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Phototropin from *Chlamydomonas reinhardtii* is Functional in *Arabidopsis thaliana*

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Phototropin, a plant blue light photoreceptor, mediates important blue light responses such as phototropism, chloroplast positioning and stomatal opening in higher plants. In *Arabidopsis thaliana*, two phototoropins, phototropin 1 and 2, are known. Recently, in the unicellular green alga, *Chlamydomonas reinhardtii*, a phototropin homolog was identified. It exhibits photochemical properties similar to those of higher plant phototropins and is involved in multiple steps of the sexual life cycle of *Chlamydomonas*. Here, we expressed *Chlamydomonas* phototropin in *Arabidopsis* to examine whether it is active in a distantly related plant species. The *Arabidopsis* mutant deficient in both phototropin 1 and 2 was transformed with a vector containing *Chlamydomonas* phototropin cDNA fused to a cauliflower mosaic virus 35S promoter. The resulting lines were classified into high, medium and low expressers based on RNA gel blot and immunoblot analyses. Typical phototropin responses were restored in high expression lines. These results demonstrate that *Chlamydomonas* phototropin is functional in higher plants. Hence, the basic mechanism of phototropin action is highly conserved, even though its apparent physiological functions are quite diverse.

Keywords: *Arabidopsis thaliana* — Blue light response — *Chlamydomonas reinhardtii* — Phototropin — Signal transduction.

Abbreviations: CaMV, cauliflower mosaic virus; phot, phototropin

Introduction

Blue light is one of the important environmental signals that control plant growth and development. Blue light controls the phototropic response (Baskin and Iino 1987), chloroplast positioning within cells (Zurzycki et al. 1983), stomatal opening (Zeiger and Field 1982), inhibition of hypocotyl elongation (Cosgrove 1981) and the expression of some photosynthetic genes (Senger and Bauer 1987). Plants utilize specific blue light photoreceptors such as cryptochrome (Ahmad and Cashmore 1993) and phototropin (phot) (Huala et al. 1997), both of which have been identified recently.

Phot binds two flavin mononucleotides as chromophores at two conserved domains, LOV1 and LOV2, which are arranged tandemly in the N-terminal half of the molecule (Huala et al. 1997, Christie et al. 1998, Christie et al. 1999). From their mutational analysis, Christie et al. (2002) suggested that the chromophore within the LOV1 domain of *Arabidopsis thaliana* phot1 is dispensable for phototropism. However, the LOV1 domain of phot1 may be involved in dimerization, interaction with anchor proteins and/or regulation of responses other than phototropism (Christie et al. 2002). The C-terminal half of phot exhibits a high homology to Ser/Thr kinases. Indeed, blue light-dependent auto-phosphorylation of phot has been observed (Christie et al. 1998).

In *Arabidopsis*, phot1 (Huala et al. 1997) and phot2 (Jarillo et al. 1998) are involved in phototropism, chloroplast positioning, blue light-activated leaf expansion and stomatal opening (Briggs and Christie 2002). However, phot1 and phot2 behave differently in phototropism and chloroplast positioning. In the phototropic response, phot2 is capable of sensing only a higher intensity of blue light, whereas phot1 senses both low and high intensity blue light (Sakai et al. 2000, Sakai et al. 2001). For chloroplast positioning, accumulation and avoidance responses are induced by low and high light, respectively. Phot2 mediates both the responses, whereas phot1 only mediates the accumulation response (Sakai et al. 2001, Kagawa and Wada 2002).

Chlamydomonas reinhardtii, a unicellular green alga, has been used intensively as an experimental tool in plant research (Harris 1989, Rochaix 1995, Harris 2001), and plant molecular techniques, such as gene cloning and transformation, have been established in this organism. Recently, *Chlamydomonas* phot was found (Kasahara et al. 2002, Huang et al. 2002). Amino acid sequence similarity between *Chlamydomonas* and higher plant phots is observed throughout the molecule except the N-

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terminal extensions, which are found only in higher plant phots (Huang et al. 2002). Biochemical and spectral analyses indicate that *Chlamydomonas* phot exhibits typical spectral properties of phototropin (Kasahara et al. 2002).

It has been shown recently that phot mediates blue light responses related to the sexual life cycle in *Chlamydomonas* (Huang and Beck 2003). Hence, the physiological roles of phots are very different in different organisms regardless of their structural and spectral similarities. It is thus important to compare the biological activity of *Chlamydomonas* phot with those of phots of higher plants. Such an analysis will give us insights into the structural basis of phot action. It will also help us to understand how phot and its signal transduction mechanism(s) have been altered during the evolution of land plants.

In the study, we examined the biological activities of *Chlamydomonas* phot in the higher plant *Arabidopsis*. The *Chlamydomonas PHOT* gene was expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in the *phot1 phot2* double mutant of *Arabidopsis*. The resulting transgenic lines were classified into high, medium and low expressers on the basis of expression levels of the *Chlamydomonas* phot protein. Here we examined whether the *Chlamydomonas* protein can complement any or all of the above-mentioned phot-mediated responses to elucidate its biological activity in higher plant cells.

Results

Expression of the Chlamydomonas phot in Arabidopsis

The LOV domains of *Chlamydomonas* phot exhibit spectral characteristics observed in those of other phots when they are expressed in *Escherichia coli* (Kasahara et al. 2002). However, it had never been tested whether *Chlamydomonas* phot protein exhibits light-dependent auto-phosphorylation. Therefore, we expressed *Chlamydomonas* phot in insect cells and examined the auto-phosphorylation activity (Fig. 1A). As expected, *Chlamydomonas* phot showed light-dependent auto-phosphorylation in vitro. Hence, it was further confirmed that *Chlamydomonas* phot can respond to light stimuli.

To examine the biological activity of the *Chlamydomonas* phot in higher plants, we constructed a gene transfer vector to express the *Chlamydomonas PHOT* gene in *Arabidopsis* under the control of the CaMV 35S promoter (Fig. 1A). An *Arabidopsis phot1-5 phot2-2* double mutant (kindly provided by Dr. Kagawa) was transformed with this vector by the *Agrobacterium*-mediated method. We selected kanamycin-resistant plants and determined the levels of the *Chlamydomonas PHOT* mRNA and *Chlamydomonas* phot protein by RNA gel blotting and immunoblotting, respectively. Consequently, we established several transformed lines that expressed *Chlamydomonas* phot at various levels. Among them, 8–3, 13–1 and 14–1 were chosen as the representative lines for low, medium and high expressers (Fig. 1C). In addition, a few more lines for each category were analyzed in most of the experiments to confirm the results.

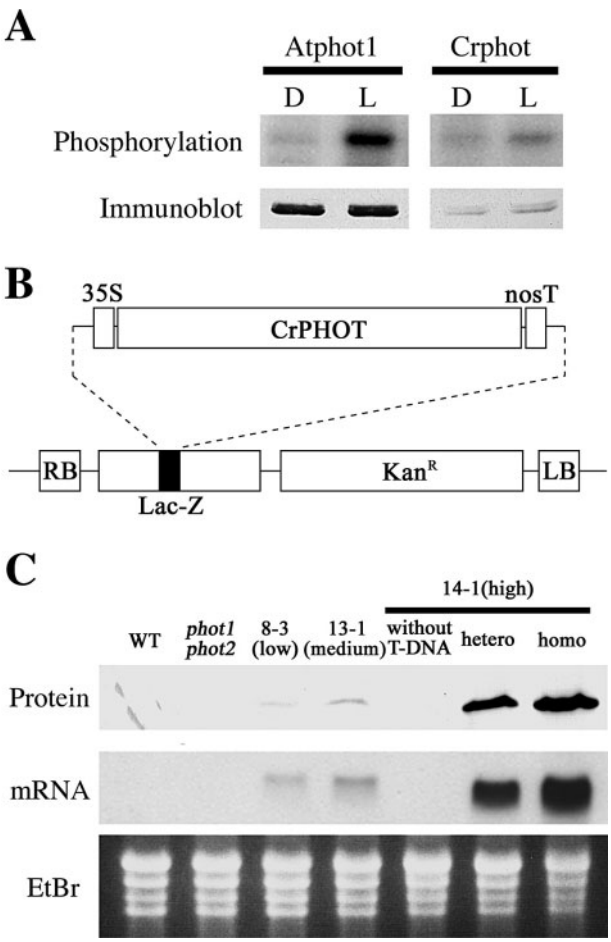

Fig. 1 Expression of *Chlamydomonas* phot in insect cells and transgenic *Arabidopsis* plants. (A) Auto-phosphorylation analysis of *Chlamydomonas* phot (right) and *Arabidopsis* phot1 (left) expressed in insect cells. The insect extracts were incubated with radiolabeled ATP and subjected to electrophoretic analysis (upper). Samples were subjected to mock irradiation, D, or irradiated with saturating white light, L, at a total fluence of 30,000 μmol m^{-2} prior to the addition of ATP. The extracts were subjected to the immunoblot analysis with anti-His antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) to confirm the expression of the introduced protein (lower). (B) A transformation vector to express *Chlamydomonas* phot in *Arabidopsis*. CrPHOT, full-length *Chlamydomonas PHOT* cDNA sequence; RB, right border of T-DNA; LB, left border of T-DNA; 35S, cauliflower mosaic virus 35S promoter; nosT, nopaline synthase terminator. (C) RNA blot (middle) and immunoblot (upper) detection of the *Chlamydomonas* phot mRNA and protein in *Arabidopsis*. The total RNA and the crude protein extracts were prepared from rosette leaves of 30-day-old plants. As loading controls, ethidium bromide staining of the RNA gel is shown (lower). For 14–1, progeny of heterozygous individuals were used. Siblings homozygous (homo) or heterozygous (hetero) for T-DNA, or segregating wild-type (without T-DNA) were chosen and subjected to the analysis.

In order to estimate the expression levels of the introduced *Chlamydomonas* phot protein in these transformant

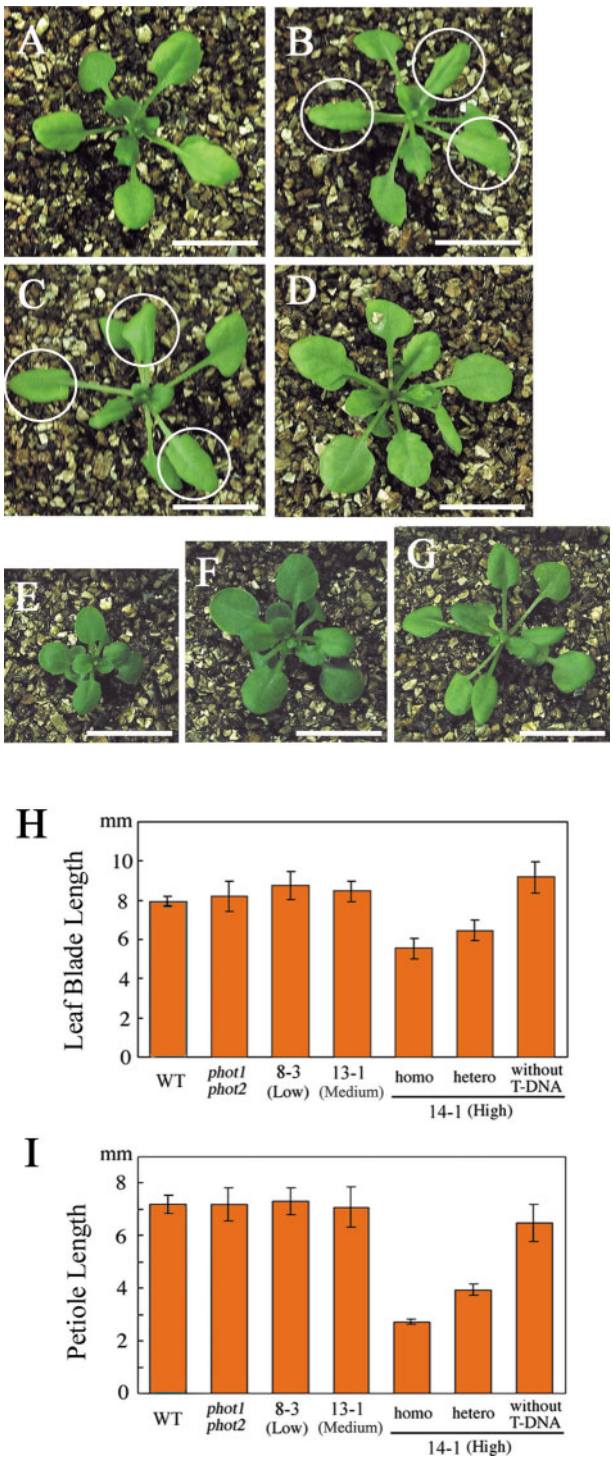

Fig. 2 Leaf morphology of transgenic *Arabidopsis* plants. (A–G) photographs of rosette leaves. Plants were grown under continuous white light for 20 d. Scale bars represent 10 mm. (A) Wild type. (B) *phot1 phot2*. (C) 8–3 (low expresser). (D) 13–1 (medium expresser). (E–G) Homozygous, heterozygous and wild-type siblings of 14–1 (high expresser). Leaves curling downward are indicated by circles. Leaf blade (H) and petiole (I) lengths of the 5th rosette leaves of 30-day-old plants are shown. Values are means ± SD of seven samples.

lines, purified recombinant *Chlamydomonas* phot proteins were used as a standard. Proteins were extracted from rosette leaves of light-grown plants and subjected to immunoblot analysis. The results indicated that the *Chlamydomonas* phot protein accumulated at about 100, 7 and 4.5 ng (mg total protein)$^{-1}$ in a homozygous high expresser 14–1, a medium expresser 13–1 and a low expresser 8–3, respectively (data not shown). In a similar manner, the endogenous level of phot1 in wild-type plants was estimated to be 200 ng (mg total protein)$^{-1}$ using an antibody raised against *Arabidopsis* phot1

Leaf shape and male sterility

Rosette leaves of the *Arabidopsis phot1 phot2* double mutant curl downward (Sakamoto and Briggs 2002; Fig. 2B). This phenotype was complemented not only in the high (Fig. 2E, F) and medium expressers (Fig. 2D) but also in the low expressers, although the complementation was partial in the low expressers (Fig. 2C). In addition, inhibition of leaf expansion was observed in the high expressers (Fig. 2E, F). The leaf blade and petiole lengths of the 5th leaves in the homozygous 30-day-old 14–1 plants averaged 2.7 ± 0.1 and 5.6 ± 0.5 mm, respectively, whereas those in the wild type averaged 7.2 ± 0.4 and 7.9 ± 0.3 mm, respectively (Fig. 2H, I). The heterozygous plants of the high expresser exhibited an intermediate phenotype (Fig. 2F, H, I), indicating that the extent of inhibition depended on expression. In the medium and low expressers, the inhibition was not observed (Fig. 2H, I). We confirmed that the siblings without T-DNA insertion exhibited normal leaf expansion (Fig. 2G–I).

In addition to the leaf shape phenotype, seed setting was highly disturbed in the high expressers. Almost no seed could be obtained from the homozygous 14–1 plants. This phenotype was observed only in the high expressers. Observation of the 14–1 flowers revealed that the anthers never opened (data not shown). In contrast, the 14–1 carpel was fertile because the 14–1 flower set seeds when it was pollinated with wild-type pollen. Because of this phenotype, the high expressers were maintained as heterozygous plants. For physiological analysis, progeny of heterozygous individuals were used.

Phototropic response

To investigate the biological activity of the *Chlamydomonas* phot, we examined whether the phototropic response was complemented in the transgenic lines. It was previously reported that the *phot1 phot2* double mutant exhibits phototropism only very weakly (Sakai et al. 2001). As shown in Fig. 3A, the wild-type seedlings curved towards unilateral blue light of 15 μmol m^{-2} s^{-1} (87.6 ± 7.5°) whereas the *phot1 phot2* mutant did not (12.5 ± 5.4°). Although the extent was reduced, the seedlings of a high expresser, 14–1, exhibited a significant phototropic response (66.0 ± 7.3°). In the medium and low expressers, no response was observed (Fig. 3A).

We further analyzed the response to a range of fluence rates of blue light in the wild type, 14–1 and the phot-deficient

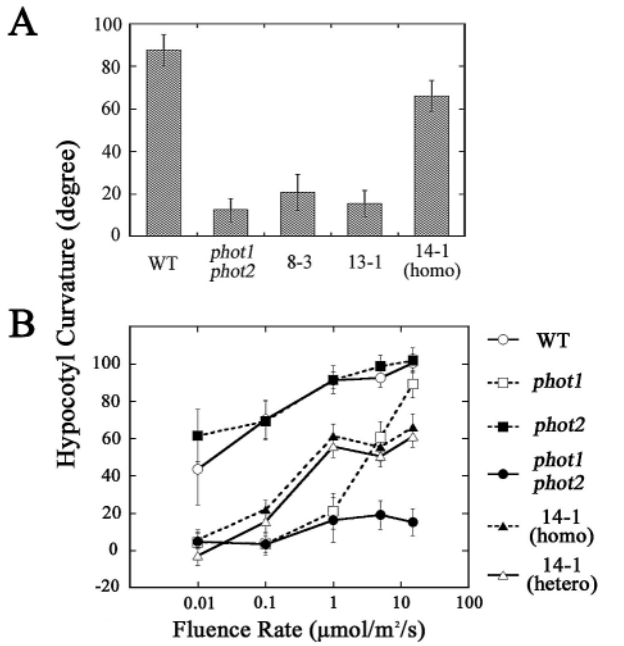

Fig. 3 Hypocotyl phototropism in the etiolated seedlings of the transgenic *Arabidopsis* plants. Etiolated seedlings were treated with unilateral blue light for 16 h and then hypocotyl curvature was determined. Values are means ± SD of at least seven independent samples. (A) Hypocotyl curvature induced by 15 μmol m^{-2} s^{-1} unilateral blue light in different lines. (B) Fluence rate response analysis. Light treatment and curvature measurement were as for A.

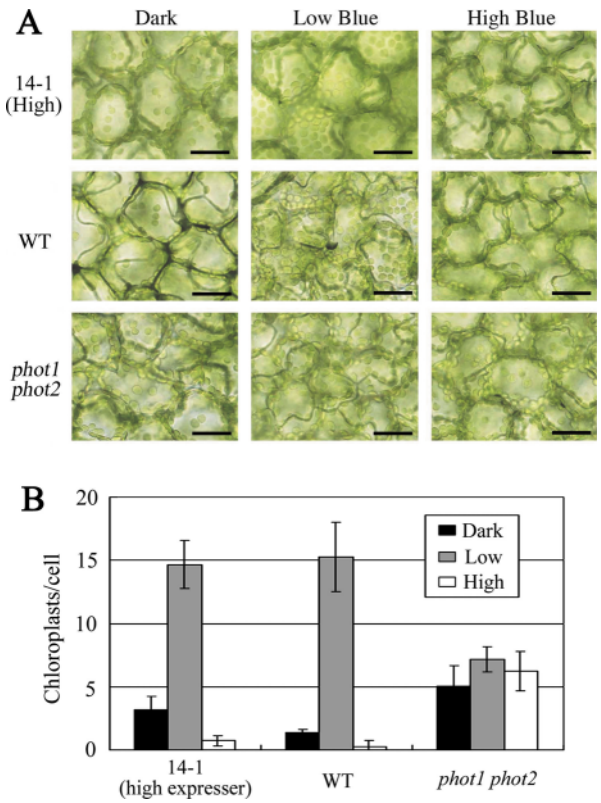

Fig. 4 Chloroplast positioning in palisade cells of rosette leaves in transgenic *Arabidopsis*. (A) Rosette leaves detached from 3-week-old plants were treated with blue light or kept in darkness for 3 h and then observed under a microscope. Fluence rates of low and high blue light were 1.5 and 50 μmol m^{-2} s^{-1}, respectively. (B) Numbers of chloroplasts at the front face per palisade cell after light treatment. Each value represents the mean ± SD of the numbers observed in at least 11 cells. Light treatment was as for (A).

mutants (Fig. 3B). As shown in the figure, 14–1 showed no response to low intensity of blue light (0.01 μmol m^{-2} s^{-1}), whereas the wild type and *phot2* mutant exhibited a partial response to the same light. The 14–1 line exhibited a significant response to medium intensity blue light (1.0 μmol m^{-2} s^{-1}), although the curvature was smaller by 29.8° compared with the wild type (Fig. 3B). The response of 14–1 appeared to be saturated under this condition, although the curvature did not reach 90°. Interestingly, 14–1 showed a higher response (61.4 ± 6.3°) to medium intensity blue light (1.0 μmol m^{-2} s^{-1}) than the *phot1* mutant (21.1 ± 9.6°), indicating that *Chlamydomonas* phot is more sensitive than the authentic phot2. In contrast to the leaf expansion phenotype, no difference was observed between homozygous and heterozygous plants (Fig. 3B).

Chloroplast positioning

Phot1 and phot2 in *Arabidopsis* regulate the distribution of chloroplasts within the cell. Under weak blue light, chloroplasts accumulate at the periclinal wall next to the epidermis (front face) in the palisade cells. Either phot1 or phot2 is required for this response (Sakai et al. 2001). Conversely, chloroplasts move away from the front face to the sides of the cells (anticlinal wall) in response to strong blue light. This response is mediated solely by phot2 (Sakai et al. 2001). To examine these responses in the transgenic lines expressing *Chlamydomonas* phot, palisade cells were observed after treatment with different intensities of blue light. The detached leaves were kept under 1.5 or 50 μmol m^{-2} s^{-1} blue light or in darkness for 3 h on agar plates and then observed with a microscope (Fig. 4A). The numbers of chloroplasts at the front face were counted in the leaves of 14–1, the parental *phot1 phot2* double mutant and the corresponding wild type (Fig. 4B).

Chloroplasts were sparsely localized at the front face regardless of the light conditions in the *phot1 phot2* double mutant (Fig. 4). The average number of chloroplasts at the front face per cell was around 6.0 under the three light conditions. In contrast, chloroplasts accumulated at the front face under weak blue light (15.3 ± 2.7 chloroplasts/cell) and moved away from the front face under the high blue light (0.3 ± 0.5 chloroplasts/cell) in the wild type. In the 14–1 leaves, both the accumulation (14.7 ± 1.9 chloroplasts/cell) and avoidance (0.7 ± 0.4 chloroplasts/cell) responses were restored. In the medium and low expressers, no clear response was observed (data not shown). In conclusion, *Chlamydomonas* phot complemented

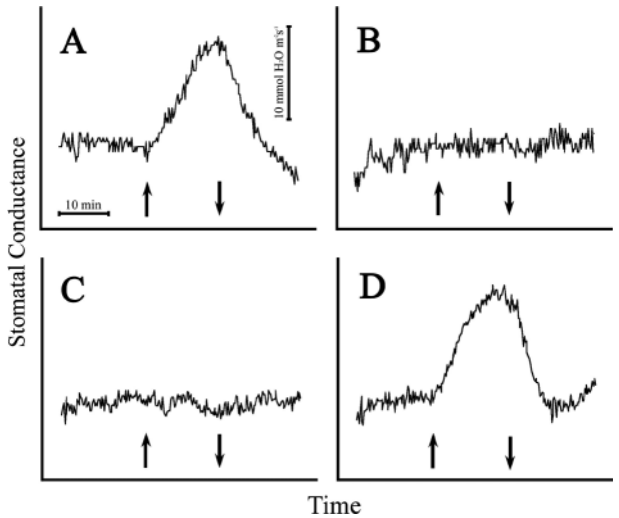

Fig. 5 Stomatal opening by blue light in transgenic *Arabidopsis*. Leaf stomatal conductance in response to blue light was measured. Weak blue light of 5 μmol m^{-2} s^{-1} was superimposed for 15 min on the background red light at 600 μmol m^{-2} s^{-1}. Lower and upper arrows represent the beginning and the end of the blue light irradiation period, respectively. (A) Wild-type plant. (B) *phot1 phot2*. (C) 13–1 (medium expresser). (D) 14–1 (high expresser). All plants were grown for 4 weeks under long day conditions (16 h/8 h, day/night).

the function of phot2 in *Arabidopsis* with respect to the chloroplast positioning.

Stomatal opening

Stomata open in response to red and blue light in *Arabidopsis* (Eckert and Kaldenhoff 2000, Kinoshita et al. 2001). Phot1 and phot2 redundantly regulate the blue light-specific response of stomata (Kinoshita et al. 2001). To examine whether *Chlamydomonas* phot can mediate this response, stomatal opening by blue light at 5 μmol m^{-2} s^{-1} was measured. Transpiration from rosette leaves was monitored by a gas exchange technique as a measure of stomatal conductance (Eckert and Kaldenhoff 2000).

When a strong red light at 600 μmol m^{-2} s^{-1} was applied to *Arabidopsis* leaves, stomatal conductance increased and reached steady state (0.8–1.0 mmol m^{-2} s^{-1}) in all plants tested. After reaching steady state, an application of weak blue light at 5 μmol m^{-2} s^{-1} superimposed on the red light further increased stomatal conductance in wild-type plants. A representative result is shown in Fig. 5A. No such increase in stomatal conductance was observed in leaves of the *phot1 phot2* double mutant (Fig. 5B) and a medium expresser 13–1 (Fig. 5C). In contrast, stomatal conductance was increased in response to weak blue light in the 14–1 high expresser plant (Fig. 5D). These results indicated that *Chlamydomonas* phot restored the blue light-specific response of stomata in the *Arabidopsis phot1 phot2* double mutant, and the complementation depended on the expression levels of *Chlamydomonas* phot in transgenic plants.

Discussion

Complementation of the phot-deficient phenotype.

The *Arabidopsis phot1 phot2* double mutant is almost totally deficient in phototropism, chloroplast positioning and blue light-induced stomatal opening (Kinoshita et al. 2001, Sakai et al. 2001). In addition, leaves of the mutant curl downwards (Sakamoto and Briggs 2002). In the present study, we have shown that *Chlamydomonas* phot complemented the lack of phot1 and phot2 in the double mutant for all the above responses, at least in high expressers [about 100 ng (mg total protein)$^{-1}$] (Fig. 2–5). Since the expression level of the endogenous phot1 was relatively high [about 200 ng (mg total protein)$^{-1}$], we concluded that the *Chlamydomonas* phot exhibited a relatively high activity in *Arabidopsis*. It should be noted here that the endogenous level of phot2 could be much lower. To compare the activity more precisely, we need to express the authentic phot1 and phot2 under the control of the CaMV 35S promoter.

The phototropic responses mediated by phot1 and phot2 were elicited with blue light of about 0.01 and 5 μmol m^{-2} s^{-1}, respectively (Sakai et al. 2000; Fig. 3B). The present analysis demonstrated that the sensitivity of *Chlamydomonas* phot was one order of magnitude lower than that of phot1 and about 50 times higher than that of phot2 (Fig. 3B). This could not be explained fully by the difference in the expression level of *Chlamydomonas* phot, because the expression level was about half of that of the endogenous phot1 (see above). With respect to chloroplast and stomatal responses, fluence rate analysis has not yet been carried out. However, we chose relatively low intensities of light to elicit these responses. Taken together, the *Chlamydomonas* phot expressed in *Arabidopsis* functioned almost normally although the activity might be somewhat lower than that of the endogenous phot1.

It is noteworthy here that *Chlamydomonas* phot functions in *Arabidopsis* regardless of its apparent structural difference from higher plant phots. The *Chlamydomonas* phot lacks the N-terminal stretch and a stretch of about 70 amino acids in the hinge region linking LOV1 and LOV2. Although *Avena sativa* phot1a shows light-activated phosphorylation of Ser27 and Ser30 about 80 residues upstream from LOV1 (Salomon et al. 2003), these serine residues are missing in *Chlamydomonas* phot. Similarly, other auto-phosphorylation sites found in the hinge region of higher plant phots (Kinoshita et al. 2003, Salomon et al. 2003) are not conserved in *Chlamydomonas* phot. Hence auto-phosphorylation of serine residues reported so far may not be essential for the signal transduction. Nevertheless, the *Chlamydomonas* phot exhibited light-dependent auto-phosphorylation (Fig 1A). It will be intriguing to examine which amino acid residues are phosphorylated in the *Chlamydomonas* phot and to know whether those residues are conserved in higher plant phots.

Although the *Chlamydomonas* phot was functional in *Arabidopsis* and its apparent sensitivity to light was almost nor-

mal, it was not as active as authentic phots. The *35S*-driven expression of the *Arabidopsis* phot1 in the *phot1* mutant complements the deficiency in phototropic response almost entirely (Christie et al. 2002). In contrast, the mutant expressing the *Chlamydomonas* phot exhibited reduced curvature even toward a high intensity of light (Fig. 3B). In the case of chloroplast positioning and stomatal opening, no such difference was observed.

Phot1 and phot2 specificity

Although phot1 and phot2 are structurally and functionally similar, there are important differences. The most prominent functional difference is that phot2 but not phot1 mediates the chloroplast avoidance response (Sakai et al. 2001). In this respect, the *Chlamydomonas* phot behaved more like phot2 than phot1 because it mediated both the avoidance and accumulation responses (Fig. 4). Another important difference is the higher sensitivity of phot1 to light (Briggs and Christie 2002). The present analysis revealed that the sensitivity of the *Chlamydomonas* phot was somewhere between those of phot1 and phot2. The *Chlamydomonas* phot exhibited phototropism in response to lower fluences than phot2 (Fig. 3B). Taken together, the *Chlamydomonas* phot behaved more like phot2 than phot1, but its biological activity was different from that of authentic phot2.

Structural differences are known between phot1 and phot2. The N-terminal extensions of phots are highly divergent. These extensions might determine the specificity of phot1 and phot2. The *Chlamydomonas* phot lacks this N-terminal extension almost totally (see above). As discussed above, the *Chlamydomonas* phot behaved more like phot2. Hence, the N-terminal domain does not appear to be required for the function of phot2.

The LOV domains of phot1 and phot2 exhibit different spectral properties (Kasahara et al. 2002). The binding of chromophore to LOV1 is not required for the function of phot1 (Christie et al. 2002). From this observation, it is suggested that LOV2 may be more important in determining the activity of phots. Spectral properties of isolated LOV domains from different phots, including the *Chlamydomonas* phot, have been examined (Kasahara et al. 2002). According to the results, the LOV2 domain of the *Chlamydomonas* phot resembles that of phot1 more than that of phot2. Nevertheless, the *Chlamydomonas* phot was not as sensitive as phot1 in *Arabidopsis* (Fig. 3B). Hence, it is difficult at this stage to relate the spectral nature directly to light sensitivity.

Light-independent phenotype

Leaves of the *Arabidopsis phot1 phot2* mutant curl downwards (Sakamoto and Briggs 2002); we do not know how the lack of phots is involved in this phenomenon. However, the expression of the *Chlamydomonas* phot restored normal flat leaves (Fig. 2D–F), indicating that the *Chlamydomonas* phot was morphologically functional with respect to this phenotype. In comparison with other responses (see above), much lower expression was sufficient to complement the phenotype (Fig. 2D), suggesting that this phenotype may represent a totally different aspect of phototropin activity.

In contrast to the restoration of leaf shape, inhibition of leaf expansion and seed setting was observed only in high expressers (Fig. 2H, I). It remains unclear whether this effect reflects the authentic function of phots because no respective phenotype such as leaf elongation is observed in the *phot1 phot2* mutant. One possible explanation is that the homeostasis of plant hormones was disturbed by overexpression of the *Chlamydomonas* phot. Phot has been proposed to alter the distribution of auxin (Briggs 1963, Sakamoto and Briggs 2002), and mutants that are deficient in auxin-related genes have shorter leaves (Christensen et al. 2000, Gil et al. 2001). Alternatively, the expression of the *Chlamydomonas* phot may have disturbed the mitogen-activated protein (MAP) kinase signal cascades. The MAP kinase signal is involved in cell proliferation in plants (Nakashima et al. 1998, Krysan et al. 2002), and the kinase domain of phototropin exhibits similarities to MAP-KKK proteins.

Concluding remarks

As expected from a distant relationship, significant structural differences are found between the higher plant phots and the *Chlamydomonas* phot. The overall amino acid identities of *Chlamydomonas* phot to *Arabidopsis* phot1 and phot2 are about 35 and 39%, respectively (Huang et al. 2002). Regardless of this relatively low homology, the *Chlamydomonas* phot was functional in *Arabidopsis*, suggesting that the basic signal transduction mechanism is relatively simple and highly tolerant to changes in structure. We need to understand more about how plants utilize such a simple mechanism to regulate divergent but specific responses.

The *Chlamydomonas* phot has been shown to be involved in multiple blue light-dependent steps in the sexual life cycle of *Chlamydomonas* (Huang and Beck 2003). As discussed above, the basic mechanism of the signal transduction should be conserved between higher plants and *Chlamydomonas*. Although *Chlamydomonas* is suitable for molecular and genetic analysis (Harris 1989, Rochaix 1995, Harris 2001), it has never been used intensively for the study of light signal transduction. The present study suggests that future analysis of phot action in this organism should help us to understand the mechanism of phot action not only in *Chlamydomonas* but also in higher plants.

Materials and Methods

Plant materials and growth conditions

The *Arabidopsis* mutant lines *phot1-5* (Huala et al. 1997), *phot2-2* (*cav1-2*) (Kagawa et al. 2001) and *phot1-5 phot2-2* (kindly provided by Dr. Kagawa) were used. The *gl-1* mutant (ecotype Columbia), which is the parental line of the above *phot* mutant lines, was used as the wild-type control. Plant growth conditions were described previously (Nakamura et al. 2000). Seeds were sown on 0.6% agar (Phytoa-

gar; Gibco-BRL, LifeTech, U.S.A.) plates containing Murashige–Skoog salts and Gamborg's B5 vitamins. Mature plants were transferred to vermiculite soil for further growth.

Plasmid construction

The cDNA fragment of the full-length *Chlamydomonas PHOT* gene (Huang et al. 2002, Kasahara et al. 2002) was amplified by polymerase chain reaction (PCR), and inserted into pPZP211 (Hajdukiewicz et al. 1994), an expression vector for *Arabidopsis*. Fragments of the CaMV 35S promoter and the nopaline synthase terminator were inserted onto the pPZP211 vector.

Auto-phosphorylation assay

Recombinant baculovirus encoding the full-length *Chlamydomonas PHOT* coding sequence was generated using the BaculoGold Transfection Kit (BD Biosciences, Pharmingen, Palo Alto, CA, U.S.A.) in accordance with the supplier's instructions. Recombinant baculovirus was used to infect Sf9 (*Spodoptera frugiperda*) insect cells. Expression of recombinant phot1 and phot2 was carried out as described previously (Christie et al. 1998, Sakai et al. 2001). As a positive control, *Arabidopsis* phot1 was examined in the same way (Sakai et al. 2001). In vitro auto-phosphorylation analysis of soluble protein extracts from insect cells (about 10 μg) was carried out in the absence of Triton X-100, as described previously (Sakai et al. 2001). Western blot analysis of soluble protein extracts from insect cells (about 10 μg) was performed as described (Sakai et al. 2001).

Arabidopsis transformation

The *Arabidopsis phot1 phot2* mutant was transformed using the MP90 strain of *A. tumefaciens* by in planta transformation (Bechtold et al. 1993, Clough and Bent 1998). Transformed plants (T1 plants) were selected on Murashige–Skoog medium containing 25 μg ml^{-1} kanamycin and 166 μg ml^{-1} cefotaxime sodium (Aventis Pharm, Tokyo, Japan). For physiological and biochemical analysis, homozygous T3 seeds were used except for the high expressers which could not be maintained as homozygous populations since they could not self-pollinate. Thus, populations segregating the homozygous, heterozygous and the wild-type siblings at a 1 : 2 : 1 ratio were used for analysis. In most cases, the result was recorded for each plant and their genotypes were determined later by PCR.

Detection of RNA and protein of the Chlamydomonas PHOT in Arabidopsis

Total RNA was prepared from the rosette leaves of 30-day-old plants, using Sepasol®-RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the instructions. For RNA gel blot analyses, 5 μg of total RNA was used.

To detect the *Chlamydomonas* phot protein, crude extract was prepared from the rosette leaves and/or the cauline leaves of 30-day-old plants. The tissues were homogenized in the following extraction buffer (Sakamoto and Briggs 2002): 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 0.1 M Tris–HCl, pH 8.5 and Protease Inhibitor Cocktail for General Use (P2714; Sigma-Aldrich, St Louis, MO, U.S.A.) with a glass mortar and pestle. The extracts were then incubated at 80°C for 3 min and centrifuged twice (16,000×*g* for 10 min at room temperature) to remove insoluble materials. For immunoblot analysis, the proteins were separated by SDS–PAGE, blotted onto a membrane and probed with a mouse anti-*Chlamydomonas* phot monoclonal antibody or a goat anti-*Arabidopsis* phot1 antibody [Nph1(aN-20), Santa Cruz Biotechnology, CA, U.S.A.]. The hybridoma line producing the anti-*Chlamydomonas* phot monoclonal antibody was established (López-Juez et al. 1992) from a mouse immunized with purified full-length *Chlamydomonas* phot protein expressed in *E. coli*.

Leaf measurement

Seedlings were grown on agar for 10 d and then on vermiculite soil for further 20 d. The 5th leaves were detached from seven plants and photographed. The leaf blade and petiole lengths were determined on a computer screen.

Phototropism

Hypocotyl curvature assay was performed as described in Sakai et al. (2001). The *Arabidopsis* seedlings were planted on square plates containing 0.6% agar medium, and grown vertically in darkness for 3 d. The etiolated seedlings were then illuminated for 16 h with unilateral blue light (0.01–15 μmol m^{-2} s^{-1}) using blue light-emitting diodes (E1L53–3BD02; Toyoda Gosei, Aichi, Japan). Seedlings were photographed for curvature measurement after the illumination period.

Microscopic observation of Arabidopsis chloroplasts

Chloroplasts in palisade cells of *Arabidopsis* rosette leaves were observed under a microscope (Kagawa and Wada 2000). Rosette leaves detached from plants grown for 3 weeks under white light were placed on agar plates and irradiated with 1.5 μmol m^{-2} s^{-1} or 50 μmol m^{-2} s^{-1} blue light from light-emitting diodes for 3 h. As a control, leaves were placed in the dark for 3 h. The leaf was then placed on a slide glass and observed under a microscope with a 40× objective lens. The images were recorded on a computer with a CCD camera (DP50; Olympus, Tokyo, Japan).

Recording of stomatal opening

Leaf stomatal conductance (mol m^{-2} s^{-1}) of *Arabidopsis* plants was measured by a gas exchange method (Lascève et al. 1999, Eckert and Kaldenhoff 2000) using LI-6400 open-flow systems (Li-Cor, NE, U.S.A.). Transpiration from rosette leaves was monitored by measuring the water-vapor pressure at the outlet. Whole *Arabidopsis* plants in the soil were placed in a 14 ml Falcon tube containing distilled water, and a leaf was clamped with a gas-tight *Arabidopsis* chamber (Li-Cor, NE, U.S.A.) designed for small leaves. Gas exchange was determined at constant concentration of CO_2 at 350 ml l^{-1} and at relative humidity of 55–60% in the chamber. The measurement was performed three times for each genotype.

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