CIPK23 regulates blue light-dependent stomatal opening in Arabidopsis thaliana

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SUMMARY

Phototropins (phot1 and phot2) are plant blue light receptor kinases that function to mediate phototropism, chloroplast movement, leaf flattening, and stomatal opening in Arabidopsis. Considerable progress has been made in understanding the mechanisms associated with phototropin receptor activation by light. However, the identities of phototropin signaling components are less well understood by comparison. In this study, we specifically searched for protein kinases that interact with phototropins by using an in vitro screening method (AlphaScreen) to profile interactions against an Arabidopsis protein kinase library. We found that CBL-interacting protein kinase 23 (CIPK23) interacts with both phot1 and phot2. Although these interactions were verified by in vitro pull-down and in vivo bimolecular fluorescence complementation assays, CIPK23 was not phosphorylated by phot1, as least in vitro. Mutants lacking CIPK23 were found to exhibit impaired stomatal opening in response to blue light but no deficits in other phototropin-mediated responses. We further found that blue light activation of inward-rectifying K+ (K+in) channels was impaired in the guard cells of cipk23 mutants, whereas activation of the plasma membrane H+-ATPase was not. The blue light activation of K+in channels was also impaired in the mutant of BLUS1, which is one of the phototropin substrates in guard cells. We therefore conclude that CIPK23 promotes stomatal opening through activation of K+in channels most likely in concert with BLUS1, but through a mechanism other than activation of the H+-ATPase. The role of CIPK23 as a newly identified component of phototropin signaling in stomatal guard cells is discussed.

INTRODUCTION

Phototropins (phot1 and phot2) are plasma membrane-associated, autophosphorylating blue light receptor kinases that induce a range of physiological responses in Arabidopsis thaliana, which help optimize photosynthetic efficiency under weak light conditions by increasing light capture and CO2 absorption in leaves (Christie et al., 1998; Takemiya et al., 2005; Inoue et al., 2010; Gotoh et al., 2018). These processes include phototropism, chloroplast photorelocation movement, leaf flattening, leaf positioning,
and stomatal opening (Christie, 2007; Inoue et al., 2008a; Demarsy and Fankhauser, 2009). Despite extensive efforts by many researchers, the primary signaling events associated with each of these responses remain largely unknown (Christie et al., 2015).

Many phototropin interaction partners have been identified (Inoue et al., 2010), four of which, ATP-BINDING CASSETTE B19 (ABCB19), PHYTOCHROME KINASE SUBSTRATE 4 (PKS4), BLUE LIGHT SIGNALING 1 (BLUS1), and CONVERGENCE OF BLUE LIGHT AND CO2 (CBC) 1, have been shown to be direct substrate targets (Christie et al., 2011; Demarsy et al., 2012; Takemiya et al., 2013; Hiyama et al., 2017). NON-PHOTOTROPIC HYPOCHOTYL 3 (NPH3), ROOT PHOTOTROPISM 2 (RPT2), and PKS1, 2, and 4 positively regulate phototropism, leaf flattening, and leaf positioning but do not regulate chloroplast movement and stomatal opening (Motchoulski and Liscum, 1999; Sakai et al., 2000; Inada et al., 2004; Lariguet et al., 2006; Inoue et al., 2008a; de Carbonnel et al., 2010; Harada et al., 2013; Tsutsumi et al., 2013). While PKS4 is phosphorylated by phot1 in a blue light-dependent manner, phosphorylation negatively regulates its action on phototropism (Demarsy et al., 2012; Schumacher et al., 2018). In addition, the auxin efflux transporter ABCB19 has been shown to be a substrate for phot1 kinase activity (Christie et al., 2011). Phosphorylation of ABCB19 is proposed to inhibit its efflux activity and indirectly promote auxin fluxes involved in phototropism (Christie et al., 2011). The guard cell-specific protein kinase BLUS1 is phosphorylated by both phot1 and phot2 in response to blue light, and phosphorylated BLUS1 mediates stomatal opening (Takemiya et al., 2013). CBC1 is phosphorylated by phot1 and phot2 in guard cells, and CBC1 and the closest homolog CBC2 regulate blue light-induced stomatal opening in both positive and negative manners (Hiyama et al., 2017; Hayashi et al., 2020). The roles of CBC1 phosphorylation are not understood at the present time. Together, these findings indicate that the primary signaling events associated with different phototropin-mediated responses are likely to be distinct, with signal propagation diverging from the phosphorylation of specific substrates.

Blue light-driven stomatal opening is one of the most characterized phototropin signaling pathways to date (Shimazaki et al., 2007; Inoue et al., 2010). Stomatal opening, which facilitates gaseous exchange between plants and the atmosphere, is driven by the swelling of stomatal guard cells in response to blue light (Zeiger and Hepler, 1977; Shimazaki et al., 2007). This swelling is achieved by increased turgor pressure in guard cells, which is induced by hyperpolarization of the plasma membrane and subsequent K\(^+\) uptake via inward-rectifying K\(^+\) (K\(^{\text{in}}\)) channels at the membrane (Schroeder et al., 1987; Schroeder and Hedrich, 1989; Schroeder et al., 2001; Marten et al., 2010). Membrane hyperpolarization is generated by activation of the guard cell plasma membrane H\(^+\)-ATPase (Assmann et al., 1985; Shimazaki et al., 1986; Blatt, 1987; Yamauchi et al., 2016). The H\(^+\)-ATPase is activated in a blue light- and phototropin-dependent manner by phosphorylation of its C-terminus and subsequent binding of 14-3-3 proteins (Kinosita and Shimazaki, 1999; Palmgren, 2001; Ueno et al., 2005; Hayashi et al., 2011). Phototropin autophosphorylation leads to H\(^+\)-ATPase activation via BLUS1 kinase activity, as well as the involvement of BLUE LIGHT-DEPENDENT H\(^+\)-ATPASE PHOSPHORYLATION (BHP) and type 1 protein phosphatases (Kinosita et al., 2001; Takemiya et al., 2006; Inoue et al., 2006b; Takemiya et al., 2013; Hayashi et al., 2017). However, knowledge of the signaling events coupling blue light sensing by the phototropins to activation of the H\(^+\)-ATPase is incomplete. For instance, the identity of the kinase responsible for phosphorylation of the H\(^+\)-ATPase is still lacking.

While it is well accepted that activation of the H\(^+\)-ATPase is the main driver of stomatal opening, there is additional evidence to support the involvement of two other modes of signaling and regulation. The first involves phototropin-mediated suppression of plasma membrane anion channels that mediate stomatal closure, whereby their inhibition promotes stomatal opening through an increase in plasma membrane hyperpolarization (Marten et al., 2007). The other pathway involves phototropin modulation of K\(^{\text{in}}\) channel activity, which is thought to facilitate H\(^+\)-ATPase-driven stomatal opening through the uptake of K\(^+\) across the plasma membrane (Zhao et al., 2012). It is therefore possible that phototropin substrates besides BLUS1 could be involved in either of these signaling pathways. Here we adopted the Amplified Luminescent Proximity Homogeneous Assay Screen (AlphaScreen) methodology (PerkinElmer Life Sciences) to determine whether phototropins could interact with protein kinases other than BLUS1. In doing so, we identified CBL-interacting protein kinase 23 (CIPK23) as a positive regulator in blue light-dependent stomatal opening in Arabidopsis. Furthermore, we propose that CIPK23 constitutes a distinct signaling pathway involved in phototropin-driven stomatal opening and contributes to this response through the activation of K\(^{\text{in}}\) channels.

RESULTS

Identification of CIPK23 as a phototropin-interacting protein

To identify novel kinase-interacting partners of Arabidopsis phototropins, we performed an in vitro protein–protein interaction screen using AlphaScreen technology in combination with a wheat germ cell-free protein synthesis system as reported previously (Hayashi et al., 2020). RIKEN Arabidopsis Full-Length (RAFL) cDNA clones were used to construct an Arabidopsis protein kinase library (562 protein
kinases) which were expressed individually by an in vitro transcription and translation system (Sawasaki et al., 2002; Kawasaki et al., 2004; Nemoto et al., 2011). In this system, a luminescent signal is generated when donor and acceptor beads are brought into close proximity. The donor beads generate singlet oxygen by excitation and the acceptor beads emit light by reacting with singlet oxygen. When two beads are in close proximity, the acceptor beads are able to receive singlet oxygen from the donor beads. The proximity of the beads depends on the interaction between proteins that have been conjugated onto the beads (Figure 1a). Phot1 or phot2 and each protein kinase from the library were bound onto acceptor and donor beads as bait and prey, respectively. We then screened this library for kinases that interact with phot1 and phot2 using the AlphaScreen approach and identified CIPK23 as a candidate. A negative control protein, dihydrofolate reductase (DHFR), showed a slight interaction with phot1 and phot2 in this system that was expressed as a luminescence signal in the AlphaScreen (Figure 1b). By contrast, CIPK23 showed a high level of interaction with both phot1 and phot2. The extent of CIPK23 binding to phot1 was similar to that of NPH3 to phot1, which served as a positive control in our analysis (Motchoulski and Liscum, 1999; de Carbonnel et al., 2010).

The interaction between phototropins and CIPK23 was confirmed by in vitro pull-down assay. FLAG-tagged CIPK1, CIPK23, or CIPK24 was expressed and purified from Escherichia coli cells using anti-FLAG antibody-conjugated agarose beads. Purified FLAG-tagged CIPK proteins were then incubated with microsomal membranes from Arabidopsis seedlings. Immunoprecipitation analysis showed that FLAG-CIPK23 co-purified with both phot1 and phot2 from microsomal membrane fractions, but CIPK1 and CIPK24 did not (Figure 1c). These results indicate that CIPK23 interacts with both phot1 and phot2 in vitro.

To verify the occurrence of the CIPK23-phototropin interactions in plant cells, we performed bimolecular fluorescence complementation (BiFC) in tobacco (Nicotiana benthamiana) leaves as reported previously (Kaiserli et al., 2009). Both phot1 and phot2 were found to interact with CIPK23 in the tobacco epidermal cells (Figure 2), whereas no BiFC signal was detected when CIPK24 and empty vectors were used in the series of co-expression experiments (Figure 2; Figures S1 and S2). Taken together, these findings further demonstrate that CIPK23 specifically interacts with phot1 and phot2 in vivo.

CIPK23 is not required for phototropin-mediated phototropism, chloroplast movement, leaf flattening, and promotion of plant growth

To investigate the functions of CIPK23 in phototropin-mediated blue light responses, we obtained two transfer DNA insertional knockout mutants, cipk23-1 (SALK_032341) and cipk23-5 (SALK_138057) (Figure S3a; Cheong et al., 2007; Nieves-Cordones et al., 2012). We confirmed the absence of transcripts of full-length CIPK23 in either of these knockout mutants by reverse transcriptase (RT)-PCR (Figure S3b).
We next confirmed the abundance of CIPK23 transcripts in various tissues from 4-week-old Arabidopsis plants. RT-PCR analysis indicated that CIPK23 transcripts were expressed in guard cell protoplasts (GCPs), mesophyll cell protoplasts (MCPs), rosette leaves, petioles, inflorescence stems, and roots with a higher level being detected in GCPs (Figure S3c). Ubiquitous expression of CIPK23 was previously shown by promoter-GUS expression assay (Cheong et al., 2007) and ubiquitous expression of phot1 and phot2 proteins was similarly confirmed (Kagawa et al., 2001; Sakamoto and Briggs, 2002). These findings suggest that CIPK23 could play a role in phototropin-mediated responses.

We explored whether phototropin-mediated responses were altered in cipk23 mutants given that CIPK23 is ubiquitously expressed. First, phototropic curvature was determined using etiolated seedlings. The hypocotyls of cipk23-1 and cipk23-5 mutants showed phototropic bending in response to unilateral blue light (0.1 µmol m⁻² sec⁻¹) comparable to that found for wild-type (Col) seedlings, unlike the phot1phot2 double mutant (Figure 3a). Next, we determined whether blue light-induced chloroplast relocation movements were altered in cipk23 mutants by slit band assay (Suetsum et al., 2005; Inoue et al., 2011) (Figure 3b). Irradiation of wild-type leaves with weak blue light (1 µmol m⁻² sec⁻¹) through a 1-mm slit promotes chloroplast accumulation in this region and the appearance of a darker green band (Figure 3b: upper panel). Conversely, a white band is produced when this area is irradiated with strong blue light (90 µmol m⁻² sec⁻¹) owing to chloroplast avoidance movement (Figure 3b: lower panel). Similarly, leaves of cipk23 mutants showed both green and white bands in response to weak and strong blue light, respectively. These results indicate that cipk23 mutants were not altered in chloroplast accumulation and avoidance responses.

The rosette leaves of the phot1phot2 double mutant are epinastic and curl downward at the side in comparison to the leaves from wild-type plants (Figure 3c). This leaf-flattening response was still apparent in cipk23 mutants. Moreover, cipk23 mutants showed normal growth under our conditions, comparable to that of wild-type plants, unlike the phot1phot2 double mutant (Figure 3d).

Taken together, the above findings demonstrate that mutants lacking CIPK23 are not altered in phototropin-mediated phototropism, chloroplast movement, and leaf flattening in the tested conditions.

**CIPK23 is required for phototropin-mediated stomatal opening**

Given the prevalence of CIPK23 expression in GCPs (Figure S3c), we determined whether blue light-dependent stomatal opening was altered in the cipk23 mutants. Stomata in the leaf epidermis of wild-type plants opened in response to blue light under a background of red light, but did not open in response to red light treatment (Figure 4a). This blue light response is mediated by the phototropins and is absent in the stomata of the phot1phot2 double mutant (Inoue et al., 2006; Takemiya et al., 2013; Inoue et al., 2011; Hayashi et al., 2017). Blue light-dependent stomatal opening was found to be impaired in epidermal peels isolated from cipk23-1 and cipk23-5 mutants (Figure 4a).

The defect in blue light-induced stomatal opening observed in cipk23 mutants was investigated in more detail. Changes in leaf temperature are known to reflect differences in stomatal aperture. For instance, water evaporation via open stomata results in a decrease in leaf temperature (Merlot et al., 2002; Hashimoto et al., 2006; Takemiya et al., 2013; Inoue et al., 2017). The leaf temperature of cipk23 mutants was higher compared to that of wild-type leaves under our growth conditions (Figure 4b), consistent with their inability to open stomata in response to blue light (Figure 4a). The result suggests that water evaporation is reduced in cipk23 mutants since stomatal opening is reduced under the light conditions examined.
Finally, we measured the stomatal conductance of intact leaves in response to light (Figure 4c). Stomatal conductance in wild-type leaves increased in response to strong red light (600 \( \mu \text{mol m}^{-2} \text{ sec}^{-1} \)) and reached a steady state after approximately 30 min (Figure 4c: upper graph). By comparison, this rate of increase was slightly reduced in the cipk23-5 mutant (Figure 4c,e). Stomatal conductance in wild-type leaves was further increased following irradiation with weak blue light (10 \( \mu \text{mol m}^{-2} \text{ sec}^{-1} \)) superimposed onto the background of red light (Figure 4c: upper graph). However, the magnitude and rate of this increase was largely reduced in the leaves of the cipk23-5 mutant (Figure 4c-e). From the analysis of stomatal responses, we conclude that CIPK23 plays a role in mediating blue light-dependent stomatal opening in Arabidopsis.

**Blue light activation of the plasma membrane H\(^+\)-ATPase is unaltered in the guard cells of cipk23 mutants**

Blue light activates the plasma membrane H\(^+\)-ATPase via phototropins through phosphorylation in guard cells. Activation of the H\(^+\)-ATPase generates the driving force for stomatal opening (Shimazaki et al., 2007; Inoue et al., 2010; Yamauchi et al., 2016; Inoue and Kinoshita, 2017). We therefore prepared GCPs from the rosette leaves of wild-type and cipk23-5 mutant plants, and measured H\(^+\) pumping activity in response to blue light (Figure S4a). GCPs from wild-type or cipk23-5 plants both showed a similar magnitude and rate of H\(^+\) pumping in response to blue light (Figure S4a; Table S1). We further determined whether blue light-dependent phosphorylation of the H\(^+\)-ATPase was altered in GCPs isolated from cipk23-5 through 14-3-3 protein binding by far-Western blotting (Figure S4b). GCPs from the cipk23-5 mutant exhibited similar levels of H\(^+\)-ATPase phosphorylation to those detected in GCPs from wild-type rosette leaves. Moreover, the abundance of H\(^+\)-ATPase was unaltered in GCPs from the cipk23-5 mutant. These results indicate that the H\(^+\)-ATPase is normally activated by blue light in the absence of CIPK23 and further suggest that signaling components downstream of the H\(^+\)-ATPase were impaired in cipk23 mutants.

The fungal toxin fusicoccin (FC) directly activates the plasma membrane H\(^+\)-ATPase and induces stomatal opening (Figure 3). Fusicoccin activates the plasma membrane H\(^+\)-ATPase and induces stomatal opening in Arabidopsis thaliana wild-type (Col), cipk23-1, cipk23-5, and phot1phot2. Etiolated seedlings were irradiated with unilateral blue light at 0.1 \( \mu \text{mol m}^{-2} \text{ sec}^{-1} \) for 14 h. Values are means ± SE (\( n = 30-54 \)). Differences from wild-type plants were evaluated using Student’s \( t \) test (\( **P < 0.01 \)).

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opening in the dark (Kinoshita and Shimazaki, 2001; Kinoshita et al., 2001). In darkness, the stomata in the epidermis of wild-type Arabidopsis opened in response to FC treatment in a dose-dependent manner and the aperture reached a maximum at 5 µM (Figure 5a). Stomata of the cipk23-1 and cipk23-5 mutants showed a similar response to 10 µM FC treatment as did those from wild-type plants, as well as the phot1phot2 double mutant in the dark (Figure S5). Interestingly, further increases in stomatal opening were observed in the epidermis of wild-type plants when blue light was introduced following FC treatment (Figure 5b). By contrast, stomata in the phot1phot2 double mutant did not show this increase in stomatal opening after blue light irradiation. These data therefore provide evidence for the presence of other factors in addition to the H⁺-ATPase that are required for phototropin-mediated stomatal opening. These likely include CIPK23 since the cipk23-5 mutant also failed to show an increase in stomatal opening when irradiated with blue light following FC treatment in darkness (Figure 5b).

CIPK23 is required for blue light activation of K⁺_in channels

Recent experiments, using a voltage-clamp technique, have demonstrated that blue light increased plasma membrane K⁺_in channel currents by about 50% in guard cells and this increment was completely lost in phot1phot2 guard cells (Zhao et al., 2012). In addition, CIPK23 promotes K⁺ uptake through direct activation of the AKT1 K⁺_in channel in roots (Li et al., 2006; Xu et al., 2006). Given our present findings, we rationalized that CIPK23 could be involved in mediating the signaling between the phototropins and K⁺_in channels. To test this possibility, we determined the activity of K⁺_in channels in GCPs isolated from the cipk23-5 mutant by monitoring the K⁺ current by

Figure 4. Effect of CIPK23 mutations on blue light-dependent stomatal opening.
(a) Blue light-dependent stomatal opening in the epidermis of wild-type (Col), phot1phot2, cipk23-1, and cipk23-5 plants. Epidermal peels were isolated from dark-adapted plants, and irradiated with red light (50 µmol m⁻² sec⁻¹; R) with or without blue light (10 µmol m⁻² sec⁻¹; R + B) for 3 h. Values are means of three independent experiments with standard deviations. In each experiment, 45 stomata were measured.
(b) Infrared thermal images of wild-type (Col), cipk23-1, and cipk23-5 plants. Plants were grown in well-watered conditions for 3 weeks under white light at 50 µmol m⁻² sec⁻¹.
(c) Stomatal conductance changes in response to red and blue light in intact leaves from wild-type (Col) and cipk23-5 plants. Red light (600 µmol m⁻² sec⁻¹; RL) and blue light (10 µmol m⁻² sec⁻¹; BL) were switched on/off at the times indicated by the arrowheads.
(d, e) Magnitude (d) and the maximum rate (e) of stomatal conductance in response to red and blue light. Data represent the means ± SD of four independent experiments. Differences were evaluated using Student’s t test (*P < 0.05, **P < 0.01).
whole-cell patch clamping. No difference in \( K^{+}_{\text{in}} \) channel activity was observed between wild-type GCPs and those from the cipk23-5 mutant under dark conditions (Figure 6a). A short pulse (30 sec) of blue light led to a rapid (within 5 min), 2-fold increase in \( K^{+}_{\text{in}} \) channel activity in wild-type GCPs, as reported previously (Figure 6a; Zhao et al., 2012). By contrast, this change in \( K^{+} \) current in response to blue light was highly diminished in GCPs from the cipk23-5 mutant (Figure 6a). Steady-state current-voltage curves also showed that \( K^{+}_{\text{in}} \) currents increased in response to blue light in wild-type GCPs, whereas this was decreased in the case of cipk23-5 (Figure 6b,c; Figure S6a,b). These findings therefore suggest that blue light-induced changes in guard cell \( K^{+}_{\text{in}} \) currents are impaired in the cipk23-5 mutant. Thus, CIPK23 likely couples phototropin activation to changes in \( K^{+}_{\text{in}} \) currents at the guard cell plasma membrane.

Recent studies have shown that the guard cell-specific kinase BLUS1, which mediates blue light-dependent stomatal opening, acts as a substrate for phototropin kinase BLUS1, which mediates blue light-dependent stomatal opening in darkness was unaffected in the cipk23-5 mutant but was affected under blue light conditions (Figure 5; Figure S5). Furthermore, the plasma membrane H\(^{+}\)-ATPase was normally activated in response to blue light in cipk23-5 guard cells (Figure S4). Thus, we assume that CIPK23 is involved in a signaling pathway which is different from that associated with phototropin-dependent activation of the plasma membrane H\(^{+}\)-ATPase for stomatal opening. Finally, we found that blue light activation of the \( K^{+}_{\text{in}} \) channel current was impaired in the guard cells of the cipk23-5 mutant compared to wild-type guard cells (Figure 6; Figure S6). From these results, we conclude that CIPK23 acts as a positive regulator in stomatal opening and mediates blue light signaling from phototropins to the \( K^{+}_{\text{in}} \) channels in guard cells (Figure S10). However, previous studies have indicated that CIPK23 functions as a negative regulator of ABA or water stress signaling in guard cells (Cheong et al., 2007; Nieves-Cordones et al., 2012). In this case, mutations in CIPK23 were found to enhance ABA- or water stress-dependent inhibition of light-induced stomatal opening. It is therefore reasonable to interpret

**DISCUSSION**

**CIPK23 is a positive regulator of phototropin-mediated stomatal opening**

Mutants lacking CIPK23 showed impaired blue light-dependent stomatal opening in both experiments using epidermal fragments and intact leaves (Figure 4). FC-induced stomatal opening in darkness was unaffected in the cipk23-5 mutant but was affected under blue light conditions (Figure S5; Figure 5b). Furthermore, the plasma membrane H\(^{+}\)-ATPase was normally activated in response to blue light in cipk23-5 guard cells (Figure S4). Thus, we assume that CIPK23 is involved in a signaling pathway which is different from that associated with phototropin-dependent activation of the plasma membrane H\(^{+}\)-ATPase for stomatal opening. Finally, we found that blue light activation of the \( K^{+}_{\text{in}} \) channel current was impaired in the guard cells of the cipk23-5 mutant compared to wild-type guard cells (Figure 6; Figure S6). From these results, we conclude that CIPK23 acts as a positive regulator in stomatal opening and mediates blue light signaling from phototropins to the \( K^{+}_{\text{in}} \) channels in guard cells (Figure S10). However, previous studies have indicated that CIPK23 functions as a negative regulator of ABA or water stress signaling in guard cells (Cheong et al., 2007; Nieves-Cordones et al., 2012). In this case, mutations in CIPK23 were found to enhance ABA- or water stress-dependent inhibition of light-induced stomatal opening. It is therefore reasonable to interpret
that CIPK23 acts as a positive regulator of phototropin-mediated stomatal opening through the activation of \( K_{\text{in}} \) channels, and this action appears to antagonize ABA-induced stomatal closure.

CIPK23 is a member of the SnRK3 subfamily that mainly acts in adaptive responses through the regulation of ion transporters (Hrabak et al., 2003; Luan, 2008; Sanyal et al., 2015). CIPK23 stimulates \( K^+ \) and nitrate uptake through phosphorylation-dependent activation of the high-affinity \( K^+ \) transporter 5 (HAK5), the \( K^+ \) in channel AKT1, and the nitrate transporter CHL1/NRT1.1 in the plasma membrane of roots under low \( K^+ \) and N conditions (Li et al., 2006; Xu et al., 2006; Ho et al., 2009; Ragel et al., 2015). Since uptake of \( K^+ \) and nitrate into guard cells contributes to stomatal opening (Schroeder et al., 2001; Guo et al., 2003; Shimazaki et al., 2007), these studies provide further support that CIPK23 acts as a positive regulator of stomatal opening through the activation of ion transporters downstream of the phototropins. The work presented here highlights an additional level of complexity associated with phototropin signaling events required for transporter regulation and stomatal opening.

A recent electrophysiological study using transient expression in Xenopus oocytes suggested that CIPK23 potentially functions as a positive regulator in ABA-induced stomatal closure (Negi et al., 2008; Vahisalu et al., 2008). In contrast, cipk23 mutants did not show such open stomata phenotypes but showed closed stomata phenotypes (Cheong et al., 2007; Nieves-Cordones et al., 2012; Figure 4). From these findings, we conclude that SLAC1 activation by CIPK23 may not strongly contribute to regulation of stomatal aperture in our experimental conditions. Further electrophysiological measurements of anion channel activity in cipk23 guard cells are therefore needed to clarify how CIPK23 regulates SLAC1 under our experimental conditions.

**Regulation of \( K_{\text{in}} \) channel activity by CIPK23 in guard cells**

It is well established that blue light activates the guard cell \( H^+ \)-ATPase via the phototropins, resulting in hyperpolarization of the plasma membrane, which in turn activates voltage-dependent \( K_{\text{in}} \) channels that are required for \( K^+ \) uptake (Schroeder et al., 2001; Shimazaki et al., 2007; Inoue et al., 2010). However, in addition to this single-scheme signaling, recent findings have demonstrated that blue light also enhances the \( K^+ \) channel current in guard cells via phototropins (Zhao et al., 2012). Since this enhancement is observed under the membrane voltage-clamped conditions, it is thought that the effect of blue light on the \( K^+ \) channel current is independent from plasma membrane \( H^+ \)-ATPase activity. In the present study, we found that cipk23-5 exhibited impaired stomatal opening and lacked \( K^+ \) channel activation in response to blue light.
(Figures 4-6; Figure S6). In addition, activation and phosphorylation of the H\(^+\)-ATPase in response to blue light were not affected in cipk23-5 guard cells (Figure S4). From these results, we conclude that CIPK23 does not mediate signaling from the phototropins to H\(^+\)-ATPase activation but activates K\(^+\) channels via the phototropins (Figure S10). Blue light-dependent stomatal opening is completely abolished in the phot1phot2 mutant but not completely in cipk23 mutants (Figure 4), suggesting that the blue light activation of K\(^+\) channels partially contributes to stomatal opening and phototropin-mediated activation of both the H\(^+\)-ATPase and K\(^+\) channels is needed for full stomatal opening.

AKT1 is a guard cell-expressing K\(^+\) channel that functions in stomatal opening together with other K\(^+\) channels, including KAT1, KAT2, and AKT2 (Véry and Sentenac, 2003; Gambale and Uozumi, 2006; Harada and Shimazaki, 2009; Takahashi et al., 2013). The kinase mutant was generated by expressing a dominant-negative variant of KAT2 in the kat2 mutant background. Guard cell K\(^+\) currents are lost in the kinase mutant (Lebaudy et al., 2008), which shows a strong impairment in blue light-dependent stomatal opening (Takahashi et al., 2013). CIPK23 has been shown to activate AKT1 by direct phosphorylation to promote K\(^+\) uptake in roots under low K\(^+\) conditions (Li et al., 2006; Xu et al., 2006; Sanchez-Barrena et al., 2020). On the basis of these findings, we propose that CIPK23 could also activate guard cell AKT1 by direct protein phosphorylation in response to blue light. Indeed, CIPK23 has been shown to specifically interact with AKT1 among other plant K\(^+\) channels in yeast (Li et al., 2006). Alternatively, CIPK23 could phosphorylate and regulate the activity of other K\(^+\) channels in guard cells either directly or indirectly via scaffold proteins. Further investigation will therefore be required to clarify the role of CIPK23 in regulating K\(^+\) channel activity in phototropin signaling.

The CIPK protein family consists of 26 members in Arabidopsis. Phylogenetic tree analysis indicates that CIPK23 is localized in the clade containing CIPK3, CIPK9, and CIPK26 (Figure S8). According to a public microarray database eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Guard_Cell), CIPK3 and CIPK9 are expressed in guard cells at similar levels to CIPK23 (Figure S9). These expression data suggest that CIPK3, CIPK9, and CIPK26 may function redundantly with CIPK23 in the regulation of stomatal opening. However, Luan's group has demonstrated that members outside this clade, namely, CIPK6 and CIPK16, have similar functions to CIPK23 with respect to AKT1 activation (Lee et al., 2007). Expression of CIPK6 is also apparent in guard cells (Figure S9), suggesting that it may also have a similar function to CIPK23 in stomatal opening.

We found that blue light increased K\(^+\) channel currents 2-fold in wild-type guard cells (Zhao et al., 2012; Figure 6a, b), and this enhancement in K\(^+\) channel activity appears to contribute to stomatal opening. However, it has already been reported that Arabidopsis guard cells possess sufficient K\(^+\) channel activity required for stomatal opening (Szyroki et al., 2001; Lebaudy et al., 2008; Takahashi et al., 2013). Stomatal opening is affected only when K\(^+\) channel activity is largely reduced (by 70–80%). It is therefore difficult to attribute the impairment in stomatal opening in cipk23 mutants solely to a failure in K\(^+\) channel activation. Other ion transporters, such as nitrate transporters activated through CIPK23 (Guo et al., 2003; Ho et al., 2009), could also play an important role in stomatal opening in response to blue light.

**Activation of CIPK23 via the phototropins**

One question arising from our findings is how the phototropins activate CIPK23 in response to blue light. Structural and biochemical analyses suggest that CIPK23 is activated by binding of the calcium sensor calcineurin B-like (CBL) 1/9 in a Ca\(^{2+}\)-dependent manner (Luan, 2008) and/or phosphorylation by another protein kinase (Chaves-Sanjuan et al., 2014). The activation of CIPK23 in guard cells may therefore require an increase in cytosolic Ca\(^{2+}\) or phosphorylation of CIPK23 by an upstream protein kinase. At least for the former, previous reports have demonstrated that cytosolic Ca\(^{2+}\) increases in response to ABA or high concentrations of CO\(_2\) in guard cells contribute to stomatal closure (McAinsh et al., 1990; Schroeder and Hagiwara, 1990; Allen et al., 1999, 2001; Kim et al., 2010). Indeed, guard cell K\(^+\) channel currents have shown to be blocked by cytosolic Ca\(^{2+}\) increases in electrophysiological experiments (Grabov and Blatt, 1998, 1999; Wang et al., 2013). In addition, the plasma membrane-anchoring of the CIPK23 kinase domain strongly increased K\(^+\) channel currents in the absence of CBL interactions (Lee et al., 2007). These observations suggest that CIPK23 activity for stomatal opening may be independent from cytosolic Ca\(^{2+}\) increases and the function of CBL1/9 may be required for the recruitment of CIPK23 to the plasma membrane.

A guard cell-specific kinase BLUS1, which acts as a substrate for phototropins, mediates blue light-dependent stomatal opening through H\(^+\)-ATPase activation (Takemiya et al., 2013). In addition, our electrophysiological experiments indicated that blue light-dependent activation of K\(^+\) channel currents was severely impaired in the blus1-3 guard cells (Figure 6a,d; Figure S6a,c). These results suggest that BLUS1 activates both the plasma membrane H\(^+\)-ATPase and the K\(^+\) channel, but CIPK23 only activates the K\(^+\) channel in stomatal opening. On the basis of these findings, we speculate that CIPK23 may regulate BLUS1 in blue light activation of the K\(^+\) channels (Figure S10: left model). In the present study, phot1 did not phosphorylate CIPK23 in vitro (Figure S7), although these proteins were shown to physically interact in vitro and in vivo (Figures 1
and 2). Since CIPK23 is likely to form a protein complex with phototropins and BLUS1, another potential signaling scenario could involve CIPK23 acting as a substrate for BLUS1 (Figure S10: right model). However, we have not been successful to date in determining whether or not CIPK23 is a substrate for BLUS1 kinase activity because of the difficulty in producing active recombinant BLUS1 (Hayashi et al., 2017). Further experiments are now needed to clarify these points and improve our understanding of how CIPK23 regulates phototropin signaling.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

*Arabidopsis thaliana* and *N. benthamiana* were grown on soil with a 16-h light/8-h dark photoperiod under white fluorescent light (50 µmol m⁻² sec⁻¹) at 22-24°C. Four- to five-week-old plants were used for stomatal bioassays, isolation of GCPs, and preparation of total RNA.

We obtained *cipk23-1* (*SALK_032341*) and *cipk23-5* (*SALK_13067*) from the Arabidopsis Biological Resource Center and isolated homozygous mutants by PCR using genomic DNA according to the SIGnAL website (http://signal.salk.edu). We used both mutants for phenotypic analyses after confirmation of knock-out mutants by RT-PCR (Figure S3b).

**Protein kinases screening by using the AlphaScreen**

All proteins for the AlphaScreen method were expressed using a wheat germ cell-free protein synthesis system (Sawasaki et al., 2002, 2004, 2005). Full-length cDNAs of *PHOT1* and *PHOT2* were amplified by RT-PCR from the wild-type (Col) cDNAs using the following oligonucleotide primers: 5'-CCCAAGCTTATGGAACCAACAGAAAAACCTATCG-3' and 5'-CCCAAGCTTTTCAAAACCATTTGTTGCAGATC TTC-3' for *PHOT1* and 5'-ATGGAGGAGGCGCAAGGAGGCCTC-3' and 5'-TTAGAAGAGGCTAATGCTCAAATGGCCT-3' for *PHOT2*. The amplified *PHOT1* and *PHOT2* fragments were cloned into the pFLAG-MAC vector (Sigma-Aldrich) via HindIII and Smal sites, respectively. The coding regions of FLAG-tagged phototropins were amplified by PCR from these vectors using the following primers: 5'-GGGTTACCATGG GACTACAGAAGGACTGAC-3' and 5'-GGGTTACCATGG GACTACAGAAGGACTGAC-3' for *PHOT1* and 5'-GGGTTACCATGG GACTACAGAAGGACTGAC-3' and 5'-GGGTTACCATGG GACTACAGAAGGACTGAC-3' for *PHOT2*. The DNA fragments were cloned into the pRLN vector (Alphalinear) via HindIII and Smal sites, respectively. The coding regions of FLAG-tagged phototropins were amplified by PCR from these vectors using the following primers: 5'-GGGTTACCATGG GACTACAGAAGGACTGAC-3' and 5'-GGGTTACCATGG GACTACAGAAGGACTGAC-3' for *PHOT1* and 5'-GGGTTACCATGG GACTACAGAAGGACTGAC-3' and 5'-GGGTTACCATGG GACTACAGAAGGACTGAC-3' for *PHOT2*. The DNA fragments were cloned into the pBE vector (Alphalinear) via HindIII and Smal sites, respectively. The DNA fragments were cloned into the pRLN vector (Alphalinear) via HindIII and Smal sites, respectively.

**Pull-down assays**

Pull-down assays were carried out using recombinant CIPKs expressed and purified from *E. coli* in combination with microsomal membranes from Arabidopsis seedlings. Full-length cDNAs of *CIPK1*, *CIPK23*, and *CIPK24* were amplified by RT-PCR using the following oligonucleotide primers: 5'-CCCAAGCTTTTATGACAAAGAAAATGAGAAGTGGGC-3' for *CIPK1*, 5'-CCCAAGTGTATGGCTTCGAGACCCGGCC-3' and 5'-CCCAAGCTTTTATGACACTTGTTTGCAATTGTCCG-3' for *CIPK23*, and 5'-CCCAAGTGTATGGCTTCGAGACCCGGCC-3' and 5'-CCCAAGCTTTTATGACAAAGAAAATGAGAAGTGGGC-3' for *CIPK24*. The amplified DNA fragment was cloned into the HindIII site of the pFLAG-MAC Expression Vector (Sigma-Aldrich). The recombinant plasmids were transformed into the *E. coli* BL21 strain. The recombinant CIPK proteins were expressed as a fusion protein with FLAG-tag and purified from *E. coli* extracts using anti-FLAG agarose (Sigma-Aldrich) according to the manufacturer's instructions (https://www.sig maaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/2126 ul.pdf). The agarose beads were then incubated with microsomal protein fractions (100 µg) for 2 h at 4°C. Proteins were solubilized from the beads by adding a half volume of SDS sample buffer containing 4.5% SDS, 30% sucrose, 22.5% β-mercaptoethanol, 0.018% Coomassie Brilliant Blue, 4.5 mM EDTA, and 45 mM Tris-Cl (pH 8.0) and subjected to SDS-PAGE. Proteins were immunodetected by using anti-phot1, anti-phot2, and anti-FLAG monoclonal antibodies (Sigma-Aldrich) according to a previously described method (Inoue et al., 2011).

**BiFC assays**

Full-length cDNAs of *CIPK23*, *CIPK24*, and *PHOT2* were amplified by RT-PCR using the following oligonucleotide primers: 5'-GCC TCTGATGCTTCGAGACCCGGCC-3' and 5'-ATCCGG GATGCTTCGAGACCCGGCC-3' for *CIPK23*, 5'-GCCCTTAG AATGAAAGAAATGAGAAGGAGTGGCC-3' and 5'-ATCCGGAGG AAGCATGGTTTGGCAATTGTCCG-3' for *CIPK24*, and 5'-GCCCTTAG AATGAAAGAAATGAGAAGGAGTGGCC-3' and 5'-ATCCGGAGG AAGCATGGTTTGGCAATTGTCCG-3' for *PHOT2*. The amplified DNA fragments were cloned into the binary vectors pSPYNE-35S and pSPYCE-35S (Walter et al., 2004) via XbaI and Smal sites. The pSPYNE-35S and pSPYCE-35S vectors bearing *PHOT1* cDNA were used as reported previously (Kaiserli et al., 2009). *Agrobacterium tumefaciens* (GV3101) was transformed with the resulting plasmid vectors and used for transformation of *N. benthamiana*. Agrobacte ria-mediated co-infiltration of *N. benthamiana* leaves with pSPYNE and pSPYCE containing the indicated inserts was performed as previously described (Walter et al., 2004; Kaiserli et al., 2009). Detection of reconstituted YFP fluorescence was monitored 2.5 days post-infiltration using a confocal microscope (Zeiss LSM510 and Leica SP8) (Walter et al., 2004; Kaiserli et al., 2009). Total YFP fluorescence from seven separate images and three independent experiments was quantified using Fiji (ImageJ) (Schindelin et al., 2012).

**Expression of CIPK23**

RT-PCR was performed as described previously (Inoue et al., 2008a). GCPs and MCPs were enzymatically prepared from rosette leaves as reported by Ueno et al. (2005) with slight modifications. Total RNAs were extracted from GCPs, MCPs, rosette leaves, petioles, inflorescence stems, and roots of 4-week-old wild-type (Col) plants or from the aerial parts of *cipk23-1* and *cipk23-5* mutants

1 mg ml⁻¹ BSA, 0.1 µl streptavidin-coated donor beads, and 0.1 µl anti-igG acceptor beads at 23°C for 1 h. Luminescent signals were analyzed by the AlphaScreen detection program (PerkinElmer Life and Analytical Sciences; Takahashi et al., 2009).

**In vitro pull-down assays**

Pull-down assays were carried out using recombinant CIPKs expressed and purified from *E. coli* in combination with microsomal membranes from Arabidopsis seedlings. Full-length cDNAs of *CIPK1*, *CIPK23*, and *CIPK24* were amplified by RT-PCR using the following oligonucleotide primers: 5'-CCCAAGCTTTTATGACAAAGAAAATGAGAAGTGGGC-3' for *CIPK1*, 5'-CCCAAGCTTTTATGACACTTGTTTGCAATTGTCCG-3' for *CIPK23*, and 5'-CCCAAGCTTTTATGACAGAGAAGGAGTGGGC-3' for *CIPK24*. The amplified DNA fragment was cloned into the HindIII site of the pFLAG-MAC Expression Vector (Sigma-Aldrich). The recombinant plasmids were transformed into the *E. coli* BL21 strain. The recombinant CIPK proteins were expressed as a fusion protein with FLAG-tag and purified from *E. coli* extracts using anti-FLAG agarose (Sigma-Aldrich) according to the manufacturer's instructions (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/2126ul.pdf). The agarose beads were then incubated with microsomal protein fractions (100 µg) for 2 h at 4°C. Proteins were solubilized from the beads by adding a half volume of SDS sample buffer containing 4.5% SDS, 30% sucrose, 22.5% β-mercaptoethanol, 0.018% Coomassie Brilliant Blue, 4.5 mM EDTA, and 45 mM Tris-Cl (pH 8.0) and subjected to SDS-PAGE. Proteins were immunodetected by using anti-phot1, anti-phot2, and anti-FLAG monoclonal antibodies (Sigma-Aldrich) according to a previously described method (Inoue et al., 2011).
with ISOGEN (Nippon Gene). First-strand cDNAs were synthesized from 2 μg of each total RNA by SuperScript III reverse transcriptase using oligo(dT)20 primer (Invitrogen). Full-length CIPK23 cDNAs were amplified by PCR using the following oligonucleotide primers: 5′-ATGGCTTCTCGAAGACGGCTTAC-3′ and 5′-TTATGTCAGCTTTGCAATTGTCGCC-3′ (Figure S3b). For amplification of the fragment of CIPK23 cDNA, PCR was performed using the following oligonucleotide primers: 5′-AGTTTCAAAACGTCTGCTCCAC-3′ and 5′-ACGAGGATTACATTTGCT-3′. As an internal standard, a fragment of ACT78 was used with the primers 5′-ACTTTACGCGAGTGTCGTACAAC-3′ and 5′-AAGGACTTCTGGGACCTGAAATCT-3′.

Measurement of stomatal aperture
Stomatal aperture in the leaf abaxial epidermis was measured according to previous methods (Inoue et al., 2008b; de Carbonnel et al., 2010) with a modification. To determine the stomatal opening, the epidermal fragments were incubated in 2 ml buffer containing 5 mM MES/bistrispropane (pH 6.5), 50 mM KCl, and 0.1 mM CaCl2, and illuminated with light for 3 h at room temperature (Figure 4a). To determine the stomatal opening by FC, the epidermis were incubated in 2 ml buffer containing 5 mM MES/bistrispropane (pH 6.5), 10 mM KCl, 0.1 mM CaCl2, and FC at indicated concentrations for 3 h (Figure 5; Figure S5).

Phenotypic analyses of phototropin-mediated responses
Phototropic curvature of etiolated seedlings, chloroplast relocalizations and leaf flattening of rosette leaves, and stomatal conductance in intact leaves were determined according to previous methods (Doi et al., 2004; Inoue et al., 2008a, 2008b, 2011, 2017; Takemiya et al., 2013).

H+ pumping and H+ -ATPase phosphorylation in guard cell protoplasts
GCPs were enzymatically prepared from rosette leaves and used for the measurements of H+ pumping, immunoblotting, and far-Western blotting according to previously described methods (Ueno et al., 2005; Inoue et al., 2008b; Takemiya et al., 2013). Immunoblotting and far-Western blotting were performed using anti-H+ -ATPase antibodies and GST-14-3-3 protein (GF14phi) to determine the amount of H+ -ATPase and the levels of H+ -ATPase phosphorylation, respectively.

Isolation of guard cell protoplasts and whole-cell K+ current recordings
GCPs for whole-cell K+ current recordings were isolated as described previously (Zhao et al., 2012). Prior to each experiment, epidermal peels were obtained carefully from the abaxial surface of the youngest and fully expanded leaves of 2-week-old Arabidopsis and cut into pieces of 5 mm length. The epidermal strips were exposed to enzyme buffer (1.3% cellulase RS, 0.0075% pectinase 4a). To determine the stomatal opening by FC, the epidermis were incubated in 2 ml of buffer containing 5 mM MES/bistrispropane (pH 6.5), 10 mM KCl, and 0.1 mM CaCl2, and illuminated with light for 3 h at room temperature (Figure S3c). As an internal standard, a fragment of ACT78 was used with the primers 5′-ACTTTACGCGAGTGTCGTACAAC-3′ and 5′-AAGGACTTCTGGGACCTGAAATCT-3′. The Plant Journal 689

CIPK23 regulates stomatal opening

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Thio-phosphorylation in in vitro phosphorylation assay
Detection of thio-phosphorylation derived from phot1 kinase activity was performed as previously described (Schnabel et al., 2018). The cDNA fragments of GST-PHOT1-T740G and GST-CIPK23 were cloned into the pSP64 vector. GST-PHOT1-T740G and GST-CIPK23 were expressed using the TNT SP6 High-Yield Wheat Germ Protein Expression System (Promega), with 2 μg of each vector for a 20 μl reaction, and 10 μM flavin mononucleotide as a chromophore. Protein expression was performed at room temperature in the dark for 2 h. The in vitro kinase assay was performed as previously described (Sakai et al., 2001) with modifications. Ten μl of in vitro protein expression extract and N2-benzyl-ATP-S (500 μM) were mixed in each reaction volume (20 μl) with phosphorilation buffer (37.5 mM Tris-HCl (pH 7.5), 5.3 mM MgSO4, 150 mM NaCl, and 1 mM EDTA). Light was irradiated to the samples for 20 sec at a total fluence of 60 000 μmol m−2 s−1. Reactions were incubated in the dark at room temperature for 5 min and terminated by adding EDTA (pH 8.0) to a final concentration of 20 mM. Thio-phosphorylated molecules were alkylated by adding p-nitrobenzyl mesylate at a final concentration of 2.5 mM and incubated for 2 h. Then, protein samples were subjected to SDS-PAGE and Western blotting as described previously (Schnabel et al., 2018). Protein thio-phosphorylation and GST protein were detected using a rabbit anti-thiophosphoester monoclonal antibody and a goat anti-GST monoclonal antibody, respectively, as primary antibodies. HRP-conjugated anti-rabbit or anti-goat secondary antibody and Pierce ECL Plus Western blotting substrate (Thermo Fisher Scientific) were used to develop the signals.

ACCESSION NUMBERS
PHOT1 (AT3G45780), PHOT2 (AT5G58140), CIPK23 (AT1G30270), BLUS1 (AT4G14480), CIPK1 (AT3G17510), CIPK24 (AT3G35410), ACT8 (AT1G94240).

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Phylogenetic relationships among kinase assay. CIPK23 is not phosphorylated by phot1 in the type (Col) and cipk23-5 totropins and CIPK24 and CIPK24 homodimerization in Nicotiana benthamiana. Channel currents in wild-type (Col), and expression of CIPK23 in various tissues. Figure S4. Blue light-induced activation of the plasma membrane H+-ATPase in GCPs from wild-type (Col) and cipk23-5 plants. Figure S5. Stomatal opening in response to fusicoccin in dark-ness. Figure S6. Effects of blue light on whole-cell inward-rectifying K+ channel currents in wild-type (Col), cipk23-5, and blus1-3 GCPs. Figure S7. CIPK23 is not phosphorylated by phot1 in the in vitro kinase assay. Figure S8. Phylogenetic relationships among Arabidopsis thaliana CIPK family members. Figure S9. Gene expression levels of CIPK23, CIPK3, CIPK9, CIPK26, CIPK6, and CIPK16 in guard cells. Figure S10. Proposed blue light signalings in guard cells. Table S1. Blue light-dependent H+-pumping in GCPs from wild-type (Col) and cipk23-5 plants.

REFERENCES
Allen, G.J., Chu, S.P., Harrington, C.L., Schumacher, K., Hoffmann, T., Tang, Y.T., Grill, E. and Schroeder, J.I. (2001) A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature, 411, 1053-1057.
Allen, G.J., Kwak, J.M., Chu, S.P., Llopis, J., Tsien, R.Y., Harper, J.F. and Schroeder, J.I. (1999) Cameleon calcium indicator reports cytoplasmic calcium dynamics in Arabidopsis guard cells. Plant J. 19, 735-747.

Assmann, S.M., Simoncini, L. and Schroeder, J.I. (1985) Blue light activates electrogenic ion pumping in guard cell proteoplasts of Vicia faba. Nature, 318, 285-287.
Blatt, M.R. (1987) Electrical characteristics of stomatal guard cells: the contribution of ATP-dependent, "electrogenic" transport revealed by current-voltage and difference-current-voltage analysis. J. Membr. Biol. 98, 257-274.
Chaves-Sanjuan, A., Sanchez-Barrena, M.J., Gonzalez-Rubio, J.M., Moreno, M., Ragel, P., Jimenez, M., Perdo, J.M., Martinez-Ripoll, M., Quintero, F.J. and Albert, A. (2014) Structural basis of the regulatory mechanism of the plant CIPK family of protein kinases controlling ion homeostasis and abiotic stress. Proc. Natl Acad. Sci. USA. 111, E4532-E4541.
Cheong, Y.H., Pandey, G.K., Grant, J.J., Batistic, O., Li, L., Kim, B.-G., Lee, S.-C., Kudla, J. and Luan, S. (2007) Two calcineurin-B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis. Plant J. 52, 223-239.
Christie, J.M., Reymond, P., Powell, G., Bernasconi, P., Railbeakas, A.A., Lis-cum, E. and Briggs, W.R. (1998) Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for phototropism. Science, 282, 1698-1701.
Christie, J.M. (2007) Phototropin blue-light receptors. Annu. Rev. Plant Biol. 58, 21-45.
Christie, J.M., Yang, H., Richter, G.L. et al. (2011) phot1 inhibition of ABCB19 primes lateral auxin fluxes in the shoot apex required for phototropism. PLoS Biol. 9, e1001076.
Christie, J.M., Blackwood, L., Petersen, J. and Sullivan, S. (2015) Plant flavoprotein photoreceptors. Plant Cell Physiol. 56, 401-413.
de Carbonnel, M., Davis, P., Roelfsema, M.R., Inoue, S., Schepens, I., Lari-guet, P., Geisler, M., Shimazaki, K., Hangarter, R. and Fankhauser, C. (2010) The Arabidopsis PHYTOCHROME KINASE SUBSTRATE 2 protein is a phototropin signaling element that regulates leaf flattening and leaf positioning. Plant Physiol. 152, 1391-1405.
Demarsy, E. and Fankhauser, C. (2009) Higher plants use LOV to perceive blue light. Curr. Opin. Plant Biol. 12, 69-74.
Demarsy, E., Schepens, I., Okajima, K., Hersch, M., Bergmann, S., Christie, J.M., Shimazaki, K., Tokutomu, S. and Fankhauser, C. (2012) Phy-tochrome kinase substrate 4 is phosphorylated by the phototropin 1 photo-receptor. EMBO J. 31, 3457-3467.
Dol, M., Shigenaga, A., Eme, T., Kinoshita, T. and Shimazaki, K. (2004) A trans-gene encoding a blue-light receptor, phot1, restores blue-light responses in the Arabidopsis phot1 phot2 double mutant. J. Exp. Bot. 55, 517-523.
Guo, F.Q., Young, J. and Crawford, N.M. (2006) Properties of shaker-type potassium channels in higher plants. J. Membr. Biol. 210, 1-19.
Gotoh, E., Suzetsu, N., Yamori, W., Ishihashi, K., Kiyabu, R., Fukuda, M., Higa, T., Shirouchi, B. and Wada, M. (2018) Chloroplast accumulation response enhances leaf photosynthesis and plant biomass production. Plant Physiol. 178, 1388-1389.
Grabov, A. and Blatt, M.R. (1998) Membrane voltage initiates Ca2+ waves and potentiates Ca2+ increases with asbscic acid in stomatal guard cells. Proc Natl Acad Sci USA. 95, 4778-4783.
Grabov, A. and Blatt, M.R. (1999) A steep dependence of inward-rectifying potassium channels on cytosolic free calcium concentration increase evoked by hyperpolarization in guard cells. Plant Physiol. 119, 277-288.
Guo, F.Q., Young, J. and Crawford, N.M. (2003) The nitrate transporter ANTR1.1 (CHL) functions in stomatal opening and contributes to drought susceptibility in Arabidopsis. Plant Cell, 15, 107-117.
Harada, A. and Shimazaki, K. (2009) Measurement of changes in cytosolic Ca2+ in Arabidopsis guard cells and mesophyll cells in response to blue light. Plant Cell Physiol. 50, 360-373.
Harada, A., Takemiyia, A., Inoue, S., Sakai, T. and Shimazaki, K. (2013) Role of RPT2 in leaf positioning and flattening and a possible inhibition of phot2 signaling by phot1. Plant Cell Physiol. 54, 36-47.
Hashimoto, M., Negi, J., Young, J., Israelsson, M., Schroeder, J.I. and Iba, K. (2006) Arabidopsis HRT1 kinase controls stomatal movements in response to CO2. Nat. Cell Biol. 8, 391-397.
Hayashi, M., Inoue, S., Takahashi, K. and Kinoshita, T. (2011) Immunohisto-chemical detection of blue light-induced phosphorylation of the plasma membrane H+-ATPase in stomatal guard cells. Plant Cell Physiol. 52, 1238-1248.
Hayashi, M., Inoue, S.I., Ueno, Y. and Kinoshita, T. (2017) A Raf-like protein kinase BPH mediates blue light-dependent stomatal opening. Sci Rep. 7, 45596.
Hayashi, M., Sugimoto, H., Takahashi, H., Seki, M., Shinozaki, K., Sawasaki, T., Kinoshita, T. and Inoue, S. (2020) Raf-like kinases CBC1 and CBC2 negatively regulate stomatal opening by negatively regulating plasma membrane H+–ATPase phosphorylation in Arabidopsis. *Photochem Photobiol Sci.* 19, 88–98.

Hiyama, A., Takeyama, A., Munemasa, S., Okuma, E., Sugiyama, N., Tada, Y., Murata, Y. and Shimazaki, K. (2017) Blue light and CO2 signals converge to regulate light-induced stomatal opening. *Nat Commun.* 8, 1284.

Ho, C.H., Lin, S.H., Hu, H.C. and Tsay, Y.F. (2009) CHL1 functions as a nitrate sensor in plants. *Cell,* 138, 1184–1194.

Hrabak, E.M., Chan, C.W., Grilbsov, M. et al. (2003) The Arabidopsis CPK-SnRK superfamily of protein kinases. *Plant Physiol.* 132, 666–680.

Inada, S., Ohashi, M., Mayama, T., Okada, K. and Sakai, T. (2004) RPT2 is a signal transducer involved in phototropic response and stomatal opening by association with phototropin1 in *Arabidopsis thaliana.* *Plant Cell,* 16, 887–896.

Inoue, S. and Kinoshita, T. (2017) Blue light regulation of stomatal opening and the plasma membrane H+–ATPase. *Plant Physiol.* 174, 531–539.

Inoue, S., Kinoshita, T., Takemiya, A., Doi, M. and Shimazaki, K. (2008a) Leaf positioning of Arabidopsis in response to blue light. *Mol Plant,* 1, 15–26.

Inoue, S., Kinoshita, T., Matsumoto, M., Nakayama, K.I., Doi, M. and Shimazaki, K. (2008b) Blue light-induced autophosphorylation of phototropin is a primary step for signaling. *Proc Natl Acad Sci USA,* 105, 5626–5631.

Inoue, S., Takemiya, A. and Shimazaki, K. (2010) Phototropin signaling and stomatal opening as a model case. *Curr Opin Plant Biol.* 13, 587–593.

Inoue, S., Matsushita, T., Tomokiyu, Y., Matsumoto, M., Nakayama, K.I., Kinoshita, T. and Shimazaki, K. (2011) Functional analyses of the activation loop of phototropin2 in *Arabidopsis.* *Plant Physiol.* 156, 117–128.

Inoue, S., Iwasita, N., Takahashi, Y. et al. (2017) Brassinosteroid involvement in *Arabidopsis thaliana* stomatal opening. *Plant Cell Physiol.* 58, 1048–1058.

Kagawa, T., Sakai, T., Suetusgu, N., Oikawa, K., Ishiguro, S., Kato, T., Tabata, S., Okada, K. and Wada, M. (2001) Arabidopsis NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. *Science,* 291, 2138–2141.

Kaiserl, E., Sullivan, S., Jones, M.A., Feeney, K.A. and Christie, J.M. (2009) Domain swapping to assess the mechanistic basis of Arabidopsis phototropin 1 receptor kinase activation and endocytosis by blue light. *Plant Cell,* 21, 3226–3239.

Kim, T.H., Bohmer, M., Hu, H., Nishimura, N. and Schroeder, J.J. (2010) Guard cell signal transduction network: advances in understanding abscisic acid, CO2, and Ca2+ signaling. *Annu Rev Plant Biol.* 61, 561–591.

Kinoshita, T. and Shimazaki, K. (1999) Blue light activates the plasma membrane H+–ATPase by phosphorylation of C-terminus in stomatal guard cells. *EMBO J.* 18, 5548–5558.

Kinoshita, T. and Shimazaki, K. (2001) Analysis of the phosphorylation level in guard-cell plasma membrane H+–ATPase in response to fusococcin. *Plant Cell Physiol.* 42, 424–432.

Kinoshita, T., Doi, M., Suetusgu, N., Kagawa, T., Wada, M. and Shimazaki, K. (2001) phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature,* 414, 566–569.

Lariguet, P., Schepens, I., Hodgson, D. et al. (2006) PHYTOCHROME KINASE SUBSTRATE 1 is a phototropin 1 binding protein required for phototropism. *Proc Natl Acad Sci USA,* 103, 10134–10139.

Lebaudi, A., Vavasseur, A., Hossy, E., Dreyer, I., Leonhardt, N., Thibaud, J.B., Very, A.A., Simonneau, T. and Sentenac, H. (2008) Plant adaptation to fluctuating environment and biomass production are strongly dependent on guard cell potassium channels. *Proc Natl Acad Sci USA,* 105, 5271–5276.

Lee, S.C., Lan, W.-Z., Kim, B.-G., Li, L., Cheong, Y.H., Pandey, G.K., Lu, G., Buchanan, B.B. and Luan, S. (2007) A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. *Proc Natl Acad Sci USA,* 104, 15959–15964.

Li, L., Kim, B.-G., Cheong, Y.H., Pandey, G.K. and Luan, S. (2006) A Ca2+ signaling pathway regulates a K+ channel for low-K response in *Arabidopsis.* *Proc Natl Acad Sci USA,* 103, 12625–12630.

Luan, S. (2006) The CBL-CIPK network in plant calcium signaling. *Trends Plant Sci.* 11, 37–42.
Schröeder, J.I., Raschke, K. and Neher, E. (1987) Voltage dependence of K+ channels in guard-cell protoplasts. Proc. Natl Acad. Sci. USA, 84, 4108–4112.

Schröeder, J.I. and Hedrich, R. (1989) Involvement of ion channels and active transport in osmoregulation and signaling of higher plant cells. Trends Biochem. Sci. 14, 187–192.

Schröeder, J.I. and Hagiwara, S. (1990) Repetitive increases in cytosolic Ca2+ of guard cells by abscisic acid: activation of nonselective Ca2+ permeable channels. Proc. Natl Acad. Sci. USA, 87, 9305–9309.

Takahashi, Y., Ebisu, Y., Kinoshita, T., Doi, M., Okuma, E., Murata, Y. and Takahashi, H., Nozawa, A., Seki, M., Shinozaki, K., Endo, Y. and Sawasaki, (2006) Protein.

Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M. and Waner, D. (1989) Involvement of ion channels and Ca2+ channels in guard-cell protoplasts of Arabidopsis thaliana in response to blue light. Plant Cell Physiol. 46, 955–963.

Schroeder, J.I. and Hedrich, R. (1990) Repetitive increases in cytosolic Ca2+ of guard cells by abscisic acid: activation of nonselective Ca2+ permeable channels. Proc. Natl Acad. Sci. USA, 87, 9305–9309.

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., Doi, M., Assmann, S.M. and Kinoshita, T. (2007) Light regulation of stomatal movement. Annu. Rev. Plant Physiol. 58, 219–247.

Suetsugu, N., Kagawa, T. and Wada, M. (2005) An auxilin-like J-domain protein, JAC1, regulates phototropin-mediated chloroplast movement in Arabidopsis. Plant Physiol. 139, 151–162.

Szyroki, A., Ivashikina, N., Dietrich, P. et al. (2001) KAT1 is not essential for stomatal opening. Proc. Natl Acad. Sci. USA, 98, 2917–2921.

Takahashi, H., Nozawa, A., Seki, M., Shinozaki, K., Endo, Y. and Sawasaki, T. (2009) A simple and high-sensitivity method for analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis. BMC Plant Biol. 9, 39.

Takahashi, Y., Ebisu, Y., Kinoshita, T., Doi, M., Okuma, E., Murata, Y. and Shimazaki, K. (2013) bHLH transcription factors that facilitate K+ uptake during stomatal opening are repressed by abscisic acid through phosphorylation. Sci. Signal. 6, ra48.

Takahashi, Y., Ebisu, Y., Kinoshita, T., Doi, M., Kinoshita, T. and Shimazaki, K. (2005) Phototropins promote plant growth in response to blue light in low light environments. Plant Cell, 17, 1120–1127.

Takahashi, Y., Kinoshita, T., Asanuma, M. and Shimazaki, K. (2006) Protein phosphatase 1 positively regulates stomatal opening in response to blue light in Vicia faba. Proc. Natl Acad. Sci. USA, 103, 13549–13554.

Takemiya, A., Sugiyama, N., Fujimoto, H., Tsutsumi, T., Yamauchi, S., Hiyama, A., Tada, Y., Christie, J.M. and Shimazaki, K. (2013) Phosphorylation of BLUS1 kinase by phototropins is a primary step in stomatal opening. Nat. Commun. 4, 2094.

Tsuetsuki, T., Takemiya, A., Harada, A. and Shimazaki, K. (2013) Disruption of ROOT PHOTOTROPISM2 gene does not affect phototropin-mediated stomatal opening. Plant Sci. 201–202, 93–97.

Ueno, K., Kinoshita, T., Inoue, S., Emi, T. and Shimazaki, K. (2005) Biochemical characterization of plasma membrane H+-ATPase activation in guard cell protoplasts of Arabidopsis thaliana in response to blue light. Plant Cell Physiol. 46, 955–963.

Vahisalu, T., Kollist, H., Wang, Y.F. et al. (2008) SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. Nature, 452, 487–491.

Very, A. and Sentenac, H. (2003) Molecular mechanisms and regulation of K+ transport in higher plants. Annu. Rev. Plant Biol. 54, 575–603.

Walter, M., Chaban, C., Schütze, K. et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J. 40, 428–438.

Wang, Y., Chen, Z.H., Zhang, B., Hills, A. and Blatt, M.R. (2013) PYR/PYL/RCAR abscisic acid receptors regulate K+ and Cl− channels through reactive oxygen species-mediated activation of Ca2+ channels at the plasma membrane of intact Arabidopsis guard cells. Plant Physiol. 163, 566–577.

Xu, J., Li, H.-D., Chen, L.-Q., Wang, Y., Liu, L.-L., He, L. and Wu, W.-H. (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K+ transporter AKT1 in Arabidopsis. Cell, 125, 1347–1360.

Yamauchi, S., Takemiya, A., Sakamoto, T., Kurata, T., Tsutsumi, T., Kinoshita, T. and Shimazaki, K. (2016) Plasma membrane H+-ATPase (AHA1) plays a major role in Arabidopsis thaliana for stomatal opening in response to blue light. Plant Physiol. 163, 560–565.

Yin, Y., Adachi, Y., Ye, W., Hayashi, M., Nakamura, Y., Kinoshita, T., Mori, I.C. and Murata, Y. (2013) Decrease in abscisic acid perception mechanisms between closure induction and opening inhibition of stomata. Plant Physiol. 163, 600–610.

Zeiger, E. and Hepler, P.K. (1977) Light and stomatal function: blue light stimulates swelling of guard cell protoplasts. Science, 20, 887–889.

Zhao, X., Qiao, X., Yuan, J., Ma, X. and Zhang, X. (2012) Nitric oxide inhibits blue light-induced stomatal opening by regulating the K+ influx in guard cells. Plant Sci. 184, 29–35.