<table>
<thead>
<tr>
<th>Title</th>
<th>Blue light and CO2 signals converge to regulate light-induced stomatal opening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Hiyama, Asami; Takemiya, Atsushi; Munemasa, Shintaro; Okuma, Eiji; Sugiyama, Naoyuki; Tada, Yasuomi; Murata, Yoshiyuki; Shimazaki, Ken-ichiro</td>
</tr>
<tr>
<td>Citation</td>
<td>Nature Communications (2017), 8</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2017-11-03</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/2433/228164">http://hdl.handle.net/2433/228164</a></td>
</tr>
</tbody>
</table>
| Rights | © The Author(s) 2017. This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.
| Type | Journal Article |
| Textversion | publisher |
Blue light and CO\textsubscript{2} signals converge to regulate light-induced stomatal opening

Asami Hiyama\textsuperscript{1}, Atsushi Takemiya\textsuperscript{1,5}, Shintaro Munemasa\textsuperscript{2}, Eiji Okuma\textsuperscript{2}, Naoyuki Sugiyama\textsuperscript{3}, Yasuomi Tada\textsuperscript{4}, Yoshiyuki Murata\textsuperscript{2} & Ken-ichiro Shimazaki\textsuperscript{1}

Stomata regulate gas exchange between plants and atmosphere by integrating opening and closing signals. Stomata open in response to low CO\textsubscript{2} concentrations to maximize photosynthesis in the light; however, the mechanisms that coordinate photosynthesis and stomatal conductance have yet to be identified. Here we identify and characterize CBC1/2 (CONVERGENCE OF BLUE LIGHT (BL) AND CO\textsubscript{2} 1/2), two kinases that link BL, a major component of photosynthetically active radiation (PAR), and the signals from low concentrations of CO\textsubscript{2} in guard cells. CBC1/CBC2 redundantly stimulate stomatal opening by inhibition of S-type anion channels in response to both BL and low concentrations of CO\textsubscript{2}. CBC1/CBC2 function in the signaling pathways of phototropins and HT1 (HIGH LEAF TEMPERATURE 1). CBC1/CBC2 interact with and are phosphorylated by HT1. We propose that CBCs regulate stomatal aperture by integrating signals from BL and CO\textsubscript{2} and act as the convergence site for signals from BL and low CO\textsubscript{2}. 

\textsuperscript{1}Department of Biology, Faculty of Science, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan. \textsuperscript{2}Graduate School of Environmental and Life Science, Okayama University, Okayama 700-8530, Japan. \textsuperscript{3}Department of Molecular & Cellular BioAnalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. \textsuperscript{4}Center for Gene Research, Nagoya University, Chikusa, Nagoya 464-8602, Japan. \textsuperscript{5}Present address: Graduate School of Sciences and Technology for Innovation, 1677-1 Yoshida, Yamaguchi 753-8512, Japan. Correspondence and requests for materials should be addressed to K.-i.S. (email: kenrcb@kyushu-u.org)
S

tomata in plants open in response to light and low concent-
trations of CO₂ and facilitate CO₂ uptake from the
atmosphere for photosynthetic CO₂ fixation and the tran-
spirational stream that delivers mineral nutrients to plant tissues
through xylem1-4. Stomata close in response to drought, Ca²⁺,
abscissic acid (ABA), other plant hormones, and high concentra-
tions of CO₂ to prevent water loss5-7. Optimal stomatal apertures
are maintained by integrating these ever-changing and antag-
onizing factors; appropriately sized apertures maximize plant
growth8-8. Furthermore, a close correlation between photo-
synthetic CO₂ fixation and stomatal conductance is often found
under various light intensities, but the underlying mechanisms for
this relationship are unknown9-10.

The opening of stomata is induced by blue light (BL), red light
(RL), and low concentrations of CO₂. Stomatal opening responses
specific to BL are enhanced by RL and are ubiquitous in land
plants except the fern species belonging to the Polypodiopsida11.
All of the signaling components for the BL response exist in guard
cells, and guard cell protoplasts (GCPs) swell in response to BL12.
BL is perceived by phototropins (phot1, phot2)13, plant-specific BL
receptor kinases14, 15. After autophosphorylation of phototropins16-18,
the signals activate plasma membrane
H⁺-ATPases via the signaling components19 that include BLUS1
kinase and a type1 protein phosphatase20, 21. H⁺-ATPase activi-
ation is induced by phosphorylation of the penultimate threo-
nine in the C terminus19, and the H⁺-ATPase hyperpolarizes the
membrane potential by pumping H⁺ out of the guard cells22-24.
The hyperpolarization drives K⁺ uptake through the inward-
rectifying K⁺ (Kᵢᵣ) channels25 with accumulation of malate25,
Cl⁻, and NO₂⁻. Simultaneous activation of Kᵢᵣ channels in the
phototropin-mediated pathway is also known26. Malate is syn-
thesized in guard cells via starch degradation that is induced by
the activated H⁺-ATPase27. The accumulated K⁺ salt in guard
cells decreases the water potential and causes water uptake,
resulting in stomatal opening. The signals from phototropins also
inhibit the S-type anion current28 that may support stomatal
opening; however, the signaling components connecting the
phototropins, the H⁺-ATPase, and the anion channels remain
unknown.

In contrast to BL-dependent stomatal opening, the mechan-
isms by which RL induces stomatal opening are a matter of
debate2-4, 29-31. RL causes membrane hyperpolarization in guard
cells of Vicia faba32 and stomatal opening in the epidermal peels
of Vicia and Commelina. Both of these responses to RL are
suppressed by DCMU, a photosynthetic electron transport inhib-
itor33. RL-induced stomatal opening is not evident in epidermal
peels of Arabidopsis plants16, 21, but opening is conspicuous in the
intact leaves. In line with this observation, recent investigations
have suggested that in the light diffusible substances are moved
from mesophyll cells to guard cells to induce leaf stomatal
opening30, 34 but the substances have yet to be identified. RL-
induced stomatal opening may be caused by a low intercellular
concentration of CO₂ (Ci) brought about by mesophyll photo-
synthesis because low CO₂ has been reported to cause stomatal
opening22, 27, 35. In contrast, other investigators have reported that
such a reduction in the Ci of leaves was insufficient to cause
stomatal opening4, 30.

Closure of stomata is driven by the release of anions from
guard cells via the S-type channels of SLAC1 (SLOW ANION
CHANNEL ASSOCIATED 1) and SLAH3 (SLAC1 HOMO-
LOGUE 3)36-38. These channels are activated in response to
ABA, Ca²⁺, and high concentrations of CO₂3, 9, 39. The release of
anions across the membrane causes depolarization, resulting in
activation of outward-rectifying K⁺ channels and, thereby,
induces stomatal closure through the simultaneous efflux of
anions and K⁺2, 26. Inhibition of the H⁺-ATPase also occurs in
response to ABA or Ca²⁺ and maintains membrane depolarization
40-42. With respect to CO₂-specific stomatal closure, carbonic
anhydrases43, 44, a MATE-type transporter RHCl1 (RESISTANT TO
HIGH CO₂)44 and HT1 (HIGH LEAF TEMPERATURE 1)45
kinase are responsible for transducing the high CO₂ signal in this
order and activate the S-type anion channels via OST1 (OPEN
STOMATA 1), a common signal transducer for ABA and CO₂46.
Recently, two mitogen-activated protein kinases (MPKs), MPK4
and MPK12, are shown to inhibit HT1 activity, thereby, stimu-
lating stomatal closure in response to CO₂47, 48. Two carbonic
anhydrases, βCA1 and βCA4, stimulate stomatal closure by
converting CO₂ to bicarbonate39. In contrast, HT1 kinase func-
tions as a negative regulator of CO₂-induced stomatal closure.
In ht1-1 and ht1-2 mutants, stomatal opening was impaired or
disrupted in response to low concentrations of CO₂ and both
mutants exhibited constitutive high CO₂ responses49, 50, but a recent report suggested that phosphor-
ylation of the transmembrane domain in SLAC1 is crucial to
CO₂-induced stomatal closure51. Furthermore, HT1 is hypothe-
sized to have an essential role in RL-induced stomatal opening
under the low Ci via the interaction with OST149, 52, but the
functional roles of HT1 in CO₂ signaling and RL-induced stomatal
opening are unclear.

In this study, we searched for proteins that are phosphorylated
in response to BL using phosphoproteome analyses to identify
missing components in the phototropin-mediated signaling
pathway in guard cells. We found that a novel protein kinase is
rapidly phosphorylated in a phototropin-dependent manner and
that the kinase and its homolog are redundantly responsible for
light-induced stomatal opening. We demonstrated that the pro-
tein kinases mediate the inhibition of the S-type anion channels
in response to BL and also provide evidence that the kinases are
required for stomatal opening in response to low concentrations
of CO₂ and function in the same signaling pathway as HT1.

Results
Phosphoproteome analyses of proteins in guard cells. To
to identify the signaling components in the pathway for
phototropin-mediated stomatal responses, we used phosphopro-
tome analyses of guard cell protoplasts (GCPs) from Arabidopsis
thaliana. The method facilitates the identification of components
that function redundantly because the mutant screening with a
single mutation does not always provide clear phenotype. GCPs
were illuminated with a short pulse of blue light (BL, 100 μmol m⁻² s⁻¹)
for 30 s superimposed on a background of high-fluence-rate red light (RL, 600 μmol m⁻² s⁻¹). Strong RL
maintains photosynthesis in GCPs at near saturation and enables
to isolate the response specific to BL. We stopped the reaction at
0.5 and 2.5 min after the start of BL pulse by adding tri-
chloroacetic acid (TCA) to GCPs and collected GCP samples
because phosphorylation levels of phototropin and the H⁺-
ATPase, respectively, reach their maximum at these times16, 53.
The samples were digested, and the resulting phosphopeptides
were subjected to mass spectrometric analyses34. We selected
phosphopeptides that were rapidly phosphorylated within 30 s
after BL exposure in a phototropin-dependent manner (Fig. 1a, b).
Phosphopeptides having the sequence RKpSLpSDGEDNVNNTR
were derived from the gene product of At3g01490 that encodes a
novel Ser/Thr protein kinase with a deduced molecular mass of
47 kDa and has a typical kinase domain in the C terminus. The

amount of this protein did not change in response to BL for 30 s (Fig. 1b, inset). The phosphopeptides were reported in PhosPhAt 4.0 database. The At3g01490 gene comprises 411 amino acids with no transmembrane domain (Fig. 1c) and was classified as a mitogen-activated protein kinase kinase kinase (MAPKKK, ref. 55). The protein was named CBC1 (CONVERGENCE OF BL AND CO2) kinase because CBC1 transduces the signals not only from BL, but also from CO2 into stomatal movement; further justification for this name will be described below. The analyses also revealed that the phototropins and the H+-ATPase isoforms of AHA1, 2, 4 (or 11), and 5 (or 8) were phosphorylated in response to BL (Supplementary Fig. 1), as has been reported16–18, and enabled the simultaneous identification of phosphorylation sites in multiple proteins.

**Stomatal opening is impaired in the cbc1 cbc2 double mutant.** To identify the role of CBC1 kinase in BL-dependent stomatal opening, we obtained a T-DNA insertion mutant (cbc1) (Fig. 1c). The temperature of wild-type (WT) plants measured by infrared thermography decreased in response to BL, a result of stomatal opening21, but the temperature of the phot1-5 phot2-1 mutant did not decline (Fig. 1d, e). The cbc1 mutant was slightly impaired in the temperature decrease. Since CBC1 belongs to subgroup C7 of the MAPKK family (ref. 55 and Supplementary Fig. 2), which
includes four other genes, we selected At5g50000 in the same clade of CBC1 and named it CBC2. CBC1 and CBC2 genes were both expressed in guard cells (Supplementary Fig. 3a). When CBC1-GFP or CBC2-GFP was expressed in the cbc1 cbc2 double mutant, GFP fluorescence was found in the cytosol of guard cells (Supplementary Fig. 3b). A T-DNA insertion mutant of At5g50000 (cbc2) exhibited partial impairment in the temperature decrease (Fig. 1d, e). The double mutant (cbc1 cbc2) was severely impaired in the temperature decrease. The impairment was partially complemented by transforming the mutant with CBC1-GFP or CBC2-GFP (Fig. 1f). When CBC1-GFP was overexpressed in cbc1 cbc2 using each own promoter, the temperature decrease was restored almost completely in proportion to the expression levels of CBC1-GFP (Fig. 1g). These results suggest that CBC1 and CBC2 have redundant roles in BL-dependent stomatal opening.

In epidermal peels, stomata scarcely opened in response to RL (50 μmol m\(^{-2}\) s\(^{-1}\)) but did substantially to BL (10 μmol m\(^{-2}\) s\(^{-1}\)) superimposed on RL in WT (Fig. 1h). BL-dependent stomatal opening was not affected in cbc1 and slightly impaired in cbc2, but severely in cbc1 cbc2. Stomatal apertures were smaller in cbc1 cbc2 than in WT under darkness, with apertures of 2.55 ± 0.10 μm for the mutant and 2.89 ± 0.11 μm for WT (P = 2.34 × 10\(^{-4}\)) (Supplementary Table 1a). Since no difference was found in stomatal size between WT and the mutant (Supplementary Table 1b), stomata closed tighter in cbc1 cbc2 than in WT plants.

In intact leaves, RL at 600 μmol m\(^{-2}\) s\(^{-1}\) induced an increase in stomatal conductance that reached a steady state within 60 min (Fig. 1i). BL at 20 μmol m\(^{-2}\) s\(^{-1}\) superimposed on the RL induced a rapid increase in stomatal conductance. The response is specific to BL and is distinct from photosynthesis-dependent stomatal opening\(^{22,56}\). Stomatal opening by both RL and BL was partially reduced in both the cbc1 and cbc2 single mutants and was severely reduced in the cbc1 cbc2 double mutant (Fig. 1i and Supplementary Table 2). To see this easily, the opening responses were normalized to the same starting values on the basis of raw data in Fig. 1i (Supplementary Fig. 4). From these results, we conclude that CBC1 and CBC2 redundantly function to open stomata in response to light. We note that stomata in leaves closed tighter in the double mutant than in WT (Fig. 1i).

We note that stomata exhibited the different responses between leaves and epidermis in Arabidopsis, with opening in leaves and negligible opening in epidermis under RL (Fig. 1h, i). Such difference is partly due to the situation where guard cells are placed. In leaves, mesophyll tissues might provide guard cells with unidentified substances that stimulate stomatal opening but in epidermal peels such tissues are absent.

We investigated BL signaling pathway that mediates stomatal opening in the double mutant. The pathway includes the signaling components of phot1, phot2, BLUS1 kinase, and H\(^{+}\)-ATPase. Unexpectedly, neither of the components nor light signaling was affected in cbc1 cbc2 (Supplementary Fig. 5). Autophosphorylation of phot1 and phot2\(^{38,57}\), phosphorylation of BLUS1\(^{38}\) and H\(^{+}\)-ATPase\(^{38,58}\) were not impaired (Supplementary Fig. 5a–c). Proton pumping in GCPs in response to BL and fusicoccin (Fc), an activator of the H\(^{+}\)-ATPase, occurred normally in cbc1 cbc2 (Supplementary Fig. 5d). We thus hypothesized that the mutation impairs a function downstream of the H\(^{+}\)-ATPase, and K\(^{+}\)in channels are candidates. Whole-cell K\(^{+}\)in current in GCPs determined by patch-clamp technique was reduced by 50% in cbc1 cbc2 (Supplementary Fig. 5e). However, such a reduction in K\(^{+}\)in current will not inhibit stomatal opening because the K\(^{+}\)in current in guard cells is sufficiently large for opening to occur\(^{57,58}\). In accord with this interpretation, stomata in the double mutant opened with a similar time course to those of WT in response to Fc (Supplementary Fig. 5i), but with a slight reduction of opening.

**S-type anion channels cause reduced stomatal opening.** The mechanisms generating a driving force for BL-dependent stomatal opening so far identified were not impaired in the cbc1 cbc2 double mutant, but light-induced stomatal opening was reduced. Such reduction is probably due to accelerated closing processes. During the measurement of stomatal apertures of light-treated epidermal peels by microscope, we noticed that opened stomata gradually closed in the mutant, but such a response was not found in WT. When 50 mM KCl was present in the bathing buffer (pH 6.5), once opened stomata remained constant in the cbc1 cbc2 double mutant (Fig. 2a), but gradually closed in cbc1 cbc2 when KCl was removed. We thus suspected that the S-type anion channels might release Cl\(^{-}\) from mutant guard cells even under light. To test this hypothesis, we investigated the effect of an anion channel blocker, anthracene-9-carboxylic acid (9-AC, ref. \(^{59}\)), on stomatal movement and found that stomatal closure in the mutant was arrested by 9-AC without KCl (Fig. 2b). The result suggests that S-type channels release Cl\(^{-}\) in cbc1 cbc2 under light and causes reduced stomatal opening.

To show genetically the involvement of S-type anion channels of SLAC1 and/or SLAH3\(^{36–38}\) in the reduced stomatal opening, we generated the triple mutants of cbc1 cbc2 slac1-4 and cbc1 cbc2 slah3-3. Stomatal apertures were larger in slac1-4 than in WT under both darkness and light (Fig. 2c). The apertures were...
Fig. 2 Enhancement of stomatal closure in the cbc1 cbc2 mutants in the absence of KCl. a Light-induced stomatal opening in WT and cbc1 cbc2. Epidermal peels in 5 mM MES-bistrispropane buffer (pH 6.5) containing 50 mM KCl were illuminated with RL (50 μmol m⁻² s⁻¹) plus BL (10 μmol m⁻² s⁻¹) for 2 h to open stomata. Stomatal apertures were measured from epidermal peels floating on the buffer. A portion of the peels was transferred to a buffer containing 50 mM KCl or no KCl and held for 15 or 30 min in the light; epidermal peels were left floating on the same buffer for measurement of stomatal apertures. Asterisks denote the significant differences in stomatal apertures in comparison with WT or cbc1 cbc2 double mutants treated with RL + BL. *P < 0.05 by Student's t test. b Inhibition of stomatal closure by anthracene-9-carboxylic acid (9-AC) in the absence of KCl. Once stomata were allowed to open as described in a, then epidermal peels were transferred to a KCl-free buffer containing 100 μM 9-AC. c, d Light-induced stomatal opening in WT, cbc1 cbc2, slac1 and slah3, their triple (cbc1 cbc2 slac1-4 and cbc1 cbc2 slah3-3) and quadruple (cbc1 cbc2 slac1-4 slah3-3) mutants. Significant differences are denoted with different lowercase letters and analyzed by ANOVA tests followed by a post hoc Tukey test. Stomatal apertures were measured for 25 stomata from floating epidermal peels, and the experiments were repeated three times in different occasions. Bars represent means ± s.d. (n = 75, pooled from triplicate experiments).

smaller in cbc1 cbc2 slac1-4 than in slac1-4, probably due to the activation of SLAH3 in cbc1 cbc2 slac1-4. Likewise, stomatal apertures were smaller in cbc1 cbc2 slah3-3 than in slah3-3 (Fig. 2d), caused by activated SLAC1 in cbc1 cbc2 slah3-3. We generated the quadruple mutant cbc1 cbc2 slac1-4 slah3-3. Stomatal apertures were much larger in slac1-4 slah3-3 than in WT (Fig. 2e). The apertures in the quadruple mutant were much larger than those in cbc1 cbc2 and were almost similar to those in slac1-4 slah3-3. The results indicate that the SLAC1 and SLAH3 genes are epistatic to the CBC1 and CBC2 genes, suggesting that SLAC1 and SLAH3 are regulated by CBC1 and CBC2. Loss of CBC function might fail to inhibit S-type anion channels under the light. Furthermore, the stomatal apertures in slac1-4 and slah3-3 were smaller than those in slac1-4 slah3-3 (Fig. 2c–e), suggesting the redundant function of SLAC1 and SLAH3 in stomatal closure. In most cases, however, the stomatal apertures in slac1-4 were larger than those in slah3-3, suggesting that the SLAH3 role in stomatal closure under our conditions was not large in our materials. We note that light-enhanced stomatal opening in these plant materials, at least be partly caused by the pump activation (Fig. 2c–e and Supplementary Fig. 5).

CBCs regulate the S-type anion channels in response to BL. BL inhibits the S-type anion currents in guard cells via phototropins in Arabidopsis leaves28. Our results suggest that gene disruption of both CBC1 and CBC2 impairs the inhibitory action of BL, thereby causing a reduction in stomatal opening. To test this hypothesis directly, the S-type anion currents were measured in Arabidopsis GCPs by whole-cell patch-clamp technique (Fig. 3). Since a large current was not observed under the conditions, we pre-exposed WT GCPs to extracellular bicarbonate as has been reported43 and
confirmed that the S-type anion current was enhanced fourfold (Fig. 3a, b). We found that the current was greatly reduced by illuminating GCPs with BL at 100 μmol m−2 s−1 under RL (Fig. 3a, b), suggesting that the reduction is BL dependent. The current enhancement by bicarbonate was also documented in cbc1 cbc2 GCPs (Fig. 3a, c). However, the enhanced current in cbc1 cbc2 was not reduced by BL. These results suggest that the signals from phototropins negate the CO2-enhanced anion current of the S-type channels, and CBC1 and/or CBC2 mediate the phototropin-dependent response. We also showed that the anion currents in GCPs from WT and cbc1 cbc2 were similarly enhanced by extracellular 40 mM Ca2+ (Supplementary Fig. 6), suggesting that the mutation of CBCs did not affect the Ca2+ sensitivity in the S-type channels.

CBC1 is phosphorylated by phot1. Phosphoproteome analyses revealed that CBC1 was phosphorylated in the phototropin-mediated pathway (Fig. 1). We indirectly showed the phosphorylation by mobility shift using Phos-tag PAGE and immunological method (Fig. 4a). The CBC1 in GCPs of WT and cbc2 was shifted upward in response to BL, and the upward shift was phototropin-dependent (Fig. 4b). No CBC1 protein was found in cbc1 and cbc1 cbc2. Antibodies against CBC2 recognized four protein bands, but the CBC2 mobility was not changed (Fig. 4a and Supplementary Fig. 7).

Since CBC1 was rapidly phosphorylated in response to BL (Fig. 1b), CBC1 possibly interacts with phot1 and is directly phosphorylated by phot1. His-CBC1 or His-CBC2 was mixed with FLAG-phot1. Pull-down assays indicated that both CBC1 and CBC2 co-precipitated with phot1, suggesting that both CBCs interacted with phot1 (Fig. 4c). To investigate the interaction in vivo, we conducted bimolecular fluorescence complementation (BiFC) assays in Arabidopsis mesophyll protoplasts (MCPs) using CBCs-nYFP and phot1-cYFP (Fig. 4d). Since CBC2-nYFP expressed in MCPs damaged to the protoplast probably due to kinase activity, we used a kinase-dead CBC2 (D245N) instead of an active CBC2. Co-infection of CBC1 or CBC2 (D245N) with phot1 produced yellow-green fluorescence on the periphery of MCPs, indicating that both CBC1 and CBC2 interacted with phot1. No signal was found in the absence of CBCs, and in the presence of both phot1-cYFP and PIN1-nYFP as a negative interactor for phot1 (Supplementary Fig. 8). We next measured the phosphorylation of CBC1 and CBC2 by phot1 in vitro. A glutathione S-transferase (GST)-tagged C-terminal fragment of phot1 (620–996) (P1C) lacking the LOV (light, oxygen, voltage) domains, which is active without BL, was combined with kinase-dead CBC1 (D271N) or CBC2 (D245N) as substrates. P1C phosphorylated CBC1 but not CBC2 (Fig. 4e), and the kinase-dead P1C (D806N) did not phosphorylate either substrate.

Phot1 phosphorylated CBC1 in in vitro and in vivo, but it is unclear whether the phosphorylation sites in vitro are the same as those in vivo. To test this, we replaced two in vivo phosphorylated Sers (S43, S45) (Fig. 1c) by Alas in the kinase-dead CBC1 (D271N) and utilized this protein as substrate for P1C. We found no phosphorylation of the CBC1 (S43A, S45A) by P1C (Fig. 4f), suggesting that the phosphorylation sites in vitro are the same as those in vivo or contain one of them.

We interpreted already that the CBC1 mobility shift was caused by phosphorylation in vivo through Phos-tag PAGE (Fig. 4a, b). If so, we can expect that the CBC1 (S43A S45A) will not show such shift in response to BL in vivo. In accord with this, CBC1 (S43A S45A)-GFP expressed in the cbc1 cbc2 double mutant did not show the shift although WT CBC1-GFP exhibited the typical shift (Fig. 4g). The result confirms that CBC1 is phosphorylated on Ser43 and Ser45 in guard cells and suggests that the mobility shift is caused by phosphorylation of these sites (Fig. 4a, b). We then investigated the interactions between CBCs. Pull-down assays indicated that both CBC1 and CBC2 interacted with either CBC1 or CBC2 (Fig. 4h).
Phot1 directly phosphorylated CBC1 in vitro. Since phototropins directly phosphorylate BLUS1 in as vivo substrate[2], we investigated the role of BLUS1 in the CBC1 phosphorylation. We found that CBC1 was phosphorylated in the blus-1-3 mutant in response to BL (Fig. 4i), suggesting that the BLUS1 does not mediate the signaling between phototropins and CBC1.

**CBC1 and CBC2 function in the same pathway as HT1.** CBCs abolish the CO2-induced activation of S-type anion channels via a phototropin-mediated pathway (Fig. 3). Since stomatal closure by high CO2 concentrations requires the S-type anion channel activation, CBCs function as negative regulators for CO2-induced stomatal closure[4]. As HT1 kinase functions as a negative regulator for CO2-induced stomatal closure[4–9], CBCs may act in the same pathway as HT1. We thus investigated stomatal responses to CO2 in thecbc1 cbc2 double mutant in comparison with those in theht1 mutant under darkness. Stomatal conductance increased with the CO2 concentration decrease from 350 to 100 ppm and decreased with the concentration rise 100 to 800 ppm in WT (Fig. 5a). Interestingly, no such response was found incbc1 cbc2. In theht1-9 mutant that was identified in our laboratory as having lost kinase activity (Supplementary Fig. 9), stomata had the same responses as those incbc1 cbc2 (Fig. 5a). The triple mutantcbc1 cbc2ht1-9 revealed the same stomatal phenotype as thecbc1 cbc2 andht1-9 mutants (Fig. 5a). These results indicate that CBC1 and CBC2 function in the same pathway as HT1. Sinceht1 mutants exhibited the normal ABA sensitivity[45], we investigated the ABA sensitivity in thecbc1 cbc2 double mutant. Stomata closed by ABA in the mutant (Fig. 5b), indicating that CBCs do not play a role in ABA-induced stomatal closure.

We determined the stomatal CO2 sensitivity in thecbc1 andcbc2 single mutants under darkness (Fig. 5c). Stomatal conductance increased in both single mutants when the CO2 concentrations were decreased from 350 ppm to 100 or 50 ppm, but the magnitudes were smaller by 50% in the mutants than in...
mutants and the μ

Co-infection of CBC1 or CBC2 (D245N) with HT1 produced Arabidopsis in To investigate this possibility in vivo, we conducted BiFC assays response to changes in CO2 concentration in the dark.

cbc1 cbc2

Stomatal opening in WT and only slightly in the double mutant in response to changes in CO2 concentration in the dark. (Fig. 5d). The degree of opening in WT was twofold larger under low CO2- (Fig. 5d). The degree of opening in WT was twofold larger under light than darkness (Fig. 5c, d). Furthermore, the low CO2-

( Fig. 5d). The degree of opening in WT was twofold larger under light than darkness (Fig. 5c, d). Furthermore, the low CO2-

opening was shown in both plant lines in response to RL (100 mol m−2 s−1) (Fig. 5d). The CO2 concentration was changed as indicated. (Fig. 5d and Supplementary Table 3). The results suggest that both CBC1 and CBC2 interacted with HT1. No signal was found without CBCs. We further investigated the interaction between CBCs and HT1 in vitro. FLAG-CBC1 or FLAG-CBC2 was mixed with His-HT1. Pull-down assays indicated that CBC1 and CBC2 co-precipitated with HT1, suggesting that CBC1 and CBC2 physically interacted with HT1 (Fig. 6b). We next determined the protein kinase activities of the CBCs. GST fusions of CBC1 (GST-CBC1) and CBC2 (GST-CBC2) were autophosphorylated (Fig. 6c). A mutation introduced in the kinase domain caused the loss of their autophosphorylation activities in the mutated CBCs (CBC1 (D271N), CBC2 (D245N)). The native CBCs phosphorylated myelin basic protein (MBP) and histone but not casein (Fig. 6d), indicating that CBCs possess protein kinase activity. We then determined whether HT1 could phosphorylate CBC1 and CBC2 and vice versa. Kinase-dead forms of all CBCs or HT1 were used as substrates. We found that HT1 phosphorylated both CBC1 and CBC2, but CBC1 and CBC2 did not phosphorylate HT1 (Fig. 6e). CBC1 and CBC2 may be the substrates of HT1 and likely function downstream of HT1. Since CBC1 can be a substrate for both HT1 and phot1, we tested whether the phosphorylation sites of CBC1 by HT1 are the same as those by phot1. We used CBC1 (D271N S43A S45A) (Figs. 1c and 4f), in which phosphorylatable SerS by phot1 in vivo were substituted by Ala, as a substrate for HT1, and found the phosphorylation of the CBC1 (Fig. 6f). The result suggests that phosphorylation sites in CBC1 by HT1 might be different from those by phot1 or have additional sites, but further investigation will be required.

Fig. 5 Stomatal responses of the cbc mutants to CO2 and ABA in Arabidopsis. a Stomatal movements of the cbc1 cbc2, htl-9, and cbc1 cbc2 htl-9 mutants in response to changes in CO2 concentration in the dark. b Stomatal closure mediated by ABA in cbc1 cbc2. c Stomatal responses of the cbc1, cbc2 single mutants and the cbc1 cbc2 double mutant in response to changes in CO2 concentration in the dark. d Stomatal conductance increases in WT plants and the cbc1 cbc2 double mutant in response to the CO2 concentration decrease under the moderate light of RL (90 μmol m−2 s−1) plus BL (10 μmol m−2 s−1) or RL (100 μmol m−2 s−1) only. The CO2 concentration was changed as indicated. a, c, d Bars represent ± s.e.m. (n = 4 for a, c and n = 8 for d). Stomatal opening in intact leaves was induced by light, and the epidermal peels were obtained. b ABA was added to the peels at the indicated concentrations and kept in the light for 2 h. Experiments were repeated three times in different occasions. Bars represent means ± s.d. (n = 75)

WT. The results indicate that CBC1 and CBC2 are required for stomatal opening in response to low concentrations of CO2 and function redundantly. We next determined stomatal opening in response to CO2 concentration changes under the moderate light. Stomata opened in WT and slightly in cbc1 cbc2 in response to RL (90 μmol m−2 s−1) plus BL (10 μmol m−2 s−1) (Fig. 5d). Less opening was shown in both plant lines in response to RL (100 μmol m−2 s−1) than RL plus BL. When CO2 concentration was reduced from 350 to 100 ppm under the light, stomata opened remarkably in WT and only slightly in the double mutant (Fig. 5d). The degree of opening in WT was twofold larger under the light than darkness (Fig. 5c, d). Furthermore, the low CO2-induced opening was larger by 25% under RL plus BL than RL (Fig. 5d and Supplementary Table 3). The results suggest that CBCs substanitate the co-operation between the light and low CO2 for stomatal opening, which enhances the uptake of CO2 for photosynthesis. It is interesting to note here that BL-dependent stomatal opening was enhanced by reduction of the intracellular CO2 concentration in Commelina.62 Taken together, these results indicate that CBC1 and CBC2 integrate and transduce the signals from both BL and CO2 into stomatal movement.

**CBC1 and CBC2 are phosphorylated by HT1 in vitro.** Since CBC1, CBC2, and HT1 function in the same pathway, we expected protein–protein interactions between CBCs and HT1. To investigate this possibility in vivo, we conducted BiFC assays in Arabidopsis MCPs using CBCs-nYFP and HT1-cYFP (Fig. 6a). Co-infection of CBC1 or CBC2 (D245N) with HT1 produced yellow–green fluorescence on the periphery of MCPs, indicating that both CBC1 and CBC2 interacted with HT1. No signal was found without CBCs. We further investigated the interaction between CBCs and HT1 in vitro. FLAG-CBC1 or FLAG-CBC2 was mixed with His-HT1. Pull-down assays indicated that CBC1 and CBC2 co-precipitated with HT1, suggesting that CBC1 and CBC2 physically interacted with HT1 (Fig. 6b). We next determined the protein kinase activities of the CBCs. GST fusions of CBC1 (GST-CBC1) and CBC2 (GST-CBC2) were autophosphorylated (Fig. 6c). A mutation introduced in the kinase domain caused the loss of their autophosphorylation activities in the mutated CBCs (CBC1 (D271N), CBC2 (D245N)). The native CBCs phosphorylated myelin basic protein (MBP) and histone but not casein (Fig. 6d), indicating that CBCs possess protein kinase activity. We then determined whether HT1 could phosphorylate CBC1 and CBC2 and vice versa. Kinase-dead forms of all CBCs or HT1 were used as substrates. We found that HT1 phosphorylated both CBC1 and CBC2, but CBC1 and CBC2 did not phosphorylate HT1 (Fig. 6e). CBC1 and CBC2 may be the substrates of HT1 and likely function downstream of HT1. Since CBC1 can be a substrate for both HT1 and phot1, we tested whether the phosphorylation sites of CBC1 by HT1 are the same as those by phot1.

We used CBC1 (D271N S43A S45A) (Figs. 1c and 4f), in which phosphorylatable SerS by phot1 in vivo were substituted by Ala, as a substrate for HT1, and found the phosphorylation of the CBC1 (Fig. 6f). The result suggests that phosphorylation sites in CBC1 by HT1 might be different from those by phot1 or have additional sites, but further investigation will be required.
Discussion

In this study, we used phosphoproteomic methods to identify phototropin-mediated phosphorylated proteins in guard cells (Fig. 1). Among these proteins, we selected one protein kinase that was phosphorylated in response to BL for detailed study. We named it CBC1 and characterized the role of this protein in stomatal responses. Since a cbc1 mutant exhibited only slight impairment in the stomatal BL response (Fig. 1d, e), we identified another homologous protein (Supplementary Fig. 2) and named it CBC2. The cbc2 mutant had slightly impaired stomatal opening by BL and the cbc1 cbc2 double mutant showed severely impaired response, suggesting that CBC1 and CBC2 function redundantly in stomatal opening (Fig. 1f, g).

How is the stomatal response to BL impaired in the cbc1 cbc2 mutant? We initially expected that activation of H+-ATPase by phototropins was compromised in the mutant. Unexpectedly, neither the components nor the signaling that leads to H+-ATPase activation was injured in the mutant (Supplementary Fig. 5c, d). Since $K^{+}$ channel activity was reduced to 50% in cbc1 cbc2 (Supplementary Fig. 5e), it might cause decreased stomatal opening (Fig. 1). However, the channel activity in guard cells is thought to be much higher than required for the opening in Arabidopsis 58, 63, 64. If the $K^{+}$ channel activity limited stomatal opening, the opening rate should be decreased in response to fusicoccin as reported 57, but the rate was not affected in cbc1 cbc2 (Supplementary Fig. 5f). The different results reported are probably caused by different species used 65.

We then suggested that altered activities of S-type anion channels were responsible for stomatal phenotype in the epidermis of cbc mutants using various channel mutants (Fig. 2). Furthermore, our patch-clamp experiments revealed that bicarbonate-activated S-type anion channels in GCPs were suppressed in response to BL in WT but not in cbc1 cbc2 (Fig. 3). From these physiological, genetic, and electrophysiological analyses, we conclude that impaired stomatal opening is due to the inability to inhibit the S-type channels in response to BL and...
CBCs mediate the phototropin-dependent inhibition of S-type anion channels. The presence of this pathway was previously reported in intact plants of *Vicia* and *Arabidopsis* 26; however, our study has now established the physiological relevance of this pathway.

It is evident that CBC1 is located in the phototropin-mediated signaling pathway (Fig. 1). CBC1 physically interacted with phot1 and was directly phosphorylated by phot1 in vitro (Fig. 4), suggesting that CBC1 can be a substrate of phot1. If so, CBC1 should be involved in a pathway distinct from BLUS1 because BLUS1 is a substrate of phototropins 21. In accord with this, the disruption of *BLUS1* (blus1-3) 23 did not affect CBC1 phosphorylation (Fig. 4i). We were unable to show the phosphorylation of CBC2 by phototropins, although CBC2 interacted with phot1 (Fig. 4). Since CBC2 functions redundantly with CBC1 for stomatal opening (Fig. 1), it is unclear how the BL signal is transmitted to CBC2. CBC2 may receive BL signal from CBC1 by protein–protein interaction or from phototropins by the direct interaction because CBC2 interacts with both CBC1 and phot1 (Fig. 4c, b).

We obtained evidence that CBC1 and CBC2 function in the same pathway as HT1 for *CO2* signaling (Fig. 5a). For example, the *cbc1 cbc2* double mutant did not respond to *CO2* concentration changes. The *cbc1 cbc2* mutant had the same stomatal response as the *ht1-9* single mutant and the *ht1-2* mutant 45 (Fig. 5 and Supplementary Fig. 5). The *cbc1 cbc2* *ht1-9* triple mutant had the same phenotype as *ht1-9*. Furthermore, both CBC1 and CBC2 interacted with HT1 in vivo (Fig. 6a). CBCs physically interacted with HT1 in vitro and were phosphorylated by HT1, but did not phosphorylate HT1 (Fig. 6e). The simple interpretation of these results is that CBCs function downstream of HT1 and ultimately inhibit the *S*-type anion channels as has been reported 44, 46, 47.

Since CBCs act not only in the phototropin-mediated BL signaling pathway, but also in the HT1-mediated *CO2* signaling pathway, we propose a working model indicating that CBCs function where BL and *CO2* signals converge (Fig. 7). In this model, red arrows favor stomatal opening in response to RL, and low *CO2*, and black arrows stimulate stomatal closure in response to high *CO2*. We noticed here that HT1 (At1g62400) and CBCs belong to the subgroups C5 and C7, respectively, in this phylogenetic tree of the MAPKKK multigene family (Supplementary Fig. 2). These three genes are likely derived from the same ancestral gene. Furthermore, CBC1 and CBC2 recently evolved by duplication.

Recent investigations have indicated that HT1 plays a role in *CO2*-dependent stomatal closure as a negative regulator 44, 49, 52. We showed that the CBC protein kinases function as negative regulators in the same pathways as HT1 and are involved in inhibiting the activities of SLAC1 and SLAH3. Furthermore, we showed that CBCs were phosphorylated by the HT1 kinase in vitro (Fig. 6). In contrast, HT1 kinase is proposed to directly phosphorylate and inactivate OST1, thereby inhibiting high *CO2*-induced stomatal closure through inactivation of SLAC1 46. High concentrations of *CO2* abolish HT1 activity and result in the activation of SLAC1 via phosphorylation of the N terminus by active OST1 44, 46 (see Fig. 7). Although CBCs are involved in the *CO2* signaling pathways of guard cells, the role(s) and substrate(s) of CBCs are unknown. CBCs may be substrates to inhibit the *S*-type channels of both SLAC1 and SLAH3 by phosphorylation or indirect regulation via other kinases and phosphatases. Furthermore, high *CO2* activates protein kinases other than OST1 and causes *CO2*-dependent stomatal closure by activation of SLAC1 through phosphorylation of the transmembrane region 31. CBCs may inhibit the unidentified kinases that activate SLAC1. The reconstitution studies in *Xenopus* oocytes that express these components, including phot1 or HT1, CBCs, S-type channels, and OST1 will confirm these findings in planta.

We showed that RL-induced stomatal opening was impaired to the same degree as BL-dependent stomatal opening in the *cbc* mutants (Fig. 1i and Supplementary Table 2). The lesion in BL-dependent stomatal opening can be accounted by the inability of the mutants to inhibit S-type channels in response to BL (Figs. 2 and 3). In contrast, how RL-induced stomatal opening is inhibited in the mutants is unknown. Since strong RL (also strong BL) reduces *Ci* by photosynthetic *CO2* fixation, we speculated that the reduced *Ci* might cause stomatal opening by inhibiting the S-type channels through CBCs, but such a response did not occur in the *cbc* mutants. Other mechanisms rather than the inhibition of the S-type channels may exist, and previous reports have suggested that HT1 plays a role in RL-induced stomatal opening 44, 49, 52 and that *H+-ATPase* is possibly involved in the response 22. We note that the *H+-ATPases* can be activated by modifying several sites in their autoinhibitory domain 46. Further investigation is required for elucidating the role of CBCs in RL-induced stomatal opening.

In conclusion, we showed that CBCs stimulate stomatal opening by inhibiting the S-type anion channels in response to BL, which is a major component of photosynthetically active radiation (PAR). CBCs also likely cause stomatal opening in response to low concentrations of *CO2* resulting from photosynthesis by suppressing the S-type channels. We thus propose that CBCs are a convergence point for the signals from PAR and *CO2* (Fig. 7). PAR, including both BL and RL, may efficiently support stomatal opening by activating CBCs via phototropins and low *Ci*.

### Methods

**Plant materials and growth conditions.** *Arabidopsis thaliana* plants were grown on soil:vermiculite (1:1) for 4 weeks. *D. viridis* (14/10 h, white light (50 μmol m⁻² s⁻¹)) dark cycle at 24 °C. To measure leaf temperature, *Arabidopsis thaliana* seeds were sown on 0.8% (w/v) agar plates containing half-strength Murashige–Skoog salts (pH 5.7), 2.3 mM MES, and 1% (w/v) sucrose and were grown under continuous white light (80 μmol m⁻² s⁻¹) at 23 °C. The 10-day-old plants were transferred to a photomicroclimate (11:13 h light:dark) cycle for 7 days. Mutants of *cbc1, cbc2, blus1-3*, *slac1-4*, and *slah3-3* were obtained from the Nottingham Arabidopsis Stock Center (NASC), Nottingham, UK. The *phot1-5 phot2-1* mutant has been described in ref. 13.

**Phosphoproteome analyses and phosphopeptide quantification.** Enzymatically isolated GCPs were illuminated with strong RL (600 μmol m⁻² s⁻¹) for 30 min, and then a BL pulse (100 μmol m⁻² s⁻¹) was applied for 30 s. We terminated the reactions at 0.5 and 2.5 min after the start of the pulse by adding TCA to the GCPs. Then, the GCPs were suspended in 1.0 M Tris-Cl (pH 8.0) containing 8 M urea, protein phosphatase inhibitors, and protease inhibitor cocktails (Sigma-Aldrich) according to the manufacturer’s protocol, and sonicated for 5 min. The suspensions were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with *Lys-C*, followed by tryptic digestion. The digested samples were desalted using StageTips with C18 Empore disk membranes (3 M). Phosphopeptides were enriched by hydroxy acid-modified metal oxide chromatography using lactic acid–modified titania 54. In brief, the digested samples were diluted with 0.1% TFA, 80% acetonitrile containing 300 mg ml⁻¹ lactic acid (solution A), and loaded to custom-made metal oxide chromatography tips preliminary equilibrated with solution A. After successive washing with solution A and 0.1% TFA, 80% acetonitrile, the peptides were eluted with 0.5% piperidine. The obtained fractions were desalted using SDB-XC-StageTips and concentrated in a vacuum evaporator for nanoLC-MS/MS analysis. NanoLC-MS/MS analyses were performed by using LTQ-Orbitrap (Thermo Fisher Scientific, Rockwell, IL, USA). Peptides and proteins were identified by means of automated database searching using Mascot version 2.3 (Matrix Science, London, UK) against the TAIR database (release 10) with a precursor mass tolerance of 3 ppm, a fragment ion mass tolerance of 0.8 Da, and strict trypsin specificity allowing for up to two missed cleavages. Cysteine carboxymethylation was set as a fixed modification, and methionine oxidation and phosphorylation of serine, threonine, and tyrosine were allowed as variable modifications. Peptides were considered to be identified if the Mascot score was over the 95% confidence limit based on “identity” score of each peptide and if at least three successive y or b ions with an additional two or more y, b ions were observed. False-positive rate was evaluated by searching against a randomized decoy database created by the Mascot Perl script, and estimated at less than 1% for phosphopeptide identification in all LC-MS data. Phosphorylation sites were unambiguously determined when b or y ions were between which the phosphorylated residue exists.
Infrared data were obtained and visualized31. Arabidopsis thaliana plants were kept in the dark before the measurements.

Measurement of stomatal aperture in epidermal peels. Epidermal peels of 4- to 6-week-old plants were detached from dark-adapted Arabidopsis leaves. Peels floating on basal reaction mixture that contained 5 mM MES-bis-trispropane (pH 6.5), 50 mM KCl, and 0.1 mM CaCl₂ were illuminated with RL (50 μmol m⁻² s⁻¹) and BL (10 μmol m⁻² s⁻¹) superimposed on the RL for 2 h to open stomata. The epidermal peels were collected on a nylon mesh, rinsed with distilled water, and the stomatal apertures were measured microscopically. In Fig. 2a, b, one stomata opened in response to light, the peels were transferred to 5 mM MES-bis-trispropane (pH 6.5) buffer containing 50 mM KCl or without KCl (control) and stomatal apertures were measured at the indicated times. To test the effect of an anion channel blocker on stomatal movement, anacrine-9-carboxylic acid (9-AC) (Sigma) dissolved in DMSO was added to the epidermal peels at a final concentration of 100 μM32. ABA induced stomatal closure was conducted according to the method of ref. 33 with modification. To preopen stomata, leaves from 4- to 6-week-old plants were floated on opening buffer containing 5 mM KCl, 50 μM CaCl₂, and 10 mM MES-Tris (pH 6.15) for 2 h in the light (80 μmol m⁻² s⁻¹). The epidermal peels were detached from the leaves and immersed in opening buffer in the presence of ABA. Stomatal apertures were measured after illuminating the epidermal peels with white light (80 μmol m⁻² s⁻¹) for 2 h.

GCPs and H⁺ pumping. GCPs were prepared enzymatically unless otherwise noted34. BL- and Fr-dependent H⁺ pumping were measured with a glass pH electrode35.

Phosphorylation assays. Phosphorylation of phototropins, BLUS1, CBC1, and CBC2 was terminated 1 min after the start of BL illumination by adding TCA to GCPs. Phosphorylation of these three proteins was determined by a mobility shift assay and phosphorylation of H⁺-ATPase was estimated from a protein blot using antiprotein36. The amounts of phosphorylated proteins were analyzed37. Unprocessed immunoblots are shown in Supplementary Fig. 10.

Protein expression and in vitro kinase assay. For expression of GST-tagged protein, CBC1, CBC1 (D271N), CBC2, CBC2 (D243N), HT, HT1 (D184N), and HT2 (D184N) were cloned into pColdTMGST DNA (TAKARA). P1C and P1C (D806N) were cloned into the pGEX-2T vector (GE Healthcare)61. All of these GST-tagged proteins were expressed in E. coli strain RosettaTM2 (DE3) (Novagen). The cells were collected at 37 °C in Luria–Bertani (LB) broth containing 50 mg ml⁻¹ ampicillin until the OD₆₀₀ reached 0.4-0.6. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.2 mM, and the culture was incubated at 15 °C overnight. The cells were collected and resuspended in 20 mM Tris- HCl (pH 7.4) and 140 mM NaCl and were disrupted by sonication in the presence of 0.1% (v/v) Triton X-100. The disrupted cells were centrifuged, and the resulting supernatant was incubated with glutathione-Sepharose 4B (GE Healthcare) for 1 h at 4 °C. The glutathione-Sepharose beads were washed three times with 20 mM Tris- HCl (pH 7.4), 140 mM NaCl, 0.1% Triton X-100, and once with 50 mM Tris- HCl buffer (pH 8.0). The GST fusion proteins were eluted with 10 mM reduced glutathione in 50 mM Tris- HCl buffer (pH 8.0). Purified proteins were stored in 50% (v/v) glycerol at −20 °C. In vitro phosphorylation assays were performed in a reaction mixture of 50 μl that contained 50 mM Tris- HCl (pH 7.5), 10 mM MgCl₂, 3.3 μM ATP, 20 μCi of [γ-³²P] ATP (3000 Ci mmol⁻¹, PerkinElmer), and purified proteins. Artificial substrates of casein (dephosphorylated from...
bovine milk), Sigma), MBP (MyBP, Sigma), and histone (type III-S from calf thymus, Sigma) of 3 μg were used. The reaction proceeded for 2 h at 15 °C was stopped by adding SDS sample buffer and then boiled at 95 °C for 3 min. The samples were subjected to SDS-PAGE, and the CBB-stained gels were dried. Phosphorylated proteins were identified by autoradiography with an image plate and analyzed with an FLA1000 Phosphoimager (Fujiﬁlm). Uncropped immunoblots are shown in Supplementary Fig. 10.

**In vitro pull-down assays.** For in vitro pull-down assays, His-tagged and FLAG-tagged proteins were synthesized.1–3 Synthesized proteins (50–100 μl) were mixed with 450–900 μl binding buffer containing 20 mM Tris–HCl (pH 7.4), 140 mM NaCl, and 0.1% (v/v) Triton X-100, and the mixtures were kept on ice for 10 min. After centrifugation at 12,000g for 10 min at 4 °C, the supernatants were mixed with anti-FLAG M2 Affinity Gel (Sigma–Aldrich) for 1 h at 4 °C. After washing the gel three times, the bound proteins were eluted and subjected to immunoblotting using the TEL, His2Tag antibody, mAb, mouse (1:4000, GenScript, #A08106-100) and mouse TrueBlot Ultra: anti-mouse Ig HRP (1:1000, Rockland Immunochemicals, #18–8817–30). FLAG-tagged proteins were detected by a monoclonal anti-Flag M2-peroxidase (HRP) antibody produced in a mouse (1:1000, Sigma-Aldrich, #A8592). Uncropped immunoblots are shown in Supplementary Fig. 10.

**BiFC experiments.** For BiFC assays, nYFP- and cYFP-fused constructs were co-transformed into mesophyll protoplasts4–5 by PEG-calcium transfection. To generate the nYFP- and cYFP-fused constructs, expression cassettes of pL100N(E173 and pSPYCE (M) were introduced into the pRI101 vector. CDS fragments of PHOT1, CBC1, CBC2, and HTI were introduced into the BambHI and SalI sites of the vector. Fluorescent images were obtained using a confocal laser scanning microscope (Digital Eclipse CI; Nikon). Waveforms for excitation and emission of YFP were 488 and 513–530 nm, and those for chlorophyll fluorescence were 543 and 590 nm long-pass.

**Data availability.** MS data files have been deposited at ProteomeXchange with the identifier PXD006586. The authors declare that all other data supporting the findings of this study are available within the manuscript and its supplementary files or are available from the corresponding author on request.

Received: 31 August 2016 Accepted: 31 August 2017

Published online: 03 November 2017

**References**


Acknowledgements

We are grateful to Dr M. Doi and Dr Y. Takahashi for their suggestions. We thank M. Aibe, E. Abe, and Dr E. Gotoh for their technical assistance. We are indebted to Dr H. Tachiya for the discussion on the evolutionary aspect. We are also grateful to the Salk Institute Genomic Analysis Laboratory, the NASC, and the Syngenta Arabidopsis Insertion Library collection for providing the seeds. This work was supported by JSPS KAKENHI Grant numbers, 21227001, 26251032 (to K.S.), 26710191, and 15K14552 (to A.T.), and MEXT KAKENHI Grant numbers, 23150251 and 25120719 (to A.T.), and Grant-in-Aid for JSPS fellows Grant number 13J05118 (to A.H.).

Author contributions

A.H., A.T., and K.S. conceived and designed the experiments. A.H. performed most of the experiments. N.S. did the mass spectrometric analysis. S.M., E.O., and Y.M. did the patch-clamp experiments and analysis. Y.T. assisted in in vitro transcription/translation experiments. A.H. and K.S. wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at doi:10.1038/s41467-017-01237-5.

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.