Phototropin2 Contributes to the Chloroplast Avoidance Response at the Chloroplast-Plasma Membrane Interface

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Phototropin2 Contributes to the Chloroplast Avoidance Response at the Chloroplast-Plasma Membrane Interface[1[CC-BY]]

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Blue-light-induced chloroplast movements play an important role in maximizing light utilization for photosynthesis in plants. Under a weak light condition, chloroplasts accumulate to the cell surface to capture light efficiently (chloroplast accumulation response). Conversely, chloroplasts escape from strong light and move to the side wall to reduce photodamage (chloroplast avoidance response). The blue light receptor phototropin (phot) regulates these chloroplast movements and optimizes leaf photosynthesis by controlling other responses in addition to chloroplast movements. Seed plants such as Arabidopsis (*Arabidopsis thaliana*) have phot1 and phot2. They redundantly mediate phototropism, stomatal opening, leaf flattening, and the chloroplast accumulation response. However, the chloroplast avoidance response is induced by strong blue light and regulated primarily by phot2. Phots are localized mainly on the plasma membrane. However, a substantial amount of phot2 resides on the chloroplast outer envelope. Therefore, differentially localized phot2 might have different functions. To determine the functions of plasma membrane- and chloroplast envelope-localized phot2, we tethered it to these structures with their respective targeting signals. Plasma membrane-localized phot2 regulated phototropism, leaf flattening, stomatal opening, and chloroplast movements. Chloroplast envelope-localized phot2 failed to mediate phototropism, leaf flattening, and the chloroplast accumulation response but partially regulated the chloroplast avoidance response and stomatal opening. Based on the present and previous findings, we propose that phot2 localized at the interface between the plasma membrane and the chloroplasts is required for the chloroplast avoidance response and possibly for stomatal opening as well.

Plants recognize UV-B, blue (BL), red (RL), and far-red light with photoreceptor molecules (Kami et al., 2010; Paik and Huq, 2019), i.e. a red/far-red photoreceptor phytochrome (Franklin and Quail, 2010), a photolyase-like BL receptor cryptochrome (Liu et al., 2016), a UV-B photoreceptor, UVR8 (Jenkins, 2017), and so on. These plant photoreceptors mediate light responses including photomorphogenesis and photoperiodic flowering by regulating gene expression. They interact with various transcription factors and activate or suppress them (Liu et al., 2016; Pham et al., 2018; Liang et al., 2019). Most plant photoreceptors localize primarily in the nucleus or cytosol.

In contrast, the plant-specific BL photoreceptor known as phototropin (phot) is localized mainly on the plasma membrane (PM; Christie, 2007). It regulates BL responses without directly affecting gene expression (Christie, 2007). Phots consist of two light-sensing LOV (light, oxygen, or voltage) domains and a C-terminal Ser/Thr kinase domain (Christie, 2007). Arabidopsis (*Arabidopsis thaliana*) has two phots, phot1 and phot2. BL-induced autophosphorylation is essential for phot function (Christie et al., 1998; Inoue et al., 2008a; Inoue et al., 2011). Phots can optimize photosynthetic activity and plant growth under fluctuating light conditions by regulating various responses (Kasahara et al., 2002; Takemiya et al., 2005; Gotoh et al., 2018). Both phot1 and phot2 are functionally redundant in phototropism (Huala et al., 1997; Sakai et al., 2000, 2001), the chloroplast accumulation response (Sakai et al., 2001), stomatal opening (Kinoshita et al., 2001), leaf flattening (Sakai et al., 2001; Sakamoto and Briggs, 2002), and leaf positioning (Inoue et al., 2008b). However, phot1 has a relatively greater contribution to these responses under weak light conditions (Suetsugu and Wada, 2013). Certain responses are regulated by phot1 or phot2 alone. Phot1 mediates rapid inhibition of hypocotyl growth (Folta and Spalding, 2001) and high-fluence, BL-mediated mRNA destabilization (Folta and Kaufman, 2003). Phot2 regulates chloroplast dark positioning (Suetsugu et al., 2005),

chloroplast and nuclear avoidance responses (Kagawa et al., 2001; Iwabuchi et al., 2007; Higa et al., 2014), and palisade cell development (Kozuka et al., 2011).

Chloroplasts move toward weak light to optimize light capture (the chloroplast accumulation response; Gotoh et al., 2018). Conversely, they evade strong light in order to reduce photodamage (the chloroplast avoidance response; Kasahara et al., 2002). Only chloroplasts directly irradiated with strong light exhibited avoidance movement (Kagawa and Wada, 1999; Tsuboi and Wada, 2011), suggesting that phot2 is closely associated with chloroplasts or localized on the PM near them. Although most phototropins are constitutively associated with the PM, we previously showed that some Arabidopsis phototropins are localized on the outer envelope of the chloroplast (Kong et al., 2013c). The amount of phot2 in the chloroplast outer membrane is much higher than that of phot1 (Kong et al., 2013c). A single species of phot of the liverwort *Marchantia polymorpha* (Mpphot) regulates chloroplast accumulation and avoidance (Komatsu et al., 2014). It is localized on the chloroplast outer envelope and the PM (Kodama, 2016). However, it has not yet been empirically demonstrated which Arabidopsis phot2 (the one localized on the chloroplast or the one on the PM) mediates the chloroplast avoidance response and other phot-mediated responses, such as phototropism, stomatal opening, and leaf flattening. To answer this question, we produced transgenic plants in which phot2 was tethered to the chloroplast outer envelope or the PM in the Arabidopsis *phot1phot2* mutant background, and we observed phot-associated phenotypes in the transgenic plants.

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E.G., N.S., and M.W. conceptualized the research; E.G. and K.I. designed the study and performed most of the experiments; T.H. and M.N. performed the immunoblot analysis of cell-fractionated proteins; H.T. contributed to the light transmittance analysis; T.H. and A.C. contributed to the microscopic analysis; S.I. analyzed the light-induced stomatal opening; N.S., T.U., and T.M. generated the transgenic mutants; T.K. contributed to analytical tools; K.I., T.H., N.S., M.W., and E.G. analyzed the data; N.S. and E.G. wrote the article; and all authors read and approved the manuscript.

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RESULTS

Generation of Transgenic Plants Expressing PM- or Chloroplast Envelope-Anchored Phot2

To reveal the functions of phot2 localized on the chloroplast outer membrane, we produced transgenic Arabidopsis plants expressing phot2-GFP fusion protein (phot2-GFP) targeted to the chloroplast outer membrane with the N-terminal, 47-amino acid sequence of OUTER ENVELOPE MEMBRANE PROTEIN7 (OEP7; Fig. 1A; Lee et al., 2001), hereafter called the CP-P2G lines. This short sequence contains all of the domains required to target the chloroplast outer membrane, including the transmembrane and C-terminal positively charged regions (Fig. 1A; Lee et al., 2001). We also generated transgenic plants expressing wild-type phot2-GFP (hereafter called the P2G lines) as a control and PM-anchored phot2-GFP (hereafter called the PM-P2G lines; Fig. 1A). The phot2-GFP protein of the PM-P2G lines contains a myristoylation sequence at the extreme N-terminus (Fig. 1A; Preuten et al., 2015). The *PHOT2-GFP* genes were expressed under the control of the *PHOT2* native promoter in *phot1phot2* double mutant plants that are phot-null (Kinoshita et al., 2001; Suetsugu et al., 2013). Two independent lines in each transgenic plant, P2G, PM-P2G, and CP-P2G, were selected for further analysis. We performed immunoblot analyses with PHOT2 antibody to verify the phot2-GFP protein levels in the selected lines. The ~120-kD PHOT2 band was detected in the wild type but not the *phot1phot2* double mutant (Fig. 1B). In the P2G, PM-P2G, and CP-P2G lines, bands of ~150 kD representing the phot2-GFP protein were detected. This finding was consistent with GFP fusion. The amounts of phot2-GFP proteins in the selected P2G and CP-P2G plants were comparable with that of the wild-type plants. However, the amounts of phot2-GFP in the PM-P2G lines were always lower than in the P2G and CP-P2G lines despite verification of multiple independent PM-P2G lines (Fig. 1B).

The subcellular localizations of phot2-GFP in the leaf mesophyll cells of P2G, PM-P2G, and CP-P2G line were observed by confocal imaging. Time gating was used to eliminate chlorophyll autofluorescence (Kodama, 2016). In our P2G transgenic plants, GFP fluorescence was found on both the PM and the chloroplast outer envelope (Fig. 1C; Supplemental Fig. S1). GFP fluorescence in the PM-P2G line was observed only on the PM (Fig. 1C). Fluorescence in the PM-P2G line strongly coincided with that of propidium iodide, which stains cell boundaries (Supplemental Fig. S1). GFP fluorescence was observed on the vacuolar side of the chloroplasts of the P2G line but not on those of the PM-P2G line (Fig. 1C; Supplemental Fig. S1). Thus, phot2-GFP proteins in the PM-P2G line were not measurably targeted to the chloroplast outer membrane. In CP-P2G transgenic plants, however, GFP fluorescence was

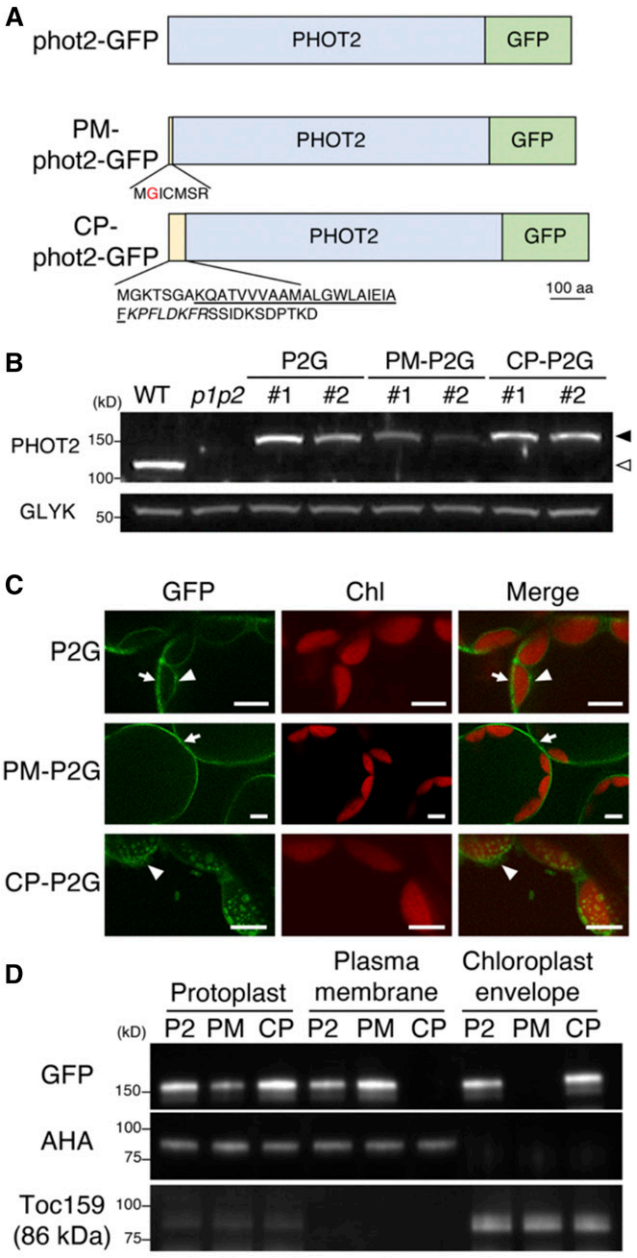

Figure 1. Targeted localization of phot2-GFP in the transgenic lines. A, Outline of phot2-GFP constructs. Protein structures of phot2-GFP, PM-phot2-GFP, and CP-phot2-GFP are indicated. The yellow box in PM-phot2-GFP is a signal sequence for PM targeting. The myristoylated Gly residue is colored red. The yellow box in CP-phot2-GFP is a chloroplast-targeting sequence from Arabidopsis OUTER ENVELOPE MEMBRANE PROTEIN 7 (OEP7). A transmembrane region (underlined) and the *C*-terminal positively charged region (italic) are indicated. Scale bar = 100 amino acids (aa). B, Western blot of phot2-GFP proteins using anti-PHOT2 antibody. Wild-type (WT) and *phot1phot2* (*p1p2*) plants were positive and negative controls, respectively. Twenty micrograms of total protein were extracted from 3-week-old plants. Black and white arrowheads indicate phot2-GFP and endogenous phot2, respectively. GLYK was the loading control. Molecular weight (kD) is indicated at the left side. C, Subcellular localizations of phot2-GFP in leaf mesophyll cells of 3-week-old P2G, PM-P2G, and CP-P2G plants. GFP fluorescence (left), chlorophyll auto-fluorescence (Chl; middle), and merged images of GFP and Chl (right) are indicated. Arrows and arrowheads indicate the localizations of phot2-GFP on the PM and chloroplast outer envelope, respectively. Scale bar = 0.5 μm. D, Immunoblot analysis of cell fraction in transgenic plants. Protoplasts were prepared from fully expanding rosette leaves of 3-week-old P2G (P2), PM-P2G (PM), and CP-P2G (CP) plants. Protoplast fractions were ruptured physically and further separated into PM and chloroplast envelope fractions. Total proteins were obtained from all fractions. The loading volume in the protoplast fraction and chloroplast envelope fraction or in the PM were determined based on the protein accumulation level of Toc159 (86-kD fragments) or AHA, respectively.

detected exclusively around the chloroplasts but not on the PM, although punctate GFP fluorescence that was not associated with chloroplasts was observed (Fig. 1C; Supplemental Figs. S1 and S2). Therefore, phot2-GFP proteins in the CP-P2G line are localized on the chloroplast envelope. However, the localization pattern of phot2-GFP on the chloroplast surface was somewhat different between P2G and CP-P2G transgenic plants. The phot2-GFP fluorescence on the chloroplast surface was diffused in the P2G line, but was punctate in the CP-P2G line (Fig. 1C; Supplemental Fig. S2), although we do not know what gave rise to this difference. To determine phot2-GFP localization in each transgenic plant, we performed immunoblot analyses on protein extracts obtained by cell fractionation (Fig. 1D). The PM or chloroplast fractions were normalized with anti-H^+-ATPASEs (AHAs; Hayashi et al., 2010) or anti-TRANSLOCONE AT THE OUTER ENVELOPE MEMBRANE OF CHLOROPLAST 159 (Toc159; Kikuchi et al., 2006) polyclonal antibodies, respectively. Consistent with a previous report (Kong et al., 2013c), phot2-GFP in P2G lines was enriched in the PM fraction and was detected also in the chloroplast fraction. In the PM-P2G lines, phot2-GFP was also enriched in the PM fraction and was hardly detected in the chloroplast fraction (Fig. 1D). In contrast, phot2-GFP was highly enriched in the CP-P2G chloroplast fraction, but we could not detect phot2-GFP in the PM fraction (Fig. 1D).

In summary, phot2-GFP was targeted to the PMs and chloroplast outer envelopes of PM-P2G and CP-P2G plants, respectively.

BL-Induced Phot2 Protein Autophosphorylation on the PM and the Chloroplast Outer Envelope

To determine whether the phot2-GFPs of the PM-P2G and CP-P2G lines function as BL-activated kinases, we investigated BL-induced autophosphorylation of phot2-GFP proteins via BL-induced electrophoretic mobility shift. BL-induced retardation in the mobility shift of phot2-GFP proteins was observed in P2G, PM-P2G, and CP-P2G plants (Fig. 2). Thus, phot2-GFP of PM-P2G and CP-P2G plants had normal autophosphorylation and kinase activity. This means that the phot2 on the chloroplast outer membrane as well as on the PM can be autophosphorylated in response to BL.

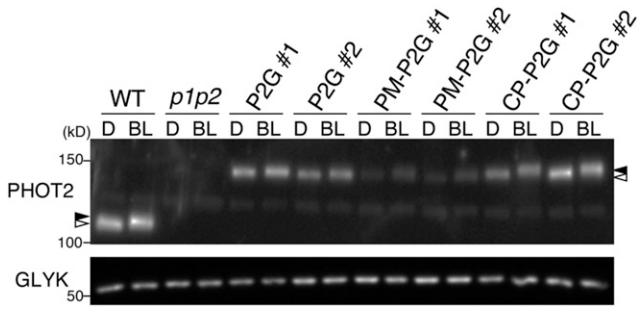

Figure 2. BL-induced autophosphorylation of phot2-GFP proteins in P2G, PM-P2G, and CP-P2G plants. BL-induced autophosphorylation of phot2-GFP proteins. Twenty micrograms of total protein were extracted from dark-adapted (D) or BL-irradiated (100 μmol m^{-2} s^{-1} for 1 min) plants for western blot. Black and white arrowheads indicate BL-dependent autophosphorylated and dark-adapted phot2-GFP (right) or endogenous phot2 (left), respectively. GLYK was the loading control. Molecular weight (kDa) is indicated at the left side. *p1p2*, *phot1phot2* double mutant.

PM-Associated Phot2 Induced Leaf Flattening and Biomass Production, Whereas Chloroplast Envelope-Associated Phot2 Did Not

We analyzed the localization of phot2-GFP in leaf epidermal cells (Fig. 3A; Supplemental Fig. S3) because phot2 in the epidermal tissues is required to regulate leaf flattening and expansion (Kozuka et al., 2011). In the P2G lines, phot2-GFP localized on the PMs of the leaf epidermal cells, and we could not detect any phot2-GFP fluorescence around the chloroplasts (Fig. 3A; Supplemental Fig. S3). In PM-P2G plants, phot2-GFP showed exclusive plasma-membrane localization. On the other hand, phot2-GFP in the CP-P2G lines was localized primarily around the chloroplasts (Fig. 3A; Supplemental Fig. S3). Furthermore, dot-like phot2-GFP structures not associated with chloroplasts were also observed in the CP-P2G lines (Fig. 3A, arrow).

Under white light at 100–120 μmol m^{-2} s^{-1}, in the P2G and PM-P2G lines, the leaves were flat and resembled those of the wild type (Fig. 3, B and C). Plant size in the P2G and PM-P2G lines was much bigger than *phot1phot2* double mutant plants, but these lines were nonetheless smaller than the wild type (Fig. 3, B and D). The size of P2G and PM-P2G plants was comparable with that of *phot1* mutant plants, because the absence of phot1 in the P2G and PM-P2G lines could influence plant size as reported previously (Takemiya et al., 2005; Inoue et al., 2008b). In contrast, CP-P2G plants had curled leaves and lower biomass production than the wild-type, P2G, and PM-P2G lines, similar to *phot1phot2* mutants (Fig. 3, B–D). Our findings indicate that PM-localized phot2, but not chloroplast-localized phot2, induced leaf flattening and biomass production.

PM-Associated Phot2 Induced Phototropism, Whereas Chloroplast Outer Membrane-Associated Phot2 Did Not

To investigate the role of differential phot2 localization in hypocotyl phototropism, we examined subcellular phot2-GFP localization in the hypocotyl epidermal (Fig. 4A) and cotyledon palisade (Supplemental Fig. S4) cells of 4-d-old etiolated transgenic seedlings. In Arabidopsis, etioplasts in etiolated seedlings could be detectable by chlorophyll autofluorescence (Wang et al., 2013). In the P2G lines, phot2-GFP was found on the PM and around the etioplast periphery. Phot2-GFP in the PM-P2G lines exhibited the exclusive PM localization in the hypocotyl and cotyledon epidermal cells (Fig. 4A; Supplemental Fig. S4). For the CP-P2G lines, phot2-GFP was detected around the etioplasts and in certain dot-like structures not associated with chloroplasts (Fig. 4A, arrows; Supplemental Fig. S4).

We explored phototropism under 10 μmol m^{-2} s^{-1} unilateral BL at which phot2 can mediate phototropism in the absence of phot1 (Fig. 4B). The wild type showed positive phototropism, whereas *phot1phot2* mutants showed no phototropic responses. The P2G and PM-P2G plants were positively phototropic and at the same level as the wild type (Fig. 4B). In contrast, no phototropic curvature was detected in CP-P2G plants (Fig. 4B).

Chloroplast Outer Membrane-Associated Phot2 Partially Induced Stomatal Opening

We investigated whether chloroplast-localized phot2 mediates BL-dependent stomatal opening. In the leaf epidermal guard cells of P2G plants, phot2-GFP was localized on the PM and around the chloroplast outer envelope (Fig. 5A). Phot2-GFP in a PM-P2G line was exclusively localized on the guard cell PMs. On the other hand, phot2-GFP in CP-P2G plants was detected around the chloroplasts and in certain dot-like structures (Fig. 5A, arrows) but not on the PM (Fig. 5A). These patterns of phot2-GFP were also observed in the epidermal cells of leaves (Fig. 3A) and etiolated seedlings (Fig. 4A; Supplemental Fig. S4).

The isolated leaf epidermis of transgenic plants was dark-adapted and irradiated either with RL at 60 μmol m^{-2} s^{-1} or RL (50 μmol m^{-2} s^{-1}) plus BL (10 μmol m^{-2} s^{-1}). RL enhancement of BL-dependent stomatal opening was observed in the wild-type and P2G plants but not in *phot1phot2* mutants (Fig. 5B). BL-dependent stomatal opening was induced in the PM-P2G line to the same extent as in the wild-type and P2G plants. However, CP-P2G plants exhibited weak but significant BL-induced stomatal opening (Fig. 5B). RL irradiation alone did not induce stomatal opening in any lines (Fig. 5B). Thus, PM-localized phot2 was sufficient to induce BL-dependent stomatal opening, but chloroplast outer membrane-localized phot2 also induced it.

Chloroplast Outer Membrane-Associated Phot2 Induced the Chloroplast Avoidance Response

We examined whether chloroplast outer membrane-localized phot2 regulates BL-induced chloroplast movements. Three-week-old plants were transferred to

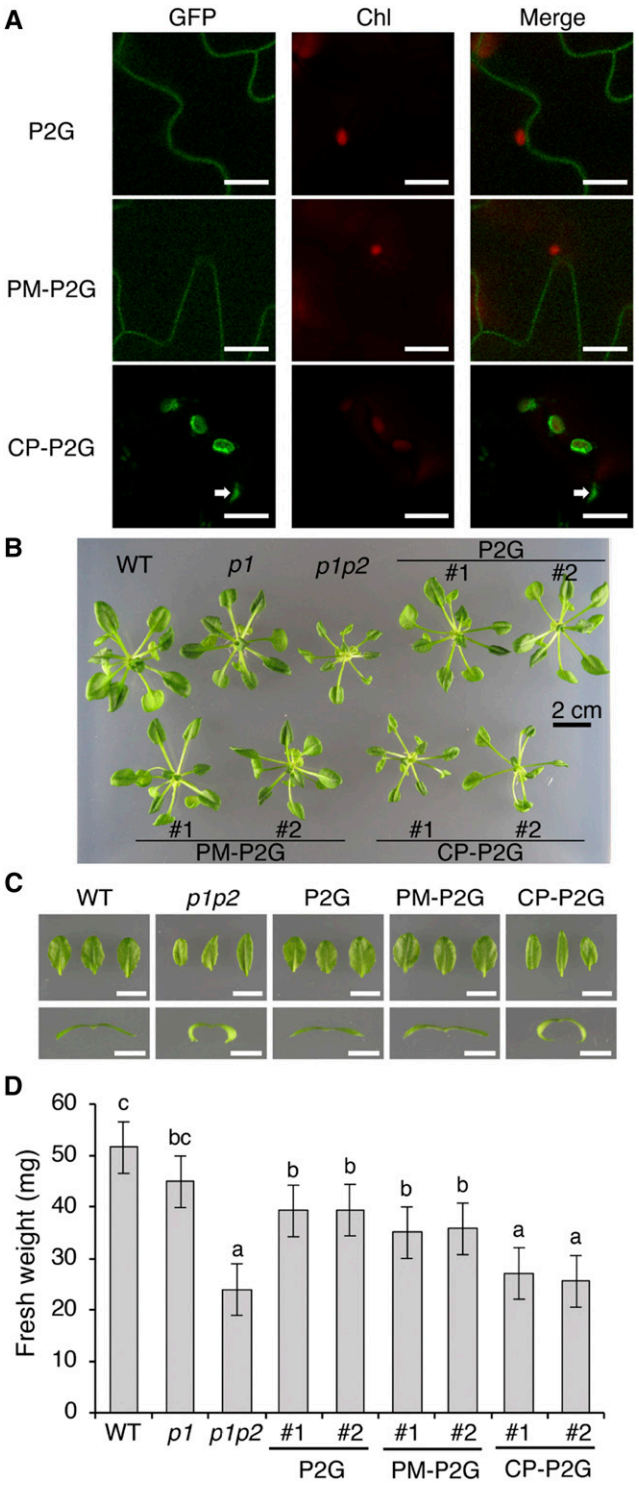

Figure 3. Plant growth and leaf flattening in P2G, PM-P2G, and CP-P2G plants. A, Localization of phot2 on the leaf epidermal tissue of transgenic plants. GFP fluorescence and chlorophyll autofluorescence (Chl) are shown in green and red, respectively. In Arabidopsis, immature epidermal cell plastids are referred to as chloroplasts, as they are detectable by chlorophyll autofluorescence (Higa et al., 2014; Barton et al., 2016). Merged images were produced by stacking the two fluorescence images. Scale bars = 10 μm. Arrows indicate dot-like phot2-GFP proteins not associated with chloroplasts in the CP-P2G lines. B, Images of whole plants. Wild type (WT), *phot1* (*p1*), *phot1phot2* (*p1p2*), and transgenic plants were grown under continuous white light at 100 μmol m^{-2} s^{-1} for 3 weeks. C, Detached leaves showing leaf flattening. Images of whole leaves (top) and leaf cross sections (bottom) from 3-week-old plants grown under continuous white light at 120 μmol m^{-2} s^{-1} are shown. Scale bars = 1 cm. D, Plant biomass production. Wild type, *phot1*, *phot1phot2*, and transgenic plants were grown for 3 weeks under continuous white light at 100 μmol m^{-2} s^{-1}. Images are representative samples. Data are means ± SD (n = 30). Lowercase letters indicate significant differences ($P < 0.01$, Tukey's honestly significant difference [HSD] mean separation test).

darkness, weak light, or strong light and chloroplast distributions were observed under a confocal microscope (Fig. 6A). In P2G and PM-P2G plants under darkness, chloroplasts accumulated on the cell bottom. Under weak BL, chloroplasts were localized on the upper and lower periclinal walls. Under strong BL, chloroplasts were localized on the side walls (Fig. 6A). Thus, phot2-GFP in P2G and PM-P2G lines mediated chloroplast dark positioning, accumulation, and avoidance responses. In a CP-P2G line, chloroplasts localized to the side walls under all light conditions. Therefore, the CP-P2G line was defective in chloroplast dark positioning and accumulation response (Fig. 6A). However, it remains to be determined whether phot2-GFP of the CP-P2G lines regulates the chloroplast avoidance response. We examined under a confocal microscope the chloroplast avoidance response to partial irradiation in leaf palisade mesophyll cells (Fig. 6). In wild-type, *phot1*, P2G, and PM-P2G plants, chloroplasts evaded the irradiated area as a result of the avoidance response (Fig. 6, B and C; Supplemental Fig. S5, left and middle). The chloroplast avoidance response in P2G and PM-P2G plants was slightly greater than that in wild-type and *phot1* plants, although statistically insignificant (Fig. 6, D and E). In CP-P2G plants, chloroplasts also escaped from the irradiated area (Fig. 6B, right), but much more slowly than in P2G and PM-P2G plants (Fig. 6, C–E). Upon induction of the chloroplast avoidance response, the total distance traveled in 15 mins was shorter in the CP-P2G line than in P2G or PM-P2G plants (Fig. 6, C and D). However, the avoidance response was never detected in *phot1-phot2* mutants (Fig. 6, D and E; Supplemental Fig. S5, right). Thus, phot2-GFP in the CP-P2G line partially rescued the defect in the avoidance response in the *phot1phot2* mutant.

We also assessed chloroplast movements by measuring light-induced transmittance changes (Fig. 7). In wild-type plants, weak light (1, 3, and 5 μmol m^{-2} s^{-1}) reduced leaf transmittance as a result of the chloroplast accumulation response (Fig. 7, A–C). At 5 μmol m^{-2} s^{-1}, a biphasic response was observed, i.e. initial transient increase in leaf transmittance and then the strong decrease (Fig. 7C) indicative of a transient chloroplast avoidance. A biphasic response was more prominent, and the clear avoidance response was induced at

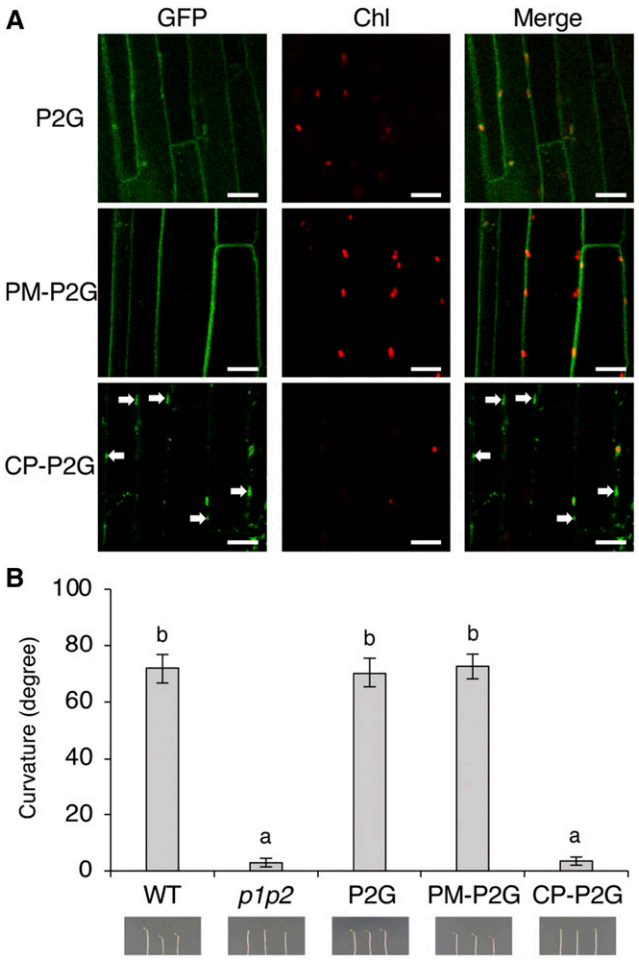

Figure 4. BL-induced phototropism in transgenic plants. A, Localization of phot2 in the hypocotyl epidermal cells of transgenic plants. GFP and chlorophyll (Chl) fluorescence are shown in green and red, respectively. Merged images were produced by stacking the two fluorescence images. Arrows indicate dot-like phot2-GFP proteins not associated with chloroplasts in the CP-P2G lines. Scale bars = 10 μm. B, Phototropism. Etiolated seedlings were irradiated with unilateral BL at 10 μmol m^{-2} s^{-1} for 12 h. Images below the graph are representative samples. Data are means ± SE (n = 90). Different letters indicate significant differences ($P < 0.01$, Tukey's HSD mean separation test).

20 μmol m^{-2} s^{-1} (Fig. 7D). At >50 μmol m^{-2} s^{-1}, only the avoidance response was detectable and the response was saturated between 100 and 300 μmol m^{-2} s^{-1} (Fig. 7, E–G). In the *phot1* mutant (that has phot2), the accumulation response was severely attenuated and the avoidance response was induced with slightly reduced amplitude (Fig. 7). Partial defect in the avoidance response in the *phot1* mutant was reported also in other studies (Luesse et al., 2010; Sztatelman et al., 2016). No detectable light-induced changes in leaf transmittance were observed in the *phot1phot2* mutants (Fig. 7) due to their lack of light-induced chloroplast movements (Sakai et al., 2001). Both P2G and PM-P2G plants basically showed chloroplast movements similar to those observed in *phot1* mutant plants, although the accumulation response was weaker than in the *phot1* mutant (Fig. 7). In CP-P2G plants, no chloroplast accumulation response was observed at any fluence rate of BL. However, the weak but significant avoidance response was observed at >20 μmol m^{-2} s^{-1} although the amplitude was less than in P2G and PM-P2G plants (Fig. 7, D–G).

Taken together, these results indicate that phot2 localized in the chloroplast outer membrane certainly induced the chloroplast avoidance response, although less effectively than that localized in the PM.

DISCUSSION

Here, we showed that PM-localized phot2 is sufficient to mediate phot2-dependent responses.

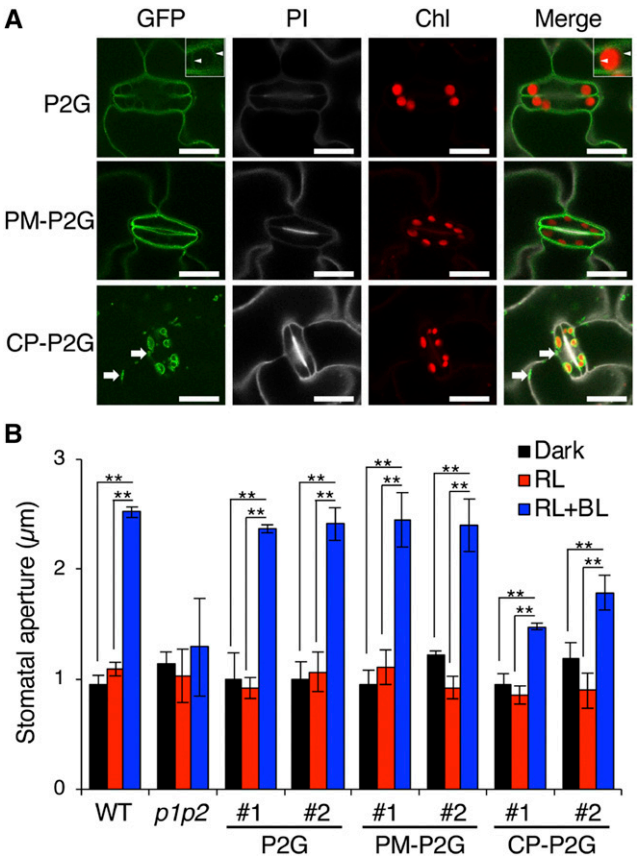

Figure 5. BL-induced stomatal opening in the P2G, PM-P2G, and CP-P2G plants. A, Intracellular localization of phot2 in guard cells. Images show GFP (green), PI (white), and Chl (red) fluorescence, respectively. Merged images were produced by stacking the three. Insets are enlarged images showing localization of phot2-GFP around the chloroplast in the P2G line (arrowheads). Arrows indicate dot-like phot2-GFP proteins not associated with chloroplasts in the CP-P2G line. Scale bars = 10 μm. B, BL-induced stomatal opening in transgenic plants. Epidermal peels were irradiated with RL or RL+BL. Thirty stomata were measured per experiment. Measurements were repeated three times. Data are means of three independent experiments ± SE (n = 90). Asterisks indicate significant differences between two groups ($P < 0.001$, Student's t test).

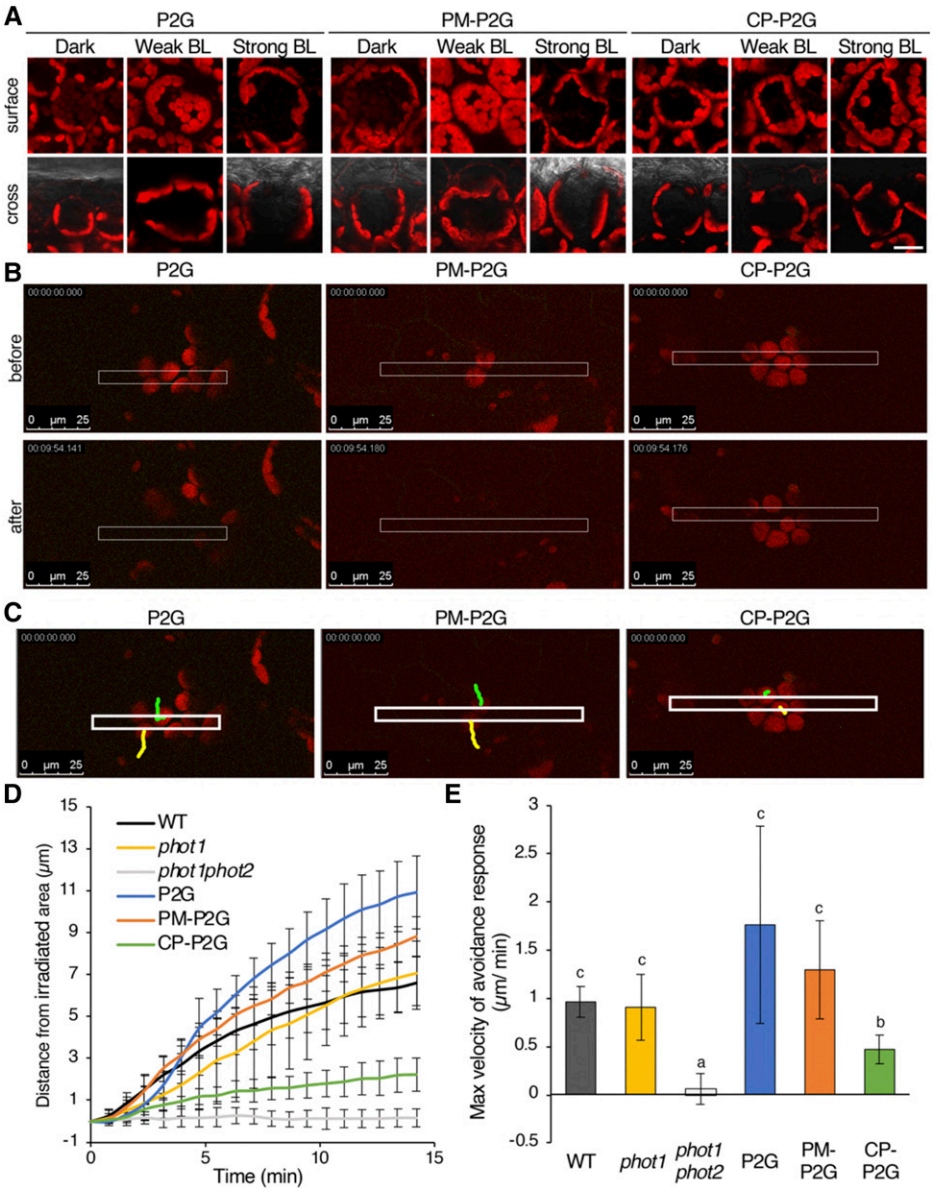

Figure 6. Analysis of BL-induced chloroplast avoidance movements with partial irradiation. A, Distribution of chloroplasts in leaf palisade mesophyll cells in the transgenic plants. Three-week-old plants were dark-adapted for 1 d (Dark) or irradiated with BL at 3.0 (weak BL) or 50 (strong BL) μmol m^{-2} s^{-1} for 3 h. Images of the upper cell surface (surface) and cross sections (cross) are shown. Scale bar = 20 μm. B, The chloroplast avoidance response in the leaf palisade mesophyll cells was induced by partial irradiation with a blue laser beam. Top and bottom rows indicate chloroplast autofluorescence before and after partial irradiation (~3 min for the P2G and PM-P2G lines and ~8 min for the CP-P2G line), respectively. Irradiated areas are indicated by white rectangles. C, Tracking chloroplast avoidance movement. Images are the same as those in the upper row of (B). Yellow and green lines indicate the center of the path of chloroplast avoidance of BL. D and E, chloroplast migration distance (D) and chloroplast avoidance movement velocity (E) in wild-type (WT), *phot1*, *phot1phot2*, P2G, PM-P2G, and CP-P2G plants. The chloroplast avoidance response was induced by a strong microbeam. Data are means ± SD ($n = 8$). Lowercase letters indicate significant differences ($P < 0.01$, Tukey-Kramer test).

Chloroplast outer membrane-localized phot2 mediates BL-dependent stomatal opening and the chloroplast avoidance response.

PM-Anchored Phot2 Mediated All Phot2-Dependent Responses

Phots are localized and function on the PM. By contrast, most plant photoreceptors function and are localized in the nucleus or cytosol. Phots have no transmembrane domain (Christie, 2007). PM-bound phots can be solubilized with detergents or alkaline solutions but not with chaotropic salt (Knieb et al., 2004; Kong et al., 2013b, 2013c). Therefore, phots are not integral membrane proteins. Moreover, mechanisms other than electrostatic interaction and covalent bonding to integral compounds are involved in the association of phots with the PM. Phot proteins are not always localized on the PM. BL causes the release of phot1 to the cytosol (Sakamoto and Briggs, 2002; Wan et al., 2008) and the movement of phot2 to the Golgi apparatus (Kong et al., 2006). The importance of phots in cytosol and in the Golgi was examined in various ways. When the cytoplasmic phots were cleared through RL-mediated inhibition of release of phot1 to the cytosol (Han et al., 2008), tethering of phot1-GFP to the PM by myristoylation or farnesylation (Preuten et al., 2015), and targeting phot2-GFP to the nucleus with a nuclear localization signal (phot2-GFP-NLS; Kong et al., 2013c), all examined phot-mediated responses were normally induced, indicating that cytosolic phots are dispensable for phot-mediated responses. Furthermore, analysis of

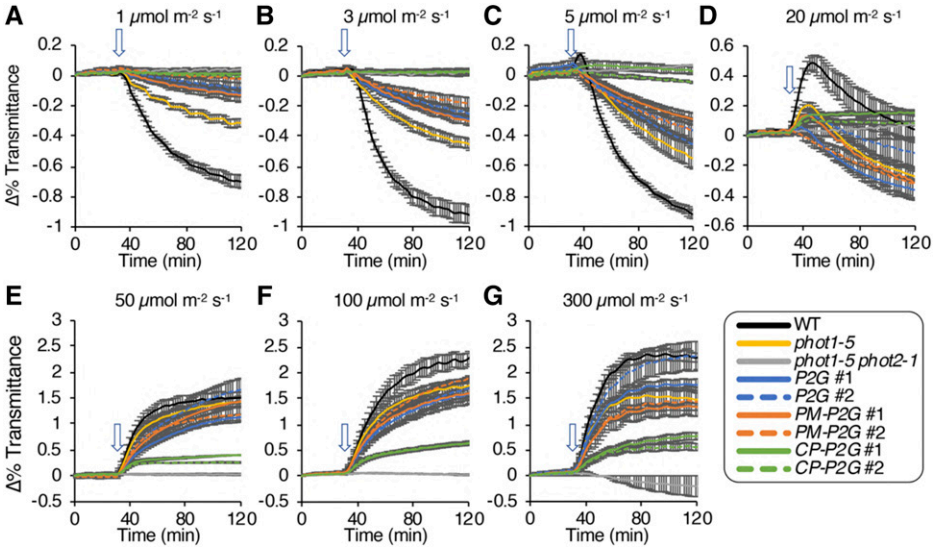

Figure 7. Analysis of BL-induced chloroplast movements through measurement of changes in leaf transmittance. BL-induced chloroplast movements in wild-type (WT), *phot1*, *phot1phot2*, P2G, PM-P2G, and CP-P2G plants were tracked by measuring light-induced changes in leaf transmittance. Detached leaves were dark-adapted for >3 h before the measurement. After measurements of leaf transmittance in darkness for 30 min, the leaves were irradiated with BL at 1 (A), 3 (B), 5 (C), 20 (D), 50 (E), 100 (F), or 300 μmol m^{-2} s^{-1} (G) for 80 min from the timepoint indicated by the white arrow. Wild-type and *phot1* plants were used as a control. At least eight leaves were used per experiment, and the experiments were repeated three times. Data are means ± SE (n = 3) of biological triplicates.

domain swapping between phot1 and phot2 revealed that targeting phot2 to the Golgi apparatus is not required for the chloroplast avoidance response (Aihara et al., 2008). A chimeric phot consisting of phot2 N-terminal light-sensing- and phot1 kinase domains could not move to the Golgi apparatus in response to BL but could mediate the chloroplast avoidance response (Aihara et al., 2008). Therefore, phots have virtually no function in the Golgi apparatus.

Our findings for the PM-P2G lines confirm that phots function primarily on the PM (Fig. 8A). The PM-P2G lines were almost normal for all phot2-dependent responses including phototropism, leaf flattening, plant biomass, stomatal opening, and chloroplast accumulation and avoidance, although slightly less phot2-GFP accumulated in the PM-P2G lines relative to the other lines (Figs. 1 and 2). However, the amount of phot2-GFP on the PM in PM-P2G lines was comparable to or higher than that in the P2G lines (Figs. 1, C and D, and 3–5; Supplemental Figs. S1–S4). That should be why phot2-GFP in the PM-P2G lines efficiently rescued all examined phot2-mediated responses. Preuten et al. (2015) showed that PM-anchored phot1-GFP mediated phot1-dependent responses including phototropism, leaf flattening and positioning, and chloroplast accumulation. Stomatal opening was not evaluated in that study. The PM is a major site of phot signaling initiated in response to BL. Most phot-interacting proteins essential for certain phot-mediated responses are also localized in the PM. These include the BTB/POZ (for Broad-Complex, Tramtrack, and Bric-a-brac/POX virus and Zinc finger)-domain proteins NON-PHOTOTROPIC HYPOCOTYL 3 (Motchoulski and Liscum, 1999), ROOT PHOTOTROPISM 2 (Inada et al., 2004), NPH3/RPT2-LIKE (NRL) PROTEIN FOR CHLOROPLAST MOVEMENT 1 (NCH1; Suetsugu et al., 2016), the PHYTOCHROME KINASE SUBSTRATE (PKS) proteins (Lariguet et al., 2006; de Carbonnel et al., 2010; Demarsy et al., 2012), and the auxin efflux carrier ATB-BINDING CASSETTE B 19 (ABCB19; Christie et al., 2011). However, they do not participate in stomatal opening or the chloroplast avoidance response. Moreover, no phot-interacting proteins on the PM have yet been associated with these responses. Stomatal opening and the chloroplast avoidance response are mediated by phot2-GFP in the CP-P2G line.

How Does Chloroplast Outer Membrane-Anchored Phot2 Promote BL-Induced Stomatal Opening?

phot2-GFP in control plant guard cells was localized on the PM and chloroplast outer envelopes (Fig. 5A). We found that chloroplast outer membrane-anchored phot2 may be partially involved in BL-induced stomatal opening (Figs. 5B and 8A). Phots mediate stomatal opening by regulating the phosphorylation and activation of PM H^+-ATPase (PM-H^+-ATPase; Kinoshita et al., 2001). Several signaling proteins downstream of phots participate in PM-H^+-ATPase activation, including protein kinases BLUE LIGHT SIGNALING1 (BLUS1; Takemiya et al., 2013a) and BLUE LIGHT-

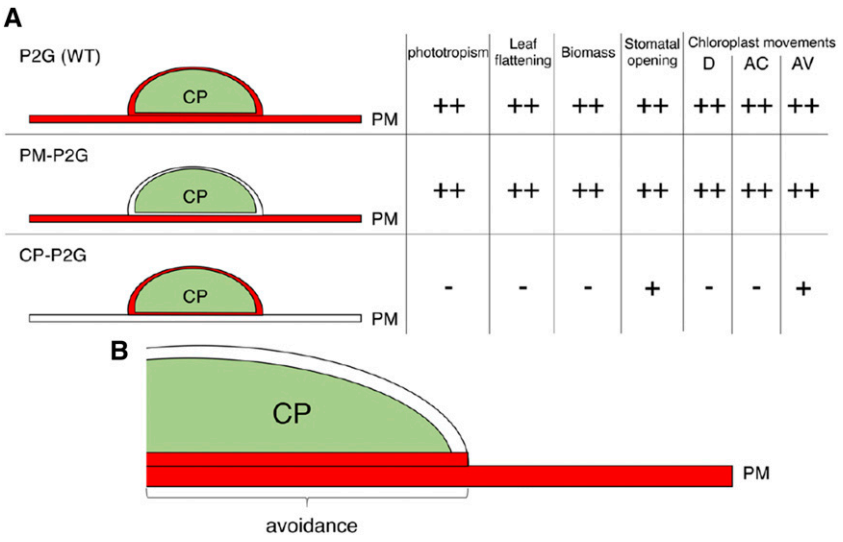

Figure 8. P2G, PM-P2G, and CP-P2G phenotypes. A, At left are schematics showing localizations of phot2-GFP in P2G, PM-P2G, and CP-P2G plants. Phot2-GFP was localized on the PM and the outer envelopes of chloroplasts in the P2G wild type (WT), as shown in red, on the PM in the PM-P2G lines, and on the chloroplast outer membrane in the CP-P2G lines. At right is a phenotypic characterization of P2G, PM-P2G, and CP-P2G plants. ++, wild-type responses; +, weak responses in the CP-P2G lines; −, no response (such as in the *phot1phot2* mutant). B, Chloroplast avoidance response mediated by phot2 at the interface between the chloroplasts and the PM is indicated by a horizontal bracket. Phot2 (and phot1) on the PM regulate the chloroplast accumulation response.

DEPENDENT H^+-ATPASE PHOSPHORYLATION (BHP; Hayashi et al., 2017), Protein phosphatase1 (PP1), and PP1 REGULATORY SUBUNIT2-LIKE PROTEIN 1 (PRSL1; Takemiya et al., 2006, 2013b). Phots also regulate BL-induced suppression of the S-type anion channel in guard cells (Marten et al., 2007). CONVERGENCE OF BLUE LIGHT AND CO_2 1 (CBC1) is essential for BL-induced suppression of the S-type anion channel along with its close homolog CBC2 (Hiyama et al., 2017). These signaling components are localized in guard cell cytosols (Takemiya et al., 2006, 2013a, 2013b; Hayashi et al., 2017; Hiyama et al., 2017). Thus, chloroplast outer membrane-associated phot2 may induce signal transduction for phot-dependent stomatal opening via these cytosolic components. We found dot-like phot2-GFP in the epidermal cells of leaves (pavement and guard cells), cotyledons, and hypocotyls. They were not associated with chloroplasts (Figs. 3–5; Supplemental Fig. S4). These dot-like phot2-GFPs might be nonfunctional in leaf flattening, plant biomass production, or phototropism, because phot2-GFP in the CP-P2G lines could not rescue these responses in the *phot1phot2* mutant background (Figs. 3 and 4). On the other hand, we cannot rule out the possibility that the dot-like phot2-GFP could mediate stomatal opening. Further analysis is required to establish how chloroplast outer membrane-associated phototropins mediate BL-induced stomatal opening.

Phots at the Interface between the Chloroplasts and the PM May Be Required for the Chloroplast Avoidance Response

Previous photobiological and physiological analyses suggested that the chloroplast avoidance response is mediated by phots localized on the chloroplast outer membrane or in the vicinity of the chloroplasts (Kagawa and Wada, 1999; Tsuboi and Wada, 2011). The chloroplast accumulation response may be mediated by PM-localized phots because chloroplasts can move to irradiated sites far from the chloroplasts. Moreover, phots may generate transmissible signals mediating the chloroplast accumulation response (Tsuboi and Wada, 2010; Higa et al., 2017). In fact, in the PM-P2G lines both chloroplast accumulation and avoidance responses could be induced, but in the CP-P2G lines only the chloroplast avoidance response was detected, although it was relatively weak (Figs. 6 and 7). No phot2-GFP was detected on the vacuolar sides of the PM-P2G chloroplasts or the CP-P2G PMs (Fig. 8A). It is therefore plausible that phot2 at the interface between the chloroplasts and the PM is required for the chloroplast avoidance response (Fig. 8B). Chloroplast-actin (cp-actin) filaments, which are necessary for photorelocation and positioning, are localized at the interface between the chloroplasts and the PM (Kadota et al., 2009; Kong et al., 2013a). During chloroplast avoidance movements, phot2 is essential for reorganization of the cp-actin filaments (Kong et al., 2013a) by which chloroplasts are tightly bound to the PM (Kadota et al., 2009; Suetsugu et al., 2010). Thus, both PM- (as in the PM-P2G lines) and chloroplast-anchored phot2 (as in the CP-P2G lines) could function at the interface between the CPs and the PM to mediate the chloroplast avoidance response. CHLOROPLAST UNUSUAL POSITIONING1 (CHUP1), localized on the chloroplast outer membrane (Oikawa et al., 2003, 2008), is required to generate and/or maintain cp-actin filaments to control chloroplast movements and positioning (Oikawa et al., 2003; Kadota et al., 2009). Overall, phot2 might mediate the chloroplast avoidance response at the interface. Indeed, several chloroplast movement regulators, including CHUP1, are phosphorylated under strong light conditions (Boex-Fontvieille et al., 2014). Thus, phot2 might phosphorylate these proteins at the interface to regulate the cp-actin filaments and the chloroplast avoidance response.

Our findings indicated that phot2 proteins at the interface between the chloroplasts and the PM may be required for the chloroplast avoidance response.

However, they do not explain why phot1 cannot efficiently mediate this response even though phot1 localizes at the interface between the chloroplasts and the PM. Thus, substrate specificity between phot1 and phot2 may also be necessary to determine the precise functions of these phototropins. Domain swapping between phot1 and phot2 revealed that neither the N-terminal light-sensing- nor the kinase domain determines the functional specificity of phot2 in chloroplast avoidance response regulation (Aihara et al., 2008). Phot2 N/phot1 kinase and phot1 N/phot2 kinase chimeras regulated the chloroplast avoidance response (Aihara et al., 2008). To elucidate the functional difference between phot1 and phot2 in chloroplast avoidance response regulation, a more detailed structure-function analysis is needed.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col) wild type and the *phot1-5 phot2-1* mutant (Kinoshita et al., 2001) had a Col-*gl1* background. *phot1-5 phot2-1* is a null mutant for both phot1 and phot2 (Kinoshita et al., 2001; Suetsugu et al., 2013). For most experiments, the seeds were sown on 0.75% (w/v) agar plates containing Murashige and Skoog (MS) medium. The plants were incubated in a growth chamber (CLE-303; TOMY Digital Biology) at 22°C under continuous white light at 40 μmol m^{-2} s^{-1}. To measure biomass and leaf flattening, the plants were grown in soil in a growth chamber (LPH-350S; NK Systems) at 22°C and 55% relative humidity under continuous white light at 120 μmol m^{-2} s^{-1}.

Plasmid Construction and Generation of Transgenic Lines

Full-length complementary DNA (cDNA) fragments of *PHOT2* were amplified with forward primer P2-Fw (CCCtctagaATGGAGAGGCCAAGAGCCCCTC; *Xba*I site in lowercase) and reverse primer P2-Rv (AAAggtaccGAAGAGGTCAATGTCCAAGTCCGTAG; *Kpn*I site in lowercase) from the vector *PHOT2pro:AtPHOT2-GFP:nosT/pRI 101-AN* (Ishishita et al., 2016).

A recognition sequence for *N*-myristoyltransferase (Preuten et al., 2015) was added by PCR to the 5′-terminus of *PHOT2* cDNA using the primers PM-P2-Fw (AAAtctagaATGGAAATATGCATGAGTAGGATGGAGAGGCCAAGAGC, with the *Xba*I site in lowercase and the myristoylation sequence underlined) and P2-Rv.

The chloroplast outer membrane-targeting sequence of *OUTER ENVELOPE MEMBRANE PROTEIN 7* (*OEP7*; Lee et al., 2001) was obtained by PCR using the primers CP-P2 Fw (CCCtctagaATGGGAAAAACTTCGGGAGCGAA, with the *Xba*I site in lowercase) and CP-P2 Rv (CTCTTGGCCTCTCCATGGGGTCTTTGGTTGG, with the 5′ sequence of *PHOT2* cDNA underlined). The *PHOT2* fragment was obtained by PCR using the primers CP-P2 Fw2 (CCAACCAAAGACCCCATGGAGAGGCCAAGAG, with the 3′ sequence of *OEP7* cDNA underlined) and P2-Rv with template DNA from *PHOT2* and *OEP7* fragments. The latter two fragments were fused by PCR using the primers CP-P2 Fw and P2-Rv. All fragments were inserted into the *Xba*I/*Kpn*I site of GFP-NosT pBIN30 (Ushijima et al., 2017). A 3-kb *PHOT2* promoter region (−3,047 from the *PHOT2* start codon; Ishishita et al., 2016) was inserted into the *Nhe*I/*Xba*I site of the vectors. The resulting binary vectors were introduced into *Agrobacterium tumefaciens* strain C58C1 (pMP90) and transformed into the *phot1-5 phot2-1* double mutant by the floral dip method (Clough and Bent, 1998). BASTA-resistant transgenic plants were selected. Homozygous T3 lines with a single transgene were used in all subsequent experiments.

Immunoblot Analysis

Rosette leaves of 3-week-old plants were homogenized in a buffer comprising 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (Wako Pure Chemical Industries). Total proteins were used for SDS-PAGE and immunoblot analysis. PHOT2, GLYCERATE KINASE (GLYK), AHAs, and Toc159 proteins were detected with anti-PHOT2- (Takemiya et al., 2005), anti-GLYK- (Ushijima et al., 2017), anti-AHA- (Hayashi et al., 2010), and anti-Toc159 polyclonal antibodies (Kikuchi et al., 2006), respectively. Phot2-GFP fusion proteins in Figure 3D were detected with anti-GFP (no. 11814460001; Roche). In our experiment, anti-Toc159 polyclonal antibodies detected mainly 86-kD fragments of Toc159 that served as a chloroplast marker (Kikuchi et al., 2006).

Isolation of PM and Chloroplast Envelope Fractions

For the isolation of chloroplast envelope and PM fractions, mesophyll cell protoplasts were isolated from the leaves of Arabidopsis transgenic plants using the Tape-Arabidopsis Sandwich method (Wu et al., 2009). Protoplasts suspended in HS buffer (50 mM HEPES-KOH [pH 7.5] and 330 mM sorbitol) with 0.1% (w/v) bovine serum albumin and 0.1% (v/v) cOmplete protease inhibitor cocktail (F. Hoffmann-La Roche) stock solution (one tablet dissolved in 1 mL of water) were ruptured through a nylon mesh (~10 μm) attached to the cut end of a disposable syringe and then centrifuged at 4,000*g* for 3 min. The pellets were used for the isolation of the chloroplast envelope, and the supernatants were used for the isolation of the PM fraction. The crude chloroplast pellets were resuspended in TE buffer (50 mM Tricine-KOH [pH 7.5] and 2 mM EDTA) with 1 mM DTT and 0.1% (v/v) protease inhibitor cocktail stock solution, and then chloroplasts were ruptured by freeze-thaw followed by homogenization in a glass homogenizer after a 3-fold dilution with TE buffer. Ruptured chloroplasts were overlaid onto a step gradient composed of 0.46 M and 1 M Suc in TE buffer and centrifuged at 54,000*g* for 2 h. Light green fractions collected from the step gradient were diluted 5-fold with TE buffer and centrifuged at 82,000*g* for 1 h. Precipitated chloroplast envelope fractions were dissolved in TE buffer with 1 mM DTT, 0.1% (v/v) protease inhibitor cocktail stock solution, and 0.2% (v/v) TritonX-100. The supernatants collected after the rupturing of protoplasts were centrifuged at 26,000*g* for 30 min. At the end of the run, the crude PM pellets were suspended in HS buffer (pH 8.0) with 0.1% (v/v) protease inhibitor cocktail stock solution. PM fractions were purified by two-phase system (Larsson et al., 1994). The PM-enriched upper phase solution was diluted 3-fold with TE buffer with 1 mM DTT and 0.1% (v/v) protease inhibitor cocktail stock solution and then centrifuged at 82,000*g* for 1 h. Precipitated PM fractions were dissolved in TE buffer with 1 mM DTT, 0.1% (v/v) protease inhibitor cocktail stock solution, and 0.2% (v/v) TritonX-100.

Light Sources

Blue or red LEDs (ISL-150×150; CCS) were used in the analysis of phot2 autophosphorylation, phototropism, stomatal opening, and chloroplast movements.

Confocal Microscopic Analysis of Phot2-GFP Localization

Subcellular localization of the phot2-GFP fusion proteins was visualized by laser scanning confocal microscopy (SP8; Leica Microsystems). GFP fluorescence was measured by the time gating method (gating time = 0.5–12 ns) to remove chlorophyll autofluorescence according to a previous study (Kodama, 2016). A blue laser (laser power 15%) was used to induce the chloroplast avoidance response. Propidium iodide (PI) solution (Molecular Probes, Invitrogen) was introduced into plants by deaeration to visualize the cell walls. Emission spectra for chloroplast autofluorescence, GFP, and PI were measured at 488 nm. Excitation spectra for chloroplast autofluorescence, GFP, and PI were measured at 620 to 685 nm, 490 to 550 nm, and 585 to 605 nm, respectively.

Measurement of the Phototropic Responses in the Hypocotyls of Etiolated Seedlings

Seeds were sown on agar plates containing Murashige and Skoog (MS) medium (Sigma-Aldrich) solidified with 0.6% (w/v) agar. The seeds were stratified at 4°C for 4 d. The seedlings were grown vertically at 22°C for 3 d in the dark. The resulting etiolated seedlings were irradiated with unilateral BL of 10 μmol m^{-2} s^{-1} for 12 h. The irradiated seedlings were photographed and their phototropic curvatures were measured with ImageJ (National Institutes of Health; http://imagej.nih.gov/ij).

Plant Biomass and Leaf Flattening

Fresh weights were determined for the shoots of whole plants grown under continuous white light at 100 μmol m^{-2} s^{-1} for 3 weeks. For leaf flattening, cross-sections of the leaves on 3-week-old plants were photographed.

BL-Induced Stomatal Opening

BL-dependent stomatal apertures were measured according to an earlier study (Inoue et al., 2008a). Fully expanded rosette leaves were harvested from dark-adapted 4-week-old plants. Epidermal tissue fragments were isolated from the leaves and suspended in a basal reaction mixture consisting of 5 mM 2-morpholinoethanesulfonic acid-*bis-tris*-propane (pH 6.5; Wako Pure Chemical Industries), 50 mM KCl, and 0.1 mM $CaCl_2$ under dim RL. The epidermal fragments were either kept in the dark or irradiated with RL (50 μmol m^{-2} s^{-1}) with or without BL (10 μmol m^{-2} s^{-1}) for 3 h. Stomatal images were obtained with a microscope (TS100; Nikon). Stomatal apertures were measured with ImageJ.

Chloroplast Photorelocation Movement

To determine chloroplast intracellular localization, 3-week-old plants were irradiated with either weak (3 μmol m^{-2} s^{-1}) or strong (50 μmol m^{-2} s^{-1}) BL for 3 h to induce chloroplast accumulation and avoidance responses, respectively. The leaves were fixed with 2.5% (v/v) glutaraldehyde (Wako Pure Chemical Industries). Cross-sections were prepared with a vibrating microtome (VT1200 S; Leica Microsystems). Chloroplast distribution patterns were visualized with a laser scanning confocal microscope (FV10i; Olympus). Projection images were constructed using z-stacks (FV10i; Olympus).

Leaf transmittance was measured using a microplate reader Multiskan GO (Thermo Fisher) according to a previous report (Wada and Kong, 2011). The detached third leaves from 3-week-old plants were placed on the solidified 1% (w/v) gellan gum in the 96-well plastic plate and kept in the dark for >3 h. Then, samples were irradiated with BL at 1, 3, 5, 20, 50, 100, or 300 μmol m^{-2} s^{-1}.

Statistical Analysis

Data were processed in Excel v. 2011 (Microsoft Corporation) with the add-in Statcel v. 3 (Yanai 2011). Comparisons between group means were performed with Student's *t* test. Comparisons among three or more group means were made by one-way ANOVA followed by the Tukey-Kramer multiple comparisons post hoc test.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT3G52420 (OEP7), AT5G58140 (PHOT2), and AT3G45780 (PHOT1).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Detailed observation of the localization of phot2-GFP in leaf mesophyll cells.

Supplemental Figure S2. Localization of phot2-GFP on the chloroplast surface in mesophyll cells.

Supplemental Figure S3. Detailed observation of localization of phot2-GFP in leaf epidermal tissues.

Supplemental Figure S4. Localization of phot2-GFP in cotyledon palisade cells of etiolated seedlings.

Supplemental Figure S5. chloroplast avoidance response in wild-type, *phot1*, and *phot1phot2* plants.

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