1
2 **Cold-induced switch in direction of chloroplast relocation occurs independently of**
3 **changes in endogenous phototropin expression**

4
5 Yuta Fujii ^{1,2¶}, Yuka Ogasawara ^{1,3¶}, Yamato Takahashi ^{1,3¶}, Momoko Sakata ^{1,4¶}, Saori
6 Tamura ^{1,4}, and Yutaka Kodama ^{1,2,4*}

7 ¹ *Center for Bioscience Research and Education, Utsunomiya University, Tochigi*
8 *321-8505, Japan*

9 ² *United Graduate School of Agricultural Science, Tokyo University of Agriculture and*
10 *Technology, Tokyo 183-8509, Japan*

11 ³ *Faculty of Agriculture, Utsunomiya University, Tochigi 321-8505, Japan*

12 ⁴ *Graduate School of Agricultural Science, Utsunomiya University, Tochigi 321-8505,*
13 *Japan*

14
15 * Corresponding author

16 E-mail address: kodama@cc.utsunomiya-u.ac.jp (YK)

17
18 ¶These authors contributed equally to this work.

19
20

21 **Abstract**

22 When exposed to fluctuating light intensity, chloroplasts move towards weak light
 23 (accumulation response), and away from strong light (avoidance response). In addition,
 24 cold treatment (5°C) induces the avoidance response even under weak light conditions
 25 (cold-avoidance response). These three responses are mediated by the phototropin
 26 (phot), which is a blue-light photoreceptor and has also been found to act as a
 27 thermosensory protein that perceives temperature variation. Our previous report
 28 indicated that cold-induced changes in phot biochemical activity initiate the
 29 cold-avoidance response. In this study, we further explored the induction mechanism of
 30 the cold-avoidance response in the liverwort *Marchantia polymorpha* and examined the
 31 relationship between changes in the amount of phot and the induction of the
 32 cold-avoidance response. The switch between the accumulation and avoidance
 33 responses occurs at a so-called ‘transitional’ light intensity. Our physiological
 34 experiments revealed that a cold-mediated decrease in the transitional light intensity
 35 leads to the induction of the cold-avoidance response. While artificial overexpression of
 36 phot decreased the transitional light intensity as much as cold treatment did, the amount
 37 of endogenous phot remained unchanged by cold treatment in wild-type *M. polymorpha*.
 38 Taken together, these findings show that the cold-avoidance response is initiated by a
 39 cold-mediated reduction of the transitional light intensity, independent of the amount of
 40 endogenous phot. This study provides a clue to understand the mechanism underlying
 41 the switch in direction of chloroplast relocation in response to light and temperature.

42

43 **Introduction**

44 Plants sense ambient light and temperature to adapt to an ever-changing environment. In
 45 response to the light and temperature changes, the intracellular chloroplast position is
 46 precisely controlled. Under weak light conditions (e.g., under canopy), chloroplasts
 47 move towards light to increase light capture for photosynthetic efficiency (accumulation
 48 response) [1]. Under strong light conditions (e.g., under direct sunlight), chloroplasts
 49 move away from light to avoid photodamage (avoidance response) [2]. Chloroplast
 50 position is also affected by temperature [3], notably cold. Under warm conditions, weak
 51 light induces the accumulation response; however, under cold conditions (5°C) the same
 52 light level induces chloroplast avoidance (Fig 1). This movement referred to as the
 53 cold-avoidance (or cold-positioning) response, has been studied in several plant species,
 54 including the moss *Funaria hygrometrica* [3], evergreen ferns (e.g., *Adiantum*
 55 *capillus-veneris*) [4], the liverwort *Marchantia polymorpha* [5] and the flowering plant
 56 *Arabidopsis thaliana* [6]. In *M. polymorpha*, the cold-avoidance response contributes to
 57 the reduction of photoinhibition under cold conditions [6].

58 Earlier studies showed that the accumulation, avoidance and cold-avoidance
 59 responses are all mediated by the blue-light (BL) photoreceptor phototropin (phot)
 60 [4,6–9]. Phot has two photosensory light, oxygen, or voltage (LOV) domains (LOV1
 61 and LOV2) at its N-terminal region and a serine/threonine kinase domain at its
 62 C-terminal region. Phot perceives BL via its LOV domains and mediates the change of
 63 chloroplast position upon photoactivation of the LOV domains and kinase-mediated
 64 autophosphorylation [10]. A decrease in ambient temperature prolongs the lifetime of
 65 the photoactivated LOV2 domain, resulting in an increase in autophosphorylation [6].
 66 The cold-induced enhancement of the photochemical and biochemical activities of phot
 67 (i.e., a prolonged lifetime of photoactivated LOV2 and increased autophosphorylation
 68 status) is essential for induction of the cold-avoidance response [6], and further study is
 69 required to fully understand this induction.

The switch in chloroplast behavior between the accumulation and avoidance responses occurs at their ‘transitional’ light intensity (Fig 1A). In the cold-avoidance response, because the chloroplast moves away from even weak BL (wBL) under cold conditions [6], it is hypothesized that the cold-avoidance response is induced by a cold-mediated reduction of the transitional light intensity (Fig 1B). However, this hypothesis remains to be tested. Artificial overexpression of phot has been suggested to decrease the transitional light intensity in *A. capillus-veneris* and *A. thaliana* [11,12]. In addition, expression levels of endogenous phot are affected by both light [7,8,13–16] and temperature [16] conditions. Based on these findings, we postulate that a variation in the endogenous amount of phot may be involved in the induction of the cold-avoidance response.

The goals of this study were two-fold: first, to determine if a cold-mediated reduction of the transitional light intensity initiates the cold-avoidance response, and second, to determine if changes in phot amounts play a role in the induction of the cold-avoidance response. *M. polymorpha* was chosen for this study because it has a single copy of the gene for phot (Mpphot) that can mediate the accumulation, avoidance, and cold-avoidance responses [6,17].

Materials and Methods

Plasmid construction

To construct a plasmid to generate the complementation line of Mpphot-Citrine (35S::Mpphot-Citrine/Mpphot^{KO}), we used the Gateway cloning system (Invitrogen) and the binary vector pMpGWB306 [18], which includes *Citrine* (a gene for yellow fluorescent protein) for fusion at the 3′-end of the target gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The LR reaction (Invitrogen) was performed with a mixture of pMpGWB306 and the donor plasmid

96 pDONR207-MpPHOT [19], and the resulting plasmid pMpGWB306-MpPHOT was
97 used for transformation of *M. polymorpha*.

98

99 **Plant materials and growth conditions**

100 Thalli of *M. polymorpha* were asexually cultured on half-strength B5 medium under 75
101 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ continuous white light (FL40SW, NEC Corporation). Light
102 intensity was measured by using a LI-250A light meter (LI-COR Biosciences). The
103 MpPHOT knockout line (Mpphot^{KO}) was generated and kindly provided by Komatsu et
104 al. [17]. In this study, transformation of *M. polymorpha* was performed by the
105 G-AgarTrap method [20,21], which is an *Agrobacterium*-mediated transformation
106 method used for gemmalings. To generate the transformants, we used the binary vector
107 pMpGWB106-MpPHOT [19] for Mpphot-Citrine overexpression lines
108 (35S::Mpphot-Citrine/WT), and pMpGWB306-MpPHOT for the complementation line
109 of Mpphot (35S::Mpphot-Citrine/Mpphot^{KO}). The Citrine-expressing line
110 (35S::Citrine/WT) was generated in our previous study [20]. G2 gemmalings were used
111 throughout.

112

113 **Observation of chloroplast positioning**

114 One-day-old gemmalings were incubated in temperature-controlled incubators (IJ100,
115 Yamato Scientific Co., LTD) and light conditions were adjusted using blue
116 light-emitting diodes (LEDs; OptoSupply Limited) and a LI-250A light meter. To
117 produce polarized weak BL, 25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of the blue LED was used as weak
118 BL [5,6], and it was filtered with a polarizing filter (HN38, Polaroid Corp.).
119 Chlorophyll fluorescence images were captured using a fluorescence microscope
120 MZ16F (Leica Microsystems) equipped with a DP73 digital camera (Olympus) and
121 quantification of chloroplast positioning was performed using the P/A ratio method with
122 ImageJ software, as previously described [5]. Briefly, fluorescence intensity was

123 measured at 30 points (0.625 μm each, equivalent to 1 pixel) along the anticlinal cell
124 walls (A) and 30 points (39.1 μm^2 each, equivalent to 10×10 pixels) along the
125 periclinal cell walls (P). This procedure was repeated five times, and then the P/A ratio
126 was calculated as a mean of the five P/A values. The standard deviation was calculated
127 using Excel software (Microsoft).

128

129 **Immunoblot analysis**

130 Immunoblot analysis was performed as previously described [6]. Briefly, four-day-old
131 gemmalings were collected in liquid nitrogen and kept at -80°C until use. The frozen
132 plants were homogenized in SDS buffer (2% SDS, 10% glycerol, 5% mercaptoethanol,
133 and 0.25 M Tris-HCl pH6.8) or Nonidet buffer (0.5% Nonidet P-40, 0.5M EDTA, 0.15
134 M NaCl, and 0.01 M Tris-HCl pH7.5) using a mortar and pestle and then boiled at 95°C
135 for 5 min. The samples were centrifuged at 14,000 g for 10 min, and the supernatants
136 used. Total protein amount was quantified by Bradford-XL assay (APRO Science).
137 Fifteen μg of protein was subjected to SDS-PAGE with 8% polyacrylamide gels and
138 transferred to a polyvinylidene difluoride (PVDF) membrane [6]. MpPHOT proteins
139 were detected with rabbit antibody raised against the custom-made synthetic peptide
140 (C+VDERAPPSKGSKE) (Eurofins Genomics) and horseradish
141 peroxidase-conjugated anti-rabbit IgG (Thermo Scientific). Both antibodies were used
142 at dilution of 1:2000. For histone detection, anti-Histone H3 antibody (Abcam) at
143 dilution of 1:5000 and horseradish peroxide-conjugated anti-rabbit IgG (Thermo
144 Scientific) at dilution of 1:2000 were used. Chemiluminescent signal was detected using
145 ECL-Select (GE Healthcare) and Light Capture (ATTO). For the image of Rubisco
146 large subunit (RBCL), fifteen μg protein was subjected to SDS-PAGE with 8%
147 polyacrylamide gels and stained with conventional Coomassie brilliant blue (CBB)
148 method.

149

150 **Results**

151 **Chloroplasts avoid weak blue light (wBL) under cold conditions**

152 Using temperature-regulated microscopy with a microbeam irradiation system, we
153 previously reported that under cold conditions (5°C), chloroplasts moved away from
154 wBL that induced the accumulation response at 22°C [6]. However, in the study,
155 physiological evidence of wBL avoidance under cold conditions was limited to
156 microbeam analysis [6]. Here, we further analyzed whether chloroplasts move away
157 from wBL under cold conditions.

158 In *M. polymorpha*, both cold and BL were required to elicit the
159 cold-avoidance response [6]. Building on the result, to explore whether the
160 cold-avoidance response is due to avoidance from wBL under cold conditions, we tested
161 the effects of changing light direction. First, to induce the cold-avoidance response,
162 wild-type (WT) cells were incubated under 25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of wBL (BL25)
163 with vertical irradiation at 5°C (Fig 2A). In WT cells, chloroplasts were positioned
164 along the anticlinal cell walls as the cold-avoidance response (Fig 2B). When the light
165 direction was changed from vertical to horizontal (Fig 2A), the chloroplasts along the
166 anticlinal cell walls moved to the periclinal cell walls (Fig 2B), indicating chloroplast
167 avoidance from BL25. To further confirm the results, we employed polarized wBL,
168 because the vertically vibrating polarized wBL could induce chloroplast localization
169 along the anticlinal wall by the accumulation response in *A. capillus-veneris* [22,23].
170 When 10-day-old sporeling cells of *M. polymorpha* were irradiated with vertically
171 vibrating polarized wBL for 3 h at 22°C, chloroplasts localized along the anticlinal wall
172 as the accumulation response (Fig 2C). When the cells were transferred to 5°C under
173 the same polarized wBL condition, chloroplasts relocated from the anticlinal wall to the
174 periclinal wall, indicating that chloroplasts avoided the polarized wBL at 5°C. These
175 results confirm that the cold-avoidance response is due to avoidance from wBL under

176 cold conditions, which is consistent with the microbeam analysis in our previous study
177 [6].

178

179 **Cold treatment decreases the transitional light intensity between the accumulation** 180 **and avoidance responses**

181 We hypothesized that the cold-avoidance response is induced by a cold-mediated
182 decrease of the transitional light intensity (Fig 1). If this is the case, the accumulation
183 response would be induced under much weaker BL in cold conditions. To investigate
184 whether the accumulation response is induced at 5°C, WT cells were incubated under
185 different intensities of wBL (5, 10, 15 and 25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; BL5, BL10, BL15
186 and BL25, respectively) (Fig 3A and 3B). Chloroplast position was quantified by a P/A
187 ratio method, developed in earlier studies [4,5]. The P/A ratio is an arbitrary unit
188 calculated by dividing the chlorophyll fluorescence intensity along the periclinal cell
189 wall by that along the anticlinal cell wall, with a higher value indicating periclinal
190 positioning of chloroplasts and vice versa. In WT cells, the cold-avoidance response
191 was observed at 5°C under BL10, BL15 and BL25 (Fig 3A), and at these higher BL
192 intensity levels, we observed a decrease in P/A ratios (Fig 3B). However, the
193 cold-avoidance response was not observed at 5°C under BL5 (Fig 3A) and the P/A ratio
194 was unchanged during the incubation of these samples (Fig 3B). These results suggest
195 that BL5 induces the accumulation response at 5°C. However, because similar
196 chloroplast positioning was observed under dark conditions at 5°C [6], it is also possible
197 that the cells were unable to respond to the extremely weak intensity of BL5. To clarify
198 whether the accumulation response is induced under BL5 or not, an additional test was
199 carried out. Following the induction of the cold-avoidance response at 5°C for 24 h
200 under BL25, the light intensity was changed to BL5 or dark conditions (Fig 3C). After
201 incubation for 24 h, BL5 conditions induced the accumulation response at 5°C, whereas
202 under dark conditions there was no change in chloroplast position (Fig 3D). These

203 results indicate that the accumulation response is induced under BL5 at 5°C. Taken
204 together, these results indicate that the transitional light intensity is modulated by the
205 temperature change (Fig 1).

206

207 **Artificial overexpression of Mpphot in *M. polymorpha* induces the avoidance** 208 **response under wBL without cold treatment**

209 We next considered the possibility that changes in endogenous phot expression may
210 play a role in the change in transitional light intensity, as previous studies using *A.*
211 *capillus-veneris* and *A. thaliana* suggest that a reduction in the transitional light
212 intensity can be the result of artificial overexpression of phot [11,12]. In *A.*
213 *capillus-veneris* at 25°C, transient overexpression of the *AcPHOT2-GFP* gene induced
214 the avoidance response under wBL [11]. Similarly, the avoidance response was
215 observed under wBL in transgenic *A. thaliana* overexpressing the *AtPHOT2-GFP* gene
216 at 23°C [12]. Consistent with these previous studies, we found that artificial
217 overexpression of Mpphot fused to the yellow fluorescent protein Citrine
218 (Mpphot-Citrine) in WT cells (35S::Mpphot-Citrine/WT) induced the avoidance
219 response under BL25 at 22°C (without cold treatment), and degree of the avoidance
220 response was dependent on Mpphot-Citrine expression levels (Fig 4A-4C). In the
221 overexpression line (OX2) under BL5, the accumulation response at 22°C and
222 cold-avoidance response at 5°C could be induced (Fig 4D and 4E), indicating that the
223 transitional intensity is reduced by overexpression of Mpphot-Citrine. Therefore, it is
224 conceivable that increase of the endogenous amount of Mpphot might play a role in the
225 induction of the cold-avoidance response.

226

227 **Alteration of endogenous Mpphot is not required for the induction of the** 228 **cold-avoidance response**

229 To examine the role of Mpphot expression in the induction of the cold-avoidance
 230 response, the endogenous amount of Mpphot was determined using immunoblot
 231 analysis. After gemmalings were transferred from the white light condition to BL25
 232 condition at 22°C, the endogenous amount of Mpphot was increased at 6 h and the
 233 increased amount was maintained until 24 h (S Fig 1). To avoid the effect of the light
 234 condition change (i.e., from white light to BL25), the gemmalings were pre-incubated
 235 under BL25 at 22°C for 24 h. When temperature was changed from 22°C to 5°C, the
 236 endogenous amount of Mpphot was not changed at 3, 6, 9 h (Fig 5A). Note that this
 237 experimental condition was not able to separate phosphorylated and dephosphorylated
 238 forms of Mpphot. When we incubated the gemmalings under the same light and
 239 temperature condition as shown in Fig 5A, the cold-avoidance response was induced
 240 (Fig 5B), and the P/A ratio was decreased (Fig 5C). Unexpectedly the pre-incubation
 241 under BL25 at 22°C for 24 h increased the P/A ratio than the white light condition at
 242 22°C (see 0 h in Fig 5C and 0 h in Fig 3B). Anyway, an increase of the endogenous
 243 amount of Mpphot is not required for induction of the cold-avoidance response in *M.*
 244 *polymorpha*. To confirm this result, we examined the complementation lines of
 245 Mpphot-Citrine under the control of the CaMV35S promoter with the genetic
 246 background of *Mpphot*^{KO} (35S::Mpphot-Citrine/*Mpphot*^{KO}), as CaMV35S promoter
 247 activity is reported to be suppressed by cold treatment in tobacco plants [24]. In
 248 transgenic *M. polymorpha* expressing Citrine under the control of the CaMV35S
 249 promoter (35S::Citrine/WT), we also confirmed that the promoter activity is slightly
 250 suppressed by 5°C treatment in *M. polymorpha* (Fig 5D). In the complementation lines
 251 (35S::Mpphot-Citrine/*Mpphot*^{KO}), the cold-avoidance response was clearly observed
 252 (Fig 5E), confirming that the induction of the cold-avoidance response does not require
 253 any increase of Mpphot amount. Given that cold treatment increased the
 254 autophosphorylation status of phot [6,25], we conclude that an increase of phot

autophosphorylation status decreases the transitional light intensity without alteration of the endogenous amount of Mpphot.

Discussion

The transitional light intensity between the accumulation and avoidance responses can be changed

Light-induced chloroplast movement is widely found in photosynthetic organisms from algae to land plants [3]. The transitional light intensity between the accumulation and avoidance responses differs among various plant species [26]. Furthermore, the transitional light intensity can be reduced in response to low temperature, inducing the cold-avoidance response [4–6]. In this study, we analyzed the light fluence rate dependency of the cold-avoidance response. Under cold conditions, chloroplasts moved in a light fluence rate-dependent manner (Fig 3A and 3B), as occurs during the avoidance response at a standard growth temperature [27]. This result supports the conclusion that there is a cold-induced reduction of the transitional light intensity (Fig 1). Because the cold-avoidance response has been found to moderate photodamage in *M. polymorpha* [6], the plasticity of the transitional light intensity is vital for plants exposed to low temperatures. In *A. thaliana*, the transitional light intensity is controlled by two types of phot: Atphot1 and Atphot2. Atphot1 specifically regulates the accumulation response, while Atphot2 regulates both the accumulation and avoidance responses [7–9]. In this context, the coordination mechanism of the transitional light intensity in *A. thaliana* appears to be more complex than that in *M. polymorpha*. Further analysis of the transitional light intensity in various plant species and genetic mutants may lead to a better understanding of the transition mechanism of chloroplast relocation movements.

Photoactivated phototropin amount changes the transitional light intensity

282 Intracellular phot proteins are more frequently activated under strong light than weak
 283 light conditions. Weak light irradiation at lower temperatures, prolongs the lifetime of
 284 photoactivated phot [6], and like the effect of strong light irradiation at a standard
 285 growth temperature, results in the increase of photoactivated phot population. Therefore,
 286 we concluded that the intracellular amount of photoactivated phot determines the
 287 transition between the accumulation and avoidance responses [6]. Furthermore,
 288 considering the effect of phot overexpression on the transitional light intensity (Fig 4),
 289 the transition mechanism may be based on the absolute amount of photoactivated phot
 290 in the cell rather than the ratio of active/inactive phot. How an increase in
 291 photoactivated phot proteins triggers the switch from the accumulation to avoidance
 292 responses remains unknown. Because phot autophosphorylation is also increased under
 293 strong-light/warm or weak-light/cold conditions [6,10,25], we speculate that to induce
 294 the avoidance response, an increase of photoactivated phot amount may change the phot
 295 phosphorylation status, possibly via intermolecular phosphorylation activity [28,29].
 296 Notably, the recruitment of phot to microdomains at the plasma membrane is involved
 297 in phot autophosphorylation [30]. Further autophosphorylation analysis regarding the
 298 spatial density of photoactivated phot may clarify the switching mechanism between the
 299 accumulation and avoidance responses.

300

301 **Alteration of phot expression is not required for induction of the cold-avoidance** 302 **response in *M. polymorpha***

303 The cold-avoidance response is initiated by the temperature-dependent biochemistry of
 304 the phot LOV2 domain [6]. However, in *A. thaliana*, alteration of the endogenous
 305 amount of Atphot1 and Atphot2 in response to temperature was reported; lower
 306 temperature decreases Atphot1 expression and increases Atphot2 expression [16]. In
 307 this study, we showed that the cold-avoidance response can be induced regardless of the
 308 change in the endogenous Mpphot amount (Fig 5A-5C) and even without its promoter

(Fig 5D and 5E), indicating that transcriptional regulation of Mpphot is not required for the cold-avoidance response in *M. polymorpha*. Mpphot is similar to Atphot2, which regulates both the accumulation and avoidance responses [17]. Consistent with Mpphot, Atphot2 expression in *A. thaliana* was reported to be unchanged at a temperature range from 4°C to 23°C [16]. Interestingly, cold treatment had a more significant impact on the decrease of Atphot1 expression than the increase of Atphot2 expression [16], implying that the expression of Atphot1 might be more sensitive to cold than that of Atphot2. The gene duplication of phot [31] and functional differentiation between phot1 and phot2 may have enabled land plants to have different transitional light intensities and chloroplast movements, allowing species to expand their range of habitat.

Acknowledgements

The authors thank Dr. Takayuki Kohchi (Kyoto University) and Dr. Kimitsune Ishizaki (Kobe University) for providing *M. polymorpha* (WT and Mpphot^{KO}) and the binary vectors (pMpGWB106 and pMpGWB306). The authors also thank Ms. Lee Kien Yong (Utsunomiya University) for helping the preparation of the manuscript. This work was supported by the Japan Society for the Promotion of Science KAKENHI [grant No. 18H02455 to Y.K.]; the Japan Science and Technology Agency, Exploratory Research for Advanced Technology, Numata Organelle Reaction Cluster [grant No. JPMJER1602 to Y.K.]; and the Plant Transgenic Design Initiative of the University of Tsukuba to Y.K.

References

1. Gotoh E, Suetsugu N, Yamori W, Ishishita K, Kiyabu R, Fukuda M, et al. Chloroplast accumulation response enhances leaf photosynthesis and plant biomass production. *Plant Physiol.* 2018;178: 1358–1369. doi:10.1104/pp.18.00484
2. Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M, Wada M. Chloroplast avoidance movement reduces photodamage in plants. *Nature.* 2002;420: 829–832. doi:10.1038/nature01202.1.

- 339 3. Senn G. Die gestalts-und lageveränderung der pflanzen-chromatophoren.
340 Leipzig: Wilhelm-Engelmann; 1908.
- 341 4. Kodama Y, Tsuboi H, Kagawa T, Wada M. Low temperature-induced
342 chloroplast relocation mediated by a blue light receptor, phototropin 2, in fern
343 gametophytes. *J Plant Res.* 2008;121: 441–448. doi:10.1007/s10265-008-0165-9
- 344 5. Ogasawara Y, Ishizaki K, Kohchi T, Kodama Y. Cold-induced organelle
345 relocation in the liverwort *Marchantia polymorpha* L. *Plant, Cell Environ.*
346 2013;36: 1520–1528. doi:10.1111/pce.12085
- 347 6. Fujii Y, Tanaka H, Konno N, Ogasawara Y, Hamashima N, Tamura S, et al.
348 Phototropin perceives temperature based on the lifetime of its photoactivated
349 state. *Proc Natl Acad Sci U S A.* 2017;114: 9206–9211.
350 doi:10.1073/pnas.1704462114
- 351 7. Jarillo JA, Gabrys H, Capel J, Alonso JM, Ecker JR, Cashmore a R.
352 Phototropin-related NPL1 controls chloroplast relocation induced by blue light.
353 *Nature.* 2001;410: 952–954. doi:10.1038/35073622
- 354 8. Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, et al.
355 *Arabidopsis* NPL1: a phototropin homolog controlling the chloroplast high-light
356 avoidance response. *Science.* 2001;291: 2138–2141.
357 doi:10.1126/science.291.5511.2138
- 358 9. Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, et al.
359 *Arabidopsis* nph1 and npl1: blue light receptors that mediate both phototropism
360 and chloroplast relocation. *Proc Natl Acad Sci U S A.* 2001;98: 6969–6974.
361 doi:10.1073/pnas.101137598
- 362 10. Inoue S-I, Kinoshita T, Matsumoto M, Nakayama KI, Doi M, Shimazaki K-I.
363 Blue light-induced autophosphorylation of phototropin is a primary step for
364 signaling. *Proc Natl Acad Sci U S A.* 2008;105: 5626–5631.
365 doi:10.1073/pnas.0709189105
- 366 11. Kagawa T, Kasahara M, Abe T, Yoshida S, Wada M. Function analysis of
367 phototropin2 using fern mutants deficient in blue light-induced chloroplast
368 avoidance movement. *Plant Cell Physiol.* 2004;45: 416–426.
369 doi:10.1093/pcp/pch045
- 370 12. Kong SG, Kinoshita T, Shimazaki KI, Mochizuki N, Suzuki T, Nagatani A. The
371 C-terminal kinase fragment of *Arabidopsis* phototropin 2 triggers constitutive
372 phototropin responses. *Plant J.* 2007;51: 862–873.
373 doi:10.1111/j.1365-313X.2007.03187.x
- 374 13. Kanegae H, Tahir M, Savazzini F, Yamamoto K, Yano M, Sasaki T, et al. Rice
375 NPH1 homologues, OsNPH1a and OsNPH1b, are differently photoregulated.
376 *Plant Cell Physiol.* 2000;41: 415–423. doi:10.1093/pcp/41.4.415
- 377 14. Jain M, Sharma P, Tyagi SB, Tyagi AK, Khurana JP. Light regulation and
378 differential tissue-specific expression of phototropin homologues from rice
379 (*Oryza sativa* ssp. *indica*). *Plant Sci.* 2007;172: 164–171.
380 doi:10.1016/j.plantsci.2006.08.003
- 381 15. Suzuki H, Okamoto A, Kojima A, Nishimura T, Takano M, Kagawa T, et al.
382 Blue-light regulation of ZmPHOT1 and ZmPHOT2 gene expression and the
383 possible involvement of Zmphot1 in phototropism in maize coleoptiles. *Planta.*
384 2014;240: 251–261. doi:10.1007/s00425-014-2082-6
- 385 16. Łabuz J, Hermanowicz P, Gabrys H. The impact of temperature on blue light
386 induced chloroplast movements in *Arabidopsis thaliana*. *Plant Sci.* 2015;239:
387 238–249. doi:10.1016/j.plantsci.2015.07.013
- 388 17. Komatsu A, Terai M, Ishizaki K, Suetsugu N, Tsuboi H, Nishihama R, et al.
389 Phototropin encoded by a single-copy gene mediates chloroplast photorelocation
390 movements in the liverwort *Marchantia polymorpha*. *Plant Physiol.* 2014;166:
391 411–427. doi:10.1104/pp.114.245100

- 392 18. Ishizaki K, Nishihama R, Ueda M, Inoue K, Ishida S, Nishimura Y, et al.
393 Development of gateway binary vector series with four different selection
394 markers for the liverwort *Marchantia polymorpha*. PLoS One. 2015;10:
395 e0138876. doi:10.1371/journal.pone.0138876
- 396 19. Kodama Y. Time gating of chloroplast autofluorescence allows clearer
397 fluorescence imaging in planta. Abraham T, editor. PLoS One. 2016;11:
398 e0152484. doi:10.1371/journal.pone.0152484
- 399 20. Tsuboyama-Tanaka S, Kodama Y. AgarTrap-mediated genetic transformation
400 using intact gemmae/gemmalings of the liverwort *Marchantia polymorpha* L. J
401 Plant Res. 2015;128: 337–344. doi:10.1007/s10265-014-0695-2
- 402 21. Tsuboyama S, Kodama Y. AgarTrap protocols on your benchtop: Simple
403 methods for *Agrobacterium*-mediated genetic transformation of the liverwort
404 *Marchantia polymorpha*. Plant Biotechnol. 2018; in press.
- 405 22. Yatsushashi H, Kadota A, Wada M. Blue- and red-light action in photoorientation
406 of chloroplasts in *Adiantum protonemata*. Planta. 1985;165: 43–50.
407 doi:10.1007/BF00392210
- 408 23. Kagawa T, Wada M. Polarized light induces nuclear migration in prothallial cells
409 of *Adiantum capillus-veneris* L. Planta An Int J Plant Biol. 1995;196: 775–780.
410 doi:10.1007/BF01106773
- 411 24. Schnurr JA, Guerra DJ. The CaMV-35S promoter is sensitive to shortened
412 photoperiod in transgenic tobacco. Plant Cell Rep. 2000;19: 279–282.
413 doi:10.1007/s002990050012
- 414 25. Okajima K, Kashojiya S, Tokutomi S. Photosensitivity of kinase activation by
415 blue light involves the lifetime of a cysteinyl-flavin adduct intermediate, S390, in
416 the photoreaction cycle of the LOV2 domain in phototropin, a plant blue light
417 receptor. J Biol Chem. 2012;287: 40972–40981. doi:10.1074/jbc.M112.406512
- 418 26. Königer M, Bollinger N. Chloroplast movement behavior varies widely among
419 species and does not correlate with high light stress tolerance. Planta. 2012;236:
420 411–426. doi:10.1007/s00425-012-1619-9
- 421 27. Kagawa T, Wada M. Chloroplast-avoidance response induced by high-fluence
422 blue light in prothallial cells of the fern *Adiantum capillus-veneris* as analyzed by
423 microbeam irradiation. Plant Physiol. 1999;119: 917–923.
424 doi:10.1104/pp.119.3.917
- 425 28. Cho HY, Tseng TS, Kaiserli E, Sullivan S, Christie JM, Briggs WR.
426 Physiological roles of the light, oxygen, or voltage domains of phototropin 1 and
427 phototropin 2 in *Arabidopsis*. Plant Physiol. 2007;143: 517–529.
428 doi:10.1104/pp.106.089839
- 429 29. Kaiserli E, Sullivan S, Jones MA, Feeney KA, Christie JM. Domain swapping to
430 assess the mechanistic basis of *Arabidopsis* phototropin 1 receptor kinase
431 activation and endocytosis by blue light. Plant Cell. 2009;21: 3226–3244.
432 doi:10.1105/tpc.109.067876
- 433 30. Xue Y, Xing J, Wan Y, Lv X, Fan L, Zhang Y, et al. *Arabidopsis* Blue Light
434 Receptor Phototropin 1 Undergoes Blue Light-Induced Activation in Membrane
435 Microdomains. Mol Plant. 2018;11: 846–859. doi:10.1016/j.molp.2018.04.003
- 436 31. Li FW, Rothfels CJ, Melkonian M, Villarreal JC, Stevenson DW, Graham SW, et
437 al. The origin and evolution of phototropins. Front Plant Sci. 2015;6: 1–11.
438 doi:10.3389/fpls.2015.00637

441 *Figure Legends*

442

443 **Fig 1. Schematic diagram showing the transitional light intensity of the**
 444 **accumulation and avoidance responses.** Solid vertical lines indicate points of the
 445 transitional light intensity under warm (A; 22°C) and cold (B; 5°C) conditions.

446

447 **Fig 2. Chloroplasts avoid weak blue light under cold conditions.** (A) Diagram of the
 448 experimental conditions used to induce the cold-avoidance response with vertical or
 449 horizontal irradiation of blue-light. After incubation under 25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of
 450 blue-light (BL25) with vertical irradiation at 5°C for 24 h, the WT gemmalings were
 451 irradiated by horizontal BL25. (B) Chlorophyll fluorescence images after irradiation of
 452 vertical (left) or horizontal (right) BL as shown in (A). The white arrow indicates the
 453 light direction. (C) Chlorophyll fluorescence images after irradiation of the polarized
 454 weak BL. Ten-day-old WT sporelings were irradiated by vertically vibrating polarized
 455 weak BL at 22°C. The cells were transferred to 5°C, followed by incubation for 24 h
 456 and 60 h.

457

458 **Fig 3. Cold-induced reduction of the transitional light intensity.** (A) Chloroplast
 459 positioning in WT gemmalings incubated at 5°C for 12 h with 5, 10, 15, and 25 μmol
 460 $\text{photons m}^{-2} \text{ s}^{-1}$ of blue-light (BL5, BL10, BL15, and BL25, respectively). After
 461 pre-incubation of 1-day-old WT gemmalings under BL25 at 22°C for 1 h to induce the
 462 accumulation response, the cells were transferred to the indicated BL conditions at 5°C.
 463 (B) The P/A ratios, calculated from chlorophyll fluorescence images after incubation for
 464 0, 2, 6, 8, 12, and 24 h. Error bars indicate standard deviations. (C) Diagram of
 465 experimental conditions used to confirm the cold-induced reduction of the transitional
 466 intensity. After pre-incubation under BL25 at 5°C for 24 h, WT gemmalings were
 467 transferred to BL5 or dark and incubated at 5°C for 24 h. (D) Representative

468 chlorophyll fluorescence images of WT gemmalings after pre-incubation (BL25) and
469 incubation under BL5 (from BL25 to BL5) or dark (from BL25 to Dark) as shown in
470 (C).

471

472 **Fig 4. Overexpression of Mpphot-Citrine decreases the transitional light intensity.**

473 (A) Immunoblot analysis of WT and Mpphot-Citrine overexpression lines
474 (35S::Mpphot-Citrine/WT: OX1, OX2, and OX3) with anti-Mpphot antibody. White
475 and black arrowheads indicate Mpphot-Citrine and Mpphot, respectively. Rubisco
476 large-subunit (RBCL) was shown as a loading control. (B) Representative images of
477 chlorophyll fluorescence in WT and Mpphot-Citrine overexpression lines. (C) The P/A
478 ratios calculated from the chlorophyll fluorescence images shown in (B) after the
479 incubation under BL25 at 22°C for 12 h. (D) Confirmation of the reduction of
480 transitional light intensity in OX2. In the 1-day-old gemmalings from OX2, the
481 accumulation response was induced under BL5 at 22°C (left panel). The cold-avoidance
482 response was induced under BL5 at 5°C for 24 h (right panel). (E) Time-course of the
483 cold-avoidance response induced under BL5 at 5°C in OX2. The P/A ratios were
484 calculated from the chlorophyll fluorescence images after incubation for 0, 6, 12, and 24
485 h.

486

487 **Fig 5. Alteration of Mpphot expression is not required for the induction of the**

488 **cold-avoidance response.** (A) Immunoblot analysis of endogenous MpPHOT amount
489 in 4-day-old WT gemmaling (white light for 3 days and BL25 for 24 h at 22°C),
490 incubated under BL25 at 5°C. The black arrowhead and asterisk indicate MpPHOT and
491 non-specific signal, respectively. Histone H3 protein is shown as a loading control. (B)
492 Representative chlorophyll fluorescence images of WT gemmalings incubated under the
493 same light and temperature conditions as shown in (A). (C) The P/A ratios, calculated
494 from chlorophyll fluorescence images after incubation for 0, 3, 6 and 9 h. Error bars

495 indicate standard deviations. (D) Immunoblot analysis of cold-induced repression of
 496 activity of CaMV35S promoter. A transformant expressing Citrine under the control of
 497 CaMV35S promoter (35S::Citrine/WT) was used. Coomassie Brilliant Blue
 498 (CBB)-stained Rubisco large-subunit (RBCL) was used as a loading control. (E)
 499 Chlorophyll fluorescence images showing the cold-avoidance response in
 500 complementation lines (35S::Mpphot-Citrine/Mpphot^{KO}). After incubation under BL25
 501 at 22°C, the gemmalings were transferred to 5°C under the same light conditions.

502

503 **S Fig 1. Effect of the light condition change on the endogenous Mpphot expression.**

504 Immunoblot analysis of endogenous Mpphot amount in WT gemmaling, incubated
 505 under BL25 at 22°C after culture under the white light condition (75 $\mu\text{mol photons m}^{-2}$
 506 s^{-1}) at 22°C for 3 days. The black arrowhead and asterisk indicate Mppot and
 507 non-specific signal, respectively. Histone H3 protein is shown as a loading control.