Plant Cell Physiol. 45(4): 416–426 (2004)
JSPP © 2004

Function Analysis of Phototropin2 using Fern Mutants Deficient in Blue Light-Induced Chloroplast Avoidance Movement

Takatoshi Kagawa [1,2,3,6,7], Masahiro Kasahara [3,8], Tomoko Abe [4], Shigeo Yoshida [4] and Masamitsu Wada [3,5]

[1] *SORST, Japan Science and Technology Corporation, 1-8, Honcho 4-chome, Kawaguchi, 332-0012 Japan*
[2] *PRESTO, Japan Science and Technology Corporation, 1-8, Honcho 4-chome, Kawaguchi, 332-0012 Japan*
[3] *Division of Biological Regulation and Photobiology, National Institute for Basic Biology, Okazaki, 444-8585 Japan*
[4] *RIKEN Institute, Hirosawa 2-1, Wako, Saitama, Japan*
[5] *Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Hachioji, 192-0397 Japan*

Gametophytes of the fern *Adiantum capillus-veneris* L. were mutagenized by heavy ion beam irradiation and screened for mutants lacking chloroplast avoidance movement under high intensity blue light. Mutants recovered include several with small deletions in the *AcPHOT2* gene. The avoidance movement response in these mutants could be restored by transient expression of non-mutant *AcPHOT2* cDNA, indicating that the chloroplast avoidance movement in this fern is mediated by the *Ac*phot2 protein. Further functional analyses of the *Ac*phot2 protein were performed using this transient assay for chloroplast avoidance movement. The results obtained suggest that the LOV2, but not the LOV1, domain of *Ac*phot2 is essential for avoidance movement, and that several residues in the C-terminus of the kinase domain contribute to the avoidance response. The rate of dark reversion of the photo-activated LOV2 domain, which was calculated photometrically, was too fast to account for the lifetime of phot2 signal estimated from physiological responses. However, the rate of dark reversion of the combined domains of LOV1 and LOV2 did correspond to the lifetime of the signal, suggesting that LOV1 might have some function in this response, although it is not essential for playing a role as a photoreceptor.

Keywords: Blue light — Chloroplast movement — Fern (*Adiantum capillus-veneris*) — LOV domain — Phototropin.

Abbreviations: B, blue light; BHC, blue high light-dependent chloroplast movement; GFP, green fluorescent protein; IR, infrared light; R, red light; FR, far-red light.

Introduction

Plants use light as a cue to perceive and adapt to environmental changes. The photoreceptors responsible for detecting the different visible wavelengths of light have been isolated and their roles analyzed using photo-response mutants, mostly in the model plant *Arabidopsis thaliana* L. Of these, the phytochromes, comprising five molecular species (phyA through phyE) in *A. thaliana*, control such responses as seed germination, shoot growth, seedling petiole growth, and flowering time, by absorption of red light (R) or far-red light (FR) (see a review of Smith 2000). The blue light (B) responses are mediated by cryptochromes (cry1 and cry2) and phototropins (phot1 and phot2). Cryptochromes mediate floral initiation and hypocotyl growth (Cashmore et al. 1999, Lin 2000), while the phototropins regulate phototropic responses, stomata opening, chloroplast photo-relocation movement, and leaf extension (Huala et al. 1997, Kagawa et al. 2001, Sakai et al. 2001, Jarillo et al. 2001, Kinoshita et al. 2001, Kagawa and Wada 2002, Kagawa 2003). Orthologs of these photoreceptors were readily isolated in other plants but few mutants have been isolated, except from a few plant species, such as tomato (Van Tuinen et al. 1995, Weller et al. 2001) and rice (Takano et al. 2001).

Chloroplast positioning is dependent upon the ambient light conditions (Wada and Kagawa 2001, Kagawa and Wada 2002, Wada et al. 2003). In *A. thaliana*, the accumulation response by which chloroplasts gather towards weak light, is mediated by both phot1 and phot2 (Sakai et al. 2001), but the avoidance response, where chloroplasts move away from areas illuminated with high intensity light, is mediated by phot2 alone (Kagawa et al. 2001). The avoidance response allows chloroplasts to escape high intensity light stress (Kasahara et al. 2002a), and concomitant photosystem damage.

The organization of fern gametophytes is very simple compared to the tissues of seed plants. Since protonemata and prothallia develop in 1- and 2-dimensions, respectively, and are not surrounded by any other tissues, precise analyses of physiological responses at the cellular level are straightforward. In gametophytes of the fern *Adiantum capillus-veneris* L., photomorphogenesis, including light-induced chloroplast relocation movement, has been studied in detail (Wada and Sugai 1995, Yatsuhashi et al. 1985, Kagawa and Wada 1996, Kagawa and Wada 1999). Several photoreceptor orthologs, phytochromes 1 to 4 (Okamoto et al. 1993, Nozue et al. 1997, Nozue et al. 1998a, Nozue et al. 1998b), cryptochromes 1 to 5 (Kanegae and Wada 1995, Imaizumi et al. 2000) and phototropin

[6] Present address: Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, 305-8572 Japan.
[7] Corresponding author: E-mail, kagawa@agbi.tsukuba.ac.jp; Fax, +81-29-853-6270.
[8] Present address: Gene Research Center, Tokyo University of Agriculture and Technology, Fuchu, 183-8509 Japan.

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024

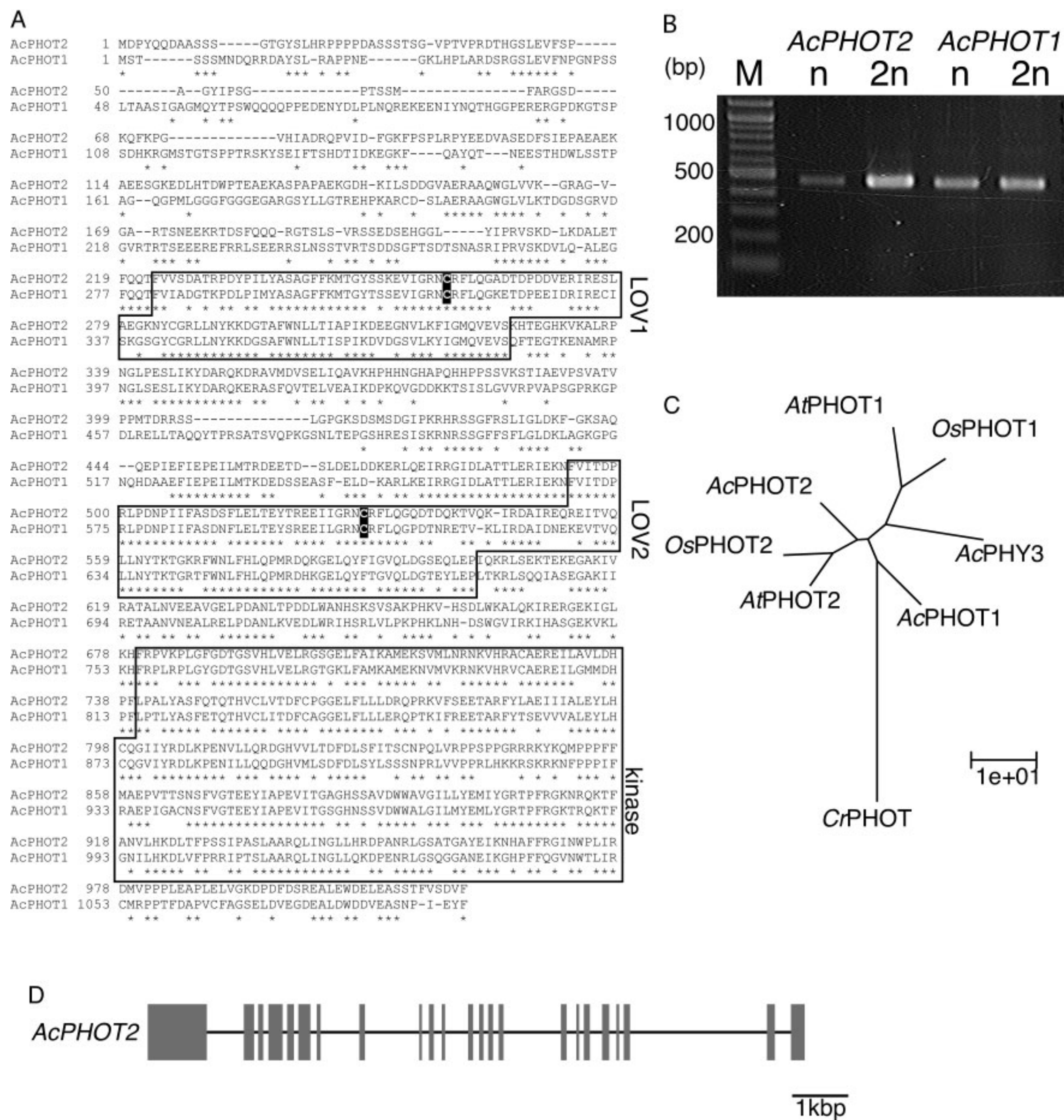


Fig. 1 Cloning of the phot2 gene in *Adiantum capillus-veneris*. (A) Comparison of the predicted *Ac*phot1 (formerly *Ac*nph1) and *Ac*phot2 amino acid sequences. Boxes show LOV1, LOV2 and kinase domains. Identical residues are indicated by asterisks (*) and FMN binding cysteines (C) in the LOV1 and LOV2 domains are shaded in black. (B) Expression of *Ac*PHOT2 and *Ac*PHOT1 genes in prothallia (n) and sporophytes (2n). M, 100 bp DNA ladder maker. (C) A phylogenic tree of the phototropins. Full-length rice *Os*PHOT1 (accession no. BAA84780), *Os*PHOT2 (accession no. BAA84779), Arabidopsis *At*PHOT1 (accession no. AAC01753), *At*PHOT2 (accession no. AAC27293), *Chlamydomonas Cr*PHOT (accession no. CAC94941), and *Ac*PHOT1 (accession no. BAA95669) were used to construct the tree. Only the phototropin region was used for *Ac*PHY3 (accession no. T30891). (D) Genomic structure of *Ac*PHOT2. Boxes represent predicted exons (23 exons in total) and lines between boxes represent introns (22 introns in total).

*Ac*NPH1 (Nozue et al. 2000), were isolated from *A. capillus-veneris*.

Previous physiological studies have revealed that R as well as B induces chloroplast accumulation movement in both gametophytes and sporophytes (Yatsuhashi et al. 1985, Augustynowicz and Gabrys 1999, Kawai et al. 2003), unlike in *A. thaliana* in which only B is effective (Trojan and Gabrys 1996, Kagawa and Wada 2000). Chloroplast avoidance movement in *A. capillus-veneris* is observed under more than 10 Wm^{-2} B irradiation or under very strong R with more than 100 Wm^{-2} (Yatsuhashi et al. 1985), although the avoidance in *A. thaliana* is observed only under strong B with more than 8 Wm^{-2} (Kagawa and Wada 2000). Recently, in *A. capillus-veneris rap* (*r*ed light *ap*hototropic) mutants deficient in R-induced phototropic responses of both chloroplast accumulation and avoidance movements were isolated (Kadota and

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024

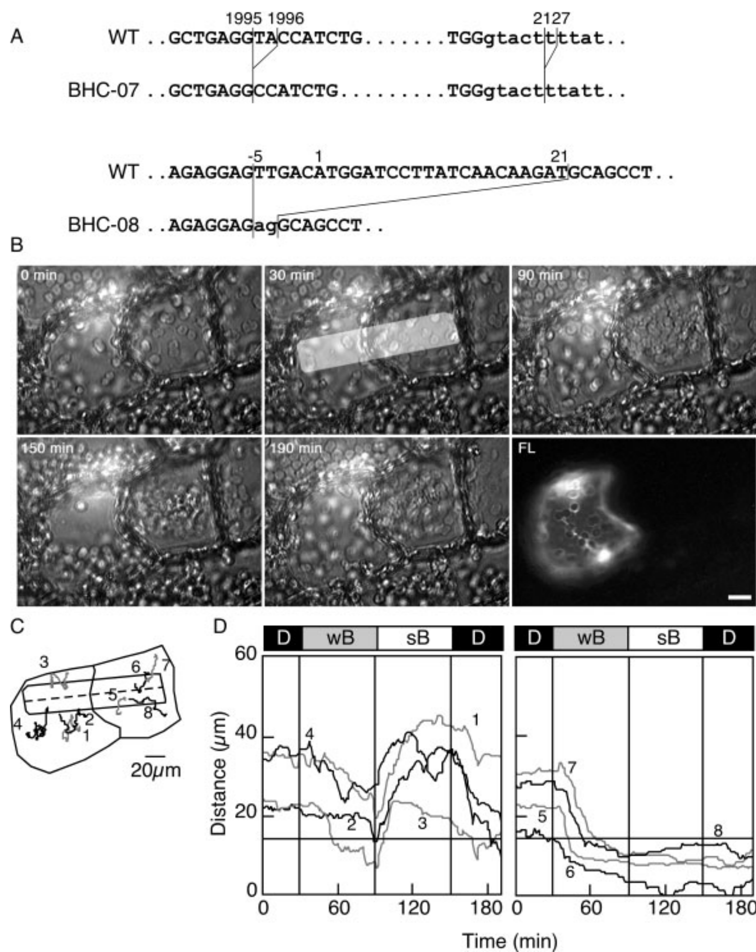


Fig. 2 Analyses of BHC-07 and BHC-08 mutants. (A) Parts of the genomic sequences encoding BHC-07 and -08 are shown. The BHC-07 mutant has two regions containing nucleotide deletions T and A at 1995 and 1996, respectively, from A of start codon in the 4th exon, and T in the 4th intron. The BHC-08 mutant has a deletion of 26 nucleotide (–5 to 21) and 2 extra nucleotides (ag) in the first exon. (B–D) Complementation test using transient gene expression in BHC-08. (B) Sequential photographs of chloroplast movement. Prothallia were co-transformed with cDNA of *AcPHOT2* and GFP using the particle bombardment delivery system and kept for 2 d in the dark (panel 0 min). The neighboring cells with (FL) and without GFP-fluorescence were partially irradiated with a microbeam (panel 30 min) of weak blue light (1 Wm^{-2}) for 60 min and then high intensity blue light (15 Wm^{-2}, panel 90 min) for 60 min (panel 150 min). Finally, the cells were kept in the dark for 40 min (panel 190 min). At panel 150 min, the avoidance response was clearly seen in the transformed cell, but chloroplast accumulation occurred rather than the avoidance response in the non-transformed cell. (C) Tracks of chloroplast movements in the same cells as (B). Each chloroplast was traced from times 0 to 190 min. Positions of the chloroplasts were plotted at 1-min intervals. The rectangle in the drawings shows the position of the irradiated area in the cells and the broken line represents the center of the longitudinal axis of the rectangle. (D) Time courses of changes of the shortest distance between each chloroplast and the broken central line (in C) of the irradiated area of the cells with (left panel) and without (right panel) GFP fluorescence. The schedule of light treatment is shown at the top of the panels. It is clear that the transformed cell on the left-hand side shows the avoidance response in the irradiated area during high intensity light irradiation, but the non-transformed cell on the right-hand side shows accumulation of chloroplasts even in high light. D, darkness; wB, low intensity blue light; sB, high intensity blue light.

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024

Wada 1999). *Ac*PHY3, a chimeric protein comprising phytochrome and phototropin sequences was found to be responsible for these phenomena (Kawai et al. 2003). However, the photoreceptor(s) mediating B-induced chloroplast movement has not yet been identified.

Here, we describe isolation of mutants deficient in the chloroplast avoidance response under high intensity B in *A. capillus-veneris* and show that the gene mediating the response is an ortholog of the *A. thaliana PHOT2* photoreceptor gene. Moreover, functional analyses of the photoreceptor were performed to identify the domains and amino acids critical for function.

Results

Mutant screening and characterization

Mutants deficient in the chloroplast avoidance response were isolated from spores irradiated with a heavy nitrogen ion beam. Protonemata grown lineally under polarized R on an agar medium were irradiated with weak white light for a few days to allow them to become 2-dimensional and for chloroplasts to gather to the cell surface, and then irradiated with high intensity white light supplied from above to induce the avoidance response. Wild-type cells showed the chloroplast avoidance response but mutant cells did not, so that mutants deficient in *b*lue *h*igh light-dependent *c*hloroplast movement were easily observed and isolated. Eleven candidates of the mutants were selected from about 20,000 protonemata as the first screening. Avoidance response of these eleven mutants was observed precisely by microbeam irradiation with B of more than 100 Wm^{-2} (data not shown) and two mutants with strong phenotypes (BHC-07 and -08) were isolated.

Phototropin cloning

Chloroplast avoidance movement was not observed in BHC-07 and -08 gametophytes, like the *phot2* mutant plants of *A. thaliana* (Kagawa et al. 2001), suggesting a possibility that these mutants are deficient in *PHOT2*. Hence, RT-PCR with

degenerate primers which were designed at phototropin LOV2 and kinase consensus regions, was performed using sporophyte RNA extracted from young leaves of wild-type *A. capillus-veneris*. The reason why we used sporophyte RNA as a template was that only *AcNPH1* and *AcPHY3* had been isolated, but no *PHOT2* related genes, in former experiments using gametophyte RNA (Nozue et al. 2000). Three fragments with different sequences were isolated in this analysis: *AcPHY3*, *AcNPH1,* and an additional novel sequence. The full-length cDNA of the novel sequence, which was isolated by 5′, 3′ RACE and RT-PCR using gene-specific primers, has a deduced amino acid sequence that contains LOV1, LOV2 and kinase domains, suggesting that the protein is an ortholog of higher plant phototropins (Fig. 1A). The novel and *AcNPH1* genes were expressed both in sporophytes and gametophytes (Fig. 1B). A phylogenic tree drawn using full-length phototropin sequences from several plant species suggests that the novel gene is closer to *AtPHOT2* than to *AtPHOT1* (Fig. 1C). Based on these results, we named the novel gene *AcPHOT2* and renamed *AcNPH1* as *AcPHOT1*. The *AcPHOT2* genomic DNA of 11.4 kbp, which was obtained by PCR based cloning, comprises 23 exons and 22 introns (Fig. 1D). All exons, except the 10th, are located in the same position as those of *AtPHOT2*. While these exons exists in a similar position, the deduced amino acid residues of the 10th exon in *AcPHOT2* and *AtPHOT2* do differ in identity.

AcPHOT2 mutant gene analysis and rescue

Since a mutation in a phototropin gene was expected, the *AcPHOT2* genes in the BCH-07 and -08 mutants were sequenced to determine the nature of the lesion. The *AcPHOT2* gene in BHC-07 had deletions of two nucleotides (TA) in the 4th exon and one nucleotide (T) in the 4th intron. The BHC-08 mutant had a deletion of 26 nucleotides (TT...AT) and an addition of two nucleotides (AG) in the first exon (Fig. 2A), resulting in loss of the start codon. Since it is still possible that the mutant plants have other mutation(s) located in different genes, a complementation test with *AcPHOT2* cDNA was performed.

As complete genetic analysis or stable transformation are not currently possible in *A. capillus-veneris*, transient assays were performed using a bombardment delivery system (Kawai et al. 2003). When prothallia of BHC-08 were co-transfected with cDNA of *AcPHOT2* and cauliflower mosaic virus 35S promoter driven green fluorescent protein (GFP), the cells with GFP fluorescence showed an avoidance response under strong B (15 Wm^{-2}), but the neighboring cells without GFP florescence showed chloroplast accumulation but not the avoidance response under the same irradiation (Fig. 2B–D). The same results were obtained in BHC-07 cells (Fig. 3). Under weak B irradiation of 1 Wm^{-2}, however, chloroplasts in the cells with GFP fluorescence were less efficient at gathering towards the irradiated area compared to the chloroplasts in the cells without the fluorescence (Fig. 2D). It is likely that over-expression of phot2 increased the sensitivity of the transformed cell to B,

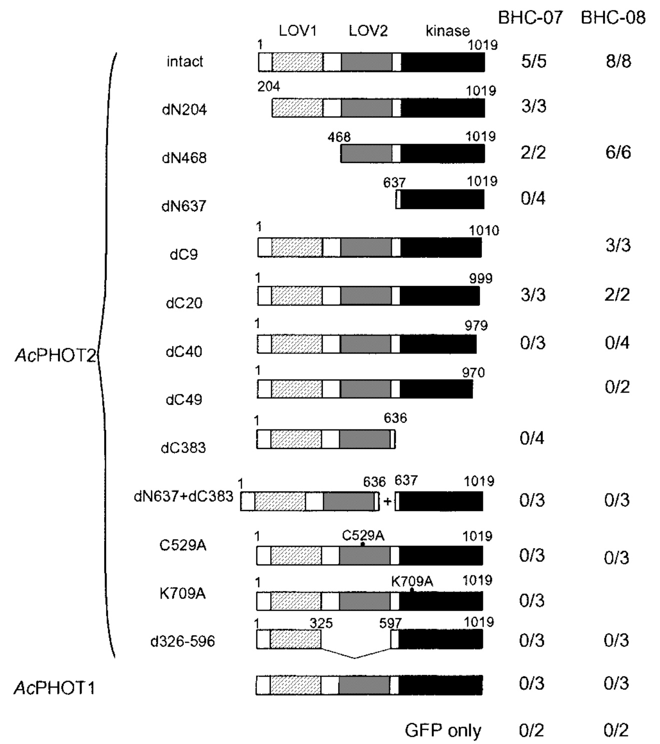


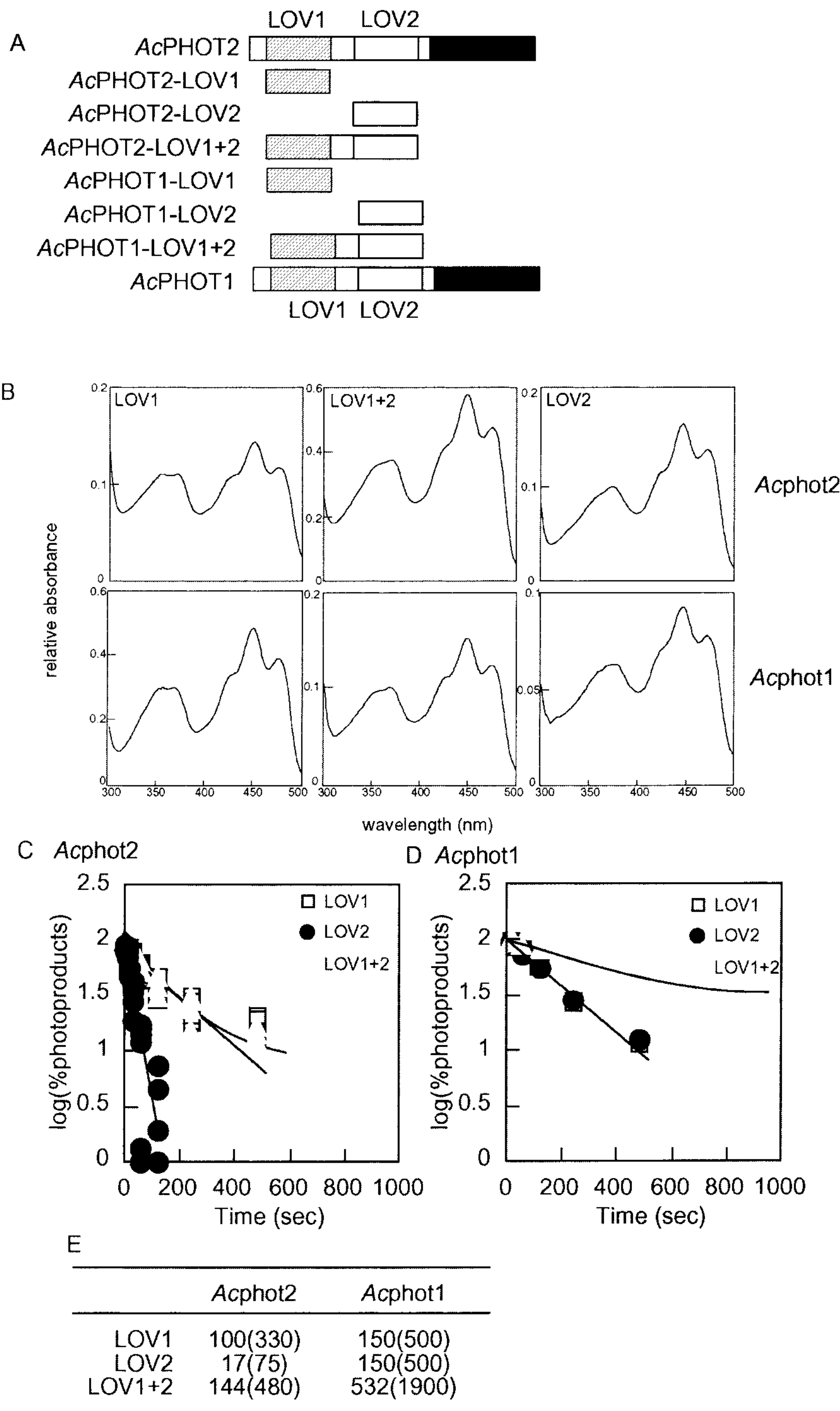
Fig. 3 Functional analyses of *Ac*PHOT2 sequence using transient expression of deletion constructs of *Ac*PHOT2 cDNA introduced into BHC mutant cells. Two adjacent cells with or without GFP-fluorescence were irradiated continuously with a microbeam of 15 Wm^{-2} B. Data are shown as (number of cells showing avoidance movement)/(number of cells tested). The dN637 + dC383 notation indicates that parts of LOV1 and LOV2 domains and the kinase domain were expressed independently. The d326–596 notation indicated that part of the LOV2 domain was deleted and the LOV1 and kinase domains were bound and expressed. The C529A notation indicated that the FMN binding cysteine was changed to an alanine residue.

so that even 1 Wm^{-2} of B was high enough to induce the avoidance response. The cells having GFP-fluorescence as a consequence of co-transformation showed only accumulation movement and no avoidance movement under strong B (Fig. 3). Taken together, these data confirm that lesions in BHC-07 and -08 reside in *AcPHOT2*.

Functional analysis of Acphot2

In order to elucidate the functional significance of particular domains in *Ac*phot2, transient expression analysis experiments were performed. First, a series of N-terminal deletions was tested (Fig. 3). When a modified *Ac*phot2 with a deletion of 204 amino acid residues from the N-terminus (dN204) or a deletion including the whole LOV1 domain from the N-terminus (dN468) was expressed in mutant cells, the modified *Ac*phot2 was still functional for the avoidance movement under B at 15 Wm^{-2}. However, without both LOV1 and LOV2 domains (dN637), *Ac*phot2 was no longer functional for the avoidance movement response. Next, a C-terminal deletion

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024



	*Ac*phot2	*Ac*phot1
LOV1	100(330)	150(500)
LOV2	17(75)	150(500)
LOV1+2	144(480)	532(1900)

Fig. 4 Spectral properties of the phototropin LOV domains. (A) Schematic representation of LOV domains used for analyses of the spectral properties of *Ac*phot2 and *Ac*phot1. These domains were expressed as calmodulin-binding peptide fusion proteins in *E. coli*. Full-length *Ac*PHOT1 and *Ac*PHOT2 are shown for comparison, but were not expressed in *E. coli*. (B) Absorption spectra of the LOV domains. Each peptide was purified using calmodulin binding resin and the absorption spectra were measured at a scan velocity of 500 nm min^{-1}. (C) The speed of dark reversion of light-activated LOV domains of *Ac*phot2. The square of regression coefficient of each curve was more than 0.9. (D) The speed of dark reversion of light-activated LOV domains of *Ac*phot1. The square of regression coefficient of each curve was more than 0.95. (E) Lifetimes of light-activated LOV domains. Half-lives and times for decreasing to the level of 10% photoproducts (in parentheses), which were calculated from the fitted curves of C and D, are shown.

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024

series revealed that deletions of less than 20 residues (dC9 and dC20) from the C-terminus did not affect *Ac*phot2 function, but deletions of more than 40 residues (dC40, dC49 and dC383) lost *Ac*phot2 function. Even when both LOV domains and the kinase domain were expressed separately (dN637+dC383) in the same cell, the BHC-07 and -08 mutants could not be rescued.

When Cys_{529} in the LOV2 domain (the residue that becomes a Cys-adduct with FMN after blue light irradiation as the primary photoresponse) or Lys_{709} in the kinase domain (one of the conserved residues in Ser/Thr kinases) were changed to alanine [(C529A) or (K709A), respectively], transient expression could not rescue *Ac*phot2 function. *Ac*phot2 mutants lacking the LOV2 domain (d326–596), with the LOV1 and kinase

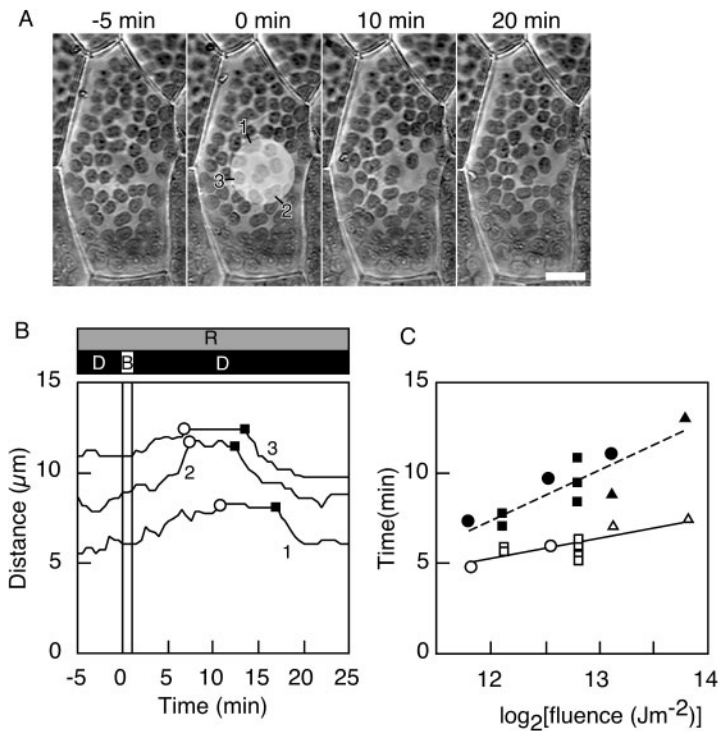


Fig. 5 Estimation of lifetimes of the signals for chloroplast avoidance movement analyzed by physiological responses. (A) Sequential photographs of chloroplast avoidance movement induced by microbeam irradiation (30 μm in diameter) of blue light (30 Wm^{-2}) for a short period (60 s). Bar = 20 μm. (B) Movement of the numbered individual chloroplasts in (A). Numbers of chloroplasts correspond to the ones shown in the photograph at 0 min in A. Open circle and closed square indicate the timing of stop and re-start of movement for each chloroplast, respectively. The schedule of light treatments is shown at the top of the panel. Red light was given continuously as background illumination. D, darkness; B, blue light; R, red light (30 Wm^{-2}). (C) The relationship between fluence irradiated with blue light of 150–600 Wm^{-2} for 15 s (circles), 30 s (squares) and 60 s (triangles), and timing of cessation of movement (open symbol) and re-start (closed symbol) of movements of each chloroplast. Half-lifetimes of the signal for chloroplast avoidance movement can be estimated from the slope of this graph (see Material and Methods and Fig. 7 for details). The half-lifetimes of the signal estimated from the data (B) of timing of stop and re-start of movement and the formulae described in Material and Methods are 1.12 (Time = 1.12 (log_2(fluence)) – 0.82) and 2.79 min (Time = 2.79 (log_2(fluence)) – 2.61), respectively.

domains connected directly, did not function either. Even when *Ac*phot1 cDNA driven by the cauliflower mosaic virus 35S promoter was expressed in mutant cells, chloroplast avoidance movement was not observed under irradiation with 15 Wm^{-2} B.

Together, these results indicate that LOV2 is more important than LOV1 for photoperception leading to B-induced chloroplast avoidance movement, and that the kinase domain must be connected directly to the LOV2 domain to function.

Photochemical analysis of LOV domains

As it is plausible that the photochemical status of the LOV domains may correlate with the physiological responses, we studied the photochemical features of LOV domains in *Ac*phot2 and *Ac*phot1. Recombinant LOV domains, such as LOV1, LOV2 and LOV1+LOV2 of *Ac*phot2 and *Ac*phot1, respectively, were expressed as a calmodulin binding peptide fusion protein in *E. coli* and the absorption spectra of the purified LOV domains were measured as described elsewhere (Kasahara et al. 2002b, Fig. 4). All absorption spectra measured had sharp peaks at 470 and 450 nm, a shoulder at 425 nm and a broad peak around 365 nm, but the LOV1 domains of *Ac*phot2 and *Ac*phot1 have an additional shoulder at 350 nm (Fig. 4B). These results suggested that all recombinant LOV domains tested could absorb B, consistent with their potential functions within blue light photoreceptors.

The rates of dark reversion of photoactivated LOV domains were measured using a fluorescence spectrophotometer by methods developed previously (Kasahara et al. 2002b). As shown in Fig. 4C, the dark reversion of *Ac*phot2-LOV2 ($T_{1/2}$ = 17 s; $T_{1/10}$ = 75 s) was faster than that of *Ac*phot2-LOV1 ($T_{1/2}$ = 100 s; $T_{1/10}$ = 330 s) and -LOV1+LOV2 ($T_{1/2}$ = 144 s; $T_{1/10}$ = 480 s). These results were similar to those measured for the LOV domains of phot1 in *A. thaliana*. In contrast, the dark reversion rate of single LOV domains in *Ac*phot1 was $T_{1/2}$= about 150 s ($T_{1/10}$ = 500 s) (Fig. 4D, E) and slower than those of other LOV domains reported in phot1 (Salomon et al. 2000, Kasahara et al. 2002b). The rate of LOV1+LOV2 domains of *Ac*phot1 was $T_{1/2}$ = 532 s, but was not a simple sum or average of the lifetimes of individual LOV1 and LOV2 domains (Fig. 4E), indicating that LOV2 is under the influence of LOV1 when both are connected.

Is the lifetime of the signal a bottleneck for timing of chloroplast avoidance movement?

When *Ac*phot2 is activated by high intensity B irradiation, a signal from *Ac*phot2 is likely to be transferred to the next reaction partner. Chloroplast avoidance movement may be regulated by several elements thereafter, through which a signal might be transferred. If the downstream signal transduction pathways have a bottleneck, the rate-limiting factor would be expected to be the one having the shortest lifetime. We therefore attempted to estimate the shortest signal lifetime by physiological analysis. When a part of a cell was irradiated with high intensity B for a short period, the chloroplasts moved away from the irradiated area. The chloroplasts continued moving for some time (even in darkness after switching off the B irradiation) and then stopped and started moving backwards towards the original location (Fig. 5A, B). If the signal generated

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024

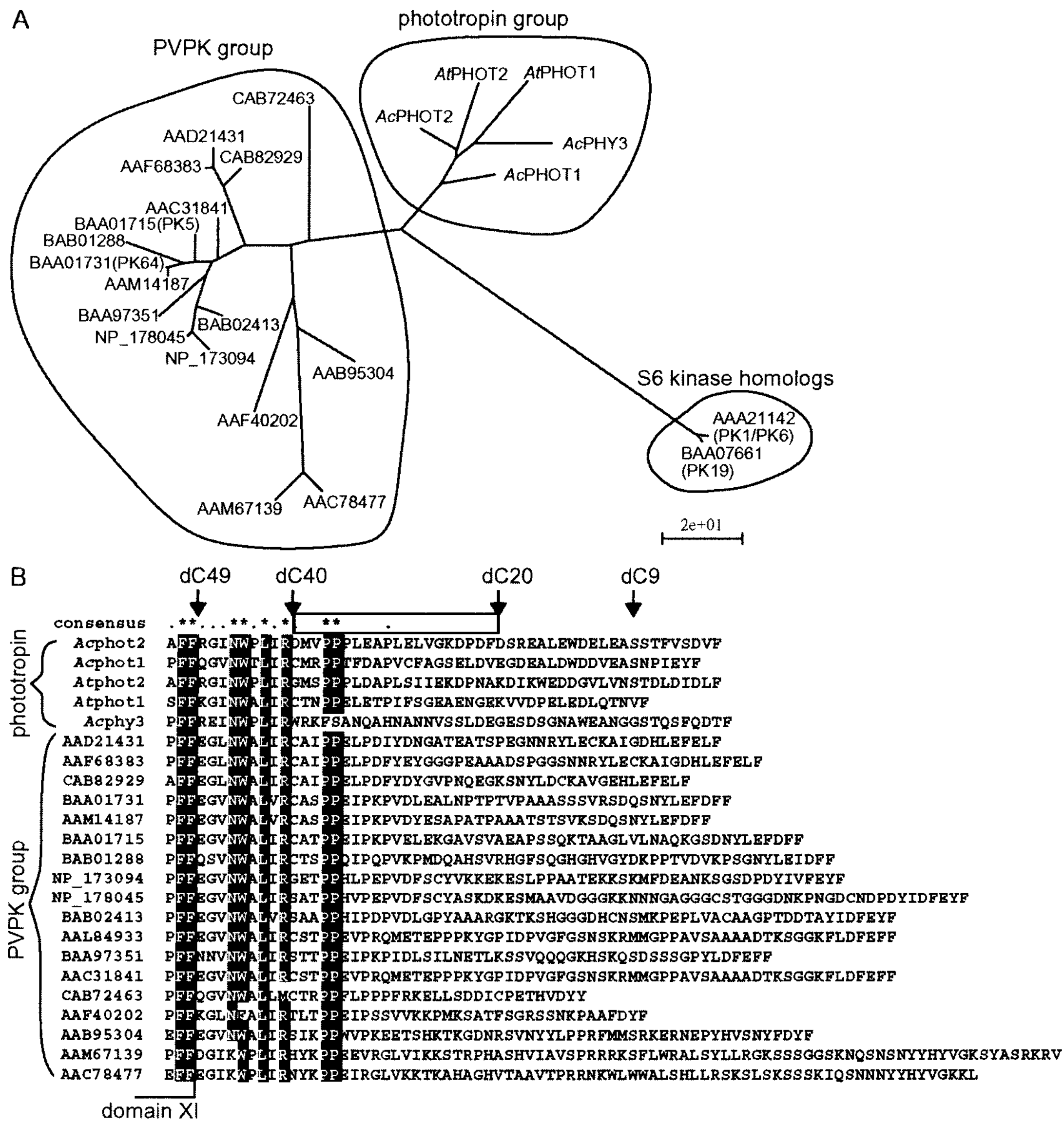


Fig. 6 Similarity of amino acid sequences in C-termini among phototropins and the PVPK group of kinases. (A) Phylogenic tree of the kinase domain of phototropins and PVPK group kinases. Proteins in GenBank are shown by their Accession Numbers. The sequences of the kinase domains were aligned using CLUSTALW and a phylogenic tree was constructed by the neighbor-joining method of MOLPHY. (B) Comparison of the C-termini of phototropins and the PVPK group of kinases. Amino acid residues differing in fewer than two of the sequences listed are shaded in black. In the consensus sequence, identical and similar residues are indicated by *(asterisks) and .(periods), respectively. Arrows at dC49, dC40, dC20 and dC9 indicate the truncated positions of *Ac*phot2 C-termini in the experiments described in Fig. 3. The hatched box indicates the region of *Ac*phot2 critical for chloroplast avoidance movement.

depends upon the fluence rate of B, it should be possible to estimate the lifetime of the signal as a period between the time when B irradiation stopped and the cessation of movement or the beginning of backward movement of the chloroplasts (Fig. 5C). Following the protocol described in "Materials and Methods", the half-lifetime of the signal was estimated to be 67 s by cessation of movement, and 167 s by the timing of backward movement (Fig. 5C). These values are similar to the half-lifetimes of activated *Ac*phot2-LOV1+LOV2 domains obtained by spectroscopy.

Discussion

Chloroplast avoidance movement under strong light is important in *A. thaliana* because it allows reduction or avoidance of chloroplast damage (Kasahara et al. 2002a). *At*phot2 mediates chloroplast avoidance movement in *A. thaliana* (Kagawa et al. 2001). In this paper, we show that *Ac*phot2 also mediates the avoidance response in *A. capillus-veneris*. The exon-intron structures in *AcPHOT2* and *AtPHOT2* are nearly identical to each other except at the 10th intron (data not

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024

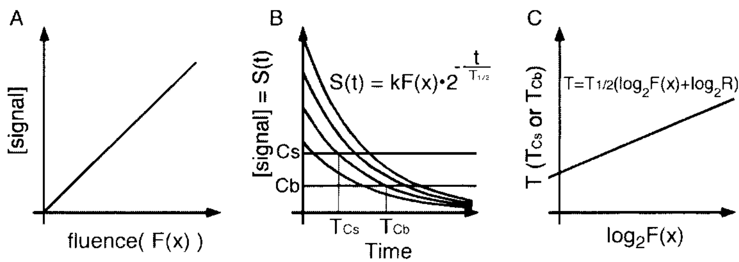


Fig. 7 Schematic basis to calculate the lifetime of a limiting factor for chloroplast avoidance movement. (A) The relationship between the amount of generated signal and fluence irradiated. (B) The relationship between the amount of remaining signal and time after the stop of newly synthesized signal. (C) The relationship between time and fluence obtained by the formula in (B). See Materials and Methods for details.

shown). Blue light-induced chloroplast avoidance movement has been reported in various lower green plants, such as the moss *Physcomitrella patens* (Kadota et al. 2000) and the green alga *Mougeotia scalaris* (Zurzycki 1980, Gabrys 1985), and a phototropin has been described in the alga *Chlamydomonas reinhardtii* (Kasahara et al. 2002b, Huang et al. 2002). These observations suggest that lower plants may have phototropins, which may work as the photoreceptors mediating the avoidance response, and which may be essential for survival.

We found that the BHC mutants used in this study are defective in *Ac*phot2 function, but it is still formally possible that these lines contain additional mutations because back-crossing of a mutant with wild-type plants are almost impossible in fern gametophytes. To show that the BHCs are bona-fide *Ac*phot2 mutants we introduced the wild-type *Ac*phot2 gene into the mutant cells by particle bombardment and found that chloroplast movement in the mutant cells could be rescued by the transient expression of this gene, although accumulation movement under weak light was reduced, probably because over-expressed *Ac*phot2 made the cells sensitive to the weak light and induced an avoidance response rather than accumulation response.

We realized that this transient assay is also a good method to test functions of domains and/or amino acids contained within the *Ac*phot2 sequence. Experiments using an N-terminal deletion series indicate that the LOV2 domain is required for photo-perception, but the LOV1 domain is not. In *A. thaliana*, phot1 mutants defective in the LOV1 region are still functional as photoreceptors for the phototropic response, but a mutant defective in the LOV2 domain is not (Christie et al. 2002). Together, these results indicate that the LOV1 domain is not required for photoperception to use light as a signal controlling chloroplast movement. However, the LOV1 domains are well conserved among members of the phototropin family as are the LOV2 domains (Kagawa 2003), suggesting that the LOV1 domain has some function other than in photoperception.

The C-terminal halves of phototropins contain a Ser/Thr kinase domain. When 40 residues from the C-terminal end of *Ac*phot2 were removed, the mutated *Ac*phot2 gene did not rescue function even though this region is outside kinase domain XI (Hanks and Hunter 1995). To identify the amino acids in the 40 residues important for function, we compared the sequences of phototropins and kinases belonging to PVPK group of *A. thaliana*, whose function is not yet known, but which represent the most similar kinases to the phototropin kinase domain (Fig. 6A, Hardie 1999). Several amino acids were found to be conserved (Fig. 6B), including two proline residues that are well conserved through out all of the sequences with the exception of *Ac*PHY3. These two proline residues are therefore likely to have an important function either for kinase activity or/and for a related function.

Physiological analyses of chloroplast avoidance movement indicate that the shortest signal half-lives for movement are 1–2.5 min. The rescue experiments using mutated *Ac*phot2 genes indicate that the functional domain for photoperception is LOV2, but not LOV1. However, the spectrophotometrically measured half-life of the activated recombinant LOV2 domain was less than 20 s, indicating that the LOV2 domain itself is unlikely to be the cause of the slow response. One of the candidates for this is the LOV1 domain; however, the LOV1 domain does not function as the photoreceptor. The lifetime of the LOV1+LOV2 measured spectrophotometrically is similar to the lifetime of the shortest signal calculated from physiological data. It is interesting that the half-lifetime of the activated LOV1+LOV2 domains together was longer than that of LOV2 domain alone. Even though our data indicate that the LOV1 domain is not necessary for playing a role as a photoreceptor, LOV1 might physically absorb B and delay (or elongate) the lifetime of the LOV1+LOV2 signal to allow chloroplast movement to serve as an effective physiological function.

Materials and Methods

Plant materials

Spores of *A. capillus-veneris* L. collected in a greenhouse at Tokyo Metropolitan University were cultured aseptically for 3 weeks or more on White's medium solidified with 0.5% agar under white light (ca. 6 Wm^{-2}) at 25°C.

Mutant isolation

Spores mutagenized using heavy ion beam irradiation with a power level of N10 were used for screening mutants defective in chloroplast avoidance movement. The dry-spores sown aseptically on the surface of 1/10 modified Murashige and Skoog medium solidified with 0.5% agar were cultured under polarized red light for a week, irradiated with white light for 2 d to let them grow 2-dimensionally, and then mutants were screened after strong white light (more than 30 Wm^{-2}) irradiation for at least 1 h. Protonemata which showed a deficiency in chloroplast avoidance movement were picked up and transferred onto White's medium solidified with 0.5% agar.

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024

DNA and RNA isolation

Genomic DNA and total RNA were obtained from both prothallia for gametophyte tissue and leaves for sporophyte tissue. For PCR, 5′ and 3′ RACE, total RNA were isolated from young leaves grown under white light using modified cetyltrimethylammonium bromide (CTAB) methods (Kanegae and Wada 1995, Imaizumi et al. 2000). Two g of young leaves frozen in liquid nitrogen were ground with 20 g of sterilized quartz by mortar and pestle. The ground tissue was incubated with CTAB solution (2% CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100mM Tris-HCl pH 8.0) at 60°C for 30 min and treated three times with chloroform/iso-amilalcohol (24 : 1). After RNA and DNA were precipitated by 2-propanol and then resolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), RNA and DNA were separated by lithium precipitation. Each RNA and DNA was washed with 70% ethanol twice and resolved in TE buffer.

Cloning of AcPHOT2

To obtain *Ac*PHOT2 cDNA, we performed RT-PCR using the degenerate primers, 5′-GGIATHGAYYTIGCIACIACIYTIGARMG-3′ and 5′-GCDATRTAYTCYTCIGTICCIACRAAISWRTT-3′. The resulting PCR products were isolated into pGEM-Easy (Clontech, U.S.A.) and sequenced. Based on the nucleotide sequence of the RT-PCR clone, both 5′ and 3′ RACE were carried out using 5′ RACE kit and 3′ RACE kit (Life Technologies). 5′ RACE: 1, AcPHOT2.02 5′-ACC-GTTTTCTGGTCAGTGTCCTGC-3′; 2, AcPHOT2.01 5′-CTGCTCC-CTGATGGCATCTCTAATC-3′. 3′ RACE: 1, AcPHOT2.03 5′-GAAC-GTGCTACTGCAAAGGGATGG-3′; 2, AcPHOT2.04 5′-CATGCAA-TCCACAGCTTGTGAGACC-3′. Both RACE PCR products were isolated using pGEM-Easy and sequenced. Again, to obtain full coding region of *Ac*PHOT2, RT-PCR cloning was performed using gene specific primers that amplify the entire region of the ORF (5′-TGGAA-CGCAGCGCCTTCTTGTTC-3′) and (5′-CTTTCAGTCGATATTCA-AGTCCAC-3′).

Detection of mRNA accumulation

Accumulation of mRNA in prothallia and sporophytes were detected by RT-PCR (Imaizumi et al. 2000).

Gene specific primer sets were sense 5′-CAGAGCCTGTAAC-CACATCT-3′ and anti-sense 5′-AAAGTTGAACTGGCCTCCAG-3′ for *Ac*PHOT2 and were sense 5′-AACCAATAGGTGCCTGCAAC-3′ and anti-sense 5′-GGATTGCTAGCCTCAACATC-3′ for *Ac*PHOT1.

Transient assay for chloroplast avoidance movement

The BHC mutant prothallia were cultured on White's medium solidified with 0.5% agar in a dish (6 cm diameter; Corning, U.S.A.) under continuous white light. Cauliflower mosaic virus 35S promoter-driven *Ac*phot2 and GFP were co-transfected into the mutant cells by particle bombardment using 1.6 µm gold particles coated with cDNA of GFP and *Ac*PHOT2 with 1,350 psi under 26 inches Hg vac using a gene delivery system (Biolistic PDS-1000/ He particle delivery system, BioRad, CA, U.S.A.). After cultivation for 1 or 2 d at 25°C, prothallia with GFP fluorescence were picked up under a fluorescent binocular dissecting microscope and mounted in a custom-made cuvette described below. Chloroplast movements in neighboring cells with and without GFP-fluorescent were observed using a microbeam irradiator described below.

Microbeam irradiation and analysis of chloroplast relocation

For partial irradiation of individual cells, a microbeam irradiation system (Olympus BX50, Tokyo) was used. Monochromatic B was obtained through an interference filter (BPF, Vacuum Optics Co. of Japan, Tokyo, Japan), which has a transmission peak at 452.4 nm and a half bandwidth of 23.3 nm. Red and infrared light (IR) was transmitted through an interference filter (BPF) and a glass filter (IR85, Hoya Corp., Tokyo, Japan), respectively. Neutral density filters of ND50, ND25, ND13 and ND3 (Hoya Corp.) were used when necessary. Part of a prothallium was cut off and was transferred into a cuvette comprising two round coverslips supported by a ring-shaped silicon-rubber spacer (outer diameter = 23 mm, inside diameter = 17.5 mm; thickness approximately 0.8 mm). The cuvette with prothallia was placed on a stage of the microbeam irradiator and an individual cell was irradiated with a B microbeam of slit or spot with 20 µm in width or 20 µm in diameter. Fluence rate of the microbeam was measured using a silicon photodiode (S1227-66BR, Hamamatsu Photonics K. K., Hamamatsu).

Chloroplast movement induced by microbeam irradiation was monitored and recorded at 1-min intervals, unless otherwise stated, using an IR-sensitive video camera (C2400–07ER, Hamamatsu Photonics K. K.) under IR condition obtained through an IR-filter (IR-85, Hoya Corp.) or R through an interference filter (DIF-BP-3, Vacuum Optics Co. of Japan), whose transmission peak is 659 nm and a half band-width 35 nm. The recoded images were analyzed by a Macintosh computer (Power Macintosh 8600, Apple Japan Inc., Tokyo, Japan) using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Time courses of chloroplast movement were shown in terms of the distance changes between the center of microbeam spot and a point of chloroplast closest to the spot. All experiments were repeated at least three times with different prothallus. The results obtained from different experiments were assembled using Adobe Photoshop (Adobe System Inc., CA, U.S.A.).

Photochemical analysis

cDNA fragments encoding LOV1, LOV2, and LOV1+LOV2 domains of *Ac*PHOT1 and *Ac*PHOT2, were cloned into a bacterial expression vector pCAL-n-EK as fusions of calmodulin binding peptide (CBP) (Christie et al. 1999). These recombinant vectors were transformed into *E. coli* BL21 (DE3)pLysS (Stratagene, U.S.A). Large-scale cultures of the cells were grown at 30°C to OD600 of approximately 0.5. Protein expression was performed under 1 mM isopropyl β-D-thiogalactopyranoside at 30°C for 3 h. Cells were harvested and kept at –80°C until use. The frozen cells were incubated at room temperature for 10 min, resuspended in 30 ml Ca-binding buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM 2-mercaptoethanol, 1.0 mM magnesium acetate, 2 mM $CaCl_2$) and then sonicated for 30 s on ice. The sample was centrifuged at 17,000×*g* for 15 min and supernatant including CBP fusion proteins was obtained. The CBP fusion proteins were purified on calmodulin resin in accordance to the instructions (Stratagene).

Absorption and fluorescence spectra of the LOV domain fusion proteins were obtained using a DU-60 spectrophotometer (Beckman, Germany) and fluorescence spectrophotometer 850 (Hitachi, Japan), respectively. The rates of dark reversion of light-activated LOV domains were measured at room temperature as described previously (Kasahara et al. 2002b). The samples were irradiated in elution buffer with monochromatic light of 380±20 nm for excitation of fluorescence until emission of fluorescence at 520 nm reached a plateau.

Curve fitting for the speed of dark reversion of photo-activated LOV domains were performed by DeltaGraph (version 4.5; SPSS, Chicago, U.S.A) software.

Calculation of lifetime of a signal at a bottle neck

The lifetime of signal was calculated from physiological response as described previously (Kagawa and Wada 2000). Blue light used for the experiments were 150, 240, 400 or 600 Wm^{-2} for 15, 30 or 60 s.

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024

When the rate-limiting signal is generated in direct proportion in relation to the fluence irradiated (F(x)) for chloroplast avoidance movement (Fig. 7A) and decreases in the dark, the half-lifetime of the signal is described as $T_{1/2}$ (Fig. 7B), and the amount of the signal (S(t)) at time t is

$$S(t) = k \bullet F(x) \bullet 2^{-t/T_{1/2}};$$

where k is a constant.

Chloroplasts stop avoidance or begin to return when the signal decrease to Cs = S(TCs) and Cb = S(TCb), respectively,

$$TCs = T_{1/2}(\log_2(F(x) + \log_2 RCs)$$
$$TCb = T_{1/2}(\log_2(F(x) + \log_2 RCb);$$

where RCs and RCb are constant, TCs and TCb are the timings of stop and start of backward movement of chloroplasts, respectively.

When the data are presented diagrammatically (as Fig. 7C), the slopes of the lines represent the half-lifetimes of the signal, because the fluence used to irradiate has a linear correlation to the amount of signal induced by the irradiation, as shown Fig. 7A.

Accession numbers

The GenBank accession numbers for cDNA and genomic DNA of *Ac*PHOT2 are AB115545 and AB115546.

Acknowledgments

We thank Prof. Yano in RIKEN Institute to irradiate spores by heavy ion beam. We thank Dr. Kinoshita in Kyusyu University to make a construct of modified *Ac*PHOT2 (K709A). We are grateful to Dr. Jane Silverthorne (University of California, Santa Cruz) and Dr. Steen Christensen (National Institute for Basic Biology, Okazaki) for critical reading of the manuscript. This work was partly supported by SORST and PRESTO, Japan Science and Technology Corporation, Japan to TK and by BRAIN (Program for Promotion of Basic Research Activities for Innovative Biosciences) and Grant-in-Aid for Scientific Research (on Priority Areas, no. 13139203, and A, no. 13304061) from the Ministry of Education, Sports, Science and Technology (MEXT) of Japan to MW.

References

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

Cashmore, A.R., Jarillo, J.A., Wu, Y.-J. and Liu, D. (1999) Cryptochromes: Blue light receptors for plants and animals. *Science* 284: 760–765.

Christie, J.M., Salomon, M., Nozue, K., Wada, M. and Briggs, W.R. (1999) LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (nph1): binding sites for the chromophore flavin mononucleotide. *Proc. Natl Acad. Sci. USA* 96: 8779–8783.

Christie, J.M., Swartz, T.E., Bogomolni, R.A. and Briggs, W.R. (2002) Phototropin LOV domains exhibit distinct roles in regulating photoreceptor function. *Plant J.* 32: 205–219.

Gabrys, H. (1985) Chloroplast movement in *Mougeotia* induced by blue light pulses. *Planta* 166: 134–140.

Hanks, S.H. and Hunter, T. (1995) The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB* 9: 576–596.

Hardie, D.G. (1999) Plant protein serine/threonine kinase: Classification and functions. *Annu. Rev. Plant Biol.* 50: 97–131.

Huala, E., Oeller, P.W., Liscum, E., Han, I.S., Larsen, E. and Briggs, W.R. (1997) *Arabidopsis* NPH1: a protein kinase with a putative redox-sensing domain. *Science* 278: 2120–2123.

Huang, K., Merkle, T. and Beck, C.F. (2002) Isolation and characterization of a *Chlamydomonas* gene that encodes a putative blue-light photoreceptor of the phototropin family. *Physiol. Plant.* 115: 613–622.

Imaizumi, T., Kanegae, T. and Wada, M. (2000) Cryptochrome nucleocytoplasmic distribution and gene expression are regulated by light quality in the fern *Adiantum capillus-veneris*. *Plant Cell* 12: 81–96.

Jarillo, J.A., Gabrys, H., Capel, J., Alonso, J.M., Ecker, J.R. and Cashmore, A.R. (2001) Phototropin-related NPL1 controls chloroplast relocation induced by blue light. *Nature* 410: 952–954.

Kadota, A., Sato, Y. and 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.

Kadota, A. and Wada, M. (1999) Red light-aphototropic (rap) mutants lack red light-induced chloroplast relocation movement in the fern *Adiantum capillus-veneris*. *Plant Cell Physiol.* 40: 238–247.

Kagawa, T. (2003) The phototropin family as photoreceptors for blue light-induced chloroplast relocation. *J. Plant Res.* 116: 77–82.

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

Kagawa, T. and Wada, M. (1996) Phytochrome- and blue-light-absorbing pigment-mediated directional movement of chloroplasts in dark-adapted prothallial cells of fern *Adiantum* as analyzed by microbeam irradiation. *Planta* 198: 488–493.

Kagawa, T. and Wada, M. (1999) Chloroplast avoidance response induced by blue light of high fluence rate in prothallial cells of the fern *Adiantum* as analyzed by microbeam irradiation. *Plant Physiol.* 119: 917–923.

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

Kagawa, T. and Wada, M. (2002) Blue light induced chloroplast relocation. *Plant Cell Physiol.* 43: 367–371.

Kanegae, T. and Wada, M. (1995) Isolation and characterization of homologues of plant blue-light photoreceptor (cryptochrome) genes from the fern *Adiantum capillus-veneris*. *Mol. Gen. Genet.* 259: 345–353.

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

Kasahara, M., Swartz, T.E., Olney, M.A., Onodera, A., Mochizuki, N., Fukuzawa, H., Asamizu, E., Tabata, S., Kanegae, H., Takano, M., Christie, J.M., Nagatani, A. and Briggs, W.R. (2002b) Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from Arabidopsis, rice, and *Chlamydomonas reinhardtii*. *Plant Physiol.* 129: 762–773.

Kawai, H., Kanegae, T., Christensen, S., Kiyosue, T., Sato, Y., Imaizumi, T., Kadota, A. and Wada, M. (2003) Responses of ferns to red light are mediated by an unconventional photoreceptor. *Nature* 421: 287–290.

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

Lin, C. (2000) Plant blue-light receptors. *Trends Plant Sci.* 5: 337–342.

Nozue, K., Christie, J.M., Kiyosue, T., Briggs, W.R. and Wada, M. (2000) Isolation and characterization of a fern phototropin (Accession No. AB037188), a putative blue-light photoreceptor for phototropism (PGR00–039). *Plant Physiol.* 122: 1457.

Nozue, K., Fukuda, S., Kanegae, T. and Wada, M. (1998b) Isolation of second phytochrome cDNA from *Adiantum capillus-veneris* (accession no. AB016232) PGR-174. *Plant Physiol.* 118: 712.

Nozue, K., Kanegae, T., Imaizumi, T., Fukuda, S., Okamoto, H., Yeh, K.C., Lagarias, J.C. and Wada, M. (1998a) A phytochrome from the fern *Adiantum* with features of the putative photoreceptor NPH1. *Proc. Natl Acad. Sci. USA* 95: 15826–15830.

Nozue, K., Kanegae, T. and Wada, M. (1997) A full length TY3/gypsy-type retrotransposon in the fern *Adiantum*. *J. Plant Res.* 110: 495–499.

Okamoto, H., Hirano, Y., Abe, H., Tomizawa, K., Furuya, M. and Wada, M. (1993) The deduced amino acid sequence of phytochrome from *Adiantum* includes consensus motifs present in phytochrome B from seed plants. *Plant Cell Physiol.* 34: 1329–1334.

Sakai, T., Kagawa, T., Kasahara, M., Swartz, T.E., Christie, J.M., Briggs, W.R., Wada, M. and Okada, K. (2001) Arabidopsis nph1 and npl1: blue light recep-

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024

tors that mediate both phototropism and chloroplast relocation. *Proc. Natl Acad. Sci. USA* 98: 6969–6974.

Salomon, M., Christie, J.M., Knieb, E., Lempert, U. and Briggs, W.R. (2000) Photochemical and mutational analysis of the FMN-binding domains of the plant blue light receptor, phototropin. *Biochemistry* 39: 9401–9410.

Smith, H. (2000) Phytochromes and light signal perception by plants—an emerging synthesis. *Nature* 407: 585–591.

Takano, M., Kanegae, H., Shinomura, T., Miyao, A., Hirochika, H. and Furuya, M. (2001) Isolation and characterization of rice phytochrome A mutants. *Plant Cell* 13: 521–534.

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

Van Tuinen, A., Kerckhoffs, L., Nagatani, A., Kendrick, R.E. and Koornneef, M. (1995) A temporarily red light-insensitive mutant of tomato lacks a light-stable, B-like phytochrome. *Plant Physiol.* 108: 939–947.

Wada, M. and Kagawa, T. (2001) Light induced chloroplast relocation. *In* Photomovement. Edited by Lebert, M. and Haeder, D.-P. pp. 897–924. Elsevier.

Wada, M., Kagawa, T. and Sato, Y. (2003) Chloroplast movement. *Annu. Rev. Plant Biol.* 54: 455–468.

Wada, M. and Sugai, M. (1995) Photobiology of fern. *In* Photomorphogenesis in Plants. 2nd edn. Edited by Kendrick, R.E. and Kronenberg, G.H.M. pp. 783–802. Kluwer Academic Publishers, Dordrecht.

Weller, J.L., Perrotta, G., Schreuder, M.E., van Tuinen, A., Koornneef, M., Giuliano, G. and Kendrick, R.E. (2001) Genetic dissection of blue-light sensing in tomato using mutants deficient in cryptochrome 1 and phytochromes A, B1 and B2. *Plant J.* 25: 427–440.

Yatsuhashi, H., Kadota, A. and Wada, M. (1985) Blue- and red-light action in photoorientation of chloroplasts in *Adiantum* protonemata. *Planta* 165: 43–50.

Zurzycki, J. (1980) Blue light-induced intracellular movements. *In* Blue Light Syndrome. Edited by Senger, H. Springer-Verlag, Berlin.

(Received October 9, 2003; Accepted January 15, 2004)

Downloaded from https://academic.oup.com/pcp/article/45/4/416/1921987 by guest on 24 April 2024