Phototropins Mediate Blue and Red Light-Induced Chloroplast Movements in *Physcomitrella patens*[1]

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Phototropin is the blue-light receptor that mediates phototropism, chloroplast movement, and stomatal opening in Arabidopsis. Blue and red light induce chloroplast movement in the moss *Physcomitrella patens*. To study the photoreceptors for chloroplast movement in *P. patens*, four phototropin genes (*PHOTA1, PHOTA2, PHOTB1*, and *PHOTB2*) were isolated by screening cDNA libraries. These genes were classified into two groups (*PHOTA* and *PHOTB*) on the basis of their deduced amino acid sequences. Then phototropin disruptants were generated by homologous recombination and used for analysis of chloroplast movement. Data revealed that blue light-induced chloroplast movement was mediated by phototropins in *P. patens*. Both photA and photB groups were able to mediate chloroplast avoidance, as has been reported for Arabidopsis phot2, although the photA group contributed more to the response. Red light-induced chloroplast movement was also significantly reduced in *photA2photB1photB2* triple disruptants. Because the primary photoreceptor for red light-induced chloroplast movement in *P. patens* is phytochrome, phototropins may be downstream components of phytochromes in the signaling pathway. To our knowledge, this work is the first to show a function for the phototropin blue-light receptor in a response to wavelengths that it does not absorb.

Blue light regulates a wide variety of photoresponses in plants, including chloroplast movement, inhibition of hypocotyl elongation, circadian timing, regulation of gene expression, and stomatal opening (Briggs and Huala, 1999; Christie and Briggs, 2001; Gyula et al., 2003). Three types of flavin-containing photoreceptors (phototropin, cryptochrome, and ZTL/FKF/ADO/LKP) have been identified as the blue-light receptors that mediate these responses (Briggs et al., 2001; Schultz et al., 2001; Briggs and Christie, 2002; Cashmore, 2003; Imaizumi et al., 2003; Lin and Shalitin, 2003; Liscum et al., 2003).

The phototropin gene *PHOT1*, first isolated in Arabidopsis, was shown to control phototropism (Huala et al., 1997). Phototropin contains a Ser/Thr protein kinase domain at the C terminus and two light, oxygen, or voltage (LOV) domains (LOV1 and LOV2) at the N terminus (Huala et al., 1997). The LOV domains function as binding sites for the chromophore FMN (Christie et al., 1999; Kasahara et al., 2002b) and belong to the PAS domain superfamily (Taylor and Zhulin, 1999). LOV1 and LOV2 are approximately 100 amino acids in length and are separated by a variable intervening sequence. Two phototropins, phot1 and phot2, function in Arabidopsis to control chloroplast movement and stomatal opening in addition to phototropism (Jarillo et al., 2001; Kagawa et al., 2001; Kinoshita et al., 2001; Sakai et al., 2001; Briggs and Christie, 2002).

Chloroplasts move to different locations in plant cells depending upon the intensity of light exposure. Under low-fluence-rate light conditions, chloroplasts spread over the cell surface perpendicular to the light direction, in order to harvest sufficient light and to maximize photosynthetic activity (Zurzycki, 1955). Under high-fluence-rate light conditions, chloroplasts become situated along the cell sides parallel to the direction of incident light, in order to minimize potential photodamage to the photosynthetic machinery (Kasahara et al., 2002a). These positions result from the movement of chloroplasts toward low-light-irradiated area (chloroplast accumulation movement) and away from strong-light-irradiated area (chloroplast avoidance movement; Haupt and Scheuerlein, 1990; Wada

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et al., 2003). In Arabidopsis, phot1 mediates the accumulation movement irrespective of fluence rates of blue light, whereas phot2 mediates the accumulation movement at low fluence rates and the avoidance movement at high fluence rates of blue light (Sakai et al., 2001; Kagawa and Wada, 2002; Wada et al., 2003). In the fern *Adiantum capillus-veneris*, blue light-induced chloroplast avoidance movement is also mediated by phot2 but not by phot1 (Kagawa et al., 2004).

In *Physcomitrella patens*, chloroplast movement is controlled by blue light. Different from seed plants, however, red light also induces this movement in *P. patens* (Kadota et al., 2000; Sato et al., 2001). This red light-induced movement is canceled by far-red light, indicating that phytochromes are the photoreceptors (Kadota et al., 2000). The motile systems for the chloroplast movement are both actin and tubulin based (Sato et al., 2001). Two cryptochromes (Ppcry1a and Ppcry1b) have been isolated in *P. patens* and are reported to regulate the blue light induction of side branches and gametophores (Imaizumi et al., 2002). Phototropins have not yet been isolated in *P. patens*.

Four phototropins from *P. patens* are reported in this study. Using phototropin disruptants, our data illustrate that both blue and red light-induced chloroplast movements are mediated by these phototropins. Several possible signaling pathways for chloroplast movement are discussed.

RESULTS

Isolation and Characterization of *P. patens* Phototropin Genes

To analyze the function of the blue-light receptor phototropin in *P. patens*, four *PHOT* cDNA sequences were determined. They exhibited the typical domain organization of the phototropin family: two LOV domains (FMN-binding domains) at the N-terminal region and a Ser/Thr protein kinase domain at the C-terminal region (Fig. 1). The N-terminal extensions (from the initiation codon to the start of LOV1 domain) are longer than those of Arabidopsis (Fig. 1). A phylogenetic analysis showed that the four phototropins form a group that is independent from the PHOT1 and PHOT2 groups (Briggs et al., 2001). The *P. patens* group is further divided into two subgroups named PHOTA (1 and 2) and PHOTB (1 and 2) on the basis of their sequences (Fig. 2). The sequences were deposited in the GenBank/EBI/DDBJ database under accession numbers AB163420, AB163421, AB163422, and AB163423, respectively. A total of 8×10^5 plaques were screened from which multiple clones for the four *PHOT* genes were obtained, suggesting that the screening scale was large enough to isolate all of the *PHOT* genes that function in protonemal cells. And also, no other phototropin gene was found in an expressed sequence tag (EST) database, PHYSCObase (Nishiyama et al., 2003). Expressions of *PHOT* genes in protonemal cells were examined under different light conditions. *PHOTA2*, *PHOTB1*, and *PHOTB2* were expressed under both light and dark conditions (Fig. 3). By contrast, *PHOTA1* expression was induced by blue and red light but was drastically reduced under dark condition (Fig. 3).

Generation of Disruptants of *PHOT* Genes

Four single disruptants (*photA1*, *photA2*, *photB1*, and *photB2*), two double disruptants (*photA1photA2* and *photB1photB2*), and one triple disruptant (*photA2photB1photB2*) were generated by homologous recombination.

Single Disruptants

A fragment of each *PHOT* gene was amplified from the genomic DNA, cloned into a plasmid, and then the cloned *PHOT* genes were digested with appropriate restriction enzymes. The excised fragments were replaced by the *nptII* gene as a selection marker. After transformation of *P. patens* protoplasts, regenerated G418-resistant plants were screened by PCR using primers flanking the *nptII* cassette to find gene disruptants by homologous recombination events (Schaefer, 2001). From gene disruptants one DNA fragment having the expected size of the *nptII* cassette

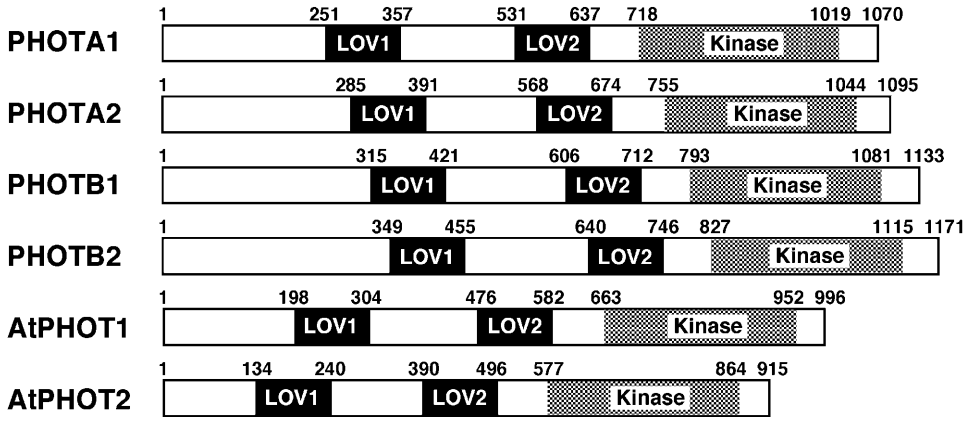

Figure 1. Domain organization of *P. patens* and Arabidopsis phototropins. Two LOV domains, LOV1 and LOV2, and Ser/Thr kinase domains are indicated. Amino acids of the start and the end of each domain are numbered.

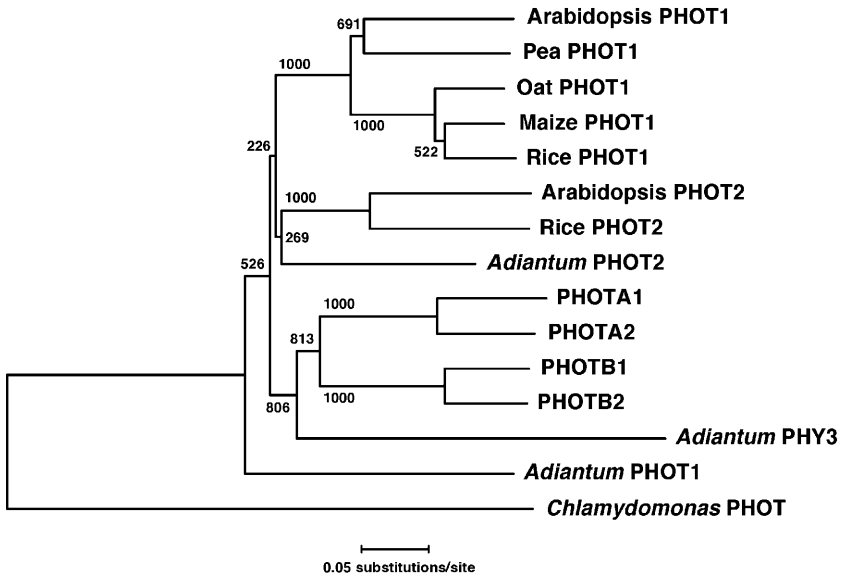

Figure 2. Phylogenetic tree of phototropins. The multiple sequence alignment and phylogenetic analysis were carried out using ClustalW program. The conserved domains in phototropins, i.e. LOV1, LOV2, and Ser/Thr protein kinase domains in Figure 1, were used for the phylogenetic analysis. Bootstrap values are indicated at the nodes.

plus the flanking sequences of *PHOT* gene was amplified, but a wild-type copy of the *PHOT* gene was also amplified from mutants in which *nptII* cassette was additionally integrated randomly (data not shown). Southern-blot analysis of genomic DNA from the gene disruptants was performed with *nptII* gene as a probe to survey whether multiple targeting constructs might be integrated into the genome. Numbers of DNA fragments hybridized by the probe are shown in Table I. Not only single but also multiple integration occurred into the genome. Expression of *PHOT* genes in representative mutants by reverse transcription (RT)-PCR was analyzed to confirm that expected genes were targeted (Fig. 4).

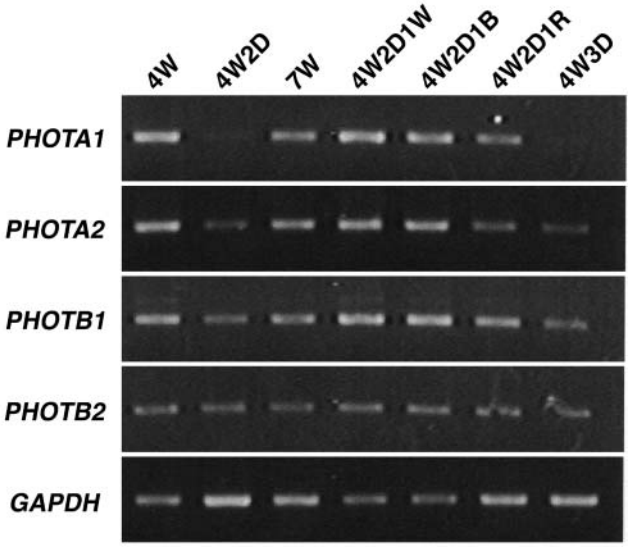

Figure 3. Expression of *PHOT* genes under different light conditions. Protonemata of *P. patens* were given different light treatments as described below: 4W, white light for 4 d; 4W2D, white light for 4 d and dark for 2 d; 7W, white light for 7 d; 4W2D1W, white light for 4 d, dark for 2 d, and white light for 1 d; 4W2D1B, white light for 4 d, dark for 2 d, and blue light for 1 d; 4W2D1R, white light for 4 d, dark for 2 d, and red light for 1 d; 4W3D, white light for 4 d and dark for 3 d. The light intensities were 20 W m^{-2} (approximately 80 μmol m^{-2} s^{-1}) for white light, 4 W m^{-2} (approximately 16 μmol m^{-2} s^{-1}) for blue light, and 5 W m^{-2} (approximately 28 μmol m^{-2} s^{-1}) for red light. RT-PCR was performed using 5 μg total RNA and specific primers for each *PHOT* gene. Glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was used as an internal control.

Double Disruptants

Double disruptants in the same class of *PHOT* genes were produced. The *photA2-1* and *photB2-1* were transformed with the targeting constructs for *PHOTA1* and *PHOTB1* interrupted by a hygromycin cassette, respectively, resulting in the production of *photA1photA2* and *photB1photB2* double disruptants. After confirming the disruption of target genes by PCR, numbers of DNA fragments integrated into the genome were analyzed by Southern blot using a hygromycin resistance gene as a probe (Table I). Expression of *PHOT* genes in *photA1photA2-1* and *photB1photB2-1* was analyzed by RT-PCR (Fig. 4).

Triple Disruptants

Zeocin was used for antibiotic selection to generate triple disruptants of *P. patens*. Growth of protonemata was reduced or arrested with 50 μg mL^{-1} of Zeocin. However, *P. patens* protonemata containing a Zeocin resistance gene cassette grew normally on the medium with Zeocin, which could be used as a third selection marker in addition to kanamycin and hygromycin. The *photB1photB2-1* mutant, which was chosen as a parental strain because *PHOTB1* and *PHOTB2* were disrupted without random integration (Table I), was transformed with the targeting construct for *PHOTA2*,

Table I. *Phototropin mutants*

Type	Mutants	Antibiotics Resistance[a]	Number of Hybridized Fragments[b]
Single	*photA1-1*	Km	3
	photA2-1	Km	1
	photA2-2	Km	6
	photB1-1	Km	1
	photB1-2	Km	1
	photB2-1	Km	1
	photB2-2	Km	3
Double	*photA1photA2-1*	Km and Hyg	2
	photA1photA2-2	Km and Hyg	2
	photB1photB2-1	Km and Hyg	1
	photB1photB2-2	Km and Hyg	1
Triple	*photA2photB1photB2-1*	Km, Hyg, and Zeo	1
	photA2photB1photB2-2	Km, Hyg, and Zeo	1

[a]Km, kanamycin; Hyg, hygromycin; Zeo, Zeocin. [b]As hybridization probes, *nptII*, *hpt*, and *Zeo* genes were used for single, double, and triple mutants, respectively.

in which the gene was interrupted with the Zeocin cassette. The integration of one copy of the Zeocin cassette into the genome of the resulting triple disruptants, *photA2photB1photB2-1* and *-2*, were confirmed by Southern blot. The expression of *PHOT* genes in *photA2photB1photB2-1* was analyzed by RT-PCR (Fig. 4).

Blue Light-Induced Chloroplast Movement in Basal Cells of Protonemata of Mutants

Chloroplast movement in protonemal cells was analyzed by microbeam irradiation with different fluence rates of blue light. The third or fourth cells from the apical tip cell of protonemal filaments were used for this analysis.

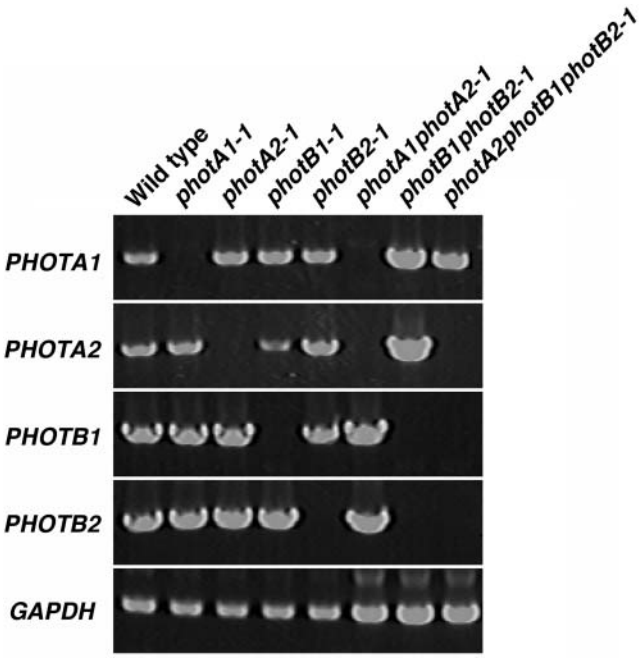

Figure 4. Expression of *PHOT* genes in phototropin mutants. RNA samples were prepared from phototropin mutants grown under white light. RT-PCR was performed using 5 μg total RNA and specific primers for each *PHOT* gene. Glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was used as an internal control.

Wild Type, photA1-1, photB1-1, and photB2-1

As shown in Figure 5A, chloroplasts in a wild-type cell moved toward the area irradiated with weak blue light (seen in the panel at 80 min) and then moved away from the area irradiated with strong blue light (at 120 min). With weak light irradiation, chloroplasts, not only around the light irradiated area but also near the septums at the cell ends, accumulated toward blue light-irradiated area. The fluence-dependent responses of blue light-induced chloroplast movement is summarized in Table II. In wild-type cells, chloroplast accumulation movement was initiated at 0.002 W m^{-2}, the transition point from accumulation movement to avoidance movement was at about 50 W m^{-2}, and avoidance movement was observed at 100 W m^{-2}. The *photA1-1*, *photB1-1*, and *photB2-1* cells showed the same fluence rate response as that obtained in wild-type cells (Table II).

photA2-1 and photA1photA2-1

In *photA2-1* cells, the lowest fluence rate at which accumulation movement was observed was 0.002 W m^{-2}, which was similar to wild-type cells. However, in *photA2-1* cells, chloroplasts stayed at the irradiated area even when light was switched to strong fluence rate (compare the panels at 120 min in Fig. 5, A and B). Unlike wild-type cells, avoidance movement was not induced at any fluence rate of blue light tested (Table II). Expression of *PHOTA2* gene using cauliflower mosaic virus 35S promoter complemented the deficiency of avoidance movement in *photA2-1* (data not shown). The *photA1photA2-1* showed similar phenotype to the *photA2-1* (Table II).

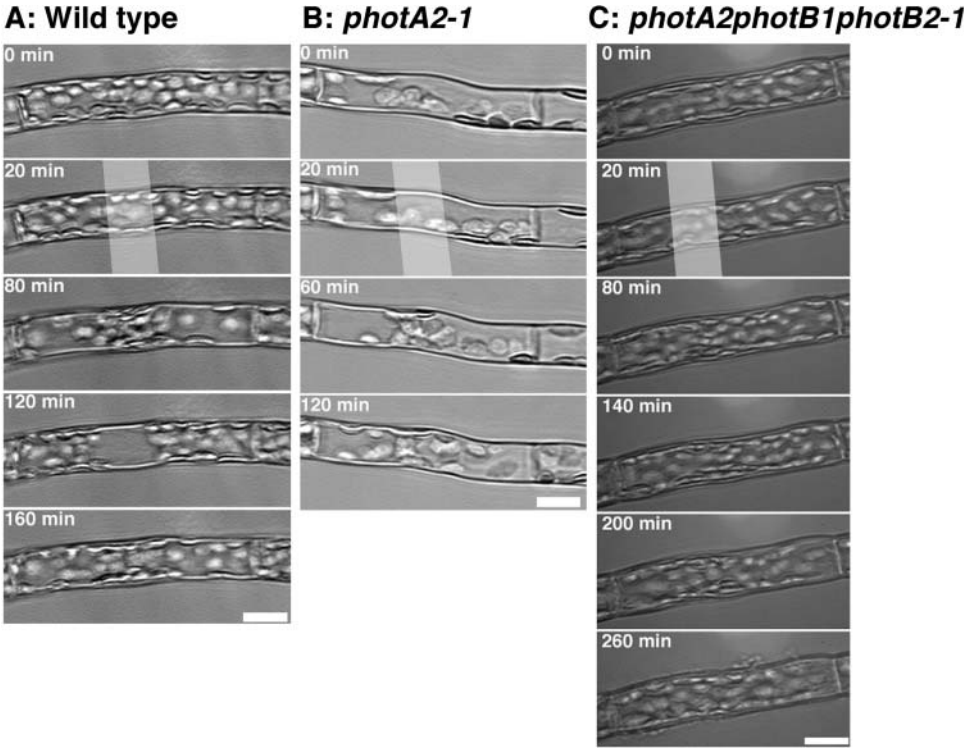

Figure 5. Blue light-induced chloroplast movement in basal cells of wild-type, *photA2-1*, and *photA2-photB1photB2-1* protonemata. A, A wild-type cell was incubated in the dark (0–20 min), irradiated with 0.01 W m^{-2} (20–80 min) and 100 W m^{-2} blue light (80−120 min), and incubated in the dark (120–160 min). B, A *photA2-1* cell was incubated in the dark (0–20 min) and irradiated with 0.01 W m^{-2} blue light (20–60 min) and 100 W m^{-2} blue light (60–120 min). C, A *photA2photB1-photB2-1* cell was incubated in the dark (0–20 min) and irradiated with 0.15 W m^{-2} (20–80 min), 1 W m^{-2} (80–140 min), 100 W m^{-2} (140–200 min), and 200 W m^{-2} blue light (200–260 min). The white bands indicate the position of the microbeam irradiation. Scale bars = 20 μm.

photB1photB2-1

In *photB1photB2-1* the transition point from accumulation movement to avoidance movement was shifted to a value between 100 and 200 W m^{-2} (Table II). This value was several times higher than that of the wild type (50 W m^{-2}).

photA2photB1photB2-1

The threshold required to induce accumulation movement in *photA2photB1photB2-1* was 100 W m^{-2}, which is 50,000-fold higher than that in the wild type (Table II). However, the accumulation movement was very weak and chloroplasts near the septums did not accumulate sufficiently to the light-irradiated area (200 and 260 min in Fig. 5C). Chloroplasts in basal cells hardly responded to blue light following the disruption of three *PHOT* genes. Chloroplast avoidance movement was not induced at any fluence rate of blue light tested.

Heterologous Expression of *PHOTA2* and *PHOTB2* in *Acphot2* Mutant

Kagawa et al. (2004) recently developed an assay system to access the function of phototropins as the photoreceptor for avoidance movement using *A. capillus-veneris phot2* mutants, which are deficient in avoidance movement. To examine whether *P. patens* phototropins could function in the *phot2* mutant,

Table II. *Blue light-induced chloroplast movement in the basal cells of protonemata*

Strain	Fluence Rate of Blue Light (W m^{-2})				
	0.001	0.002	50	100	200
Wild type	–[a]	Ac[b]	Ac or weak Av[c]	Av	Av
photA1-1	–	Ac	Ac or weak Av	Av	
photA2-1	–	Ac	Ac	Ac	Ac
photB1-1	–	Ac	Ac or weak Av	Av	
PhotB2-1	–	Ac	Ac or weak Av	Av	
photA1photA2-1	–	Ac	Ac	Ac	Ac
photB1photB2-1	–	Ac	Ac	Ac	Av
photA2photB1photB2-1	–	–	–	Weak Ac	Weak Ac

[a]–, No response. [b]Ac, Chloroplast accumulation movement. [c]Av, Chloroplast avoidance movement.

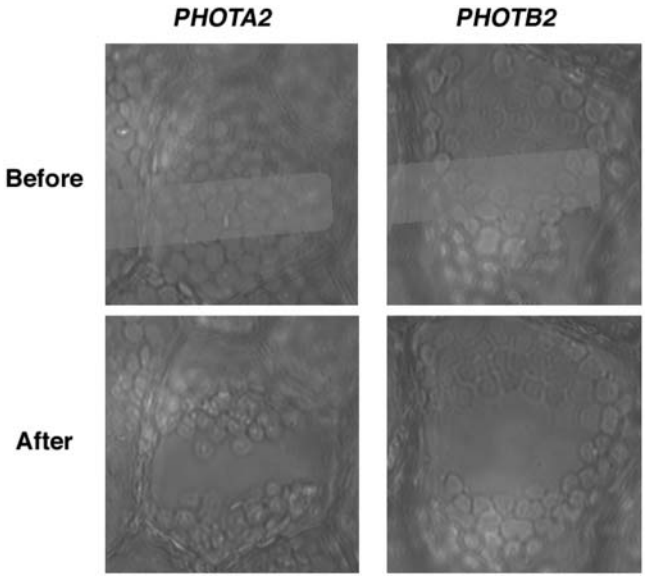

Figure 6. Heterologous expression of *PHOTA2* and *PHOTB2* in *A. capillus-veneris phot2* mutant cells. Prothallia of *A. capillus-veneris phot2* mutants were transformed with cDNA of *PHOTA2* or *PHOTB2* using a particle bombardment delivery system (Kawai et al., 2003; Kagawa et al., 2004) as described in the text. Transformed cells were partially irradiated with a microbeam of 100 W m^{-2} blue light. The micrographs show the transformed cell before and after the irradiation. The white bands indicate the position of the microbeam irradiation.

PHOTA2 and *PHOTB2* were transiently expressed using this system. Prothallial cells of *A. capillus-veneris phot2* mutants were cotransfected with cDNAs of *PHOTA2* or *PHOTB2* and green fluorescent protein (GFP) both driven by cauliflower mosaic virus 35S promoter. GFP was used as a marker to identify transformed cells. Chloroplasts moved away from areas irradiated with strong blue light (Fig. 6), showing that transient expression of *PHOTA2* and *PHOTB2* can complement the deficiency of avoidance movement of *A. capillus-veneris phot2* mutants. *PHOTA2* and *PHOTB2* have the ability to induce avoidance movement in the heterologous system.

Blue Light-Induced Chloroplast Avoidance Movement in the Tip Cells of Protonemata of Mutants

Although basal cells of *photA2-1* and *photA2photB1-photB2-1* protonemata did not exhibit avoidance movement (Table II), the tip cells of these mutants did (Table III). However, avoidance movement did not occur in the tip cells of *photA1photA2-1*.

Red Light-Induced Chloroplast Movement in Basal Cells of Protonemata of Mutants

Red light as well as blue light induced chloroplast movement in protonemata of *P. patens* (Kadota et al., 2000; Sato et al., 2001). Because the induction by red light is canceled by far-red light irradiation, the photoreceptor for red light-induced chloroplast movement is most likely phytochrome (Kadota et al., 2000). In spite of this fact, we examined red light-induced chloroplast movement in phototropin mutants. In wild-type cells, red light at 1 and 30 W m^{-2} induced accumulation and avoidance movements, respectively (Fig. 7; Table IV). The *photA2-1*, *photA1photA2-1*, and *photB1photB2-1* showed normal fluence rate responses (Table IV). However, in *photA2photB1photB2-1*, accumulation movement was not induced at 1 W m^{-2} but was induced at 30 W m^{-2} of red light, the intensity for inducing avoidance movement in wild-type cells (Fig. 7; Table IV). Because the accumulation of chloroplasts was very weak at 30 W m^{-2} (and even at 400 W m^{-2}) of red light, chloroplasts located near the septums did not gather sufficiently around the light-irradiated area (Fig. 7). No avoidance movement occurred under red light in *photA2photB1photB2-1*.

DISCUSSION

Four phototropin genes from the moss *P. patens* were isolated and divided into two groups (*PHOTA* and *PHOTB*) on the basis of the deduced amino acid sequences. This is similar to seed-plant phototropins, which are classified into two groups, PHOT1 and PHOT2 (Briggs et al., 2001). However, the *P. patens* phototropins form a separate clade from PHOT1 and PHOT2 groups in a phylogenetic tree (Fig. 2). Thus, the evolution of two groups of *P. patens* phototropins is likely to be independent of that of PHOT1 and PHOT2 groups of seed-plant phototropins.

It has been reported that phototropins control chloroplast movement in a seed plant, Arabidopsis (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001) and in a fern, *A. capillus-veneris* (Kagawa et al., 2004). The results in this article, using *P. patens phot* mutants, clearly show that chloroplast movements are also mediated by phototropins in this moss. This function of phototropin was conserved in plants.

When chloroplast movement in basal cells of protonemata was analyzed, *photA1-1*, *photB1-1*, and *photB2-1* showed the same fluence rate response as the wild type, whereas *photA2-1* lacked chloroplast avoidance movement at any fluence rate examined (Table II). This result illustrates that chloroplast

Table III. *Blue light-induced avoidance movement in the tip cells of protonemata*

Strain	Avoidance Movement[a]
Wild type	+
photA1-1	+
photA2-1	+
photA1photA2-1	−
photA2photB1photB2-1	+

[a]Chloroplast avoidance movement was analyzed in tip cells of protonemata. Cells were irradiated with blue-light microbeam at 200 W m^{-2}.

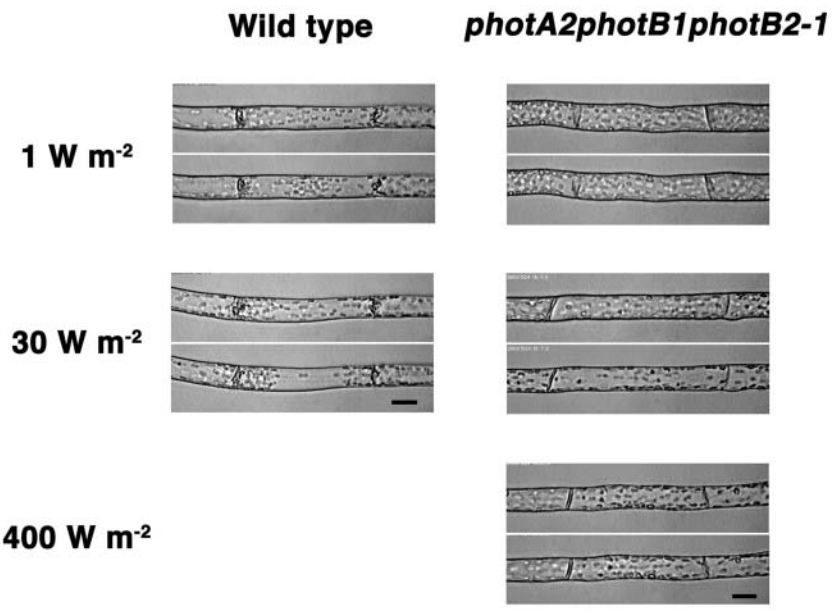

Figure 7. Red light-induced chloroplast movement in basal cells of wild-type and *photA2-photB1photB2-1* protonemata. Each section shows chloroplast distribution in the same cell before (top image) and after (bottom image) irradiation of red light. Fluence rates used (1, 30, and 400 W m^{-2}) are shown. Scale bars = 20 μm.

avoidance movement is predominantly mediated by photA2 in basal cells. On the other hand, although single disruptants of *PHOTB1* and *PHOTB2* showed normal responses, *photB1photB2* double mutants required a higher fluence rate than wild type to induce avoidance movement. photB1 and photB2 redundantly function and contribute, to some extent, to the photoperception for avoidance movement.

In Arabidopsis, phot2 mediates avoidance movement at high fluence rate of blue light, whereas phot1 mediates accumulation movement but not avoidance movement at any light fluence rate (Sakai et al., 2001). Because two classes (A and B classes) of *P. patens* phototropins can mediate avoidance movement, signaling mechanisms of phototropin itself or downstream pathway are likely more similar to those of Arabidopsis phot2. The ancestral origin of *P. patens* phototropins may be the phot2 type of Arabidopsis.

Although *photA2-1* and *photA2photB1photB2-1* were deficient in avoidance movement in basal cells, the response was induced in the tip cells of the same mutants with strong blue-light irradiation (Tables II and III). When examined in the tip cell of *photA1photA2-1*, avoidance movement did not occur. These results indicate that *PHOTA1* contributes to the induction of avoidance movement much more in the tip cells than in the basal cells and might predominantly be expressed in the tip cells of protonemal filaments.

Chloroplast movement is regulated by blue light in most plants, and is regulated by red light (in addition to blue light) in the moss *P. patens*, the fern *A. capillus-veneris*, and the algae *Mougeotia scalaris* (Haupt and Scheuerlein, 1990; Kadota et al., 2000; Sato et al., 2001; Wada and Kagawa, 2001). The photoreceptor controlling the red light-induced chloroplast movement of *A. capillus-veneris* is phy3, a chimera protein consisting of a phytochrome chromophore-binding domain and phototropin (Nozue et al., 1998; Kawai et al., 2003). In *P. patens*, because the red-light induction of chloroplast movement is canceled by far-red light irradiation, the photoreceptor for this response is probably phytochrome (Kadota et al., 2000). Because no phy3-type chimera protein was isolated from *P. patens* in our screening (data not shown), conventional phytochromes, four of which are registered in the GenBank/EBI/DDBJ database and a previous report (Kolukisaoglu et al., 1993), are likely the primary photoreceptor for red light-induced chloroplast movement of *P. patens*.

Unexpectedly, the *photA2photB1photB2-1* was deficient in both red light-induced accumulation and avoidance movements (Table IV). This result indicates that phototropins may be components of signal transduction pathways for the phytochrome-dependent chloroplast movement in *P. patens*. It should be noted that phytochrome genes were not disrupted by random integration of targeting constructs because no randomly integrated DNA fragments were detected in the *photA2photB1photB2-1* (Table I). It is also possible that phototropins could control expression of

Table IV. *Red light-induced chloroplast movement in the basal cell of protonema*

Strain	Fluence Rate of Red Light (W m^{-2})		
	1	30	400
Wild type	Ac[a]	Av[b]	
photA2-1	Ac	Av	
photA1photA2-1	Ac	Av	
photB1photB2-1	Ac	Av	
photA2photB1photB2-1	–[c]	Weak Ac	Weak Ac

[a]Ac, Chloroplast accumulation movement. [b]Av, Chloroplast avoidance movement. [c]–, No response.

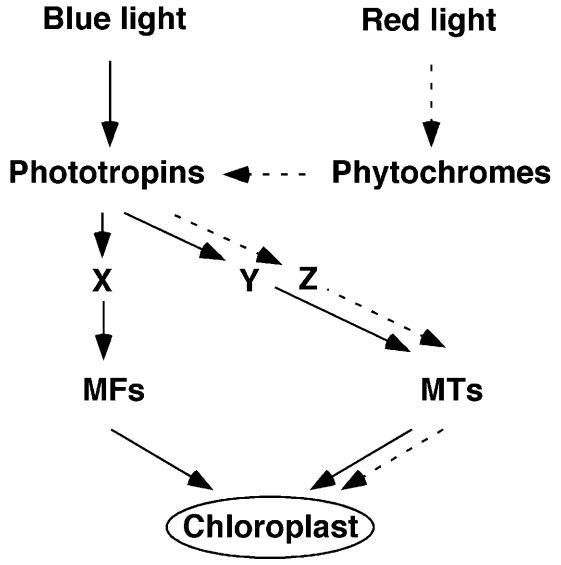

Figure 8. A model for chloroplast movement. Blue and red light-induced signaling pathways are shown in plane and broken lines, respectively. X, Y, Z, Unknown signaling components; MFs, microfilaments; MTs, microtubules.

phytochrome genes. However, the phytochrome-dependent chloroplast movement of *P. patens* takes place in red light-grown cells, but not in white light-grown cells (Kadota et al., 2000), and is enhanced by pretreatment of cells in the dark for 1 d (Sato et al., 2001), suggesting that the phytochrome that mediates red light-induced chloroplast movement predominantly expresses under dark and red-light conditions. Thus, expression of the phytochrome seems to be independent of phototropin. It has also been shown that phototropins play little role in transcriptional regulation in Arabidopsis (Ohgishi et al., 2004). For these reasons, an effect of the phototropin mutations on the expression of phytochrome in *P. patens* is unlikely. In *A. capillus-veneris*, a point mutation in the protein kinase domain of phy3 abolishes red light-induced chloroplast movement, indicating that the red-light signal received by the phytochrome domain is transmitted to downstream signaling components via the phototropin protein kinase domain of phy3 (Kawai et al., 2003). Thus, signaling mechanism, which is intramolecularly performed by phy3 in *A. capillus-veneris*, is performed directly or indirectly with phytochrome and phototropin molecules in *P. patens*. Regardless of light quality, phototropin could be an essential component for light-induced chloroplast movement. It will be interesting to test this in *M. scalaris*.

Sato et al. (2001) demonstrated that the blue light-induced chloroplast movement in *P. patens* utilizes both microfilaments and microtubules (MTs), whereas the red light-induced movement uses only MTs as traveling tracks. Figure 8 represents an updated model of the signaling mechanism of chloroplast movement in *P. patens*. There are two changes from a previous model (Sato et al., 2001): (1) the unidentified blue-light receptors are phototropins, and (2) the red-light signal received by phytochromes is transmitted to MTs system via phototropins (broken lines in Fig. 8). Although there are three pathways downstream of phototropins in this model, signaling components to control MTs system could be common in blue- and red-dependent pathways, that is Y = Z in Figure 8.

There are several reports showing an interaction between the phototropin and phytochrome signaling pathways. Chloroplast movement and phototropism are enhanced by red-light irradiation in Arabidopsis, and phytochromes are involved in both responses (Janoudi et al., 1997; Lasceve et al., 1999; Kagawa and Wada, 2000; DeBlasio et al., 2003). Phytochromes function as modulators because they are not essential for the induction of chloroplast movement and phototropic curvature. They modify the magnitude of the responses. On the other hand, the correlation between phytochromes and phototropins in the signaling pathway of red light-induced chloroplast movement in *P. patens* seems to be different. Because the disruption of phototropins abolishes the phytochrome-induced response, phototropins appear to be essential components, rather than modulators, for transmitting signals in the phytochrome pathway. This involvement of phototropins in phytochrome-signaling pathways is unique.

Chloroplast movement of *P. patens* is regulated by phototropins, like that of Arabidopsis. Availability of homologous recombination technique and easier observation of chloroplast movement because of simple cell organization are advantages for the use of *P. patens* as an experimental material. *P. patens* will be a good system to elucidate molecular mechanisms of chloroplast movement and phototropin signaling.

MATERIALS AND METHODS

Plant Materials

Protonemata of *Physcomitrella patens* subsp. *patens* were cultured aseptically at 26°C on BCDAT medium, which is BCD medium (1 mM $MgSO_4$, 10 mM KNO_3, 45 μM $FeSO_4$, 1.8 mM KH_2PO_4, pH 6.5) supplemented with 1 mM $CaCl_2$, 5 mM ammonium tartrate, and 0.8% (w/v) agar (Nishiyama et al., 2000).

Screening of *P. patens* Phototropin Genes

Partial phototropin fragments were amplified using cDNA prepared from protonemata of *P. patens* and degenerate primers specific to phototropin. Two sets of primer pairs used were F1 (5′-AARTTYATIGGIATGCARGTIGARGT-3′) and R1 (5′-CATYTCRTAIARIARIATICCIARIGCCCACCARTC-3′), or F2 (5′-GAYCCIMGIYTICCIGAYAAYCCIATIATITTYGC-3′) and R2 (5′-TCIGGIGCIATRTAYTCYTCIGTICCIACRAA-3′). A 1.5-kb fragment or a 1.1-kb fragment was amplified by PCR with F1 and R1, or F2 and R2, respectively. Sequencing analyses revealed that both amplified fragments had striking similarity to phototropin genes but were different from each other. We screened cDNA libraries produced from mRNA of *P. patens* protonemata using the PCR fragments as probes.

DNA Cloning, Sequencing, and Analysis

The positive clones were classified into four groups. The representative clones of each group were sequenced using the BigDye terminator sequencing

kit using a DNA sequencer (model 377; Applied Biosystems, Foster City, CA). The 5′ regions of the cDNA sequences were obtained by 5′ RACE method using a kit (Invitrogen, Carlsbad, CA). BLAST search was performed using the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov) to analyze DNA sequences.

RT-PCR

Total RNA was prepared from protonemata with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RT of total RNA was carried out using oligo(dT) as a primer and SuperScript II RT (Invitrogen). PCR was performed using ExTaq polymerase (TaKaRa, Kyoto).

Construction of Phototropin Gene Disruption Vectors

The hygromycin cassette containing the E7133 promoter (Mitsuhara et al., 1996), the hygromycin B phosphotransferase (*hpt*) gene, and the nopaline synthase (*nos*) terminator was excised from pE7133-hpt vector, which was kindly gifted by R. Kofuji (Kanazawa University, Japan), and used as an antibiotics selection marker.

The *Bam*HI (blunted)-*Not*I fragment of pE7133-hpt was cloned into the *Pst*I (blunted)-*Not*I site of pMBL5 (Nishiyama et al., 2000). The resulting vector was named pE7133-nptII. The kanamycin cassette containing the E7133 promoter, the neomycin phosphotransferase II gene (*nptII*), and the 35S terminator of cauliflower mosaic virus (T_{35S}) was excised from the pE7133-*nptII* vector and used as an antibiotics selection marker.

Zeocin resistance gene was amplified by PCR using the primers 5′-ATGGATCCATGGCCAAGTTGACCAGT-3′ (P1) and 5′-AGAGTCCCGCTCAGTCCTGCTCCTCGG-3′ (P2) and pCR-BluntII-TOPO (Invitrogen) as template. T_{35S} was amplified using the primers 5′-GCAGGACTGAGCGGGACTCTGGGGTTC-3′ (P3) and 5′-ATCTCGAGGATCCCCGTCACCGGTG-3′ (P4) and pMBL5 as template. The PCR products of Zeocin resistance gene and T_{35S} were mixed, and PCR was performed using the primers P1 and P4. The resulting PCR product was digested with *Bam*HI and *Xho*I and cloned into *Bam*HI-*Xho*I site of pE7133-hpt. The resulting plasmid pE7133-Zeo contains the E7133 promoter, Zeocin resistance gene, and T_{35S}.

PHOTA1 targeting vector. The genomic DNA fragment of *PHOTA1* was amplified using the primers 5′-ACGCAATGGTTGTTGAACTCTTC-3′ and 5′-GGATGATTCTTGATGTCGTTTGC-3′ and cloned into a vector, pGEM-T Easy (Promega, Madison, WI). The resulting vector was named pGEM-gPHOTA1. The kanamycin and the hygromycin cassettes were blunted and inserted into the blunted *Kpn*I-*Xho*I site of the pGEM-gPHOTA1, and the resulting vectors were named pGEM-gPHOTA1-Km and pGEM-gPHOTA1-Hyg, respectively.

PHOTA2 targeting vector. The genomic DNA fragment of *PHOTA2* was amplified using the primers 5′-GGACGAATTTGGGAGAGTGAGTT-3′ and 5′-TGTTTTCTGGCTTCAGGTCTCTG-3′ and cloned into a vector, pGEM-T Easy (Promega). The resulting vector was named pGEM-gPHOTA2. The kanamycin and the Zeocin cassettes were blunted and inserted into the *Sma*I-*Eco*RV site of the pGEM-gPHOTA2, and the resulting vectors were named pGEM-gPHOTA2-Km and pGEM-gPHOTA2-Zeo, respectively.

PHOTB1 targeting vector. The genomic DNA fragment of *PHOTB1* was amplified using the primers 5′-CTACATTTGCAAGCAACGAGGAC-3′ and 5′-ACGAGACAAATGACTGCGAAAAA-3′ and cloned into a vector, pGEM-T Easy (Promega). The resulting vector was named pGEM-gPHOTB1. The kanamycin and the hygromycin cassettes were blunted and inserted into the blunted *Hpa*I-*Bgl*II site of the pGEM-gPHOTB1, and the resulting vectors were named pGEM-gPHOTB1-Km and pGEM-gPHOTB1-Hyg, respectively.

PHOTB2 targeting vector. The genomic DNA fragment of *PHOTB2* was amplified using the primers 5′-TGATGTTTGACTTTGGTGTGGTG-3′ and 5′-GGGTCCCTACAAAACCACACATT-3′ and cloned into a vector, pGEM-T Easy (Promega). The resulting vector was named pGEM-gPHOTB2. The kanamycin cassette was blunted and inserted into the blunted *Eco*RV-*Mun*I site of the pGEM-gPHOTB2.

Transformation of Moss Protoplasts

Isolation of protoplasts and polyethylene glycol-mediated transformation were performed according to Nishiyama et al. (2000). To obtain stable transformants, in which single phototropin gene is disrupted, all phototropin targeting vectors containing the kanamycin resistant cassette were digested with *Not*I to separate its insert fragment from the vector sequences, and the digested plasmids were introduced into protoplasts. Transformed protoplasts were incubated for 4 to 5 d on BCDAT medium supplemented with 8% (w/v) mannitol without antibiotics and then transformed to BCDAT medium containing 50 μg mL^{-1} of G418 sulfate (Geneticin; Invitrogen) for 3 weeks.

To obtain double phototropin gene disrupted mutants, pGEM-gPHOTA1-Hyg and pGEM-gPHOTB1-Hyg were digested with *Not*I, and the digested plasmids were introduced into protoplasts prepared from *photA2-1* or *photB2-1* mutants, respectively. Transformed protoplasts were cultured as described above except for using 30 μg mL^{-1} of hygromycin B for antibiotic selection.

To obtain triple phototropin gene disrupted mutants, pGEM-gPHOTA2-Zeo was digested with *Not*I, and the digested plasmid was introduced into protoplasts prepared from *photB1photB2*-1 mutants. Transformed protoplasts were cultured as described above except for using 50 μg mL^{-1} of Zeocin (Invitrogen) for antibiotic selection.

Assay of Chloroplast Movement

Protonemal cells were inoculated between two layers of agar-gelatin film on a coverslip. The film was made from 0.5% (w/v) agar and 0.05% (w/v) gelatin. They were cultured under continuous dim red light for 1 to 2 weeks in the liquid BCDAT medium (Sato et al., 2001).

For partial irradiation of individual cells, a microbeam irradiation system (Olympus BX50; Tokyo) or one previously described (Kadota et al., 2000) were used. Monochromatic blue and red light was obtained through interference filters (Vacuum Optics of Japan, Tokyo), which have their transmission peaks at 452.4 and 663.2 nm and half-band widths of 23.3 and 32 nm, respectively. Neutral density filters of ND50, ND25, ND13, and ND3 (Hoya, Tokyo) were used when necessary. Fluence rate of the microbeam was measured using a siliconphotodiode (S1227-66BR; Hamamatsu Photonics K. K., Hamamatsu, Japan). One W m^{-2} of blue- and red-light microbeams used in this experiment are approximately 3.8 and 5.5 μmol m^{-2} s^{-1}, respectively. Chloroplast movement induced by microbeam irradiation was monitored and recorded using an IR-sensitive video camera (C2400-07ER; Hamamatsu Photonics K. K.), a Macintosh computer (Power Macintosh 8600; Apple Japan, Tokyo), and the public domain NIH Image program (developed at the United States National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) as described in Kagawa et al. (2004).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AB163420, AB163421, AB163422, and AB163423.

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