments covering 1123–1983 and 2683–3022 bp of VHA1, and 52–855 bp of 14-3-3 protein were cloned into the *Bam*HI site of pGEX-2T (Pharmacia, Biotech, Tokyo, Japan), respectively, and were used to transform *E.coli* JM 109. Three peptides were expressed as fusion proteins with GST in *E.coli* by addition of isopropyl-β-D-thiogalactopyranoside (IPTG), and were purified with Bulk and RediPack GST Purification Modules (Pharmacia Biotech, Tokyo, Japan). VHA1 polypeptide was purified further by SDS-PAGE. Antibodies against these polypeptides were raised in rabbits by subcutaneous injection of 0.5–1 mg of the purified polypeptide every 2 weeks. The specificity of all these antibodies for the antigens was confirmed. The antiserum recognized the individual antigenic protein in the guard cell extract, and the pre-serum did not react with any other proteins (data not shown), except that antiserum against the hydrophilic loop sometimes reacted with a few polypeptides with molecular masses of ~65 kDa.

Immunological detection of specific proteins

The plasma membrane H⁺-ATPase, the C-terminal region of H⁺-ATPase and the 14-3-3 protein were detected immunologically with their respective antibodies according to the method of Gallagher *et al.* (1992), unless otherwise stated. After separation of proteins from guard cells on SDS-PAGE, the proteins were electroblotted onto nitrocellulose membranes (Hybond-C, Amersham, UK) using Trans-blot (Bio-Rad, CA). The membranes were incubated for 1 h in the blocking medium containing 0.05% Tween-20, 5% non-fat dry milk, 20 mM Tris-HCl pH 7.4, 140 mM NaCl, and then reacted with polyclonal antibodies at 5000-fold dilution for 2 h in the same medium at room temperature. The membrane was rinsed three times for 5 min in T-TBS containing 0.05% Tween-20, 20 mM Tris-HCl pH 7.4 and 140 mM NaCl, and reacted with a goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase (Bio-Rad, CA) at 3000-fold dilution in the blocking medium. The alkaline phosphatase reaction was developed by 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The specificity of individual antibodies was examined using pre-serum as a control. To determine the concentration of 14-3-3 protein and H⁺-ATPase in the

immunoprecipitate, these proteins were estimated by an immunological method. Known amounts of 14-3-3 protein (GF140) and the C-terminus of H⁺-ATPase on the membranes were immunostained by the specific antibodies, and the standard curves for the proteins were obtained. For the immunostaining of H⁺-ATPase, the antibodies against the C-terminus of H⁺-ATPase, which is conjugated to alkaline phosphatase (Sigma-Genosys Ltd., Cambridge, UK), was used at 500-fold dilution. On the basis of the standard curve, the amounts of 14-3-3 protein and H⁺-ATPase were determined in the immunoprecipitate which was subjected to SDS-PAGE followed by Western blotting. The signal was analysed by the NIH image software system.

Fragmentation of H⁺-ATPase and the recombinant C-terminus

The immunoprecipitated H⁺-ATPase and recombinant C-terminus of VHA2 were digested by CNBr at 100 mg/ml in 70% (v/v) formic acid for 1.5 h at room temperature according to a previous method (Luo *et al.*, 1991) with slight modifications. The fragmented protein solution was dried, and the resulting peptides were dissolved and subjected to SDS-PAGE (Laemmli 1970). Radiolabelled and recombinant C-terminus peptides were determined by autoradiography and Western blotting, respectively. To obtain the recombinant C-terminus of VHA2 [AB022442], it was expressed in *E.coli*. The cDNA fragment of the C-terminus of VHA2 (2617–2970) was amplified from the first strand cDNA by PCR using two primers (5'-CCGGATCCCGGACTCGACGTGATG-3' and 5'-CCGGATCCCTCACAGTGTAGTGCT-3'), which contained the *Bam*HI site, and cloned in-frame with GST into pGEX-2T. *Escherichia coli* cells of the JM109 strain transformed with the plasmid having the fusion protein construct were grown at 37°C in 2× YTA medium. When the culture showed a turbidity of A_{600 nm} = 0.6, IPTG at 0.5 mM was added to induce generation of the C-terminus of VHA2. The cells were harvested 16 h after the induction and were subjected to SDS-PAGE in 12% gels. The fusion protein was eluted with 10 mM Tris-HCl pH 8.0 and 1% SDS, and then precipitated with 10% (w/v) trichloroacetic acid for treatment with CNBr.

Phosphoamino acid analysis

Proteins separated on SDS-PAGE were transferred to PVDF membranes (Bio-Rad, CA) by electroblotting. The membranes were washed with distilled water and dried in air, then exposed to X-ray film to localize ³²P-labelled peptides. Proteins on the membrane were hydrolysed in 6 M HCl at 110°C for 3 h. The hydrolysate was dried, and phosphoamino acids were analysed on a cellulose thin-layer plate (20×20 cm, Merck, Darmstadt, Germany) after one-dimensional electrophoresis (Schaller and Sussman, 1988). Phosphoamino acids were detected using a BAS-1500 image screen (Fuji Film, Tokyo, Japan).

Far Western blot analysis

Protein-protein interaction was analysed by far Western blotting according to Fullone *et al.* (1998) with modifications. Immunoprecipitated H⁺-ATPase or GCP proteins were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. Proteins on the membranes were denatured by 6.0 M guanidine-HCl containing 25 mM HEPES-NaOH pH 7.7, 25 mM NaCl, 5 mM MgCl₂ and 1 mM DTT, and then renatured by step-wise reduction of the concentration of guanidine-HCl to 0.3 M. The membranes were kept in the medium containing 20 mM HEPES-KOH pH 7.7, 75 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2% non-fat dry milk and 0.04% Tween-20, and then reacted with 0.1 μM GST-14-3-3 fusion protein or GST overnight at 4°C in the same medium. After washing the membrane three times with T-TBS, it was reacted with anti-GST antibodies (Pharmacia Biotech, Tokyo, Japan) at 1000-fold dilution for 1 h at room temperature in the blocking medium. The membrane was washed three times for 5 min in T-TBS, and reacted with anti-goat IgG secondary antibodies conjugated to alkaline phosphatase (Sigma-Aldrich, Tokyo, Japan) at 5000-fold dilution in the blocking medium. The alkaline phosphatase reaction was developed as described above.

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References

- Ahmad, M. and Cashmore, A.R. (1993) *HY4* gene of *A.thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature*, **366**, 162–166.
- Ahmad, M. and Cashmore, A.R. (1996) Seeing blue: the discovery of cryptochrome. *Plant Mol. Biol.*, **30**, 851–861.
- Amodio, G., Srivastava, A. and Zeiger, E. (1992) Vanadate inhibits blue light-stimulated swelling of *Vicia* guard cell protoplasts. *Plant Physiol.*, **100**, 1567–1570.
- Assmann, S.M. (1993) Signal transduction in guard cells. *Annu. Rev. Cell Biol.*, **9**, 345–375.
- Assmann, S.M. and Shimazaki, K. (1999) The multisensory guard cell. Stomatal responses to blue light and abscisic acid. *Plant Physiol.*, **119**, 809–815.
- Assmann, S.M., Simoncini, L. and Schroeder, J.I. (1985) Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba* L. *Nature*, **318**, 285–287.
- Axelsen, K.B., Venema, K., Jahn, T., Baunsgaard, L. and Palmgren, M.G. (1999) Molecular dissection of the C-terminal regulatory domain of the plant plasma membrane H⁺-ATPase *AHA2*: mapping of residues that when altered give rise to an activated enzyme. *Biochemistry*, **38**, 7227–7234.
- Baunsgaard, L., Venema, K., Axelson, K.B., Villalba, J.M., Welling, A., Wollenweber, B. and Palmgren, M.G. (1996) Modified plant plasma membrane H⁺-ATPase with improved transport coupling efficiency identified by mutant selection in yeast. *Plant J.*, **10**, 451–458.
- Baunsgaard, L., Fuglsang, A.T., Jahn, T., Korthout, H.A.A.J., de Boer, A.H. and Palmgren, M.G. (1998) The 14-3-3 proteins associate with the plasma membrane H⁺-ATPase to generate a fusicoccin binding complex and a fusicoccin-responsive system. *Plant J.*, **13**, 661–671.
- Becker, D., Zeilinger, C., Lohse, G., Depta, H. and Hedrich, R. (1993) Identification and biochemical characterization of the plasma-membrane H⁺-ATPase in guard cells of *Vicia faba* L. *Planta*, **190**, 44–50.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
- Briskin, D.P. and Reynolds-Niesman, I. (1991) Determination of H⁺/ATP stoichiometry for the plasma membrane H⁺-ATPase from red beet (*Beta vulgaris* L.) storage tissue. *Plant Physiol.*, **95**, 242–250.
- Camoni, L., Fullone, M.R., Marra, M. and Aducci, P. (1998) The plasma membrane H⁺-ATPase from maize root is phosphorylated in the C-terminal domain by a calcium-dependent protein kinase. *Physiol. Plant.*, **104**, 549–555.
- Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
- Cristie, J.M., Raymond, P., Powell, G.K., Bernasconi, P., Raibekas, A.A., Liscum, E. and Briggs, W.R. (1998) *Arabidopsis* NPH1: a flavoprotein with the properties of a photoreceptor for phototropism. *Science*, **282**, 1698–1701.
- Dewitt, N.D. and Sussman, M.R. (1995) Immunocytological localization of an epitope-tagged plasma membrane proton pump (H⁺-ATPase) in phloem companion cells. *Plant Cell*, **7**, 2053–2067.
- Desbrosses, G., Steling, J. and Renaudin, J.-P. (1998) Dephosphorylation activates the purified plant plasma membrane H⁺-ATPase: possible function of phosphothreonine residues in a mechanism not involving the regulatory C-terminal domain of the enzyme. *Eur. J. Biochem.*, **251**, 496–503.
- Frechilla, S., Zhu, J., Talbott, L.D. and Zeiger, E. (1999) Stomata from *npq1*, a zeaxanthin-less *Arabidopsis* mutant, lack a specific response to blue light. *Plant Cell Physiol.*, **40**, 949–954.
- Fullone, M.R., Visconti, S., Marra, M., Fogliano, V. and Aducci, P. (1998) Fusicoccin effect on the *in vitro* interaction between plant 14-3-3 proteins and plasma membrane H⁺-ATPase. *J. Biol. Chem.*, **273**, 7698–7702.
- Gallagher, S., Winston, S.E. and Hurrell, J.G.R. (1992) Immunoblotting and immunodetection. In Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds), *Current Protocols in Molecular Biology*. Greene Publishing and Wiley-Interscience, New York, pp. 10.8.1–10.8.16.
- Gautier, H., Vavasseur, A., Lasceve, G. and Boudet, A.M. (1992) Redox processes in the blue light response of guard cell protoplasts of *Commelina communis* L. *Plant Physiol.*, **98**, 34–38.
- Hedrich, R. and Schroeder, J.I. (1989) The physiology of ion channels and electrogenic pumps in higher plants. *Annu. Rev. Plant Physiol.*, **40**, 539–569.
- Hentzen, A.F., Smart, L.B., Wimmers, L.E., Fang, H.H., Schroeder, J.I. and Bennet, A.B. (1996) Two plasma membrane H⁺-ATPase genes expressed in guard cells of *Vicia faba* are also expressed throughout the plant. *Plant Cell Physiol.*, **37**, 650–659.
- Huala, E., Oeller, P.W., Liscum, E., Han, I.S., Larsen, E. and Briggs, W.R. (1997) *Arabidopsis* NPH1: a protein kinase with a putative redox-sensing domain. *Science*, **278**, 2120–2123.
- Iino, M., Ogawa, T. and Zeiger, E. (1985) Kinetic properties of blue light response of stomata. *Proc. Natl Acad. Sci. USA*, **82**, 8019–8023.
- Jahn, T., Fuglsang, A.T., Olsson, A., Bruntrup, I.M., Colling, D.B., Volkmann, D., Sommarin, M., Palmgren, M.G. and Larsson, C. (1997) The 14-3-3 protein interacts directly with C-terminal region of the plasma membrane H⁺-ATPase. *Plant Cell*, **9**, 1805–1814.
- Johansson, F., Sommarin, M. and Larsson, C. (1993) Fusicoccin activates the plasma membrane H⁺-ATPase by a mechanism involving the C-terminal inhibitory domain. *Plant Cell*, **5**, 321–327.
- Kaufman, L.S. (1993) Transduction of blue-light signals. *Plant Physiol.*, **102**, 333–337.
- Kinoshita, T., Nishimura, M. and Shimazaki, K. (1995) Cytosolic concentration of Ca²⁺ regulates the plasma membrane H⁺-ATPase in guard cells of fava bean. *Plant Cell*, **7**, 1333–1342.
- Korthout, H.A.A.J. and de Boer, A.H. (1994) A fusicoccin binding protein belongs to the family of 14-3-3 brain protein homologs. *Plant Cell*, **6**, 1681–1692.
- Laemmli, U.K. (1970) Cleavage of structural protein during assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lino, B., Baizabal-Aguirre, V.M. and de la Vara, L.E.G. (1998) The plasma membrane H⁺-ATPase from beet root is inhibited by a calcium-dependent phosphorylation. *Planta*, **204**, 352–359.
- Lu, G., Rooney, M.F., Wu, K. and Ferl, R.J. (1994) Five cDNAs encoding *Arabidopsis* GF14 proteins. *Plant Physiol.*, **105**, 1459–1460.
- Luo, K., Hurley, T.R. and Sefton, B.M. (1991) Cyanogen bromide cleavage and proteolytic peptide mapping of proteins immobilized to membrane. *Methods Enzymol.*, **201**, 149–152.
- Marra, M., Fullone, M.R., Fogliano, V., Pen, J., Mattei, M., Masi, S. and Aducci, P. (1994) The 30-kilodalton protein present in purified fusicoccin receptor preparations is a 14-3-3-like protein. *Plant Physiol.*, **106**, 1497–1501.
- Mawson, B.T. (1993) Modulation of photosynthesis and respiration in guard and mesophyll cell protoplasts by oxygen concentration. *Plant Cell Environ.*, **16**, 207–214.
- Michelet, B. and Boutry, M. (1995) The plasma membrane H⁺-ATPase. A highly regulated enzyme with multiple physiological functions. *Plant Physiol.*, **108**, 1–6.
- Morrison, D.K., Kaplan, D.R., Rapp, U. and Roberts, T.M. (1998) Signal transduction from membrane-bound oncogene products increases Raf-1 phosphorylation and associated protein kinase activity. *Proc. Natl Acad. Sci. USA*, **85**, 8855–8859.
- Morsomme, P., de Kerchove d'Exaerde, A., De Meester, S., Thiemes, D., Goffeau, A. and Boutry, M. (1996) Single point mutations in various domains of a plant plasma membrane H⁺-ATPase expressed in *Saccharomyces cerevisiae* increase H⁺ pumping and permit yeast growth at low pH. *EMBO J.*, **15**, 5513–5526.
- Muslin, A.J., Tanner, J.M., Allen, P.M. and Shaw, A.S. (1996) Interaction of 14-3-3 protein with signaling proteins is mediated by the recognition of phosphoserine. *Cell*, **84**, 889–897.
- Nakajima, N., Saji, H., Aono, M. and Kondo, N. (1995) Isolation of cDNA for a plasma membrane H⁺-ATPase from guard cells of *Vicia faba* L. *Plant Cell Physiol.*, **36**, 919–924.
- Oecking, C., Eckerskorn, C. and Weiler, E.W. (1994) The fusicoccin receptor of plants is a member of the 14-3-3 superfamily of eukaryotic regulatory proteins. *FEBS Lett.*, **352**, 163–166.
- Oecking, C., Piotrowski, M., Hagemeyer, J. and Hageman, K. (1997) Topology and target interaction of the fusicoccin-binding 14-3-3 homologs of *Commelina communis*. *Plant J.*, **12**, 441–453.
- Olsson, A., Svennelid, F., Ek, B., Sommarin, M. and Larsson, C. (1998) A phosphothreonine residue at the C-terminal end of the plasma membrane H⁺-ATPase is protected by fusicoccin-induced 14-3-3 binding. *Plant Physiol.*, **118**, 551–555.
- Palmgren, M.G. (1991) Regulation of plant plasma membrane H⁺-ATPase activity. *Physiol. Plant.*, **83**, 314–323.
- Palmgren, M.G. and Christensen, G. (1994) Functional comparisons between plant plasma membrane H⁺-ATPase isoforms expressed in yeast. *J. Biol. Chem.*, **269**, 3027–3033.

- Palmgren,M.G., Larsson,C. and Sommarin,M. (1990) Proteolytic activation of the plant plasma membrane H^+ -ATPase by removal of a terminal segment. *J. Biol. Chem.*, **265**, 13423–13426.
- Parvathi,K. and Raghavendra,A.S. (1995) Bioenergetic process in guard cells related to stomatal function. *Physiol. Plant.*, **93**, 146–154.
- Piotrowski,M., Morsomme,P., Boutry,M. and Oecking,C. (1998) Complementation of the *Saccharomyces cerevisiae* plasma membrane H^+ -ATPase by a plant H^+ -ATPase generates a highly abundant fusicoccin binding site. *J. Biol. Chem.*, **45**, 30018–30023.
- Pockman,W.T., Sperry,J.S. and O'Leary,J.W. (1995) Sustained and significant negative water pressure in xylem. *Nature*, **378**, 715–716.
- Rasi-Caldogno,F., Pugliarello,M.C., Olivari,C. and De Michelis,M.I. (1993) Controlled proteolysis mimics the effect of fusicoccin on the plasma membrane H^+ -ATPase. *Plant Physiol.*, **103**, 391–398.
- Roelfsema,M.R.G., Staal,M. and Prins,H.B.A. (1998) Blue light-induced apoplastic acidification of *Arabidopsis thaliana* guard cells: inhibition by ABA is mediated through protein phosphatases. *Physiol. Plant.*, **103**, 466–474.
- Schaller,A. and Oecking,C. (1999) Modulation of plasma membrane H^+ -ATPase activity differentially activates wound and pathogen defense responses in tomato plants. *Plant Cell*, **11**, 263–272.
- Schaller,G.E. and Sussman,M.R. (1988) Phosphorylation of the plasma membrane H^+ -ATPase of the oat plasma membrane. *Planta*, **173**, 509–518.
- Schroeder,J.I. (1988) K^+ transport properties of K^+ channels in the plasma membrane of *Vicia faba* guard cells. *J. Gen. Physiol.*, **92**, 667–683.
- Schwartz,A., Illan,N. and Assmann,S.M. (1991) Vanadate inhibition of opening in epidermal peels of *Commelina communis*. Cl^- interferes with vanadate uptake. *Planta*, **183**, 590–596.
- Sekler,I., Weiss,M. and Pick,U. (1994) Activation of the *Dunaliella acidiphila* plasma membrane H^+ -ATPase by trypsin cleavage of a fragment that contains a phosphorylation site. *Plant Physiol.*, **105**, 1125–1132.
- Serrano,R. (1989) Structure and function of plasma membrane ATPase. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **40**, 61–94.
- Shimazaki,K. and Zeiger,E. (1987) Red light-dependent CO_2 uptake and oxygen evolution in guard cell protoplasts of *Vicia faba* L.: evidence for CO_2 fixation. *Plant Physiol.*, **84**, 7–9.
- Shimazaki,K., Iino,M. and Zeiger,E. (1986) Blue light-dependent proton extrusion by guard-cell protoplasts of *Vicia faba*. *Nature*, **319**, 324–326.
- Shimazaki,K., Kinoshita,T. and Nishimura,M. (1992) Involvement of calmodulin and calmodulin-dependent myosin light chain kinase in blue light-dependent H^+ pumping by guard cell protoplasts from *Vicia faba* L. *Plant Physiol.*, **99**, 1416–1421.
- Shimazaki,K., Omasa,K., Kinoshita,T. and Nishimura,M. (1993) Properties of the signal transduction pathway in the blue light response of stomatal guard cells of *Vicia faba* and *Commelina benghalensis*. *Plant Cell Physiol.*, **34**, 1321–1327.
- Short,T.W., Raymond,P. and Briggs,W.R. (1993) A pea plasma membrane protein exhibiting blue light-induced phosphorylation retains photosensitivity following Triton solubilization. *Plant Physiol.*, **101**, 647–655.
- Short,T.W. and Briggs,W.R. (1994) The transduction of blue light signals in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **45**, 143–171.
- Spanswick,R.M. (1981) Electrogenic ion pumps. *Annu. Rev. Plant Physiol.*, **32**, 267–289.
- Sussman,M.R. (1994) Molecular analysis of proteins in the plant plasma membrane. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **45**, 211–234.
- Suzuki,Y.S., Wang,Y. and Takemoto,J.Y. (1992) Syringomycin-stimulated phosphorylation of the plasma membrane H^+ -ATPase from red beet storage tissue. *Plant Physiol.*, **99**, 1314–1320.
- Sze,H., Li,X. and Palmgren,M.G. (1999) Energization of plant cell membranes by H^+ -pumping ATPases: regulation and biosynthesis. *Plant Cell*, **11**, 677–689.
- Vera-Estrella,R., Barkla,B.J., Higgins,V.J. and Blumwald,E. (1994) Plant defense response to fungal pathogens; activation of host-plasma membrane H^+ -ATPase by elicitor-induced enzyme dephosphorylation. *Plant Physiol.*, **104**, 209–215.
- Villalba,J.M., Lutzelschwab,M. and Serrano,R. (1991) Immunocyto-localization of plasma-membrane H^+ -ATPase in maize coleoptiles and enclosed leaves. *Planta*, **183**, 458–461.
- Xing,T., Higgins,V.J. and Blumwald,E. (1996) Regulation of plant defense response to fungal pathogens: two types of protein kinases in the reversible phosphorylation of the host plasma membrane H^+ -ATPase. *Plant Cell*, **8**, 555–564.
- Yaffe,M.B., Rittinger,K., Volinia,S., Caron,P.R., Aitken,A., Leffers,H., Gamblin,S.J., Smerdon,S.J. and Cantley,L.C. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell*, **91**, 961–971.
- Zeiger,E. (1983) The biology of stomatal guard cells. *Annu. Rev. Plant Physiol.*, **34**, 441–475.
- Zeiger,E. and Zhu,J. (1998) Role of zeaxanthin in blue light photoreception and the modulation of light- CO_2 interactions in guard cells. *J. Exp. Bot.*, **49**, 433–442.

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