Possible Involvement of Phototropins in Leaf Movement of Kidney Bean in Response to Blue Light

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The leaf of kidney bean (Phaseolus vulgaris) moves in response to blue light. The movement is induced by a decrease in the turgor pressure of pulvinar motor cells on the irradiated side. In this study, we investigated the initial event of the movement with respect to function of phototropin and the plasma membrane H\textsuperscript{+}-ATPase in the motor cells. The results indicated that, in dark conditions, phototropin existed in a dephosphorylated state and the H\textsuperscript{+}-ATPase existed in a phosphorylated state. A pulse of blue light (30 s) induced the phosphorylation of phototropin and the dephosphorylation of the H\textsuperscript{+}-ATPase as determined by the binding behavior of 14-3-3 protein. Phototropin phosphorylation occurred rapidly, followed by the transient gradual dephosphorylation of the H\textsuperscript{+}-ATPase. When the specific flavoprotein inhibitor diphenyleneiodonium and the protein kinase inhibitors K-252a and staurosporine were administered to pulvinar cells, both phototropin phosphorylation and H\textsuperscript{+}-ATPase dephosphorylation were inhibited. The phosphorylation and dephosphorylation exhibited similar fluence rate dependencies to blue light. These results indicated that phototropin may function upstream of the plasma membrane H\textsuperscript{+}-ATPase and increase the activity of H\textsuperscript{+}-ATPase by dephosphorylation. We provide evidence for the existence of three kinds of phototropins in pulvinar motor cells.

Many leguminous plants orient their leaves in response to blue light, and this response is independent of leaf movement by circadian rhythms (Koller, 1990; Coté, 1995; Nishizaki, 1996). In kidney bean (Phaseolus vulgaris), blue light is absorbed by the laminar pulvinus, the leaf-moving site located between the petiole and leaf blade (Fig. 1F), and induces leaf movement through pulvinar bending (Koller, 1990; Coté, 1995). This bending is mediated by a decrease in turgor pressure of pulvinar motor cells, which results in a decrease in cell volume on the irradiated side of the pulvinus (Wang et al., 2001). The decrease in turgor pressure is caused by water efflux from the motor cells, which is brought about by effluxes of K\textsuperscript{+} and Cl\textsuperscript{−} (Lowen and Satter, 1989; Okazaki et al., 2000). This net K\textsuperscript{+}-salt efflux is driven by depolarization-activated K\textsuperscript{+} channels in the plasma membrane (Moran et al., 1988; Moran, 1996; Suh et al., 2000), and the depolarization is likely triggered by inactivation of the plasma membrane H\textsuperscript{+}-ATPase in response to blue light (Kim et al., 1992; Nishizaki, 1994; Okazaki et al., 1995; Wang et al., 2001). Recently, Okazaki (2002) used pulvinar protoplasts from Phaseolus to demonstrate that blue light decreased ATP hydrolysis by inactivating the H\textsuperscript{+}-ATPase. However, biochemical evidence for this response is insufficient, and the blue light receptor responsible for the inactivation of H\textsuperscript{+}-ATPase has yet to be identified.

Nishizaki (1988) suggested that a molecular mechanism similar to the blue light response of stomatal guard cells may operate in pulvinar motor cells, although the direction of electrical response is the opposite. Stomatal opening, which facilitates gas exchanges between the leaf and the atmosphere, is driven by increased turgor pressure in guard cells. This increase is induced by membrane hyperpolarization through the activation of the plasma membrane H\textsuperscript{+}-ATPase and the subsequent influx of K\textsuperscript{+} via hyperpolarization-activated K\textsuperscript{+} channels (Hedrich and Schroeder, 1989; Schroeder et al., 2001). In guard cells, blue light activates the H\textsuperscript{+}-ATPase via phosphorylation of the C terminus with subsequent binding of 14-3-3 protein to the H\textsuperscript{+}-ATPase (Assmann and Shimazaki, 1999; Kinoshita and Shimazaki, 1999; Palmgren, 2001).

In recent studies using Arabidopsis (Arabidopsis thaliana) mutant plants, phototropins, which are receptor-type protein kinases with two light, oxygen, and voltage (LOV) domains (Huala et al., 1997; Briggs and Christie, 2002), were identified as blue light receptors in guard cells (Kinoshita et al., 2001; Doi et al., 2004). Since Phaseolus pulvinar motor cells respond to blue light via protein components similar to those in guard cells, including the H\textsuperscript{+}-ATPase and K\textsuperscript{+} channels, it is most likely that phototropins function also in these cells. Phototropins undergo autophosphorylation in response to blue light (Christie et al., 1998; Sakai et al., 2001), and the phosphorylation occurs on multiple Ser residues (Short et al., 1994; Salomon et al., 2003). Recently, Kinoshita et al. (2003) found that 14-3-3 protein bound to the hinge region between LOV1 and LOV2 in broad bean (Vicia faba) phototropins (Vfphot) upon their phosphorylation in Vicia guard cells and suggested that the binding of 14-3-3 protein to phototropins is a key step in phototropin-mediated responses.

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In this study, we provide biochemical evidence for inactivation of the plasma membrane H\textsuperscript{+}-ATPase by blue light in pulvinar motor cells and suggest that phototropin may operate in this response. We also found the expression of three phototropin mRNAs in pulvini from kidney bean.

RESULTS

Blue Light Induces Pulvinar Bending of Kidney Bean

To show leaf movement, the plant was kept in the dark for 10 h before being irradiated with continuous blue light at 100 µmol m\textsuperscript{-2} s\textsuperscript{-1} for 120 min from above (Fig. 1A and B). Before the irradiation, the leaves were closed and pointing completely down (Fig. 1A, 0 min) and rose to the horizontal position in response to the blue light, greatly increasing the light-receptive area (Fig. 1, A and B, 120 min). The leaf reached its highest position at 90 min of blue light, and this position was maintained for the rest of the irradiation period (Fig. 1E). When the dark-adapted plant was irradiated at the same intensity of blue light from the side, the leaves oriented to the source of light, although the orientation was limited by the contact of two leaves (Fig. 1D). Such leaf movement maximized the light-receptive area (Fig. 1C). These leaf movements were brought about by the bending of a laminar pulvinus located on the base of each leaf blade (Fig. 1F). The properties of leaf movement may account for...
those of solar tracking in Phaseolus plant (Ritter and Koller, 1994; Briggs and Christie, 2002).

The Plasma Membrane H+-ATPase Was Dephosphorylated by Blue Light in Pulvini

Since 14-3-3 protein binds to the C terminus of the H+-ATPase in a phosphorylation-dependent manner and the binding is essential for activation of the H+-ATPase (Fuglsang et al., 1999; Kinoshita and Shimazaki, 1999, 2002; Svennelid et al., 1999), we investigated the phosphorylation status of the H+-ATPase by the binding of 14-3-3 protein in pulvini. As shown in Figure 2A, 14-3-3 protein was already bound to the H+-ATPase in the dark. The amount of 14-3-3 protein started to decrease between 1 and 2.5 min after the initiation of a pulse of blue light and reached the minimum, less than 40% of initial level, at 5 min after the pulse began, and then returned to the original level in another 5 min. The blue light did not affect the amount of the H+-ATPase (Fig. 2B). The amounts of 14-3-3 protein bound to the H+-ATPase shown here were closely related to the levels of ATP hydrolytic activity in response to blue light as shown by Okazaki (2002). The results suggest that the H+-ATPase exists in a phosphorylated state with high activity in the motor cells under darkness and that the H+-ATPase is inactivated by dephosphorylation with the dissociation of 14-3-3 protein from the H+-ATPase in response to blue light.

Phosphorylation of Phototropin Precedes Dephosphorylation of the H+-ATPase in Response to Blue Light in Pulvini

We investigated the phosphorylation status of phototropin in the pulvinar motor cells through the binding of 14-3-3 protein to phototropin. Using the antibodies for Vicia phototropins, we found initially that phototropin with molecular mass of 118 kD was present in the motor cells (Fig. 2D). When the pulvini were irradiated with a pulse of blue light, 14-3-3 protein rapidly bound to phototropin and reached a maximum within 1 min after the pulse began, after which the binding decreased gradually, returning to the basal levels within 10 min (Fig. 2C). When the pulvini were irradiated with a second blue light pulse 20 min after the first pulse start, 14-3-3 protein bound again (data not shown). The blue light irradiation did not affect the amount of phototropin (Fig. 2D). The phototropin also exhibited a mobility shift to the upper side of the nonirradiated position, suggesting that the phototropin was phosphorylated in response to blue light as described previously (Short et al., 1993; Salomon et al., 2003; Knieb et al., 2004; Fig. 2, C and D). According to the time courses of 14-3-3 protein binding to phototropin, the maximum binding occurred earlier (at 1 min) than the maximum dissociation of 14-3-3 protein from the H+-ATPase (5 min; Fig. 2E).

Light Dependencies of Dissociation of 14-3-3 Protein from the H+-ATPase and Binding of 14-3-3 Protein to Phototropin

We determined the amounts of 14-3-3 protein bound to the H+-ATPase and to phototropin in pulvini in response to blue light with various fluence rates. Dark-adapted pulvini were irradiated with a pulse of blue light, and the reaction was stopped 1 min after the start of a pulse for phototropin and 5 min after the start for the H+-ATPase. As the fluence rate of blue light increased, the amount of 14-3-3 protein bound to the H+-ATPase decreased (Fig. 3A), whereas the amount

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![Figure 2. Time courses of 14-3-3 protein binding to phototropin and H+-ATPase in pulvini in response to blue light pulse. Dark-adapted pulvini were irradiated with a pulse of blue light for 30 s at 100 μmol m⁻² s⁻¹, then reactions were stopped by disrupting the pulvini at 1, 2.5, 5, and 10 min after the start of a pulse. Microsomal membranes obtained from pulvini (4 μg protein for the H+-ATPase and 30 μg protein for phototropin) were subjected to SDS-PAGE. A, Binding of 14-3-3 protein to the H+-ATPase. Protein-blot analysis was performed using GST-14-3-3 fusion protein as a probe. B, Immunoblotting of the H+-ATPase. Anti-Vicia-H+-ATPase antibodies were used. C, Binding of 14-3-3 protein to phototropin. Protein-blot analysis was done with GST-14-3-3 fusion protein as a probe. D, Immunoblotting of phototropin. Anti-Vphphot1a antibodies were used. A broken line shows the initial size of phototropin in the dark. The GST probe was bound to neither the H+-ATPase nor phototropin (data not shown). E, Time courses of 14-3-3 protein binding to phototropin and the H+-ATPase in response to a pulse of blue light. The amount of 14-3-3 protein bound was quantified by the public domain NIH Image program (developed at the United States National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image) from images of the protein-blot analysis. Values presented are means of three independent experiments with st.s.](http://rsb.info.nih.gov/nih-image)
bound to phototropin increased (Fig. 3A). Both reactions were saturated near 50 μmol m⁻² s⁻¹ when the pulse duration was 30 s (Fig. 3E). The half saturation of the fluence rates for the dissociation of 14-3-3 protein from the H⁺-ATPase and the binding of 14-3-3 protein to phototropin were 11.2 μmol m⁻² s⁻¹ and 7.4 μmol m⁻² s⁻¹, respectively, suggesting that both reactions have essentially the same fluence rate dependency (Fig. 3E). The mobility of phototropin on SDS-PAGE decreased with increase in the fluence rate (Fig. 3D), suggesting that the levels of phototropin phosphorylation increased by blue light (Kinoshita et al., 2003; Salomon et al., 2003).

**Phosphorylation Levels of the H⁺-ATPase and Phototropin in Response to Blue Light in Pulvini**

We considered that the binding of 14-3-3 protein to the plasma membrane H⁺-ATPase reflected phosphorylation levels of the H⁺-ATPase. However, there is no direct evidence for this in pulvinar motor cells. We tried to radiolabel the H⁺-ATPase by administration of ³²P to the cells to determine the phosphorylation status, but it was not successful probably due to insufficient incorporation of ³²P into the motor cells. We thus tested phosphorylation levels of the H⁺-ATPase by blue light-dependent mobility shift of the C terminus on SDS-PAGE as shown in Vicia guard cells (Kinoshita and Shimazaki, 1999). We expected that a 16-kD peptide would be released from the C terminus when the Phaseolus H⁺-ATPase was fragmented by cyanogen bromide (CNBr) on the basis of amino acid sequence (CAA59799). The peptide would show the lower mobility when it was phosphorylated. We digested microsomal fractions according to the previous method (Kinoshita and Shimazaki, 2001). We obtained two peptides with molecular masses of 16 and 16.5 kDa, both of which were recognized by the antibodies against the C terminus (Fig. 4A, left), and found that the amount of upper 16.5-kD protein decreased by blue light with a slight increase in the amount of 16-kD peptide (Fig. 4A, right). The result suggests that 16.5-kD peptide is the phosphorylated C terminus of Phaseolus H⁺-ATPase and that the phosphorylated peptide was decreased by blue light. Furthermore, we indicated that only the 16.5-kD peptide had the binding ability to 14-3-3 protein and that the amount of this binding decreased by blue light (Fig. 4B). These results indicate that dissociation of 14-3-3 protein from the H⁺-ATPase by blue light is closely related to the phosphorylation and dephosphorylation of the C terminus of H⁺-ATPase.

Phosphorylation levels of phototropin were also determined by the mobility shift of the protein on SDS-PAGE in response to blue light (Short et al., 1993; Salomon et al., 2003; Knieb et al., 2004). In this study, we have already shown the mobility shift of phototropin by blue light (Figs. 2D and 3D). We confirmed the mobility shift of phototropin by blue light and that the shift was strongly suppressed by a Ser/Thr kinase inhibitor K-252a (Fig. 4C). The binding of 14-3-3 protein to phototropin was inhibited by K-252a in the same way (Fig. 4D). The results suggest that the binding of 14-3-3 protein to phototropin depends on the phosphorylation levels of phototropin.

**Evidence for Dissociation of 14-3-3 Protein from the H⁺-ATPase and Binding of 14-3-3 Protein to Phototropin in Vivo**

To investigate whether the binding and dissociation of 14-3-3 protein in both the H⁺-ATPase and phototropin occurs in vivo, we immunoprecipitated these proteins with individual antibodies and inspected the coprecipitated proteins. Microsomal fractions were isolated from the pulvini that were kept in darkness or irradiated with blue light. As shown in Figure 5A, 14-3-3 protein was coprecipitated with the H⁺-ATPase, and the amount of the 14-3-3 protein decreased by 58% in response to blue light. The result indicates that the 14-3-3 protein dissociated from the H⁺-ATPase in vivo.

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**Figure 3.** Fluence rate dependencies in the binding of 14-3-3 protein to the H⁺-ATPase and phototropin in response to blue light. Pulvini were irradiated with a pulse of blue light for 30 s at indicated fluence rates. Reactions were stopped at 1 min for phototropin and 5 min for the H⁺-ATPase after the pulse start. Other procedures were the same as in Figure 2. A, Binding of 14-3-3 protein to the H⁺-ATPase. B, Immunoblotting of the H⁺-ATPase. C, Binding of 14-3-3 protein to phototropin. D, Immuno blotting of phototropin. A broken line shows the size of phototropin without blue light irradiation. E, Fluence rate dependencies of 14-3-3 protein binding to phototropin and the H⁺-ATPase. Relative values are represented and were obtained by three independent experiments with six.
Figure 4. Mobility shift and binding of 14-3-3 protein in the C terminus of H^+\text{-}ATPase and phototropin in pulvini by blue light. A and B, Phaseolus pulvini were irradiated with a pulse of blue light for 30 s at 100 \mu mol m^{-2} s^{-1}. Reactions were stopped at 5 min after the pulse start. Microsomal fractions were immediately isolated and were treated with CNBr, and then subjected to SDS-PAGE. A, The C terminus of H^+\text{-}ATPase was visualized by immunoblotting with anti-C-terminus antibodies. B, Binding of 14-3-3 protein to the C terminus of H^+\text{-}ATPase. Protein-blot analysis was done with GST-14-3-3 fusion protein as a probe. C and D, A Ser/Thr kinase inhibitor, K-252a at 10 \mu M, was administered to excised pulvini in Murashige and Skoog medium, and the pulvini were incubated for 30 min in the dark. After irradiation of the pulvini with blue light for 30 s, microsomal fractions were prepared at 1 min after the pulse start. The isolated microsomal proteins were separated on SDS-PAGE. C, Phototropin was visualized by immuno blotting with anti-Vfphot1a antibodies. D, Binding of 14-3-3 protein to phototropin. Protein-blot analysis was done with GST-14-3-3 fusion protein as a probe. K-252a was dissolved in dimethyl sulfoxide (DMSO). Final concentrations of DMSO were 0.25%.

by blue light. We confirmed that the same amount of H^+\text{-}ATPase was used in this experiment (Fig. 5B).

We next immunoprecipitated the proteins using antibodies against 14-3-3 protein with microsomal fractions. We found that phototropin was coprecipitated together with 14-3-3 protein only when the pulvini were irradiated by blue light (Fig. 5C). The result demonstrates that the 14-3-3 protein bound to phototropin in vivo by blue light. The same amounts of 14-3-3 protein were used for the immunoprecipitation (Fig. 5D). If we did the immunoprecipitation using antibodies against phototropin, we could not find 14-3-3 protein in the immunoprecipitate in Phaseolus pulvini as shown previously in Vicia guard cells (Kinoshita et al., 2003).

Requirement of Phototropin Phosphorylation for the Dephosphorylation of the Plasma Membrane H^+\text{-}ATPase

If phototropin functions in blue light signaling upstream of the plasma membrane H^+\text{-}ATPase in pulvinar motor cells, the suppression of phototropin activity would inhibit dephosphorylation of the H^+\text{-}ATPase. To test this, we investigated the effect of diphenylene iodonium (DPI), a flavoprotein inhibitor (O'Donnell et al., 1993; Kinoshita et al., 2003), on the binding of 14-3-3 protein to phototropin and on the dissociation of the protein from the H^+\text{-}ATPase. DPI at 100 \mu M inhibited the binding of 14-3-3 protein to phototropin by 57% and the dissociation of 14-3-3 protein from the H^+\text{-}ATPase by 53% (Fig. 6, A and B). The degrees of inhibition in these two reactions by DPI were almost the same. We next investigated the effect of K-252a, an inhibitor of Ser/Thr protein kinase, on both reactions. K-252a at 10 \mu M inhibited the binding to phototropin by 69% and the dissociation from the H^+\text{-}ATPase by 70% (Fig. 6, C and D). Similarly, staurosporine, a Ser/Thr protein kinase inhibitor, at 10 \mu M inhibited the binding of 14-3-3 protein to phototropin by 47% and its dissociation from the H^+\text{-}ATPase by 49% (Fig. 6, E and F). These observations indicate that these three lines of inhibitors inhibit phototropin function, thereby interrupting the signaling to the H^+\text{-}ATPase in pulvinar motor cells. They also suggest that phosphorylation of phototropin is required for dephosphorylation of the H^+\text{-}ATPase.

Cloning of Phototropins from Pulvini of Kidney Bean

We cloned phototropins from pulvini of kidney bean by reverse transcription (RT)-PCR and obtained three cDNAs encoding P. vulgaris phototropin 1a (PvPHOT1a), PvPHOT1b, and PvPHOT2. All Pvphots contained LOV1, LOV2, and Ser/Thr kinase domains, which were typically conserved in phototropins of other plants (Fig. 7A). The deduced protein sequences of PvPHOT1a, PvPHOT1b, and PvPHOT2 consisted of 976, 987, and 996 amino acids, respectively, and had molecular masses of 110, 111, and 112 kD, respectively.

We compared the full-length amino acid sequences of Pvphots with those of phototropin homologs in other higher plants, which are classified into two groups, phot1 and phot2, by ClustalX (Fig. 7B). PvPHOT1a and PvPHOT1b had identities of 68% and 69% with AtPHOT1, and 57% and 61% with AtPHOT2, respectively. PvPHOT2 had an identity of 72% with both AtPHOT1 and

Figure 5. Blue light-dependent coprecipitation of 14-3-3 protein with the H^+\text{-}ATPase and phototropin with 14-3-3 protein by immunoprecipitation. A and B, Immunoprecipitation for the microsomal membranes was done using antibodies against the H^+\text{-}ATPase. A, Coprecipitated 14-3-3 protein in the immunoprecipitate was visualized by immunoblotting with anti-VfPHOT1a antibodies. B, Immunoblotting of H^+\text{-}ATPase in microsomal fractions used in immunoprecipitation. C and D, Immunoprecipitation for microsomal membranes was done using antibodies against 14-3-3 protein. C, Coprecipitated phototropin in the immunoprecipitate was visualized by immunoblotting with anti-VfPHOT1a antibodies. D, 14-3-3 protein in the immunoprecipitate was visualized with anti-VfPHOT1a antibodies.
Osphot2, and 60% with Atphot1. Therefore, Pvphot1a and Pvphot1b were placed into a phot1 group, and Pvphot2 was placed into a phot2 group. Pvphot1a and Pvphot1b have high (77%) identities to Vfphot and Psphot1 in leguminous plants, but lower (about 68%) identities to phot1 in Arabidopsis (Fig. 7B). A phototropin placed in the phot2 group had not been identified in leguminous plants thus far.

Expression of PvPHOTs

To investigate the expression of PvPHOTs, we performed the RT-PCR analysis in leaves, roots, pulvini, and etiolated seedlings using specific primers for PvPHOT1a, PvPHOT1b, and PvPHOT2 (Fig. 7C, left sections). PvPHOT1a transcripts were found in etiolated seedlings, leaves, and pulvini. PvPHOT1b transcripts were found in all tissues investigated. PvPHOT2 transcripts were detected only in green tissue, such as leaves and pulvini.

We studied the effect of light on the expression of PvPHOTs mRNAs (Fig. 7C, right sections). Etiolated seedlings of 4-d-old plants, which were irradiated with white light at 75 μmol m^-2 s^-1 for 180 min, revealed an increase in PvPHOT2 mRNA with no detectable change in PvPHOT1a and PvPHOT1b mRNAs. This result agrees with the previous observations that light induces the expression of the phot2 group (Kanegae et al., 2000; Jarillo et al., 2001; Kagawa et al., 2001).
Figure 7. Cloning of phototropin cDNAs from pulvini in kidney bean. A, Alignment of the predicted Ppphot1a (AB204872), Ppphot1b (AB204873), and Ppphot2 (AB204874) amino acid sequences. Asterisks (*) show identical amino acid residues, double dots (:) show the conserved residues, and single dots(.) indicate the semiconserved residues. Dashes indicate gaps introduced to allow for optimal alignment of the sequences. The alignment was performed using ClustalX. The LOV1, LOV2, and kinase domains are underlined. The Cys residues for FMN binding are shaded black. B, A phylogenetic tree of the phototropin proteins from kidney bean and other plant species. This relation tree was constructed using the ClustalX program. Full-length amino acid sequences of Atphot1 (Arabidopsis; AF030864), Atphot2 (Arabidopsis; AF035391), Vfphot1a (broad bean; AB095909), Vfphot1b (broad bean; AB095910), Psphot1 (Pisum sativum; U83281), Zmphot1 (Zea mays; AF033263), Asphot1a (Avena sativa; AF033096), Asphot1b (A. sativa; AF033097), Osphot1 (Oryza sativa; AB018444), and Osphot2 (O. sativa; AB018443) were used to construct the tree. The phototropins from kidney bean are shaded black. The scale represents 0.1 substitutions/site. C, Expression of PpPHOT transcripts. RT-PCR analyses of PpPHOT1a, PpPHOT1b, and PpPHOT2 mRNA expression in etiolated seedlings, leaves, pulvini, and roots (left sections). Effect of light on expression levels of PpPHOT mRNAs. Etiolated seedlings were kept in the dark (D) or were exposed to white light at 75 μmol m⁻² s⁻¹ for 3 h (L), then total RNA was extracted and RT-PCR analysis was performed (right sections). ACTIN was used as an internal standard for cDNA amounts. Separate experiments repeated three times gave similar results.
DISCUSSION

Biochemical Evidence for the Inactivation of the Plasma Membrane H\(^{+}\)-ATPase by Blue Light in Pulvinar Motor Cells

The plasma membrane H\(^{+}\)-ATPase is known to be activated by phosphorylation of the penultimate Thr in the C terminus followed by 14-3-3 protein binding on this site (Fuglsang et al., 1999; Kinoshita and Shimazaki, 1999, 2002; Svennelid et al., 1999). In this study, we determined the phosphorylation and the amount of 14-3-3 protein bound to the C terminus of the H\(^{+}\)-ATPase in pulvinar motor cells in response to blue light (Figs. 2A and 4A). We found that the bound 14-3-3 protein transiently dissociated in response to a pulse of blue light (Fig. 2A), and that the degree of dissociation depended on the intensity of the light (Fig. 3A). The transient dissociation of 14-3-3 protein agrees with the transient decrease in ATP hydrolytic activity in pulvinar protoplasts in response to blue light as reported previously (Okazaki, 2002). These results demonstrate that the H\(^{+}\)-ATPase is inactivated by dephosphorylation of the phosphorylated Thr in the C terminus and by the subsequent dissociation of 14-3-3 protein in the motor cells by blue light.

Inactivation of the H\(^{+}\)-ATPase by blue light causes the depolarization of the plasma membrane (Nishizaki, 1994; Okazaki, 2002), leading to the efflux of K\(^+\) through the depolarization-activated K\(^+\) channels (Moran, 1996; Suh et al., 2000). The sustained efflux of K\(^+\) finally results in decreased turgor pressure of motor cells exposed to blue light, thereby inducing leaf movement. The membrane depolarization is likely elicited mainly by the inactivation of the plasma membrane H\(^{+}\)-ATPase because the depolarization is completely inhibited by the addition of vanadate, a specific inhibitor of the H\(^{+}\)-ATPase (Nishizaki, 1994). However, the membrane depolarization can be induced also by the activation of an anion channel in the motor cells. We note that the membrane depolarization is brought about by a blue light-activated anion channel in Arabidopsis hypocotyls, and the activation seems to be induced by cry1 and cry2 as blue light receptors (Spalding and Cosgrove, 1989; Cho and Spalding, 1996; Parks et al., 1998; Folta and Spalding, 2001).

Involvement of Phototropin in Blue Light-Induced Inactivation of the H\(^{+}\)-ATPase

Photoreceptors that mediate blue light-induced leaf movement in leguminous plants have not been identified. In Arabidopsis, phototropins mediate phototropism (Huála et al., 1997; Christie et al., 1998; Sakai et al., 2001), chloroplast relocations (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001), rapid inhibition of stem growth (Folta and Spalding, 2001; Parks et al., 2001), and leaf expansion (Sakamoto and Briggs, 2002). It is becoming clear that these responses mediated by phototropins optimize photosynthesis by increasing the efficiency of light energy capture through the integration of phototropin-mediated responses. Very recently, Takemiya et al. (2005) demonstrated that phototropins dramatically enhance the growth of Arabidopsis plants under low-light environments, a finding that indicates the improvement of photosynthetic performance by phototropins. In this study, in accord with the functional role of phototropins, we found that the leaf surface of kidney bean was oriented perpendicular to the light direction, and that the light-capturing area of the leaf was greatly increased in response to blue light (Fig. 1, A–D). Therefore, phototropins most likely play a role in kidney bean leaf movement.

In this study, we obtained three separate lines of evidence supporting the function of phototropin in the pulvinar motor cells as a photoreceptor in the leaf movement response (Figs. 2, 3, and 6). First, the phosphorylation of phototropin preceded the dephosphorylation of the H\(^{+}\)-ATPase in response to blue light (Fig. 2E). Second, the binding of 14-3-3 protein to phototropin and the dissociation of the protein from the H\(^{+}\)-ATPase had similar fluence rate dependencies (Fig. 3E). Third, the flavoprotein inhibitor and protein kinase inhibitors, which inhibit the activity of phototropin, also suppressed dephosphorylation of the H\(^{+}\)-ATPase (Fig. 6).

We recognized phototropin proteins by an immunological method (Figs. 2D, 3D, 4C, and 5C) and identified three 
\textit{PopHOT} cDNAs (Fig. 7C) in pulvini. However, we could not distinguish these isoproteins from each other because of their similar molecular masses.

Phototropin-Mediated Blue Light Signal Transduction to the Plasma Membrane H\(^{+}\)-ATPase

These results suggest that the phototropin-mediated signal inactivates the H\(^{+}\)-ATPase in pulvinar motor cells, while the same signal activates the H\(^{+}\)-ATPase in stomatal guard cells (Kinoshita et al., 2001). This difference in response between motor cells and guard cells seems to be generated by the downstream components in these distinct cell types. In this study, we found that the activation of phototropin resulted in dephosphorylation of the H\(^{+}\)-ATPase in motor cells (Figs. 2, 3, and 6). This observation clearly indicates that the H\(^{+}\)-ATPase is not a direct substrate for phototropin kinase and that there must be other components between phototropin and the H\(^{+}\)-ATPase.

It is noteworthy that the phosphorylation statuses of the H\(^{+}\)-ATPase are different between motor cells and guard cells. In pulvinar motor cells, the greater portion of the H\(^{+}\)-ATPase was phosphorylated on the C terminus in the dark (Fig. 4A), but in stomatal guard cells most of the H\(^{+}\)-ATPase were dephosphorylated under the same condition (Kinoshita and Shimazaki, 1999). This difference may be produced by the activity balance between a set of unidentified protein kinase(s) and protein phosphatase(s) whose substrates are the C terminus of plasma membrane H\(^{+}\)-ATPase. When the pulvini or stomata are irradiated with blue light,
activated phototropin would cause the change of activities in the opposite direction between motor cells and guard cells. For example, phototropin-mediated light signal may inactivate protein phosphatase in guard cells but activate the phosphatase in motor cells. From these, we hypothesize the presence of some regulatory proteins, other than the protein kinase(s) and phosphatase(s) mentioned above, between phototropin and the H⁺-ATPase in the blue light signaling pathway. It will be intriguing to identify such a regulator in order to understand the difference in phototropin-mediated regulation of the H⁺-ATPase between pulvini and stomata.

Inactivation of the H⁺-ATPase by blue light ultimately elicits shrinkage of the motor cells in the pulvinus of kidney bean. If this reaction occurs in plant cells of other tissues, it may bring about growth inhibition. For example, Folta and Spalding (2001) demonstrated that phot1 mediates the early phase of growth inhibition in Arabidopsis hypocotyls in response to blue light. This growth inhibition was not inhibited by 5-nitro-2-(3-phenylpropylamino)-benzoic, an anion channel blocker (Parks et al., 1998). Very recently, similar transient growth inhibition mediated by phototropin was also found in coleoptiles of rice (Oryza sativa) in response to blue light (Haga et al., 2005). Therefore, the early phase growth inhibition might be caused by inactivation of the H⁺-ATPase by blue light. Further investigation will be needed to generalize our hypothesis.

**Materials and Methods**

**Plant Materials and Growth Conditions**

Plants of kidney bean (Phaseolus vulgaris L.) cv Kentucky Wonder were grown for 2 weeks at 24°C with a relative humidity of 70% under a 12/12-h light-dark cycle or continuous light in a growth chamber (CU-250, Tomy, Tokyo). The light was obtained from fluorescent lamps (FL28S W/18, Toshiba, Tokyo) at 100 μmol m⁻² s⁻¹ on the leaf surface. Laminar pulvini from a pair of first leaves (Fig. 1F) were excised with a razor blade and were used in all experiments. We used these whole pulvini without dividing them into flexor and extensor cells because blue light induces membrane depolarization in motor cells on both the flexor and extensor sides (Nishizaki, 1988).

**Light Source**

A pulse of blue light was obtained from a tungsten lamp (EXR 150W, Sylvania, Danvers, MA) by passing the light through a blue glass filter (5-60, Corning, Corning, NY) and was used for the irradiation of excised pulvini. Continuous blue light was obtained from light-emitting photodiodes (Stick-B-32, Eyela; maximum intensity at 470 nm) and was used for the irradiation of the intact plant. Photon flux densities were measured with a quantum meter (model 185A, LI-COR, Lincoln, NE).

**Microsomal Membrane Preparation from Pulvini**

We isolated microsomal membrane fractions from pulvini to determine of the phosphorylation statuses of both phototropin and the plasma membrane H⁺-ATPase. Thirty to 35 pulvini were placed on a wet paper towel in a petri dish and illuminated with a 30-s blue light pulse, and were homogenized at indicated times in an ice-cold buffer (50 mM MOPS-KOH pH 7.5, 2.5 mM EDTA, 100 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 1 mM dithiothreitol, 1 mM ammonium molybdate, 10 mM NaF; 50 mM Calyculin A) using a mortar and pestle. The homogenate was centrifuged at 12,000 rpm for 5 min, and the resulting supernatant was further centrifuged at 45,000 rpm for 45 min. Microsomal membranes obtained as a pellet were suspended in the same buffer. All procedures were performed under dim red light. These microsomal membranes were used for immunoblotting and protein-blot analysis.

**Immunoblot Analysis**

Immunoblotting was performed according to a previous method (Kinoshita et al., 2003). The polyclonal antibodies against Vhpho1A, Vicia H⁺-ATPase, the C terminus of H⁺-ATPase, and Vh4-3-3a were described previously (Kinoshita and Shimazaki, 1999; Kinoshita et al., 2003).

**Protein-Blot Analysis**

Protein-blot analysis was performed according to a previous method (Kinoshita et al., 2003). The pGEK14-3-3-3 plasmid, containing full-length GI4phi cDNA (L09111), was constructed according to a previously described method (Kinoshita and Shimazaki, 1999). This 14-3-3 protein fused to glutathione S-transferase (GST) was used as a probe in this analysis.

**Immunoprecipitation**

Immunoprecipitation of the H⁺-ATPase and 14-3-3 protein was done using the individual antibodies as described previously (Kinoshita and Shimazaki, 1999) with minor modifications. Isolated microsomal membranes were incubated with antibodies at 0.5% (v/v) and protein A-agarose at 3% (v/v; Santa Cruz Biotech, Santa Cruz, CA) in the reaction buffer (50 mM MOPS-KOH pH 7.5, 2.5 mM EDTA, 100 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 1 mM dithiothreitol, 50 mM Calyculin A, 0.3% (v/v) Triton X-100) at 4°C for 3 h with gentle mixing. Immunoprecipitates were washed three times with Tris-buffered saline. Each sample was solubilized with SDS-PAGE sample buffer and subjected to SDS-PAGE.

**Digestion of the H⁺-ATPase in Phaseolus by CNBr**

The microsomal proteins were digested by CNBr at 100 μg/ml in 70% (v/v) formic acid for 4 h at room temperature with gentle rotation according to the previous method (Kinoshita and Shimazaki, 2001). The fragmented proteins were dried, and the dried peptides were dissolved in 200 μL of water, and then dried again. The resulting peptides were subjected to SDS-PAGE.

cDNA Cloning of PvPHOT1α, PvPHOT1b, and PvPHOT2 from Pulvini

Total RNA was isolated from pulvini with ISOGEN (Nippon Gene, Toyko) according to the manufacturer’s instructions. The first-strand cDNAs were synthesized from the total RNA of pulvini by the SuperScript III RT First-Strand Synthesis System (Invitrogen, Carlsbad, CA) using Oligo(dT) primer. Two degenerate oligonucleotide primers (5’-CCNGAYAYCCNATHATHTGYGC-3’ and 5’-TAYCTYCNGTNCCACRAA-3’) were designed on the sequences of consensuses in phototropin LOV2 and kinase as described previously (Kinoshita et al., 2003). These oligonucleotide primers were used for degenerate PCR using the first-strand cDNAs as a template. The cDNA fragments obtained were inserted into pcR4-TOPO (Invitrogen) and the sequences were determined. Gene-specific primers for 5’ and 3’ RACE were designed on the basis of these fragment sequences. The 5’ and 3’ RACE were performed using the GeneRacer Kit (Invitrogen) according to the manufacturer’s instructions with the following gene-specific primers: for 5’ RACE, 5’-CTCAGTTGCTGTACGTGTCAG-3’ (for PvPHOT1α), 5’-TTCTTAC- TATCAGAAGTGCTTACCTC-3’ (for PvPHOT1b), 5’-CTTGCACCTTTGACATTAACGTACATTGTC-3’ (for PvPHOT2); and for 3’ RACE, 5’-CTCAACAAGAATGCTGTCAG-3’ (for PvPHOT1α), 5’-AGGTTCTAAAGGGAAAGACGGCGAG-3’ (for PvPHOT1b), and 5’-TGGAACTCAAAGGTACTGGT-3’ (for PvPHOT2). The RACE PCR products were inserted into pcR4-TOPO and sequenced. To obtain full-length coding regions of PvPHOT1α, PvPHOT1b, and PvPHOT2, RT-PCRs were performed using gene-specific primers annealing to 5’ and 3’-untranslated regions: for PvPHOT1α, 5’-AGACTCGCAAAATGCTCGACACCT-3’ and 5’-TGAGACCTTTAAAGATCGACGGAC-3’; for PvPHOT1b, 5’-GACGAGGAGGCGGATGCTG-3’.
and 5′-GATCAAACTTGGACACCAGTGAAGGG-3′; and for PvPHOT2, 5′-CCCTTTGGTCCGTAGTTATCATCAAC-3′ and 5′-GCTCTGGTGCGAGAATGCCAGATCG-3′. The amplified full-length cDNAs were cloned into pCR-TOPO and sequenced.

Tissue-Specific Expression of PvPHOTs Determined by RT-PCR Analysis

Leaves, roots, and pulvini were harvested from 2-week-old plants. Ectopically seedlings were grown for 4 d in darkness. Total RNA was extracted from these tissues using ISOGEN (Nippon Gene). The first-strand cDNAs were synthesized from 5 μg of total RNAs by the SuperScript III RT First-Strand Synthesis System (Invitrogen) using Oligo(dT) primer. The obtained cDNAs were diluted 10-fold, and 0.5 μL was used in each PCR reaction. PvPHOTs cDNAs were amplified using the following oligonucleotide primers: for PvPHOT1α, 5′-ATTACAATATTTCTCCACAAGCCGCTAATTTCC-3′ and 5′-GTT-ACAACAGTGGCTCAGTGGGAAC-3′; for PvPHOT1β, 5′-TAAACCTAG-ACTGCTCCAGGGAAAG-3′ and 5′-CAGCAGACAGAATAATATATTGGCTTG-3′; and for PvPHOT2, 5′-TCTGTGCCCGCACTGTGATATG-3′ and 5′-TATATATTGTAATTGTGGACATATG-3′. As an internal standard for RT-PCR, we used ACTIN homolog (AB067722) cDNA of kidney bean using oligonucleotmers (5′-ATGGGCCAGAAATGCTATGTTG-3′ and 5′-TACCTTCATAGATGGGAGCGTGT-3′). PCR reactions were performed in 27 cycles on the left panels and 30 cycles on the right panels.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AB204872 to AB204874.

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