# Supplementary Information

## A Raf-like protein kinase BHP mediates blue light-dependent stomatal opening

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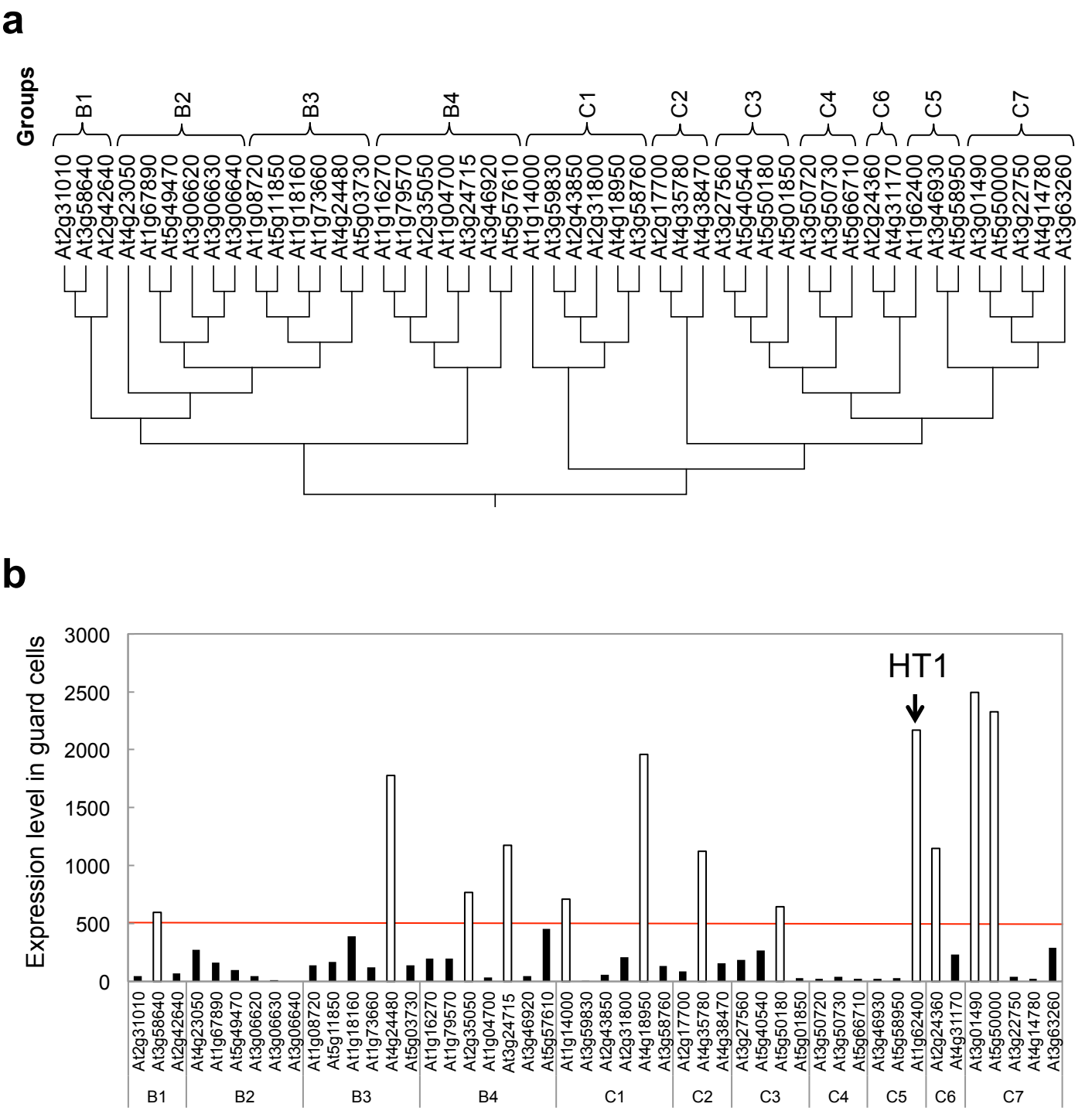
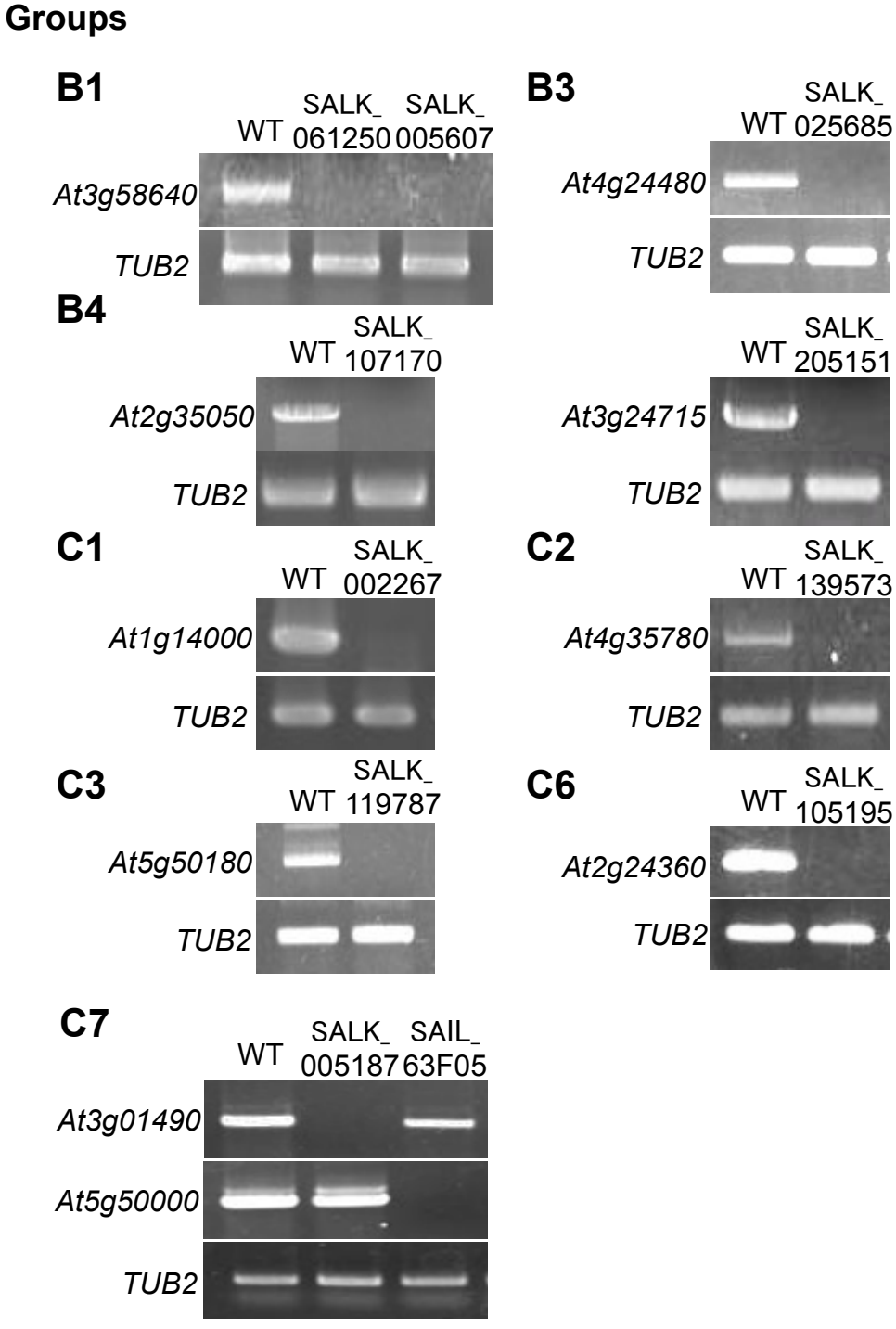


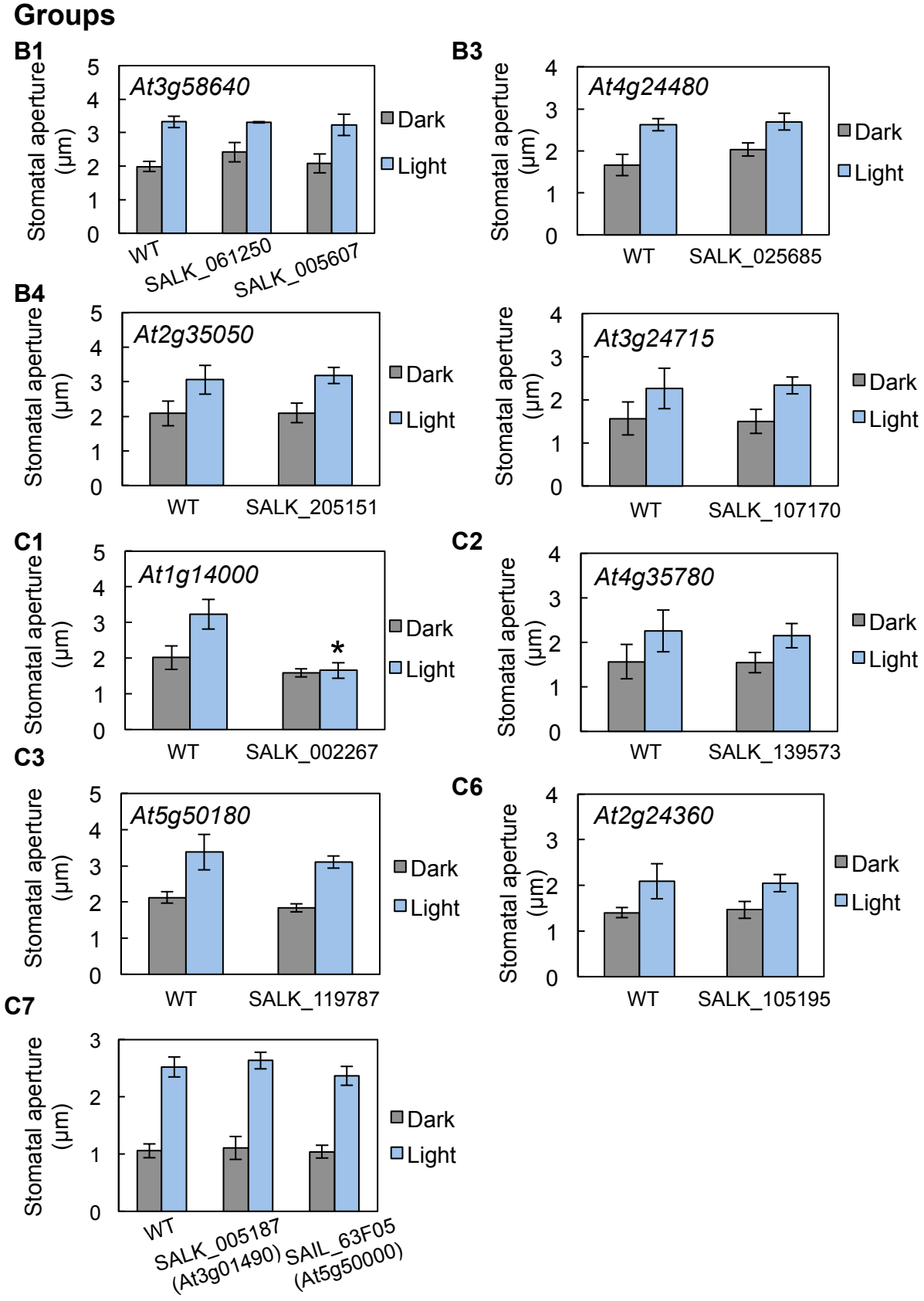

**Supplementary Figure 1. Expression levels of Arabidopsis Raf-like kinase genes in guard cells.**

(**a**) Phylogenetic relationship in the Arabidopsis Raf-like kinases. The tree was similarly created as described in Fig. 4b. Amino acid sequences of the kinase domains from the members were used. The Raf-like kinases subfamily was classified as B1-B4 and C1-C7 groups, as reported previously[1]. (**b**) Gene expression levels of Raf-like kinases in guard cells. Data of the expression levels in guard cells were obtained from a public microarray database eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Guard_Cell). White columns indicate the high expression genes in guard cells over 500 in the database.



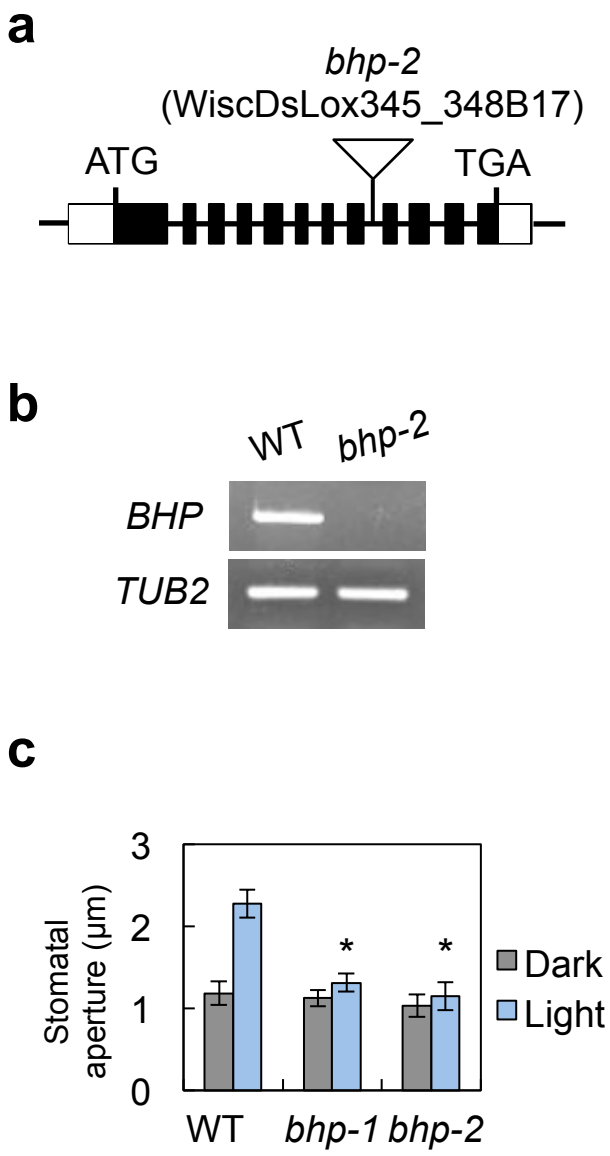

**Supplementary Figure 2. Confirmation of gene knockouts in the T-DNA insertion mutants in this study.**

RT-PCR was performed using total RNAs from the rosette leaves in wild-type (WT) and T-DNA insertion mutants. All genes were amplified by PCR using the specific primer sets (see Supplementary Table 2). *TUB2* was amplified as an internal control.




**Supplementary Figure 3. Measurements of the blue light-dependent stomatal opening in the T-DNA insertion mutants.**

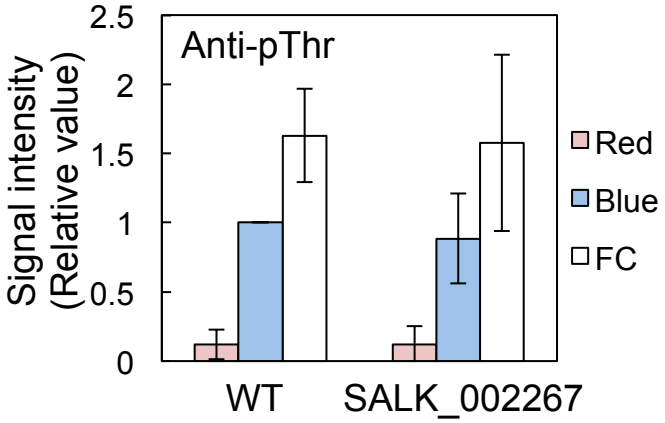
Values represent means±s.d. (*n*=3); measurement of 30 stomata in each experiment. * indicates values that statistically differ from WT blue light sample (Student's *t* test; *$p$<0.01).



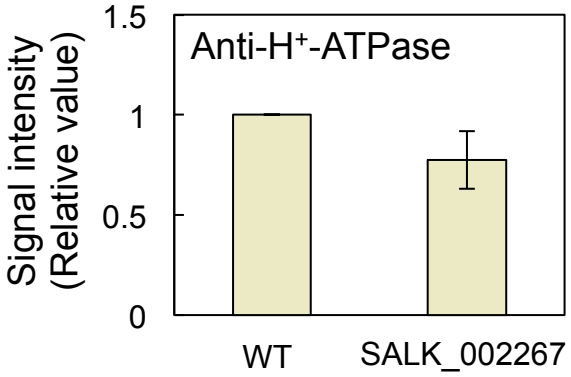

**Supplementary Figure 4. Blue light-dependent stomatal opening in T-DNA insertion alleles of *BHP.***

(**a**) Schematic representation of the *BHP* gene (*At4g18950*) and position of the T-DNA insertion in the *bhp-2* mutant. Boxes and lines indicate exons and introns, respectively. (**b**) Knockout of the *BHP* gene in the *bhp-2* mutant. (**c**) Blue light-dependent stomatal opening in leaves from WT, *bhp-1*, and *bhp-2*. The experiment was performed as described in Fig. 2d. Values represent means ±s.d. (*n*=4); measurement of 30 stomata in each experiment. * indicates values that statistically differ from the corresponding WT (Student's *t* test; *$p$<0.01).

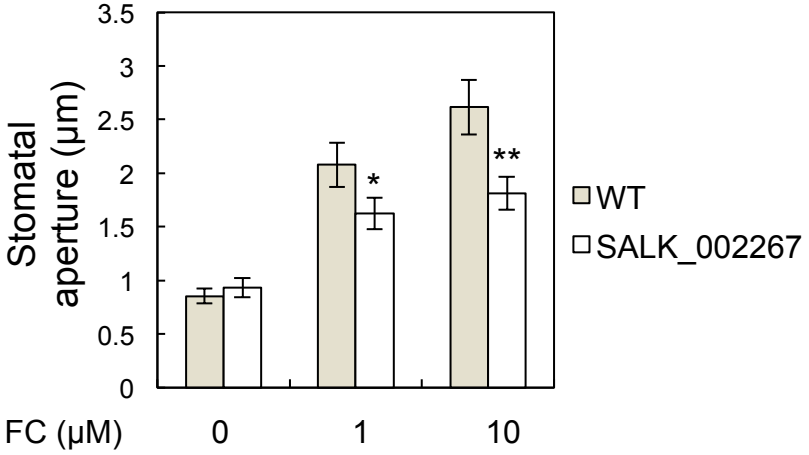
**a**


Anti-pThr
Signal intensity (Relative value)
2.5
2
1.5
1
0.5
0
Red
Blue
FC
WT
SALK_002267

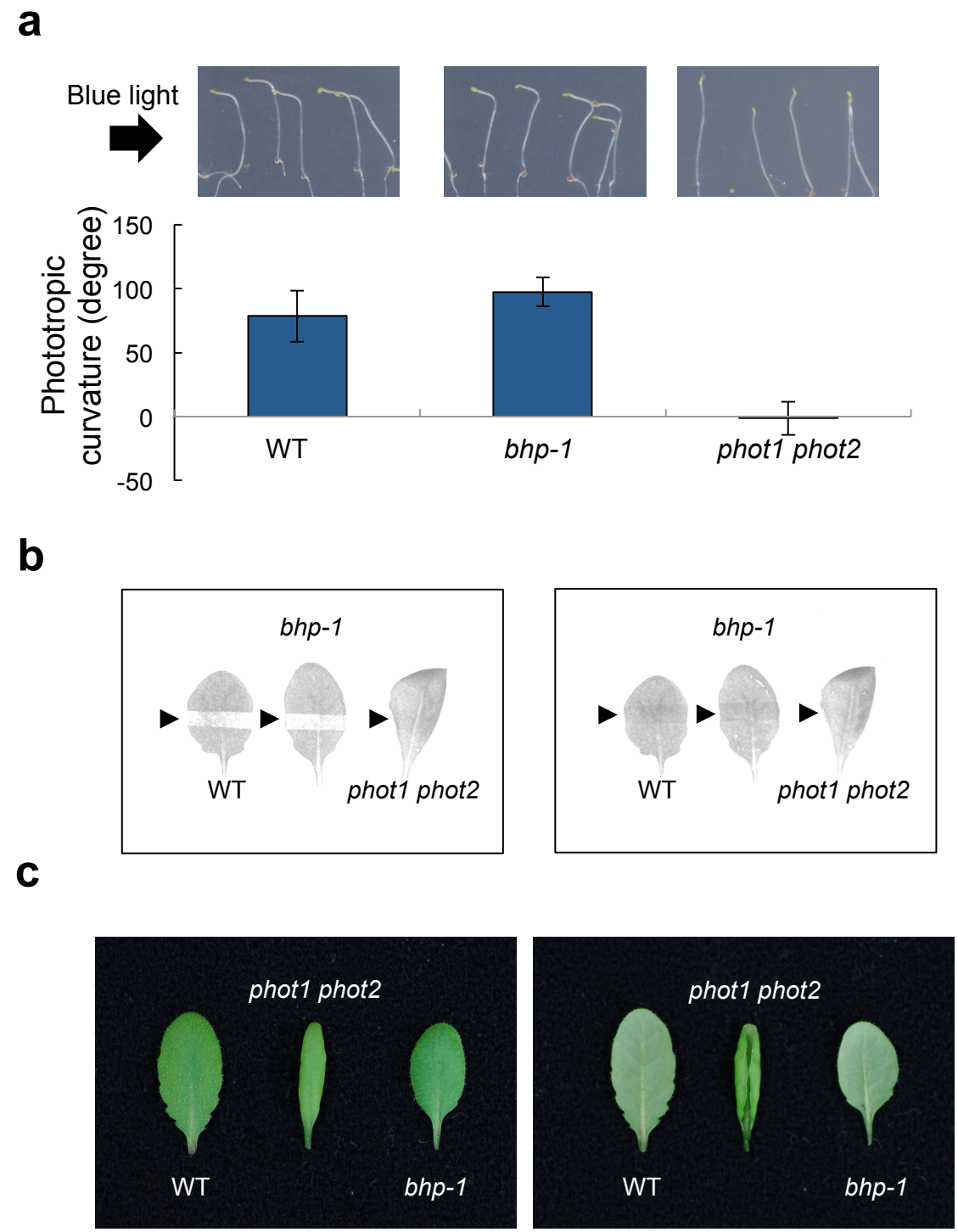
**b**


Anti-H+-ATPase
Signal intensity (Relative value)
1.5
1
0.5
0
WT
SALK_002267

**c**

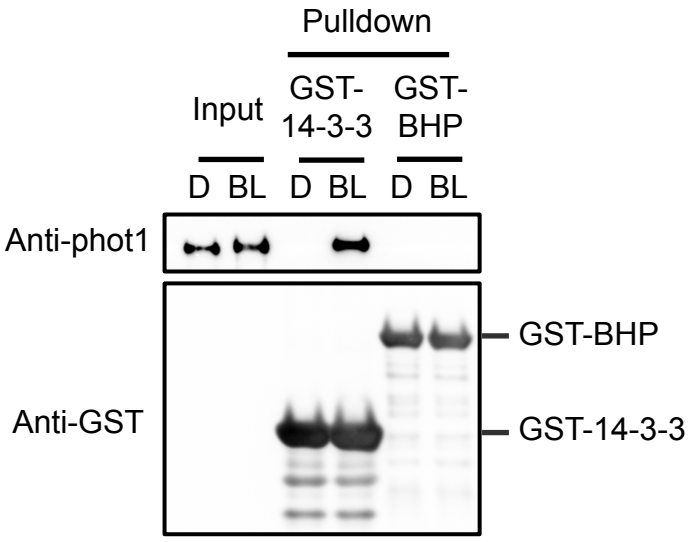

Stomatal aperture (μm)
3.5
3
2.5
2
1.5
1
0.5
0
*
**
WT
SALK_002267
FC (μM)
0
1
10

**Supplementary Figure 5. Immunohistochemical detection of guard cell $H^+$-ATPase and fusicoccin (FC) induced-stomatal opening in the *At1g14000* knockout mutant.**
(**a**) Phosphorylation of the guard cell $H^+$-ATPase in SALK_002267 in response to blue light and fusicoccin (FC). The immunohistochemical experiments using anti-pThr antibody were performed and are shown in Fig. 2h. Values represent means±s.d. ($n$=3); measurement of 30 stomata in each experiment. (**b**) Immunohistochemical detection of the amount of guard cell $H^+$-ATPase in WT and SALK_002267 using anti-$H^+$-ATPase antibody. The relative signal intensity was expressed as the ratio of the signal intensity from the SALK_002267 mutant to that from WT. Values indicate means ±s.d. ($n$=3), and measurements of 30 stomata in each experiment. (**c**) Stomatal opening in response to FC in WT and SALK_002267 epidermis. Epidermal fragments from dark-adapted plants were incubated in reaction buffer 2 (see Methods) containing FC at the indicated concentrations for 3 h in the dark. Data represent means±s.d. ($n$=4). Thirty stomata were measured in each experiment. * and ** indicate values that statistically differ from the corresponding WT (Student's $t$ test; **$p$<0.01, *$p$<0.05).

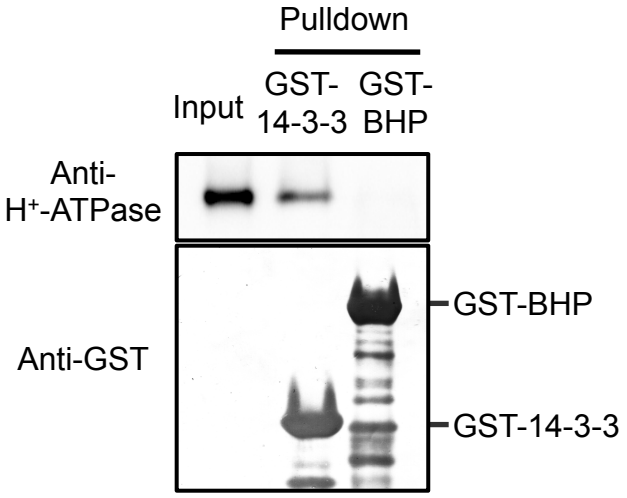



**Supplementary Figure 6. Phot-mediated blue light responses in *bhp-1* mutant.**
(**a**) Phototropism in WT, *bhp-1*, and *phot1-5 phot2-1* (*phot1 phot2*). Etiolated seedlings were irradiated with unilateral blue light (0.5 μmol $m^{-2}$ $s^{-1}$) for 14 h. Values represent means±s.d. (*n*=18-19). (**b**) Slit band assays for the chloroplast movement in WT, *bhp-1*, and *phot1 phot2*. Left and right panels indicate the chloroplast avoidance and accumulation responses, respectively. Detached leaves were irradiated with blue light at 100 μmol $m^{-2}$ $s^{-1}$ or 5 μmol $m^{-2}$ $s^{-1}$ for 30 min through a slit to induce the avoidance and accumulation responses, respectively. Arrowheads indicate the irradiated areas. (**c**) Leaf flattening. Rosette leaves were detached from 5-week-old plants, and photos were taken from the adaxial (left panel) and abaxial (right panel) sides.

**a**


Pulldown
Input
GST-
14-3-3
GST-
BHP
D BL D BL D BL
Anti-phot1
Anti-GST
GST-BHP
GST-14-3-3

**b**


Pulldown
Input
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BHP
Anti-
H+-ATPase
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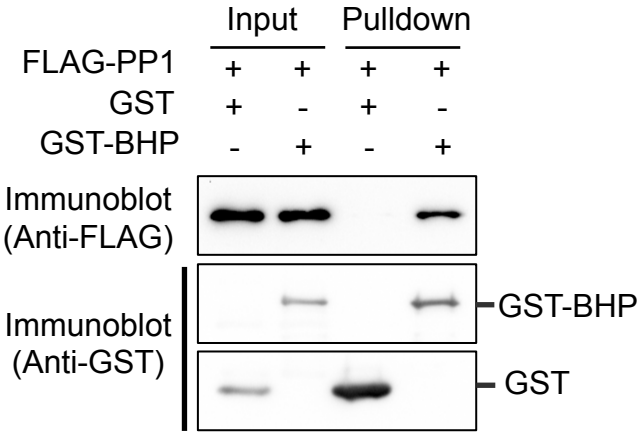
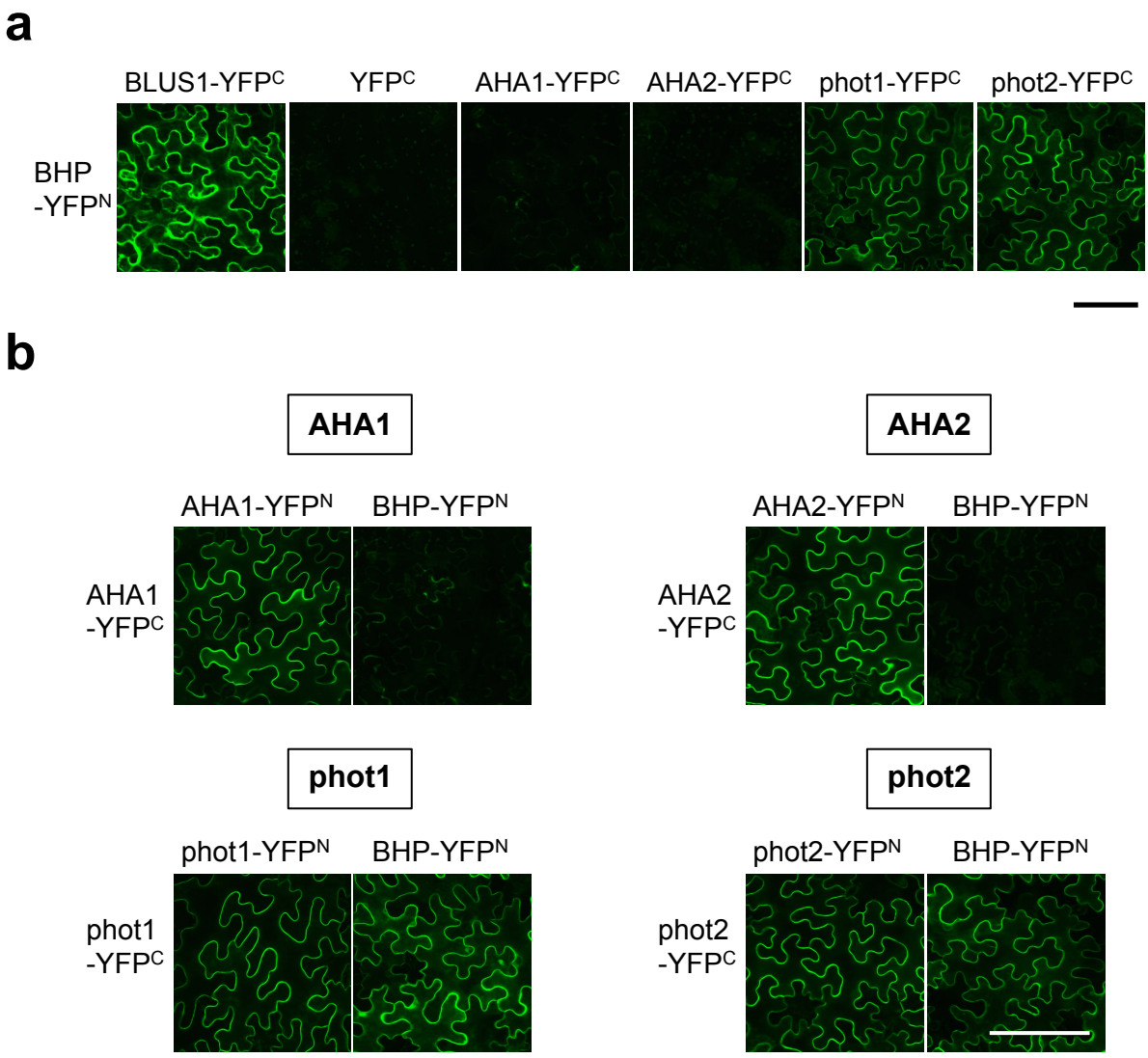
**c**


Input
Pulldown
FLAG-PP1 + + + +
GST + - + -
GST-BHP - + - +
Immunoblot
(Anti-FLAG)
Immunoblot
(Anti-GST)
GST-BHP
GST

**Supplementary Figure 7. *In vitro* pull-down assays for interaction of BHP with phot1, $H^+$-ATPase, and PP1.**

(**a,b**) The recombinant GST-14-3-3 protein or GST-BHP was purified from *E. coli* cells using glutathione-Sepharose 4B beads, and the proteins were reacted with microsomal proteins from Arabidopsis etiolated seedlings. The boundary matrices were subjected to SDS-PAGE and then immunoblot using anti-GST, anti-phot1, and anti-$H^+$-ATPase antibodies. (**a**) Pull-down of assay of phot1 and BHP. (**b**) Pull-down of assay of $H^+$-ATPase and BHP. The GST-14-3-3 was used as a positive control for the binding to phot1 and the $H^+$-ATPase. (**c**) *In vitro* pull-down assay for interaction of BHP with PP1. Both proteins were expressed in *E. coli* and used. Extract from *E. coli* cells expressing GST or GST-BHP was mixed with that expressing FLAG-PP1 and reacted with glutathione-Sepharose 4B beads. Proteins on the beads were subjected to SDS-PAGE and then immunoblotting using anti-GST and anti-FLAG antibodies.




**Supplementary Figure 8. *In vivo* interactions of BHP with signaling components in blue light-dependent stomatal opening by BiFC assay.**
(**a**) Fluorescence images of the interactions between BHP and BLUS1, AHA1, AHA2, phot1, and phot2 in BiFC assays. Experiments were performed as shown in Fig. 5b. For negative control, the C-terminal half of YFP ($YFP^C$) only was co-expressed with BHP-$YFP^N$. Scale bar is 150 μm. (**b**) Comparison of BHP-$H^+$-ATPase or -phototropin interactions with the positive interaction controls in BiFC assays. Because $H^+$-ATPase and phototropins form multimer complexes[2,3], co-expression of AHA1-$YFP^N$/AHA1-$YFP^C$, AHA2-$YFP^N$/AHA2-$YFP^C$, phot1-$YFP^N$/phot1-$YFP^C$, and phot2-$YFP^N$/phot2-$YFP^C$ were used as positive controlsScale bar represents 150 μm.

**Supplementary Table 1. Putative targets of the selected inhibitors in Arabidopsis.**

| Inhibitor | Mammalian target | Putative targets in Arabidopsis | Identity (%) |
|---|---|---|---|
| Tyrphostin 9 | PDGFRK | Raf-like kinase At4g31170 | 39 |
| | | Raf-like kinase At2g24360 | 37 |
| | | Raf-like kinase At4g35780 | 32 |
| Sphingosine | PKC | S6 kinase-1 At3g08730 | 38 |
| | | S6 kinae-2 At3g08720 | 41 |
| | | Putative protein kinase At1g48490 | 39 |
| GW5074 | c-Raf | Raf-like kinase At4g35780 | 38 |
| | | Raf-like kinase At2g17700 | 38 |
| | | Raf-like kinase At1g73660 | 37 |
| BML-265 | EGFRK | Raf-like kinase At4g35780 | 32 |
| | | Raf-like kinase At4g38470 | 32 |
| | | Raf-like kinase At2g17700 | 33 |

Tyrphostin 9, Sphingosine, GW5074 and BML-265 were screened from the Inhibitor library (Enzo; BML-2832) as inhibitors that suppress effectively the blue light-dependent $H^+$-ATPase phosphorylation and stomatal opening. Detection of the guard cell $H^+$-ATPase was performed by the immunohistochemical method (Fig. 1a). The targets of the inhibitors are known in the mammalian cells. Putative targets in Arabidopsis were assumed by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the full-length amino acid sequences, and three proteins with high similarity are shown in the list.

## Supplementary Table 2. Primers used in RT-PCR and BiFC assay.

| Gene | Experiments | Primer sequence |
|---|---|---|
| At3g58640<br>B1 | for RT-PCR | F: ATGGCGATCAAAGAGGAGAC<br>R:TAGGCCTGAAAAGTAACCAC |
| At4g24480<br>B3 | for RT-PCR | F: TCAGAGTGAGATTTTAACGAAATGC<br>R:CTATAGTATGGGAGCTGATTTAGTTGG |
| At2g35050<br>B4 | for RT-PCR | F: ATGGATCAAGCAAAAGGTTATGAACATG<br>R: TTACTTGTGGATTTGGTGGTTGACAG |
| At3g24715<br>B4 | for RT-PCR | F: ATGTCAGATAGATGGGCTCGAC<br>R: AGGAAATTGGCAATGGGAGAG |
| *VIK* At1g14000<br>C1 | for RT-PCR | F: CAATGGTGATCTGATGGTAG<br>R: TCTGAATCGGTTTTGAGATG |
| *BHP* At4g18950<br>C1 | for RT-PCR | F: ATGGAAGAGGATTATCAACAGC<br>R: TCACAAATGTGAACCGGATG |
| At4g35780<br>C2 | for RT-PCR | F: ATGGCGATCAAAGAGGAGAC<br>R: TAGGCCTGAAAAGTAACCAC |
| At5g50180<br>C3 | for RT-PCR | F: AATCTTGCAATGGATTCTTTGACTG<br>R: CAAGGTTAATAACATTGATTGAAGCAG |
| At2g24360<br>C6 | for RT-PCR | F: AGTGAATCTTAGATTGGGAAGATGC<br>R: TTCTCGCTGTCGTCATTATCTCTG |
| At3g01490<br>C7 | for RT-PCR | F: AAATGAAGGAGAAGGCGGAGAGTG<br>R: ATCCCAAAGCTATACACATCGCAC |
| At5g50000<br>C7 | for RT-PCR | F: ATGAAAGAAGGAAAGGATGGGTTTG<br>R: TTAAGGACCACGTTTCCTTCGG |
| **BHP**<br>BHP-YFP$^{N}$ | for BiFC | F: GAGGGTACCGCTCCCATGGAAGAGGATTATCAACAGC<br>R: CTTTTGCTCCATCCCCAAATGTGAACCGGATGATG |
| **BLUS1**<br>BLUS1-YFP$^{C}$ | for BiFC | F: GAGGGTACCGCTCCCATGGCTCGGAACAAGCTCGAGTTC<br>R: GTATGGGTACATCCCACCCAAAACACTATCTTTATCAGC |
| **PHOT1**<br>PHOT1-YFP$^{N}$ | for BiFC | F: GAGGGTACCGCTCCCATGGAACCAACAGAAAAACCATCG<br>R: CTTTTGCTCCATCCCAAAAACATTTGTTTGCAGATCTTCTAGC |
| PHOT1-YFP$^{C}$ | for BiFC | F: GAGGGTACCGCTCCCATGGAACCAACAGAAAAACCATCG<br>R: GTATGGGTACATCCCAAAAACATTTGTTTGCAGATCTTCTAGC |
| **PHOT2**<br>PHOT2-YFP$^{N}$ | for BiFC | F: GAGGGTACCGCTCCCATGGAGAGGCCAAGAGCCCCT<br>R: CTTTTGCTCCATCCCGAAGAGGTCAATGTCCAAGTCCG |
| PHOT2-YFP$^{C}$ | for BiFC | F: GAGGGTACCGCTCCCATGGAGAGGCCAAGAGCCCCT<br>R: GTATGGGTACATCCCGAAGAGGTCAATGTCCAAGTCCG |
| **AHA1**<br>AHA1-YFP$^{N}$ | for BiFC | F: GAGGGTACCGCTCCCATGTCAGGTCTCGAAGATATCAAG<br>R: CTTTTGCTCCATCCCCACAGTGTAGTGATGTCCTGC |
| AHA1-YFP$^{C}$ | for BiFC | F: GAGGGTACCGCTCCCATGTCAGGTCTCGAAGATATCAAG<br>R: GTATGGGTACATCCCCACAGTGTAGTGATGTCCTGC |
| **AHA2**<br>AHA2-YFP$^{N}$ | for BiFC | F: GAGGGTACCGCTCCCATGTCGAGTCTCGAAGATATCAAG<br>R: CTTTTGCTCCATCCCCACAGTGTAGTGACTGGGAGTTTC |
| AHA2-YFP$^{C}$ | for BiFC | F: GAGGGTACCGCTCCCATGTCGAGTCTCGAAGATATCAAG<br>R: GTATGGGTACATCCCCACAGTGTAGTGACTGGGAGTTTC |
| **PP1**<br>TOPP4-YFP$^{C}$ | for BiFC | F: GAGGGTACCGCTCCCATGGCGACGACGACGACG<br>R: GTATGGGTACATCCCAATCTTTGTGGACATCATGAACTTG |
| **INHIBITOR3**<br>INHIBITOR3-YFP$^{N}$ | for BiFC | F: GAGGGTACCGCTCCCATGAGCACAGCAACAAGGCCTTCC<br>R: CTTTTGCTCCATCCCGTCAACGGCTTTAGAATCATTAGAAG |

## Supplementary References


1. Ichimura, K. *et al*. Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends Plant Sci* **7**, 301-308 (2002).

2. Kanczewska, J. *et al*. Activation of the plant plasma membrane $H^+$-ATPase by phosphorylation and binding of 14-3-3 proteins converts a dimer into a hexamer. *Proc Natl Acad Sci USA* **102**, 11675-11680 (2005).

3. Katsura, H., Zikihara, K., Okajima, K., Yoshihara, S. & Tokutomi, S. Oligomeric structure of LOV domains in Arabidopsis phototropin. *FEBS Lett* **583**, 526-530 (2009).