Journal of Experimental Botany, Vol. 61, No. 15, pp. 4387–4397, 2010
doi:10.1093/jxb/erq242 Advance Access publication 6 August, 2010
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Integration of phot1, phot2, and PhyB signalling in light-induced chloroplast movements

Darron R. Luesse[1,*], Stacy L. DeBlasio[2] and Roger P. Hangarter[2]

[1] Department of Biological Sciences, Science Laboratory Building 3326, Southern Illinois University, Edwardsville, IL 62026, USA
[2] Department of Biology, Indiana University, Bloomington, IN 47405, USA

* To whom correspondence should be addressed. E-mail: dluesse@siue.edu

Received 3 June 2010; Revised 14 July 2010; Accepted 15 July 2010

Abstract

In *Arabidopsis thaliana*, chloroplasts move towards the periclinal cell walls upon exposure to low blue light intensities and to anticlinal walls under high light. The regulation of these chloroplast movements involves members of both the phototropin and phytochrome families of photoreceptors. Examination of fluence-rate response dependencies in *phot1* and *phot2* mutants revealed that although both photoreceptors are capable of inducing chloroplast accumulation under low-light conditions, the signals from these photoreceptors appear to be antagonistic. Chloroplast movements in wild-type plants were intermediate between those of the single *phot* mutants, consistent with each operating through separate signalling cascades. Mutants in *phot2* showed transient chloroplast avoidance responses upon exposure to intense blue light, and slow but sustained chloroplast avoidance under intense white light, indicating that in the absence of phot2, phot1 is capable of generating both a low and a high-light response signal. Mutations in phytochrome B (*phyB*) caused an enhanced avoidance response at intermediate and high light intensities. Examination of *phyB, phot1phyB*, and *phot2phyB* mutants indicated that this enhancement is caused by PhyB inhibition of the high-light avoidance response in wild-type plants. In addition, our results suggest that the inhibition by PhyB is not exclusive to either of the phot1 or phot2 signalling pathways.

Key words: *Arabidopsis thaliana*, chloroplast movement, phototropin, phytochrome.

Introduction

As their sole source of energy, sunlight is one of the most important resources for plants, and they have evolved many responses to facilitate its capture (Franklin *et al.*, 2005). Low-light conditions can limit plant growth due to an inadequate energy supply, while too much light can damage plants through the production of reactive oxygen species, and lead to the breakdown of the photosynthetic apparatus (Melis, 1999). Plants have evolved many developmental and physiological strategies to regulate light absorption under these less than ideal conditions, such as phototropism, shade avoidance, variations in size of light-harvesting complexes, changes in cell density, and leaf morphology, but most occur slowly and are not easily reversed (Boardman, 1977; Galen *et al.*, 2004; Vandenbussche *et al.*, 2005).

Plants have also evolved mechanisms to achieve more rapid and reversible responses to changing light intensities. Light-induced chloroplast rearrangements are one example of a rapid response that contributes to a plant's abilty to regulate the amount of light captured (Zurzycki, 1961; Wagner *et al.*, 1972; Inoue and Shibata, 1974; Lechowski, 1974; Walczak and Gabrýs, 1980; Augustynowicz and Gabrýs, 1999). Specifically, under low-intensity light, chloroplasts accumulate along periclinal cell walls, where they cover more of the surface area that is being illuminated. This is referred to as the accumulation, or low-light response. When exposed to high-intensity light, the chloroplasts relocate along anticlinal cell walls, reducing their exposure and allowing more light to pass through the cells. This is referred to as the avoidance, or high-light response.

Abbreviations: Col, Arabidopsis thaliana Columbia ecotype; *Ler*, *Arabidopsis thaliana* Landsberg *erecta* ecotype; Phot, phototropin; Phy, phytochrome.

© 2010 The Author(s).

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

At intermediate light intensities between those that cause robust accumulation and avoidance responses, the chloroplasts display biphasic movement, which involves a brief period of avoidance followed by an extended accumulation phase (Kagawa and Wada, 1999; DeBlasio *et al.*, 2003). These light-induced chloroplast movements are thought to represent an adaptive advantage for plants exposed to varying light conditions (Zurzycki, 1955; Augustynowicz and Gabrýs, 1999). Consistent with this hypothesis, mutants that are unable to move their chloroplasts showed severe bleaching and developed necrotic lesions more rapidly than the wild type after transfer to high-intensity light (Jeong *et al.*, 2002; Kasahara *et al.*, 2002; Koniger *et al.*, 2008).

In some non-flowering plant species, chloroplast movement can be initiated by both blue and red light, with the signals acting additively during simultaneous exposure (Kraml and Herrmann, 1991; Kagawa and Wada, 1996; Augustynowicz and Gabrýs, 1999; Kadota *et al.*, 2000). The response to red light is far-red reversible, indicating that a phytochrome serves as the photoreceptor (Yatsuhashi *et al.*, 1987; Kagawa and Wada, 1996; Nozue *et al.*, 1998) Available mutants in fern and moss indicate that the phototropin family of blue-light photoreceptors is required for the response to blue light in these species (Kagawa *et al.*, 2004). In addition, mutations in three of the four phototropin genes in the moss *Physcomitrella patens* also caused reduced chloroplast movement in response to red light, indicating that the phototropins may play a role in the downstream signalling from phytochrome in addition to acting as blue-light photoreceptors (Kagawa *et al.*, 2004).

In angiosperms such as *Arabidopsis thaliana*, chloroplast movement is strictly a blue-light response, mediated by the phot1 and phot2 blue-light photoreceptors (Kagawa *et al.*, 2001; Sakai *et al.*, 2001). The phototropins are also involved in phototropism, light-regulated hypocotyl elongation, stomatal opening, leaf expansion and localization of the nucleus (Liscum and Briggs, 1995; Kinoshita *et al.*, 2001; Folta *et al.*, 2003; Kosei *et al.*, 2007). Knockout of both phototropins is sufficient to eliminate all chloroplast movement in *Arabidopsis* (Sakai *et al.*, 2001), although each has a unique impact on the modulation of the response. Partially irradiated *phot1* single mutants exposed to fluence rates of blue light from 16 μmol m^{-2} s^{-1} to 100 μmol m^{-2} s^{-1} were able to induce an avoidance response while *phot2* mutants were not, suggesting that phot2 is responsible for the high-light response (Sakai *et al.*, 2001). In addition, increases in cellular levels of PHOT2 have been correlated with an increased rate of chloroplast movement during the avoidance response (Kimura and Kagawa, 2009). By contrast, both photoreceptors were able to induce the low-light response, with *phot1* and *phot2* mutants showing accumulation between 2 μmol m^{-2} $s^{-1}$and 16 μmol m^{-2} s^{-1} and 0.4–100 μmol m^{-2} s^{-1} of blue light, respectively (Kagawa *et al.*, 2001; Sakai *et al.*, 2001). Measurements of the change in light transmittance through leaves of *phot2* mutants indicated that phot1 was able to initiate a low-light response under as little as 0.08 μmol m^{-2} s^{-1} blue light and continued to produce chloroplast accumulation at light intensities up to 120 μmol m^{-2} s^{-1} (Jarillo *et al.*, 2001).

The mechanism of signal transduction from the phototropins to downstream effectors involved in chloroplast movements is unknown, but it has been hypothesized that the signals for accumulation and avoidance are distinct and act through separate pathways. The low- and high-light movement responses have been shown to display different kinetic characteristics, with the low-light response signal being generated slower and lasting longer than the high-light response signal (Malec, 1994; Trojan and Gabrýs, 1996; Kagawa and Wada, 1999). However, results from *phot1* mutants indicate that phot2 can induce both chloroplast accumulation and avoidance, and therefore may feed into both of these pathways (Sakai *et al.*, 2001). Phot1 has only been shown to cause a low-light response, but it is unclear if this is through the same pathway as phot2-induced accumulation.

Although red light has no effect on chloroplast movement in the angiosperms studied (Walczak and Gabrýs, 1980), the red/far-red photoreceptors PhyA and PhyB appear to be involved in the response. Mutants in *phyA* and *phyB* showed an enhanced avoidance response after exposure to blue-light intensities from 10–60 μmol m^{-2} s^{-1}, and overexpression of these photoreceptors caused reduced chloroplast accumulation under low light (DeBlasio *et al.*, 2003). It is unlikely that phytochromes are used as a red-light photoreceptors in this situation, since the presence or absence of red or far-red light had no significant impact on the magnitude or direction of chloroplast movement (DeBlasio *et al.*, 2003). However, the observation that the phytochrome mutants display altered chloroplast avoidance (DeBlasio *et al.*, 2003) and phototropism (Whippo and Hangarter, 2004) suggests that the phytochromes may influence blue-light responses in the absence of red light.

To understand better how the phototropins and phytochromes affect chloroplast movements, we conducted detailed fluence-rate response experiments in *phot1*, *phot2*, *phyB,* and *photphy* double mutants. Our results indicated that, in *phot1* mutants, phot2 signals for an accumulation response at blue-light intensities between 0.1 μmol m^{-2} s^{-1} and 5 μmol m^{-2} s^{-1} and produces a high-light response at 20 μmol m^{-2} s^{-1} and above. In *phot2* mutants, light intensities between 0.1 μmol m^{-2} $s^{-1}$and 100 μmol m^{-2} s^{-1} produced an enhanced low-light response compared with the wild type, but the higher fluence rates caused transient chloroplast avoidance before the onset of accumulation. Exposure of *phot2* mutants to intense light during time-lapse microscopy revealed the presence of a slow, attenuated high-light response. In addition, analysis of *phyB*, *phot1phyB*, and *phot2phyB* leaves suggest that the enhancement of the high-light response in *phyB* mutants is caused by loss of PhyB-dependent inhibition of chloroplast avoidance that may serve a regulatory role in wild-type plants.

Materials and methods

Mutant lines and growth conditions

Arabidopsis ecotype Columbia *gl1* was the wild type in this study and all mutants were in the Columbia background. *Arabidopsis phyB-9* seeds were obtained from Jason Reed (University of North Carolina, Chapel Hill). Phototropin mutants were obtained from Emmanuel Liscum (University of Missouri, Columbia; *phot1-1, phot1-5,* and *phot2-5*) and Takatoshi Kagawa (University of Tsukuba, Tsukuba City, Japan; *phot2-1*) Unless otherwise noted, *phot1* indicates allele *phot1-1*. True-breeding homozygous double mutants were generated by crossing *phyB* and either *phot1-1* or *phot2-1*. Double mutants were confirmed by using the following physiological markers: elongated hypocotyl in *phyB* seedlings grown in red light, chloroplast accumulation under high blue light in *phot2-1*, and through the low-blue-light phototropism defect in *phot1*.

Seeds were sown on moist Scott's plug mix (Scott's Sierra, Marysville, OH) and incubated at 4 °C for 48 h. Plants were germinated and grown in a growth room under 60–70 μmol m^{-2} s^{-1} of light provided by a combination of warm-white and cool-white fluorescent bulbs (General Electric, Louisville KY) with 12 h photoperiods at 23 °C. Plants were fertilized with K-Grow all-purpose plant food (Kmart, Troy, MI) every 2 weeks after germination.

Photometric measurements of chloroplast movement

Chloroplast movement was measured photometrically as described previously (DeBlasio *et al.*, 2003, 2005). Leaves were excised from 5–6-week-old adult plants at the end of their 12 h night period and placed in a dark humid container with their petioles placed in microfuge tubes filled with water. In order to make transmittance readings, individual leaves were sandwiched between two glass microscope slides (VWR International, West Chester, PA) with the petiole sticking out into a moist paper towel to maintain hydration. The assembly was then arranged on a stage so the leaf covered a 5 mm diameter red Plexiglas window (Rohm and Haas No. 2423; Dayton Plastics, Columbus, OH) above the sensor from a Li-Cor 1800 spectroradiometer (Li-Cor Inc., Lincoln, NE). A red light emitting diode (660 nm) (LED; Radio Shack, Fort Worth, TX) was mounted directly above the leaf to provide 20–30 μmol m^{-2} s^{-1} red light for measurements of leaf transmittance. Blue light to induce movement was provided by shining light from a halogen fibre optic light microscope illuminator (Cole Palmer, Chicago, IL) through a blue interference filter 450±25 nm (Melles Griot, 03FIB304) at an angle of 60° relative to the surface of the leaf. Changes in blue-light intensity were produced by placing neutral density filters between the light source and the leaf. In order to measure red-light transmittance through the leaf, a Li-Cor 1800 quantum sensor recorded and integrated the quantum flux between 650 nm and 670 nm for each time point. For each leaf, the change in % red-light transmittance was calculated as $((I_t/I_o)\times100/I_A)$ where I_t and I_o are the incident and transmitted red light fluence rate, respectively, and I_A is the average red-light transmittance value measured prior to the blue light treatments. Results are presented as the average change in % red-light transmittance ±standard error.

Time-lapse microscopy

Dark-acclimated leaves were excised from 5–6-week-old *Arabidopsis* plants. Pieces of the leaf blades of approximately 2 mm^2 were cut with a razor, excluding the midvein, and mounted abaxial side down, on a slide in water under a cover slip. The mounted leaf pieces were exposed from below to constant white light (about 3000 μmol m^{-2} s^{-1}) generated from the brightfield lamp on a Nikon E800 microscope (Melville, NY). Images of the adaxial surface of the palisade cell layer were captured every 30 s with MetaMorph software (Universal Imaging, Downingtown, PA) and a Hamamatsu ORCA-ER charge-coupled device camera (Hamamatsu City, Japan).

Results

Chloroplast movements in phototropin mutants

Because red light does not affect chloroplast movement in *Arabidopsis*, it can be used to monitor the relative position of chloroplasts in cells by measuring the amount of red light transmitted through a leaf (Walczak and Gabryś, 1980; Jarillo *et al.*, 2001; DeBlasio *et al.*, 2003, 2005; Luesse *et al.*, 2006). In order to understand better the roles of phot1 and phot2 signalling in both the accumulation and avoidance responses, detailed fluence-rate response curves for chloroplast movement were created for the wild type and the *phot1-1* and *phot2-1* mutants by recording changes in red-light transmittance through leaves during treatment with blue light intensities between 0.1 μmol m^{-2} s^{-1} and 100 μmol m^{-2} s^{-1}. Exposure of wild-type leaves to 0.1 μmol m^{-2} s^{-1} or 1 μmol m^{-2} s^{-1} of blue-light induced a chloroplast accumulation response which resulted in an average change in red-light transmittance of –1 to –1.6% (Fig. 1A). At 5 μmol m^{-2} s^{-1} and 10 μmol m^{-2} s^{-1}, the magnitude of the change in red-light transmittance was reduced with exposure to 10 μmol m^{-2} s^{-1} causing a biphasic response (Figs 1A, 2B). Fluence rates above 20 μmol m^{-2} s^{-1} induced avoidance responses that increased in magnitude with higher fluence rates. These findings with wild type are consistent with previously published results (DeBlasio *et al.*, 2005; Luesse *et al.*, 2006).

Compared with the wild type, the *phot1* mutant showed a reduced chloroplast accumulation response at fluence rates from 0.1–5 μmol m^{-2} s^{-1} (Fig. 1B, D). At 0.1 μmol m^{-2} s^{-1} of blue light, the *phot1* mutant showed a –0.4% change in transmittance, indicating that although the response is weak, when acting alone phot2 can induce chloroplast accumulation under very low fluence rates of light. Chloroplast avoidance in *phot1* mutants was similar to the wild type under high blue light intensities of 20–100 μmol m^{-2} s^{-1}. The *phot2* mutant displayed an accumulation response under all light conditions as previously observed (Fig. 1C) (Jarillo *et al.*, 1998; Kagawa *et al.*, 2001; Sakai *et al.*, 2001), but the magnitude of the light transmittance change in *phot2* mutants (about –2.4%) was about twice as large as the maximum seen in the wild-type accumulation response (Fig. 1D). Moreover, the change in red light transmittance induced by low blue light in the wild type was intermediate between the responses observed in the *phot1* and *phot2* mutants under the same conditions (Figs 1D, 2A). These data suggest that the signals generated by phot1 and phot2 for chloroplast accumulation may be acting antagonistically to one another.

Closer analysis of the kinetics of the chloroplast movement at intermediate fluence rates also supports the existence of distinct phot1 and phot2-mediated accumulation signalling pathways. Upon exposure to 10 μmol m^{-2} s^{-1} of

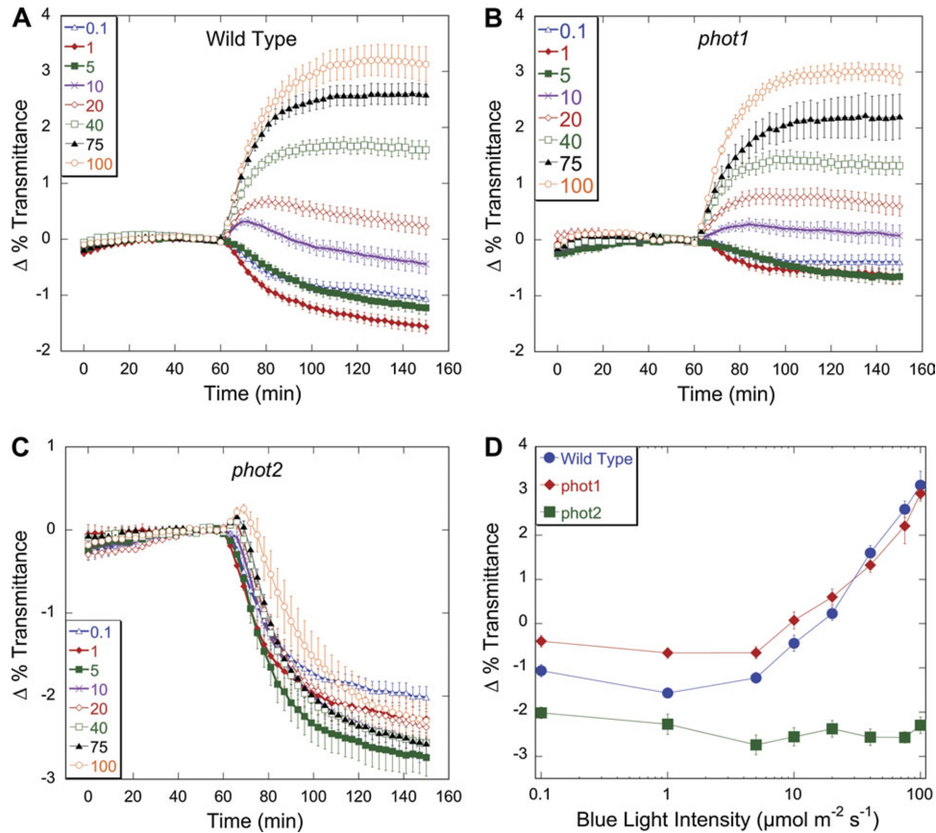

Fig. 1. Time-course of fluence responses of light-induced chloroplast movements in leaves of wild type and *phot1* and *phot2* mutants. (A–C) Red-light transmittance was measured for 60 min in dark-acclimated leaves to establish a baseline before a 90 min blue-light treatment was initiated. Red-light transmittance was recorded every 3 min. The data are the average ±SE of 5–15 leaves per light treatment. (D) Fluence response curves from the final transmittance changes from (A), (B), and (C) of wild type, *phot1*, and *phot2* after 90 min blue light treatments.

continuous blue light, wild-type leaves produced a biphasic change in light transmittance, characterized by an initial phase of chloroplast avoidance followed by a prolonged period of accumulation (Fig. 2B). However, *phot1* leaves failed to show a significant biphasic response under any tested light intensity, instead producing a weak chloroplast avoidance response of 0.2% at 10 μmol m^{-2} s^{-1}. Since *phot1* also showed attenuated chloroplast accumulation under low light, it seems likely that signalling from phot1 is responsible for the accumulation phase of the biphasic response seen in the wild type at 10 μmol m^{-2} s^{-1}. In addition, analysis of *phot2* mutants at 20 μmol m^{-2} s^{-1} revealed a 6 min lag before the onset of the accumulation response, while at lower fluence rates chloroplast accumulation could be detected within 3 min of the initiation of the light treatment (Fig. 2C). High fluence rates (40–100 μmol m^{-2} s^{-1}) produced a small biphasic response in *phot2*, with the length of the initial chloroplast avoidance phase increasing in response to higher light intensities (Figs 1C, 2D; see Supplementary Fig. S1 at *JXB* online). Similar trends were also seen with the *phot2-5* allele (data not shown).

Visual examination of chloroplast movements in *phot2* and *phot1phot2* double mutants during exposure to 60 min of 3000 μmol m^{-2} s^{-1} white light, generated from a bright-field lamp on a microscope, consistently showed slow but steady movement of chloroplasts toward the anticlinal cell walls in *phot2* mutants, indicative of an attenuated high-light avoidance response (Fig. 3; see Supplementary video S1 at *JXB* online). An identical response was also produced by the *phot2-5* (data not shown). As expected, *phot1phot2* double mutants displayed no chloroplast movement, indicating that phot1 is required to stimulate the weak avoidance response observed in the *phot2* mutant.

Chloroplast movements in phyB *mutants*

Previous work showed that mutations in *phyB* enhanced the chloroplast avoidance response under both intermediate and high fluence rates of blue light (DeBlasio *et al.*, 2003). To understand better how PhyB regulates light-induced chloroplast movements, detailed fluence rate dependencies were analysed for *phyB*, *phot1phyB*, and *phot2phyB* mutants to allow observation of the loss of PhyB signalling in backgrounds with altered phototropin-dependent chloroplast movements.

Consistent with previous results, *phyB* mutants exhibited wild-type levels of chloroplast movement upon exposure to low light (0.1–1 μmol m^{-2} s^{-1}), and an enhanced avoidance response under high light intensities (20–100 μmol m^{-2} s^{-1}) (Fig. 4A, D). Like the wild type,

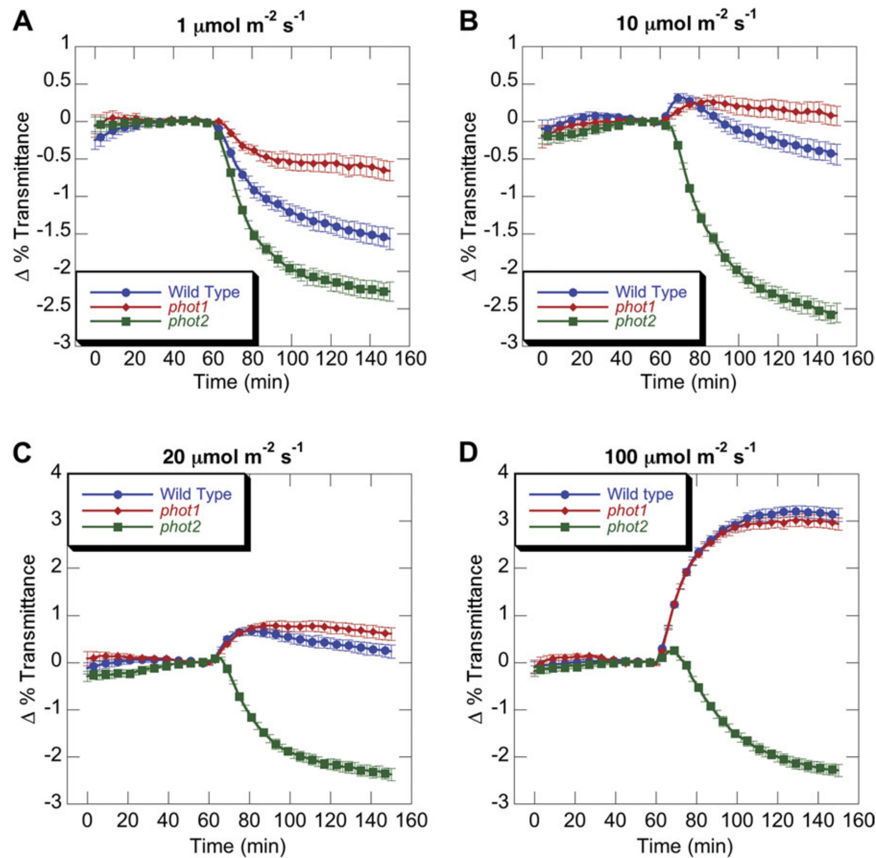

Fig. 2. Time-course of chloroplast movements in *phot1* and *phot2* under low and transitional intensities of blue light. Data from Fig. 1 are presented to allow comparisons of chloroplast movement at individual light intensities.

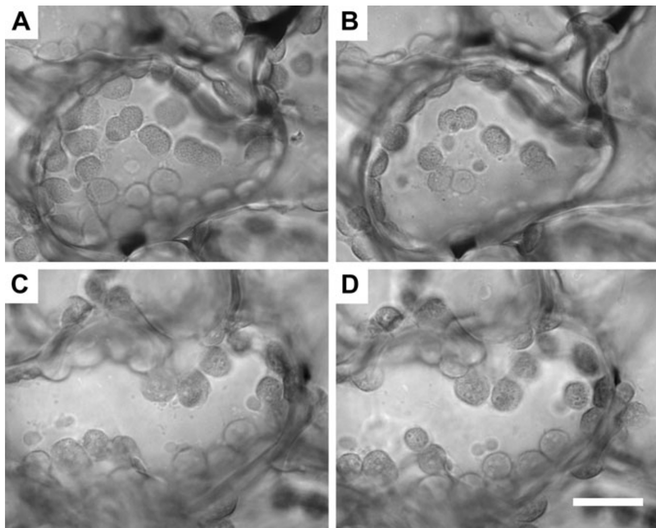

Fig. 3. High-light-induced chloroplast movements in *phot2* and *phot1phot2* mutants. The *phot2* mutant (A, B) and *phot1phot2* double mutant (C, D) were exposed to intense white light generated by the brightfield lamp on a microscope for 60 min. Images captured at 0 min (A, C) and 60 min (B, D) are shown. Some chloroplasts in the *phot2* mutant have moved to the anticlinal cell walls (see Supplementary video S1). The bar is equal to 5 μm.

phyB mutants also displayed a biphasic response when exposed to 10 μmol m^{-2} s^{-1} of blue light, but the *phyB* avoidance phase was significantly enhanced (Fig. 5A). A robust biphasic response was also observed when *phyB* leaves were illuminated with 5 μmol m^{-2} s^{-1} of blue light (Fig. 6). At this fluence rate, wild-type leaves exhibited a strict accumulation response, suggesting that, in wild-type plants, PhyB acts to repress the avoidance phase of the biphasic response. Furthermore, at 5 μmol m^{-2} s^{-1} the overall change in light transmittance (from the highest to the lowest) associated with the accumulation phase of the biphasic response in *phyB* mutants was almost identical in magnitude to the size of the accumulation response produced by the wild type (Fig. 6). This indicates that, once initiated, the chloroplast accumulation response was unperturbed in the absence of PhyB.

To determine the epistatic relationship of PhyB to phot1 and phot2, the chloroplast movement responses of *phot1phyB* and *phot2phyB* double mutants were examined under various light conditions. When exposed to blue light intensities of less than 10 μmol m^{-2} s^{-1}, *phot1phyB* and *phot2phyB* double mutants behaved like the *phot1* and *phot2* single mutants, respectively (Fig. 4D). Interestingly, the *phot1phyB* double mutant failed to develop the avoidance phase of the biphasic response seen in the *phyB* single mutant at 5 μmol m^{-2} s^{-1}, further supporting the

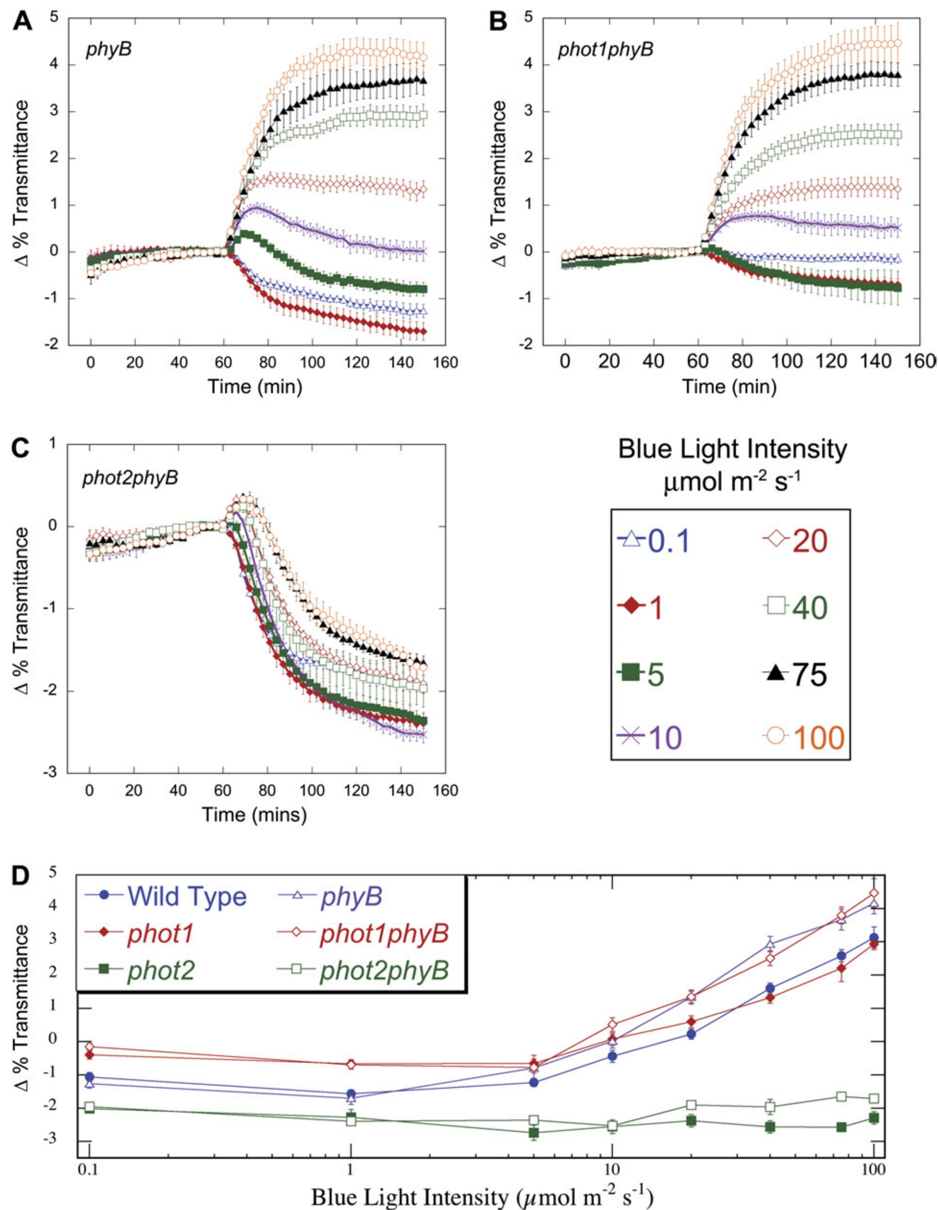

Fig. 4. Time-course of fluence responses of light-induced chloroplast movements in leaves of wild type and *phyB*, *phot1phyB*, and *phot2phyB* mutants. (A–C) Red-light transmittance was measured for 60 min in dark-acclimated leaves to establish a baseline before a 90 min blue-light treatment was initiated. Red-light transmittance was recorded every 3 min. The data are the average ±SE of 5–15 leaves per light treatment. (D) Final red-light transmittance changes in, *phot1*, *phot2*, *phyB*, *phot1phyB*, and *phot2phyB* after 90 min exposures to single fluence-rates of blue light (from Fig. 1A–C and Fig. 4A–C).

hypothesis that phot1 is required for the accumulation phase of the biphasic response under intermediate light conditions (Fig. 4A, B). At 10 µmol m^{-2} s^{-1}, *phot1phyB* displayed elements of both the *phyB* and *phot1* single mutants but with a final change in light transmittance distinct from either of these (Fig. 5A). Like *phot1*, the *phot1phyB* mutants failed to initiate the accumulation phase of the biphasic response seen in the wild type and *phyB*. At 20 µmol m^{-2} s^{-1}, *phyB* took only 12 min to reach its maximum transmittance change of 1.5%, while *phot1phyB* took approximately twice as long, but eventually attained an overall response similar to the *phyB* mutant (Fig. 5B). Under higher light intensities (>20 µmol m^{-2} s^{-1}), *phyB* and *phot1phyB* mutants displayed similarly enhanced chloroplast avoidance responses when compared with the wild type (Fig. 5B–E).

In *phot2* and *phot2phyB* leaves, low blue light induced similarly enhanced accumulation responses, but under high light the magnitude of the overall change in transmittance was decreased in the double mutant (Figs 4, 5) At blue light intensities greater than 10 µmol m^{-2} s^{-1}, the initial avoidance portion of the biphasic response normally seen in *phot2* showed increased magnitude and duration in the *phot2phyB* double mutant, indicating an enhancement of chloroplast avoidance and could be the reason for the difference in magnitude of the response between *phot2* and

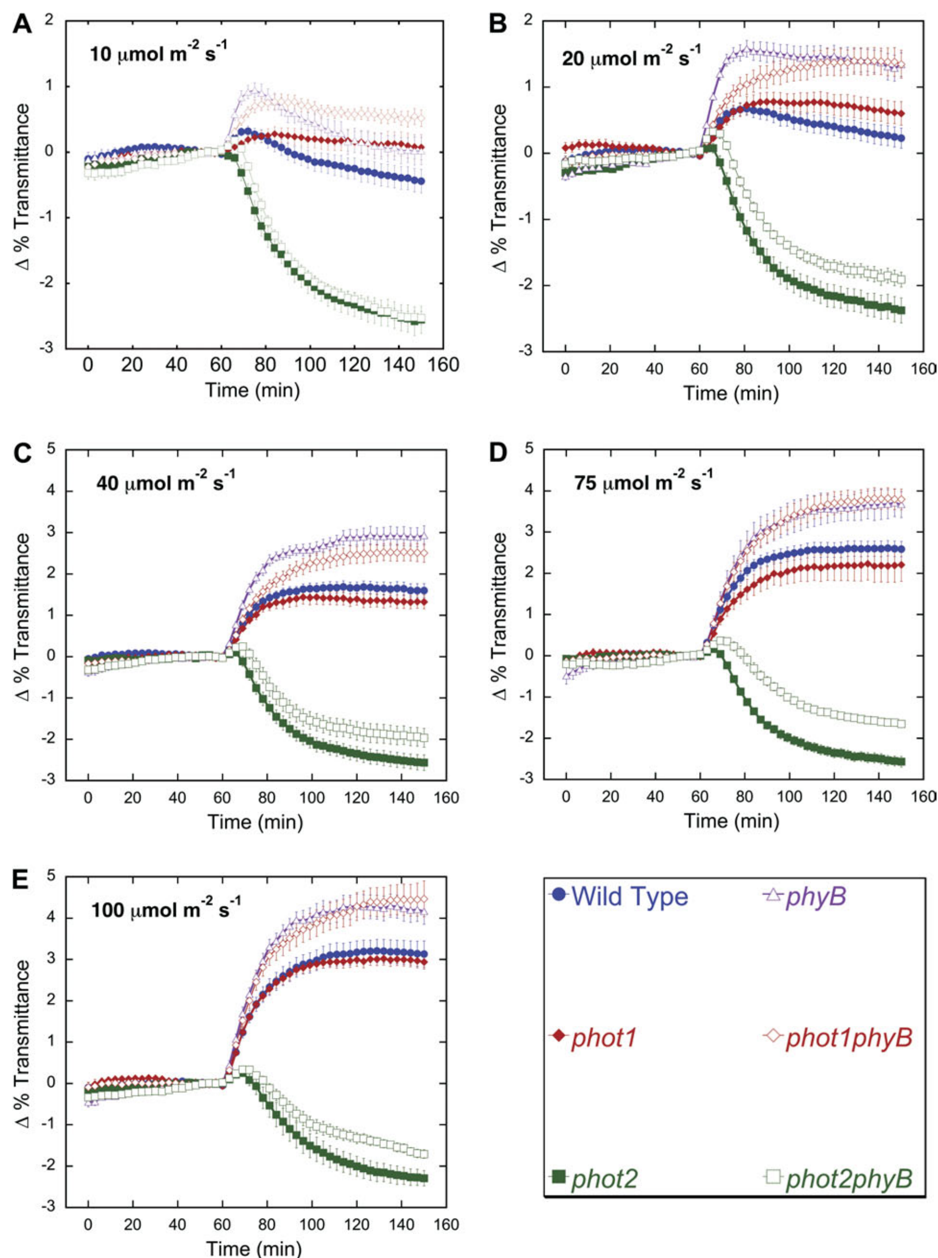

Fig. 5. Time-courses of chloroplast movements in *phyB*, *phot1phyB*, and *phot2phyB* under transitional and high blue-light intensities. Data were replotted from Fig. 4 to facilitate comparisons between genotypes at individual light intensities.

phot2phyB mutants (Fig. 5). Since the *phyB* mutation resulted in the enhancement of chloroplast movement in response to high light in both the *phot1* and *phot2* mutant backgrounds, it may indicate that PhyB does not work exclusively with either photropin signalling pathways but affects chloroplast avoidance in general.

Discussion

Phototropin signalling for chloroplast movement

In *Arabidopsis*, chloroplast movements were shown to be mediated somewhat redundantly by the activity of both phot1 and phot2 (Kagawa and Wada, 2000; Sakai *et al.*, 2001). To examine the relationship of the signals produced by these photoreceptors for chloroplast movement, detailed fluence rate response curves were created for *phot1* and *phot2* mutants. These results indicated that when exposed to low intensity blue light (0.1–5 μmol m^{-2} s^{-1}), *phot1* mutants exhibited attenuated chloroplast accumulation while *phot2* mutants displayed an enhanced accumulation response (Fig. 1D). Under the same light conditions, wild-type leaves produced an accumulation response that was intermediate between the responses observed for *phot1* and *phot*2. If the phototropins use the same signalling pathway for chloroplast accumulation, it would be expected that their signals would act additively, and mutations in either phototropin would lead to a reduction in movement under low-light conditions. However, since *phot2* mutants showed an

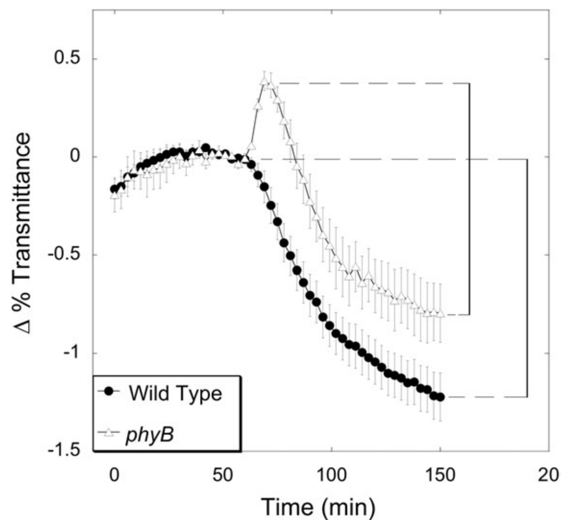

Fig. 6. Biphasic chloroplast movement response in *phyB* at 5 μmol m^{-2} s^{-1}. Red-light transmittance through wild-type and *phyB* leaves was measured every 3 min. for 60 min before and during exposure to 5 μmol m^{-2} s^{-1} blue light. At this blue light intensity, *phyB* displays a biphasic response while the wild type shows only a low-light response. The lines drawn on the graph demark the equal magnitude (1.2%) of the accumulation response in both genotypes.

enhanced accumulation response compared with the wild type, it suggests that phot2 actually inhibits phot1-dependent chloroplast accumulation under low light. In this case, even though phot1 and phot2 can each induce an accumulation response under low light, their signalling pathways for chloroplast movement appear to be distinct, and the final chloroplast position is a compromise between those two signals.

Analysis of the phototropin mutants also provided information about chloroplast movement at transitional light intensities between those that cause the accumulation response and those that cause avoidance (typically around 10 μmol m^{-2} s^{-1} in the wild type). These intermediate light intensities result in a biphasic change in light transmittance, characterized by a rapid onset of chloroplast avoidance followed by a prolonged period of chloroplast accumulation. The biphasic behaviour is probably caused by the simultaneous initiation of both the high and low-light responses, possibly by distinct phot1- and phot2-mediated pathways. Because the signal and movement for chloroplast accumulation are slower than for avoidance (Kagawa and Wada, 1999), the biphasic movement may reflect a rapid initiation of chloroplast avoidance which is eventually reversed by the slower process of accumulation. This is also supported by microbeam irradiation studies of prothallial cells in fern. When a region of a single cell was treated with a blue light microbeam of constant intensity of 5 W m^{-2} (about 20 μmol m^{-2} s^{-1}) the chloroplasts initially moved out the light, but after about 10 min returned to the irradiated area, indicating that the response involves antagonism between the signal for the high and low-light responses (Kagawa and Wada, 1999). In our studies, intermediate light intensities failed to induce a biphasic response in *phot1* mutants, instead showing only slight chloroplast avoidance (Fig. 2B). This could indicate that, in wild-type plants exposed to blue light intensities of 10 μmol m^{-2} s^{-1}, phot2 signals for a high-light response while phot1 signals for a low-light response. This suggests that not only is the biphasic response caused by separate signals for accumulation and avoidance but that at 10 μmol m^{-2} s^{-1} phot1 and phot2 pathways produce competing signals. Because *phot1* mutants do not show robust biphasic movements, the response is probably not caused by desensitization of the photoreceptors.

Signalling by phot1 can induce chloroplast avoidance

It has been thought that the high-light-induced chloroplast avoidance response is mediated completely by phot2 since previous experiments showed that *phot2* mutants display a chloroplast accumulation response at blue light intensities between 0.8 μmol m^{-2} s^{-1} and 100 μmol m^{-2} s^{-1} (Jarillo *et al.*, 2001; Sakai *et al.*, 2001). However, our results provide three pieces of evidence suggesting that phot1 is also capable of signalling for an avoidance response. (i) When exposed to high-intensity light, *phot2* mutants displayed a modest but reproducible biphasic response (Figs 1, 2). Although the response was transient, it indicates that, without the aid of phot2, phot1 can induce a weak high-light response. (ii) Double mutants in *phot1phyB* showed a reduced rate of movement under 10–40 μmol m^{-2} s^{-1} when compared to *phyB* single mutants (Fig. 5), suggesting that phot1 can enhance the rate of avoidance movement in this background. (iii) When exposed to intense white light, the chloroplasts in *phot2* mutants moved slowly towards the anticlinal cell walls, while *phot1phot2* double mutants failed to show any movement when observed through microscopy (Fig. 3). This response was seen in both *phot2-1* and *phot2-5* suggesting that it is not allele specific. Taken together, these observations indicate that, under certain light conditions, phot1 can initiate a chloroplast avoidance response in the absence of phot2. However, because *phot1* mutants and the wild type show similar chloroplast movements under high light intensities (Fig. 1), phot2 clearly plays the dominant role in driving the avoidance response. It is also possible that the avoidance movement seen in the *phot2* mutant, but not in the *phot1phot2* double mutant is driven by temperature as opposed to light intensity. It has been shown that phot2 mediates chloroplast relocation to the anticlinal walls of *Adiantum capillus-veneris* under low-temperature conditions (Kodama *et al.*, 2008). It is possible that phot1 is mediating a similar response under high-temperature conditions in *Arabidopsis*.

Phyb inhibition of the high-light avoidance response

Although previous work showed that mutants in *phyB* displayed enhanced chloroplast avoidance in response to intermediate and high blue light (DeBlasio *et al.*, 2003), it was not clear if PhyB acted to inhibit the high-light

response or if it enhanced the antagonistic low-light response. The analysis presented here of the response of *phyB* mutants to transitional blue-light intensities (5–20 μmol m^{-2} s^{-1}) suggests that PhyB acts to inhibit chloroplast avoidance in wild-type plants rather than to enhance accumulation. Specifically, under 5 μmol m^{-2} s^{-1} of blue light, *phyB* displayed a biphasic response while the wild-type showed only accumulation (Fig. 6). The total change between the maximum and minimum levels of light transmittance was similar in both lines, indicating that the avoidance response was enhanced while the low-light accumulation response was unchanged. The *phyB* mutant showed a similarly enhanced avoidance response at 10 μmol m^{-2} s^{-1}. If PhyB were inhibiting chloroplast avoidance by enhancing accumulation, it would be expected that the magnitude of the chloroplast accumulation phase of the biphasic response would be decreased in the *phyB* mutant. These observations suggest that the PhyB-mediated repression of the high-light response is limited to inhibition of the chloroplast avoidance signalling pathway.

Because phot1 and phot2 appear to act through independent and distinct signalling pathways to regulate chloroplast movement and other blue-light responses (Fig. 1) (Sakai *et al.*, 2000; Inada *et al.*, 2004) it is possible that PhyB activity could interact with one or both of these pathways to inhibit chloroplast avoidance under high light. If PhyB worked with phot1 signalling exclusively, it would be expected that the *phot1* mutants would respond exactly like *phot1phyB* double mutants. The same is true for *phot2* and *phot2phyB* double mutants. However, if PhyB signalling interacts with both phot1 and phot2, or functions independently of them, then the *phot1phyB* and *phot2phyB* double mutants would be expected to continue to display the *phyB* phenotype. Examination of *phot1phyB* and *phot2phyB* showed that the enhanced movement caused by the loss of PhyB is present in both double mutants, indicating that the PhyB inhibition of the chloroplast avoidance response is not exclusive to phot1 or phot2 signalling (Figs 4, 5).

The exact role of PhyB in light-dependent chloroplast movements in *Arabidopsis* remains unclear. Since neither red nor far-red light exposure appears to affect chloroplast movement in angiosperms, it is unlikely that PhyB acts as a red light photoreceptor in its modulation of chloroplast movement (Walczak and Gabrýs, 1980; DeBlasio *et al.*, 2003). However, because blue light can mediate PhyA-dependent very low fluence responses, PhyB may also be activated by blue light to induce suppression of chloroplast avoidance (Hamazato *et al.*, 1997). Although red light does not cause hypocotyl phototropism, phytochrome appears to modulate the response in a similar manner to what has been observed for chloroplast movement. In the absence of red light, PhyB enhanced the response at blue light intensities above 1 μmol m^{-2} s^{-1} and PhyA caused inhibition under higher fluence rates (Whippo and Hangarter, 2004). In the fern *Adiantum capillus-veneris*, mutations in the blue-light-absorbing phototropins inhibited red-light-induced chloroplast movement, suggesting a role for *Adiantum* phototropins in phytochrome signal transduction. In *Arabidopsis*, PhyB can function in signal transduction downstream of the phototropins. One potential source of Phot and Phy signalling overlap is found in the PHYTOCHROME KINASE SUBSTRATE (PKS) family of proteins, which have been shown to interact directly with both phot1 and PhyA and play a role in both phot1 and phot2 signalling (de Carbonnel *et al.*, 2010). However, triple knockout mutants in *pks1*, *pks2*, and *pks4* show normal levels of chloroplast movement, indicating that these three genes are not required for a normal response. It is possible that PKS3, for which no null mutants were available, may be involved.

In conclusion, detailed analysis of fluence response relationships for light-induced chloroplast movments in *phot1* and *phot2* mutants indicate that, although both phot1 and phot2 can induce chloroplast accumulation, they operate through integrated but antagonistic pathways to control this movement. Evidence is also presented that, under very high-light conditions, the phot1 photoreceptor can signal for chloroplast avoidance, although phot2 is the primary regulator of this response. In addition, results obtained with *phot1phyB* and *phot2phyB* double mutants indicates that PhyB acts to attenuate the high light avoidance response in concert with both phot1 and phot2.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary video S1. Time-lapse analysis of high light-induced chloroplast movement in palisade *phot2* mutant.

Supplementary Fig. S1. Comparisons between genotypes immediately after exposure to individual fluence rates of blue light for analysis of the biphasic response: data redrawn from Figs 1C and 4C.

Acknowledgement

This work was supported by a grant from the National Science Foundation (IOB-0416741).

References

Augustynowicz J, Gabrý s H. 1999. Chloroplast movements in fern leaves: correlation of movement dynamics and environmental flexibility of the species. *Plant, Cell and Environment* **22,** 1239–1248.

Boardman NK. 1977. Comparative photosynthesis of sun and shade plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **28,** 355–377.

DeBlasio SL, Mullen JL, Luesse DR, Hangarter RP. 2003. Phytochrome modulation of blue light-induced chloroplast movements in Arabidopsis. *Plant Physiology* **133,** 1471–1479.

DeBlasio SL, Luesse DR, Hangarter RP. 2005. A plant-specific protein essential for blue-light-induced chloroplast movements. *Plant Physiology* **139,** 101–114.

de Carbonnel M, Davis P, Roelfsema MRG, Inoue S-i, Schepens I, Lariguet P, Geisler M, Shimazaki K-i, Hangarter R, Fankhauser C. 2010. The Arabidopsis PHYTOCHROME KINASE SUBSTRATE2 protein is a phototropin signalling element that regulates leaf flattening and leaf positioning. *Plant Physiology* **152,** 1391–1405.

Folta KM, Lieg EJ, Durham T, Spalding EP. 2003. Primary inhibition of hypocotyl growth and phototropism depend differently on phototropin-mediated increases in cytoplasmic calcium induced by blue light. *Plant Physiology* **133,** 1464–1470.

Franklin KA, Larner VS, Whitelam GC. 2005. The signal transducing photoreceptors of plants. *International Journal of Developmental Biology* **49,** 653–664.

Galen C, Huddle J, Liscum E. 2004. An experimental test of the adaptive evolution of phototropins: blue-light photoreceptors controlling phototropism in *Arabidopsis thaliana*. *Evolution* **58,** 515–523.

Hamazato F, Shinomura T, Hanzawa H, Chory J, Furuya M. 1997. Fluence and wavelength requirements for arabidopsis CAB gene induction by different phytochromes. *Plant Physiology* **115,** 1533–1540.

Inada S, Ohgishi M, Mayama T, Okada K, Sakai T. 2004. RPT2 is a signal transducer involved in phototropic response and stomatal opening by association with phototropin 1 in *Arabidopsis thaliana*. *The Plant Cell* **16,** 887–896.

Inoue Y, Shibata K. 1974. Comparative examination of terrestrial plant leaves in terms of light-induced absorption changes due to chloroplast rearrangements. *Plant and Cell Physiology* **15,** 717–721.

Jarillo J, Ahmad M, Cashmore A. 1998. NPL1: a second member of the NPH serine/threonine kinase family of Arabidopsis. *Plant Physiology* **117,** 719.

Jarillo JA, Gabrys H, Capel J, Alonso JM, Ecker JR, Cashmore AR. 2001. Phototropin-related NPL1 controls chloroplast relocation induced by blue light. *Nature* **410,** 952–954.

Jeong WJ, Park YI, Suh K, Raven JA, Yoo OJ, Liu JR. 2002. A large population of small chloroplasts in tobacco leaf cells allows more effective chloroplast movement than a few enlarged chloroplasts. *Plant Physiology* **129,** 112–121.

Kadota A, Sato Y, Wada M. 2000. Intracellular chloroplast photorelocation in the moss *Physcomitrella patens* is mediated by phytochrome as well as by a blue-light receptor. *Planta* **210,** 932–937.

Kagawa T, Kasahara M, Abe T, Yoshida S, Wada M. 2004. Function analysis of phototropin2 using fern mutants deficient in blue light-induced chloroplast avoidance movement. *Plant and Cell Physiology* **45,** 416–426.

Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, Tabata S, Okada K, Wada M. 2001. Arabidopsis NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. *Science* **291,** 2138–2141.

Kagawa T, Wada M. 1996. Phytochrome and blue-light-absorbing pigment-mediated directional movement of chloroplasts in dark-adapted prothallial cells of fern *Adiantum*. *Planta* **107,** 389–398.

Kagawa T, Wada M. 1999. Chloroplast-avoidance response induced by high-fluence blue light in prothallial cells of the fern *Adiantum capillus-veneris* as analyzed by microbeam irradiation. *Plant Physiology* **119,** 917–923.

Kagawa T, Wada M. 2000. Blue light-induced chloroplast relocation in *Arabidopsis thaliana* as analyzed by microbeam irradiation. *Plant and Cell Physiology* **41,** 84–93.

Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Wada M. 2002. Chloroplast avoidance movement reduces photodamage in plants. *Nature* **420,** 829–832.

Kimura M, Kagawa T. 2009. Blue light-induced chloroplast avoidance and phototropic responses exhibit distinct dose dependency of PHOTOTROPIN2 in *Arabidopsis thaliana*. *Photochemistry and Photobiology* **85,** 1260–1264.

Kinoshita T, Doi M, Suetsugu N, Kagawa T, Wada M, Shimazaki K-i. 2001. phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature* **414,** 656–660.

Kodama Y, Tsuboi H, Kagawa T, Wada M. 2008. Low temperature-induced chloroplast relocation mediated by a blue light receptor, phototropin 2, in fern gametophytes. *Journal of Plant Research* **121,** 441–448.

Koniger M, Delamaide JA, Marlow ED, Harris GC. 2008. *Arabidopsis thaliana* leaves with altered chloroplast numbers and chloroplast movement exhibit impaired adjustments to both low and high light. *Journal of Experimental Botany* **59,** 2285–2297.

Kosei I, Tatsuya S, Shingo T. 2007. Blue light-dependent nuclear positioning in *Arabidopsis thaliana* leaf cells. *Plant and Cell Physiology* **48,** 1291–1291.

Kraml M, Herrmann H. 1991. Red blue-interaction in *Mesotaenium* chloroplast movement: blue seems to stabilize the transient memory of the phytochrome signal. *Photochemistry and Photobiology* **53,** 255–259.

Lechowski Z. 1974. Chloroplast arrangement as a factor of photosynthesis in multilayered leaves. *Acta Societatis Botanicorum Poloniae* **63,** 531–540.

Liscum E, Briggs WR. 1995. Mutations in the nph1 locus of Arabidopsis disrupt the perception of phototropic stimuli. *The Plant Cell* **7,** 473–485.

Luesse DR, DeBlasio SL, Hangarter RP. 2006. *Plastid movement impaired 2*, a new gene involved in normal blue-light-induced chloroplast movements in arabidopsis. *Plant Physiology* **141,** 1328–1337.

Malec P. 1994. Kinetic modeling of chloroplast phototranslocations in *Lemna trisulca* L. 2. Rate-limiting components. *Journal of Theoretical Biology* **169,** 189–195.

Melis A. 1999. Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage *in vivo*? *Trends in Plant Science* **4,** 130–135.

Nozue K, Kanegae T, Imaizumi T, Fukuda S, Okamoto H, Yeh KC, Lagarias JC, Wada M. 1998. A phytochrome from the fern *Adiantum* with features of the putative photoreceptor NPH1. *Proceedings of the National Academy of Sciences, USA* **95,** 15826–15830.

Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, Wada M, Okada K. 2001. Arabidopsis nph1 and npl1: Blue light receptors that mediate both phototropism and chloroplast

relocation. *Proceedings of the national Academy of Sciences, USA* **98,** 6969–6974.

Sakai T, Wada T, Ishiguro S, Okada K. 2000. RPT2: A signal transducer of the phototropic response in Arabidopsis. *The Plant Cell* **12,** 225–236.

Trojan A, Gabrys H. 1996. Chloroplast distribution in *Arabidopsis thaliana* (L.) depends on light conditions during growth. *Plant Physiology* **111,** 419–425.

Vandenbussche F, Pierik R, Millenaar FF, Voesenek LA, Van der Straeten D. 2005. Reaching out of the shade. *Current Opinion in Plant Biology* **8,** 462–468.

Wagner G, Haupt W, Laux A. 1972. Reversible inhibition of chloroplast movement by cytochalasin B in the green alga *Mougeotia*. *Science* **176,** 808–809.

Walczak T, Gabrýs H. 1980. New type of photometer for measurements of transmission changes corresponding to chloroplast movements in leaves. *Photosynthetica* **14,** 65–72.

Whippo CW, Hangarter RP. 2004. Phytochrome modulation of blue-light-induced phototropism. *Plant, Cell and Environment* **27,** 1223–1228.

Yatsuhashi H, Wada M, Hashimoto T. 1987. Dichroic orientation of phytochrome and blue-light photoreceptor in *Adiantum* protonemata as determined by chloroplast movement. *Acta Physiologiae Plantarum* **9,** 163–173.

Zurzycki J. 1955. Chloroplast arrangement as a factor in photosynthesis. *Acta Societatis Botanicorum Poloniae* **24,** 27–63.

Zurzycki J. 1961. The influence of chloroplast displacements on the optical properties of leaves. *Acta Societatis Botanicorum Poloniae* **30,** 503–527.