Both Phototropin 1 and 2 Localize on the Chloroplast Outer Membrane with Distinct Localization Activity

Sam-Geun Kong1,4, Noriyuki Suetsugu1,4, Shingo Kikuchi2, Masato Nakai2, Akira Nagatani3 and Masamitsu Wada1,*

1Department of Biology, Graduate School of Science, Kyushu University, Fukuoka, 812-8581 Japan
2Institute for Protein Research, Osaka University, Osaka, 565-0871 Japan
3Department of Botany, Graduate School of Science, Kyoto University, Kyoto, 606-8502 Japan
4These authors contributed equally to this work.

*Corresponding author: E-mail, wadascb@kyushu-u.org; Fax, +81-92-642-7258.

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Chloroplasts change their position to adapt cellular activities to fluctuating environmental light conditions. Phototropins (phot1 and phot2 in Arabidopsis) are plant-specific blue light photoreceptors that perceive changes in light intensity and direction, and mediate actin-based chloroplast photorelocation movements. Both phot1 and phot2 regulate the chloroplast accumulation response, while phot2 is mostly responsible for the regulation of the avoidance response. Although it has been widely accepted that distinct intracellular localizations of phototropins are implicated in the specificity, the mechanism underlying the phot2-specific avoidance response has remained elusive. In this study, we examined the relationship of the phot2 localization pattern to the chloroplast photorelocation movement. First, the fusion of a nuclear localization signal with phot2, which effectively reduced the amount of phot2 in the cytoplasm, retained the activity for both the accumulation and avoidance responses, indicating that membrane-localized phot2 but not cytoplasmic phot2 is functional to mediate the responses. Importantly, some fractions of phot2, and of phot1 to a lesser extent, were localized on the chloroplast outer membrane. Moreover, the deletion of the C-terminal region of phot2, which was previously shown to be defective in blue light-induced Golgi localization and avoidance response, affected the localization pattern on the chloroplast outer membrane. Taken together, these results suggest that dynamic phot2 trafficking from the plasma membrane to the Golgi apparatus and the chloroplast outer membrane might be involved in the avoidance response.

Keywords: Arabidopsis thaliana • Blue light • Chloroplast • Chloroplast movement • Photoreceptor • Phototropin.

Abbreviations: cp-actin, chloroplast actin; GFP, green fluorescent protein; LOV, light, oxygen or voltage; mRFP, monomeric red fluorescent protein; MS, Murashige and Skoog; NES, nuclear export signal; NLS, nuclear localization signal; P2C, C-terminal 534–915 amino acid fragment of Arabidopsis phototropin 2; P2N, N-terminal 1–533 amino acid fragment of Arabidopsis phototropin 2; P2NG, N-terminal domain of phot2 fused to GFP; P2G, full-length phot2 fused to GFP; phot1, phototropin 1; phot2, phototropin 2; TAP, tandem affinity purification; TAPI, improved tandem affinity purification; YFP, yellow fluorescent protein.

Introduction

As sessile organisms, plants have evolved various physiological responses to utilize sunlight as both an energy source for photosynthesis and an environmental signal under fluctuating conditions. Light regulates almost all aspects of the plant growth and development from seed germination to flowering (Kami et al. 2010). Higher plants possess several families of photoreceptors that monitor the wavelength from UV-B to the near infrared (far-red), the intensity and the direction of incident light. Light signals are perceived by at least five distinct families of photoreceptors in Arabidopsis: UV RESISTANCE LOCUS8 (UVR8) perceives UV-B light (Rizzini et al. 2011), cryptochromes (cry1 and cry2), phototropins (phot1 and phot2) and members of the Zeitlupe family (ZTL, FKF1 and LKP2) absorb UV-A and blue light (Christie 2007, Demarsy and Fankhauser 2009, Yu et al. 2010), and phytochromes (phyA–phyE) primarily absorb red and far-red light (Chen and Chory 2011).

The intracellular localization of chloroplasts is crucial for plant growth and development. Chloroplasts relocate within a cell in response to light conditions, e.g. accumulation at the cell surface (periclinal wall), to optimize their photosynthetic ability under low light conditions (accumulation response) and movement away from strong light to the anticlinal sides to evade chloroplast damage (avoidance response) (Kasahara et al. 2002, Wada et al. 2003). Phototropins are plant-specific blue light photoreceptors that are found in various organisms...
from unicellular algae to higher plants (Suetsugu and Wada 2007) and mediate chloroplast photorelocation movement (Wada et al. 2003). Both phot1 and phot2 share well-conserved molecular structures that consist of two LOV (light, oxygen or voltage) domains (LOV1 and LOV2) in their N-termi

us and a Ser/Thr kinase domain in their C-terminus (Briggs et al. 2001). The two phototropins in Arabidopsis are functionally redundant and have different light sensitivities in chloroplast photorelocation movement, phototropism, stomatal opening, leaf flattening and palisade cell development (Hualá et al. 1997, Jarillo et al. 2001, Kagawa et al. 2001, Kinoshita et al. 2001, Sakai et al. 2001, Kozuka et al. 2011). In chloroplast photorelocation movement, both phot1 and phot2 redundantly mediate the accumulation response, but phot2 is mainly responsible for the strong light-induced avoidance response (Jarillo et al. 2001, Kagawa et al. 2001, Sakai et al. 2001). The photoreceptor phot1 is also involved in the avoidance response, but the response is not as significant compared with that of phot2 (Luesse et al. 2010).

Several components have been identified as essential for chloroplast photorelocation movements, which involve signal transducers from light perception through photoreceptors to actin-based movements (Kong and Wada 2011). Chloroplast movement is mediated through the rapid reorganization of chloroplast actin (cp-actin) filaments at the interface between the plasma membrane and chloroplast outer membrane (Kadota et al. 2009). The rapid disappearance of cp-actin filaments at the rear part followed by extensive actin polymerization at the front part is a prerequisite for chloroplast movement under strong blue light (Kadota et al. 2009). The disappearance of cp-actin filaments is restricted to the region of the chloroplast where continuous, strong blue light illuminates, which is specifically regulated through the activity of phot2 but not phot1 (Kadota et al. 2009, Ichikawa et al. 2011, Kong and Wada 2011). In addition, the rate of cp-actin disappearance depends on the intensity of blue light (Kadota et al. 2009). Because the avoidance response is also regulated in a phot2 dose- and blue light intensity-dependent manner (Kagawa and Wada 2004, Kimura and Kagawa 2009), the phototropin-mediated dynamics of cp-actin filaments are consistent with the physiological characteristics of chloroplast movement (Kong and Wada 2011).

Phototropins are primarily localized to the plasma membrane in darkness, and some fractions of them translocate within the cell in response to blue light (Sakamoto and Briggs 2002, Kong et al. 2006): phot1 is internalized into the cytoplasm in a blue light-dependent manner (Sakamoto and Briggs 2002, Knieb et al. 2004, Wan et al. 2008, Kaiserl et al. 2009), while phot2 exhibits a blue light-dependent association with the Golgi apparatus (Kong et al. 2006, Kong and Nagatani 2008). The kinase domain is responsible for plasma membrane and Golgi localizations regardless of blue light irradiation (Kong et al. 2006). Furthermore, the kinase domain in transgenic plants constitutively activates downstream signaling for the phototropin responses including phototropism, stomatal opening and the chloroplast avoidance response (Kong et al. 2007). Hence, the N-terminal photosensory domain functions as a light switch that regulates the kinase activity through reversible conformational changes between the dark and light states in which LOV2 functions as a dark-state repressor (Christie et al. 2002, Harper et al. 2003, Harper et al. 2004). However, the mechanisms underlying the phototropin-mediated regulation of downstream signal transduction remain largely unknown.

The results of many previous physiological analyses suggest that the light-perceptive site for the avoidance response is closely associated with chloroplasts (Kagawa and Wada 1996, Kagawa and Wada 2000, Tsuboi and Wada 2011). Consistently, the avoidance response occurs specifically where the chloroplasts are irradiated with strong blue light, while the accumulation response is mediated through long-distance signal transfer (Tsuboi and Wada 2010, Kong and Wada 2011, Tsuboi and Wada 2011). Hence, it is an intriguing possibility that photoreceptors might be localized to the chloroplasts, especially for the avoidance response.

In this study, we thoroughly examined the relationship between phot2 localization patterns and chloroplast photorelocation movement. We clearly showed that membrane-localized phot2 but not cytoplasmic phot2 was essential for chloroplast photorelocation movement. Importantly, we also found that both phot1 and phot2 were localized on the chloroplast outer membrane, but the phot2 content was more abundant than that of phot1, with strong correlation with the expression levels in leaf cells. The different amounts of phototropins in the chloroplasts might reflect the functional activity of the two photoreceptors to mediate the high light-induced avoidance response.

**Results**

**The cytosolic localization of phot2 is not essential for chloroplast movements**

phot2 shows multiple localization patterns such as the main plasma membrane localization, the light-induced Golgi association and the cytoplasmic distribution (Kong et al. 2006, Kong et al. 2007, Kong et al. 2013). To characterize the functional role of the cytosolic fraction of phot2, we produced transgenic plants expressing PHOT2-green fluorescent protein (GFP) (P2G) and P2G fused with the nuclear localization signal (NLS) (P2G–NLS) or nuclear export signal (NES) (P2G–NES) in the phot1phot2 double mutant background under the authentic PHOT2 promoter (P2-P2G, P2-P2G–NLS and P2-P2G–NES lines). We obtained several independent lines of P2-P2G, P2-P2G–NLS and P2-P2G–NES in the T3 generation.

Immunoblot analysis confirmed that these transgenic plants expressed P2G, P2G–NLS and P2G–NES at the expected sizes (around 130 kDa) (**Supplementary Fig. S1A, B**). P2G expression was in a range of no expression (19-3), low expression (5-3, 11-2), moderate expression (10-1, 21-2) and high expression...
Fig. 1 Functional analysis of P2-P2G–NLS and P2-P2G–NES transgenic plants. (A) Immunoblot analysis of protein fractions for phot2 and P2G. Total protein extracts (T) were prepared from the rosette leaves of 3-week-old wild-type (WT), phot1 mutant and P2-P2G transgenic plants. The T fractions were further fractionated into soluble (S) and pelleted (P) fractions by high-speed ultracentrifugation (100,000×g) for 30 min at 4°C. Protein samples corresponding to 50 μg of T fractions were subjected to 7.5% SDS–PAGE and immunoblotted with an anti-phot2 polyclonal antibody (Kong et al. 2006). Molecular weight markers (MW) in kDa are shown in the left lane. (B) Immunoblot analysis of protein fractions for P2G–NLS and P2G–NES. The other details are the same as in (A). (C) Intracellular localizations of P2G and P2G–NLS proteins in com-}

transmittance in rosette leaves was traced separately under various blue light intensities (peak wavelength at 470 nm) of 3.2 μmol m–2 s–1 (BL3.2) for 100 min, 25 μmol m–2 s–1 (BL25) for 40 min and 60 μmol m–2 s–1 (BL60) for 40 min, respectively. The transmittance differences between the starting point and after light treatments with the indicated intensities of blue light are shown. The data are given as the means ± SD obtained from 8–10 independent leaves.

Careful protein fractionation analysis of P2G in P2-P2G transgenic plants indicated that certain amounts of P2G were detected in the soluble fraction compared with endogenous phot2 in the wild type and phot1 mutant, although the majority of P2G was recovered in the pelleted fraction, with increased amounts to some extent (Fig. 1A). As P2G expression in the P2-P2G 4-1 line was slightly higher than that in P2-P2G 21-2, the ratios of P2G in the soluble fraction proportionally increased dependent on the expression levels of P2G. Similarly, both P2G–NLS and P2G–NES were also detected mainly in the pelleted fraction rather than the soluble fraction (Fig. 1B), suggesting that the fusion of the NLS and NES did not affect the membrane localization activity of phot2. However, the soluble fraction of P2G–NLS could be contributed by the nuclear-localized rather than the cytoplasmic-localized P2G–NLS (see below).

We next verified the intracellular localizations of P2G, P2G–NLS and P2G–NES proteins in the mesophyll and epidermal cells of rosette leaves. The GFP fluorescence of P2G was strongly observed in the cell periphery and faintly in the cytoplasm (Fig. 1C, i–iii). The GFP fluorescence of P2G–NLS was observed not only in the plasma membrane but also in the nucleus, but no cytosolic signal was observed (Fig. 1C, iv–vi). On the other hand, P2G–NES exhibited localization patterns essentially identical to those of P2G (Fig. 1C, vii–ix). We further verified the intracellular localizations of P2G and P2G–NES proteins in comparison with marker proteins using transient expression in onion epidermal cells. P2G was strongly observed in the cell periphery and the nucleus, and also faintly in the cytoplasm (Supplementary Fig. S2A). The cytoplasmic and nuclear localization of P2G was consistent with the distribution of monomeric red fluorescent protein (mRFP), a cytosolic and nuclear localization marker (Supplementary Fig. S2B). It is noted here that the nuclear localization of P2G might be artifactual by overexpression in onion epidermal cells, because no such distribution was observed in the P2-P2G and P2-P2G–NES
transgenic Arabidopsis cells. In contrast, strong P2G–NLS expression was observed in the cell periphery and nucleus, but not in the cytoplasm (Supplementary Fig. S2A). In addition, P2G–NLS was co-localized with mRFP in the nucleus but not in the cytosol (Supplementary Fig. S2B). Both fusion proteins in the periphery were well co-localized with the distribution of the plasma membrane marker H⁺-ATPase–mRFP (Supplementary Fig. S2C). Collectively, these results indicated that the addition of NLS to P2G effectively reduced the cytoplasmic fraction of phot2.

The physiological activities of P2G–NLS and P2G–NES on chloroplast movements were assessed by monitoring changes in the red light transmittance in rosette leaves in response to low and high blue-light treatments (Wada and Kong, 2011). P2G–NES fully complemented the low-light-induced as well as the high-light-induced chloroplast movements; the higher expressor (P2-P2G–NES 12-1) exhibited a more rapid avoidance response than the moderate expressor (P2-P2G–NES 24-2) as observed in the P2-P2G lines 4-1 and 21-2 (Fig. 1D), indicating that P2G–NES is fully functional not only in the accumulation response but also in the avoidance response. Interestingly, P2G–NLS was fully functional in the accumulation response, but its activity for the avoidance response was slightly attenuated compared with those of P2G–NES and P2G (Fig. 1D). Collectively, these data indicate that cytoplasmic phot2 is not essential for chloroplast photorelocation movement, although we could not rule out the possibility that the reduced expression and membrane-localized extent of P2G–NLS also affect the activity for the avoidance response.

The C-terminal domain of phot2 is responsible for the chloroplast avoidance response

In the accompanying paper (Kong et al. 2013), we demonstrated that the C-terminal deletion mutants P2(Δ42)G and P2(Δ65)G, but not P2(Δ25)G, showed some attenuation on targeting to the plasma membrane and the Golgi apparatus. To assess further the relationship between the localization patterns and functionality of phot2 in chloroplast photorelocation movement, the C-terminal deletion mutants P2(Δ25)G, P2(Δ42)G and P2(Δ65)G were expressed in phot2 single and phot1phot2 double mutants under the control of the authentic phot2 promoter. Unfortunately, all P2-P2(Δ42)G and P2-P2(Δ65)G transgenic lines showed reduced expression levels compared with the amount of the endogenous phot2 in the wild-type plants, as described by Kong et al. (2013). Hence, we used P2-P2G lines with various expression levels as controls (Supplementary Fig. S1A). Although the P2-P2(Δ42)G and P2-P2(Δ65)G transgenic plants exhibited a slightly lower expression of the respective fusion proteins compared with that of endogenous phot2 in the wild-type plants, the expression was comparable with that of P2G in P2-P2G 5-3 (Supplementary Fig. S1B).

Because the 35-P2(Δ42)G transgenic plants showed an accumulation response rather than an avoidance response, even under strong light (Kong et al. 2013), we investigated whether the amount of phot2 influenced the threshold between the accumulation and avoidance responses. Chloroplast movements in P2-P2G lines were first detected from the changes of red light transmittance through the leaves under various light intensities. Both the accumulation and avoidance responses were normally observed, even in the low P2G expression line 5-3 (Fig. 2A; Supplementary Fig. S3B). In contrast, no response was observed in the no P2G expression line 19-3. These results suggest that some amount of P2G is necessary and the low P2G amount in P2-P2G 5-3 has enough activity to induce both accumulation and avoidance responses.

As described by Kong et al. (2013), the P2-P2(Δ25)G transgenic plants were fully functional in both the accumulation and avoidance responses, and the P2-P2(Δ65)G transgenic plants were not functional (Fig. 2B; Supplementary Fig. S3C, E). In contrast, the P2-P2(Δ42)G transgenic plants were only partially functional during the accumulation response; there was almost no observed accumulation response under a low intensity blue light (Fig. 2B, BL3.2; Supplementary Fig. S3D), but reduced accumulation responses were detected under high intensity blue light (Fig. 2B, BL25 and BL60; Supplementary Fig. S3E).
Fig. S3D), indicating that the C-terminal region of phot2 is involved in the regulation of phot2 activity. It is noted that P2(A42)G was only functional for the accumulation response under high intensity blue light while the expression of P2(A42)G is comparable with that of P2G in the low P2G expression line 5-3 (Supplementary Fig. S1A, D).

With a different purpose, we transformed PHOT2-TAP and PHOT2-TAPI fusion genes into the phot1phot2 double mutants under the control of the authentic PHOT2 promoter, and the resulting transgenic plants were named P2-P2TAP and P2-P2TAPI, respectively. The tandem affinity purification (TAP) method is widely used for the high-level purification of protein complexes containing a TAP-tagged protein (Rigaut et al. 1999, Puig et al. 2001). Although this TAP tag method has proven its usefulness for the identification of proteins in heterocomplexes in yeast, mammalian and insect cells using mass spectrometry, its application in plants has been limited to the purification of fully functional protein complexes for the mass spectrometric analysis of proteins because of its NLS in the calmodulin-binding protein (CBP) domain. A new improved TAP (TAPI) tag has improved the usefulness of the TAP tag through the elimination of NLS signal activity (Rohila et al. 2004). We found that phot2–TAP fusion proteins partially complemented the high-light avoidance response in phot1phot2 double mutants (see below). Hence, we asked whether the defective phenotype in these lines could be related to the membrane localization activity.

The expressions of P2TAP and P2TAPI were similar to or less than that of endogenous phot2 (Supplementary Fig. S1E). Chloroplast movements were measured using red light transmittance on leaves to determine the physiological activities of the phot2 fusion proteins. Interestingly, the P2-P2TAP 34-3 transgenic plant leaves with a low expression (Supplementary Fig. S1E) exhibited an accumulation response similar to that of the phot2 mutants (Wada and Kong 2001) not only under a low intensity (BL3.2) but also under high intensity blue light (BL25 and BL60) (Fig. 3A). However, the P2-P2TAP 9-4 transgenic plant leaves with moderate expression (Supplementary Fig. S1E) exhibited an accumulation response under low and middle intensity blue light (BL3.2 and BL25) and an avoidance response under high intensity blue light (BL60) (Fig. 3A). Similarly, the P2-P2TAPI transgenic plants were functional in both chloroplast movements, but the higher intensity of blue light was necessary for the avoidance response as in P2-P2TAP 9-4 (Fig. 3A). In addition, all of these lines exhibited a normal phototropism response similar to the phot1 mutant plants (data not shown). Interestingly, the protein fractionation analysis showed that P2TAP was more sensitive to chaotropic salt (300 mM NaCl) treatment compared with endogenous phot2 in the wild-type plant (Fig. 3B). These data suggest that the membrane association activity of P2TAP is reduced compared with that of endogenous phot2. Collectively, these results showed a positive relationship between membrane association and physiological activity of phot2.

Both phot1 and phot2 associate with the chloroplast outer membrane but in different amounts

To address further the localization dynamics of phototropins during chloroplast movement, we examined the intracellular localization of both phot1 and phot2 in the palisade cells of P1-P1G and P2-P2G transgenic plants, respectively (Sakamoto and Briggs 2002, Kong et al. 2006). As shown in Fig. 4 (see also Supplementary Movies S1, S2), the avoidance response was effectively induced by microbeam irradiation using 458 nm laser beam scans in the intervals between image acquisitions using 488 nm laser scans for GFP fusion proteins. The chloroplasts moved away from the area where the strong blue light was applied. Interestingly, clear GFP fluorescence of P1G and P2G was observed not only in the plasma membrane but also in the chloroplast envelope during the chloroplast movement (Fig. 4, chloroplasts 1, 2, 3).

To compare directly the localization patterns and the endogenous levels of phot1 and phot2 in leaf cells, we produced...
transgenic plants expressing phot1–yellow fluorescent protein (YFP; P1Y) and phot2–YFP (P2Y) fusion proteins in the phot1 and phot2 single mutant backgrounds under the control of the authentic PHOT1 and PHOT2 promoters (P1-P1Y/p1, P2-P2Y/p2 lines), respectively. The immunoblot analysis confirmed that the expression of P1Y and P2Y fusion proteins was closely comparable with that of endogenous phot1 and phot2 in the wild-type cells (Fig. 5A, B). Consequently, the serial dilution analysis with P2Y showed that the expression level of P2Y was higher than that of P1Y, by around 2- to 4-fold (Fig. 5C). The extents of P1Y and P2Y expression levels in the leaf cells were consistently confirmed in the plasma membrane using confocal microscopic analysis (Fig. 5D). Similarly, the chloroplast association of P2Y was more easily detected than that of P1Y in the intact mesophyll cells (Fig. 5D).

To confirm phototropin localization on the chloroplast, immunoblot analyses of protein extracts from the chloroplast fraction were performed. Intact chloroplasts were isolated from 3-week-old rosette leaves of wild-type and phot1phot2 double mutant plants. Crude protein extracts from the same leaves were used as a control. Both phot1 and phot2 were detected in the isolated chloroplast fractions (Fig. 6A). In several independent experiments, the phot2 band was consistently detected in the chloroplast fraction, but the phot1 band in the chloroplast fraction was consistently weak or occasionally could not be detected. Collectively, the phot1 band in the chloroplast fraction was much weaker than that in the total protein fraction; however, the phot2 band in the chloroplast fraction showed a similar intensity as that in the total protein fractions (Fig. 6A).

To verify the fractionation, we also identified some representative marker proteins in the same fractions. The well-known chloroplast outer membrane protein Toc75 (Kikuchi et al. 2006) was enriched in the isolated chloroplast fractions from both the wild-type and phot1phot2 mutant plants; however, this marker was undetectable in our total protein fractions (Fig. 6A). Moreover, the plasma membrane marker H+–ATPase (Kinoshita et al. 2001) was detected in the total protein fractions but not in the isolated chloroplast fractions from the wild-type and phot1phot2 double mutant plants (Fig. 6A), indicating that the plasma membrane contamination in this isolated chloroplast fraction is not significant. Phot2 was not detected when the isolated chloroplasts were treated with the protease thermolysin and was recovered from both pelleted (P) and soluble (S) fractions after treatment with a hypotonic agent (Fig. 6B), indicating that phot2 was peripherally associated with the chloroplast outer membrane. In addition, phot2 was released from the isolated chloroplasts into the soluble fraction by alkaline treatment with an alkaline buffer (100 mM Na2CO3, pH 11.5), indicating that phot2 is associated with the chloroplast outer membrane via ionic interaction. Collectively, these results indicate that a part of phototropins is associated with the chloroplast outer membrane.

We further evaluated the amounts of phot1 and phot2 using the isolated chloroplasts of the P1-P1Y/p1 and P2-P2Y/p2 transgenic plants. As expected, the YFP fluorescence of P1Y and P2Y was clearly observed in the isolated chloroplast envelope (Fig. 6C). The immunoblot analysis further demonstrated that the amount of P2Y was larger than that of P1Y, but the amounts of CHUP1 detected in these lines were similar (Fig. 6D), indicating that the portion of phot2 is larger than that of phot1 in the chloroplast fraction. Because phot2 activity on the avoidance response is dose dependent (Kagawa and Wada 2004, Kimura and Kagawa 2009), we further asked...
whether the amount of chloroplast-associated phot2 would be correlated to chloroplast movement. To answer this, we tried to compare P2G amounts in P2-P2G 4-1 and 21-2 lines that exhibited a correlation between P2G expression and physiological activity on the avoidance response (Figs. 1A, D, 2A).

Immunoblot analysis consistently showed that P2G in the P2-P2G 4-1 line was more than that in the P2-P2G 21-2 line not only in the total and pelleted fractions but also in the chloroplast fraction (Figs. 1A, 6E). In contrast, CHUP1 proteins were detected at similar levels in both the total protein and chloroplast fractions between these two lines (Fig. 6E). These results suggested a correlation between the amount of phot2 associated with chloroplasts and the magnitude of the avoidance response.

The C-terminal region of phot2 is responsible for the correct association with the chloroplast outer membrane

The C-terminal domain of phot2 is responsible for phot2 localization to the plasma membrane, and the blue light-dependent association of phot2 with the Golgi apparatus (Kong et al. 2013). Moreover, the chloroplast avoidance response was defective in our transgenic plants expressing the C-terminal deletion of phot2 (Fig. 2B). Hence, it is plausible that the C-terminal region of phot2 is involved in the chloroplast localization. To assess whether the differences in the responsiveness of our transgenic plants in terms of the avoidance response could be due to their varying affinities for the chloroplasts, we examined the amounts of phot2 in isolated chloroplasts and total crude protein extracts. Although the expression levels of P2G, P2(Δ42)G, P2TAP and P2G–NLS proteins were different, some amounts of these proteins were detected in all chloroplast fractions, and the ratios of the amounts in the chloroplast fractions to those in the total protein fractions were not significantly changed (Fig. 7A).

Next, we employed transient expression in mesophyll protoplasts to clarify further the association of phot2 with the chloroplast outer membrane. The uniform fluorescence of GFP-tagged phot2 (GP2) was observed in the chloroplast outer membrane of the burst protoplasts (Fig. 7B).
Interestingly, the uniform localization of GP2 in the chloroplast outer membrane was disrupted in the C-terminal deletion mutants GP2(C142) and GP2(C165), i.e. GP2(C142) was in aggregates and localized near the chloroplast outer membrane but GP2(C165) accumulated mainly in the cytoplasm (Fig. 7B).

Furthermore, P2C84G (GFP fused to the C-terminal 84 amino acid fragment of phot2) was observed in the cell periphery and near the chloroplast outer membrane of mesophyll cells (Fig. 7C), although there was no significant phenotypic effect on phot2 function in the 35-P2C84G line (Kong et al. 2013). These results suggest that the C-terminal region of phot2 is responsible for the localization pattern of phot2 on the chloroplast outer membrane.

Discussion

The physiological significance of the association of phototropin with chloroplasts

Phototropins exhibit different photosensitivities in the modulation of a range of physiological responses. The responses can be classified into two categories, long-term and comprehensive intercellular responses, such as phototropism, leaf flattening, and cellular and seedling development, and short-term responses, such as stomatal opening and chloroplast movement to adapt to rapid environmental changes (Briggs and Christie 2002, Wada et al. 2003, Christie 2007, Shimazaki et al. 2007, Kong and Wada 2011). The mechanisms underlying the downstream signaling in these responses involve various and distinct downstream components and their considerable specific interactions with phototropins (Inoue et al. 2010, Kong and Wada 2011). The different intracellular localization of phototropins and/or their interactions with cellular components would be important for the precise regulation of these responses under fluctuating environmental light conditions.

The results of many of the previous physiological analyses using microbeam irradiation suggest that the light-perceptive site for the avoidance response is associated closely with chloroplasts (Kagawa and Wada 1996, Kagawa and Wada 2000, Tsuboi and Wada 2011). Chloroplasts translocate in any direction to avoid strong light or to accumulate towards weak light without rotating and rolling with a short time lag (1–2 min) (Tsuboi et al. 2009, Tsuboi and Wada 2011). Consistently, the avoidance response occurs specifically where the chloroplasts are irradiated with strong blue light, while the accumulation response is mediated through long-distance signal transfer (Kagawa and Wada 2000, Tsuboi and Wada 2010, Kong and Wada 2011, Tsuboi and Wada 2011). Indeed, biochemical analysis of isolated chloroplasts and the confocal microscopic...
analysis suggested that both phot1 and phot2 are associated with the chloroplasts, while phot2 is detected more abundantly than phot1 in the chloroplast fraction (Figs. 5D, 6A, C, D) and on moving chloroplasts (Fig. 4). The phot2 localization on the chloroplasts was also revealed through the database analysis of plastid proteomics (AT_CHLORO Database, http://www.grenoble.prabi.fr/at_chloro/). These results consistently suggest that phot2 is more abundantly associated with the chloroplast outer membrane than phot1. In addition, the results of the biochemical analyses using chaotropic agents support the association of phot2 with the chloroplast outer membrane via ionic rather than covalent interactions, as previously described (Knieb et al. 2004, Kong et al. 2013).

Distinct chloroplast outer membrane localizations of phototropins imply their functional specificity in chloroplast photorelocation movements. The physiological aspects of the avoidance response have strongly correlated with the localizations of photoreceptors on the plasma membrane and chloroplasts. Moreover, the avoidance response is primarily mediated through phot2 (Kagawa et al. 2001, Sakai et al. 2001). Although phot1 is capable of generating both a low and a high light response signal for the accumulation response, phot1 is also involved in the regulation of a strong light-induced avoidance response, which is specifically detectable in phot2 mutants as a transient avoidance response under high light conditions (Luesse et al. 2010). Hence, the different amounts of phot1 and phot2 on chloroplasts are consistent with the relevant physiological roles of phot2 in the avoidance response compared with that of phot1.

P2G–NLS was functional in chloroplast photorelocation movement, but the activity for the avoidance response was slightly reduced compared with the P2G and P2G–NES proteins (Fig. 1D). Because the rate of response is regulated in phot2 in a dose- and light intensity-dependent manner (Kagawa and Wada 2004, Kimura and Kagawa 2009), the reduced P2G–NLS activity for the avoidance response would result from its reduced residence at the functional sites, as observed with P2G (Fig. 2A; Supplementary Figs. S1A, S3B). Indeed, the resident signals of P2G–NLS at the plasma membrane and chloroplasts were reduced (Figs. 1B, C, 7A). In contrast, the expression of P2TAP and P2TAPi proteins was comparable with that of endogenous phot2 in the wild-type plants (Fig. 3B; Supplementary Fig. S1E), whereas the reactivity of these proteins was almost normal for the accumulation response but defective for the high-light-induced avoidance response (Fig. 3A). This defectiveness was more significant in the P2TAP lines than in the P2TAPi lines, in which the NLS activity in P2TAP could reduce the resident amount of P2TAP compared with that of P2TAPi in the cytosol and the plasma membrane, as observed in P2G–NLS expression. Hence, the reduced activities of P2TAP and P2TAPi proteins, especially for the chloroplast avoidance response, might result from the fusion of the TAP tag that affected the membrane associations of these proteins (Fig. 3B), although the ratio of chloroplast localization to the total protein was not changed compared with other transgenic lines (Fig. 7A). Taken together, these results suggest that cytosolic phot2 is dispensable for chloroplast photorelocation movements and that the amount on
the membrane including the chloroplast outer membrane is important to retain phot2 activity.

**Molecular structure and domain responsible for chloroplast and Golgi membrane localization**

The LOV domains modulate the light quality and sensitivity to function in downstream signaling, in which the LOV2 domain serves as a light switch in the activation of the kinase domain (Christie et al. 2002, Cho et al. 2007, Aihara et al. 2008, Kaiserli et al. 2009). Blue light-induced autophosphorylation of phototropins and kinase activation are necessary to stimulate phot1 internalization and phot2 association with the Golgi apparatus (Sakamoto and Briggs 2002, Kong et al. 2006, Kong and Nagatani 2008, Wan et al. 2008, Kaiserli et al. 2009). To date, it is not known whether light-induced phototropin trafficking represents a mode of signaling or is associated with other processes, such as phototropin desensitization and adaptation. However, the internalization of phot1 into the cytosol is induced through the light illumination of dark-grown seedlings, resulting in the down-regulation of phot1 (Sakamoto and Briggs 2002). The results obtained in the present study implied that cytosolic phot2 is not essential for chloroplast movement (Fig. 1C, D; Supplementary Fig. S5). However, the internalization of phot1 into the cytosol could contribute to a transient avoidance response under high light conditions (Kuehle et al. 2010) through increasing its association with the chloroplast outer membrane, but consequently leads to the down-regulation of activity for the strong light-induced avoidance response.

The kinase domains of phot1 and phot2 are responsible for their localization to the plasma membrane (Kong et al. 2006, Kong et al. 2013). Furthermore, the phot2 C-terminal fragment is necessary for the proper localization of phot2 to the Golgi apparatus and the chloroplast outer membrane, and the chloroplast avoidance response (Figs. 2B, 7B; Supplementary Fig. S3D, E; Kong et al. 2013). These data suggest that the chloroplast avoidance response is strongly correlated with phototropin localization to the plasma membrane, the Golgi apparatus and the chloroplast outer envelope. Collectively, phot2 localization to the chloroplasts might be regulated by light-driven phototropin activation and trafficking. Light-dependent changes in the intracellular localization of phototropins should contribute to spatial and temporal activation of diverse downstream signals (see below).

**Molecular mechanism and regulation of actin-based chloroplast photorelocation movements**

The signaling mechanism underlying chloroplast photorelocation movement comprises several components involved in the light perception of phototropins and cp-actin-based movement (Kong and Wada 2011). The protein components CHUP1, KACs, WEB1/PMI2 and THRUMIN1 are involved in chloroplast photorelocation movements via the regulation of actin-based movements (Kadota et al. 2009, Kodama et al. 2010, Suetsugu et al. 2010, Whippo et al. 2011). Although these proteins differ in intracellular localizations, they function in the vicinity of the plasma membrane and/or the chloroplast outer membrane, i.e. the rapid reorganization of cp-actin filaments at the chloroplast periphery facing the plasma membrane (Kadota et al. 2009), the localization of CHUP1 to the chloroplast outer membrane via its hydrophobic region (Oikawa et al. 2003, Oikawa et al. 2008) and the localization of WEB1 and THRUMIN1 to the plasma membrane (Kodama et al. 2010, Whippo et al. 2011). Chloroplast-localized CHUP1 mediates chloroplast anchoring to the plasma membrane via the interaction of its coiled-coil region with an unknown component(s) localized on the plasma membrane (Oikawa et al. 2008). Hence, CHUP1-mediated chloroplast anchoring should be terminated prior to the initiation of chloroplast movement associated with the reorganization of cp-actin filaments. It is intriguing how and where phototropins regulate the status of these components.

The autophosphorylation of phototropins and subsequent kinase activation are necessary to activate downstream signaling cascades of chloroplast photorelocation movement (Kong et al. 2007, Inoue et al. 2008, Inoue et al. 2011). Moreover, the proteomic analysis of the membrane proteins suggests that phosphoproteins are the major components involved in chloroplast photorelocation movement (Kong and Wada 2011). Phototropins are localized near the phosphoproteins; thus, it is intriguing that chloroplast-localized phototropins could be involved in the regulation of the components through direct phosphorylation. Solid evidence in support of this hypothesis will be obtained in the future.

**Materials and Methods**

**Plant materials and growth conditions**

The gl-1 mutant (ecotype Columbia) is the parental line of the phot mutant lines and was used as the wild-type control. The phot mutant lines phot1-5 (Huala et al. 1997), phot2-1 (Kagawa et al. 2001) and phot1-Sphot2-1 (Kinoshita et al. 2001), and T3 homogeneous transgenic plants (see below) were used. The plants were grown on 0.8% (w/v) agar plates containing half-strength Murashige and Skoog (MS) medium supplemented with 2% (w/v) sucrose. The plates were incubated in the dark at 4°C for 72 h and subsequently transferred to a growth chamber under 16 h/8 h (day/night) periods at 23°C.

**Plasmid construction and plant transformation**

The plasmids were constructed by replacing the GFP gene of P2-P2G/pPZP211 (Kong et al. 2006) with either GFP–NLS, GFP–NES (Matsushita et al. 2003), YFP, TAP or TAPI (Rohila et al. 2004) using the Sall and Psst restriction enzyme sites, and the resulting binary vectors were named P2-P2G–NLS/pPZP211.
Intact chloroplast isolation

Intact chloroplasts were isolated as previously described (Asakura et al. 2004) with minor modifications. Briefly, the aerial parts of 3-week-old Arabidopsis seedlings were grown on MS medium and homogenized in a blending buffer [50 mM HEPES-KOH (pH 7.8), 330 mM sorbitol, 2 mM EDTA, 1 mM MnCl₂, 1 mM MgCl₂, 50 mM sodium ascorbate, 5 mM dithiothreitol and a protease inhibitor cocktail (Sigma)]. The homogenate was centrifuged in a swing-bucket rotor at 700 g for 15 min at 4 °C and then fractionated into P and S fractions by centrifugation at 100,000 g for 1 h at 4 °C. The pellet was resuspended in blending buffer and overlaid onto a 30% Percoll solution [30% (w/v) Percoll, 50 mM HEPES-KOH (pH 7.8), 330 mM sorbitol and a protease inhibitor cocktail (Sigma)] and centrifuged in a swing-bucket rotor at 700 × g for 15 min at 4 °C. The pellet containing the intact chloroplasts was washed [50 mM HEPES-KOH (pH 7.8), 330 mM sorbitol and a protease inhibitor cocktail (Sigma)] and resuspended in blending buffer. To digest the outer envelope proteins, the isolated chloroplasts were treated with 100 μg ml⁻¹ thermoslysin for 20 min on ice. In addition, the isolated chloroplasts were treated with a hypotonic buffer (10 mM HEPES-KOH, pH 7.8) or an alkaline buffer (100 mM Na₂CO₃, pH 11.5) for 20 min on ice and then fractionated into P and S fractions by centrifugation at 1,500 × g for 1 min at 4 °C.

Protein fractionation and immunoblot analysis

Total protein samples were prepared from 3-week-old rosette leaves as previously described (Kong et al. 2006). For protein fractionation, the total protein fraction (T) was further fractionated into soluble (S) and pelleted (P) fractions using ultracentrifugation at 100,000 × g for 1 h at 4 °C as described previously (Kong et al. 2006). Approximately 50 μg of total protein and 15 μl of the chloroplast samples at 1 μg μl⁻¹ Chl were separated using SDS–PAGE and then transferred onto nitrocellulose membranes (Hybond C; Amersham). The blots were probed with anti-phot1 (Suetsugu et al. 2010), phot2 (Kong et al. 2006) and Vicia H⁺-ATPase (Kinoshita et al. 2001) polyclonal antibodies, respectively.

Chloroplast movement

Changes in red light transmittance were measured to determine chloroplast photorelocation movement essentially as previously described (Wada and Kong 2011). For microbeam irradiation, confocal microscopy (SPS, Leica Microsystems) was used to capture time-lapse images. The rosette leaves of 4-week-old plants were detached and air spaces in the leaves were eliminated by gentle evacuation using a syringe that was filled with an evacuation solution (0.01% Silwet L-77). The leaves were placed into a cuvette that was composed of two round coverslips and a ring-shaped silicon–rubber spacer. The time-lapse images (at a 1.2 μm depth with three images) were taken at the indicated time intervals using 488 nm laser scanning. During the intervals, the circular area was irradiated with a strong blue light (458 nm laser scanning) to induce the chloroplast avoidance response (for details see Fig. 2). The maximized images were traced using ImageJ software (http://rsbweb.nih.gov/ij/).

Transient expression and observation

Arabidopsis mesophyll protoplasts were prepared from the rosette leaves of 4-week-old phot1phot2 double mutants essentially as previously described (Kong et al. 2006). A total of 10 μg of each plasmid construct for GP2, GP2(Δ42) and GP2(Δ65) (Kong et al. 2013) was transiently expressed in the protoplasts using polyethylene glycol (PEG)-mediated transformation. After incubation at 23 °C for 16 h in the dark, the protoplasts were burst mechanically by pipetting and viewed under a confocal microscope (SPS, Leica Microsystems). The fluorescence was observed at 500–550 nm for GFP or 630–750 nm for Chl autofluorescence.

Supplementary data

Supplementary data are available at PCP online.

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