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Phototropin LOV domains exhibit distinct roles in regulating photoreceptor function

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Summary

Phototropins (phot1 and phot2) are autophosphorylating serine/threonine kinases that function as photoreceptors for phototropism, light-induced chloroplast movement, and stomatal opening in *Arabidopsis*. The N-terminal region of phot1 and phot2 contains two specialized PAS domains, designated LOV1 and LOV2, which function as binding sites for the chromophore flavin mononucleotide (FMN). Both LOV1 and LOV2 undergo a self-contained photocycle, which involves the formation of a covalent adduct between the FMN chromophore and a conserved active-site cysteine residue (Cys39). Replacement of Cys39 with alanine abolishes the light-induced photochemical reaction of LOV1 and LOV2. Here we have used the Cys39Ala mutation to investigate the role of LOV1 and LOV2 in regulating phototropin function. Photochemical analysis of a bacterially expressed LOV1 + LOV2 fusion protein indicates that LOV2 functions as the predominant light-sensing domain for phot1. LOV2 also plays a major role in mediating light-dependent autophosphorylation of full-length phot1 expressed in insect cells and transgenic *Arabidopsis*. Moreover, photochemically active LOV2 alone in full-length phot1 is sufficient to elicit hypocotyl phototropism in transgenic *Arabidopsis*, whereas photochemically active LOV1 alone is not. Further photochemical and biochemical analyses also indicate that the LOV1 and LOV2 domains of phot2 exhibit distinct roles. The significance for the different roles of the phototropin LOV domains is discussed.

Keywords: *Arabidopsis thaliana*, blue light receptor, flavoprotein, LOV domain, phototropin, cysteinyl adduct.

Introduction

Molecular genetic analysis, using the model plant *Arabidopsis thaliana*, has shown that the effects of blue (390–500 nm) and UV-A (320–390 nm) light on plant growth and development are mediated by at least four different blue light receptors: cryptochrome 1 (cry1); cryptochrome 2 (cry2); phototropin 1 (phot1); and phototropin 2 (phot2) (Briggs and Huala, 1999; Cashmore *et al.*, 1999; Christie and Briggs, 2001; Lin, 2000). The phototropins represent a newly characterized family of flavoprotein photoreceptors (Briggs *et al.*, 2001a) that mediate both phototropism and light-induced chloroplast movement in *Arabidopsis* (Jarillo *et al.*, 2001b; Kagawa *et al.*, 2001; Sakai *et al.*, 2001). Recent genetic studies have also shown that phot1 and phot2 share redundant roles in regulating blue light-induced stomatal opening (Kinoshita *et al.*, 2001). Thus, phot1 and phot2 function to regulate not only phototropism, after which they were named (Briggs and Christie, 2002; Briggs *et al.*, 2001a; Christie *et al.*, 1999), but also other photomovement responses.

Arabidopsis PHOT1 and *PHOT2* encode proteins of 996 and 915 amino acids, respectively, and share approximately 60% sequence identity (Huala *et al.*, 1997; Jarillo *et al.*, 1998). Phot1 and phot2 contain a serine/threonine kinase domain located at the C-terminus and have been shown to undergo blue light-induced autophosphorylation (Christie *et al.*, 1998; Sakai *et al.*, 2001). The N-terminal region of phot1 and phot2 contains a repeated motif of approximately 110 amino acids that belong to the PER/ARNT/SIM (PAS) domain superfamily (Taylor and Zhulin, 1999). The PAS domains of phot1 and phot2 belong to a subset of proteins within the PAS domain superfamily that are regulated by light, oxygen and voltage. Hence the PAS domains

of phototropin were named LOV1 and LOV2, respectively (Huala *et al.*, 1997).

The individual LOV domains of phot1 and phot2 can be expressed and purified from *Escherichia coli* in amounts suitable for biochemical characterization (Christie *et al.*, 1999; Kasahara *et al.*, 2002; Sakai *et al.*, 2001; Salomon *et al.*, 2000; Swartz *et al.*, 2001). In each case, the LOV domains function as non-covalent binding sites for the chromophore flavin mononucleotide (FMN). LOV1 and LOV2, expressed alone or in tandem, undergo a self-contained photocycle involving the formation of a covalent adduct between the FMN chromophore and an active-site cysteine residue conserved in LOV1 and LOV2 (Salomon *et al.*, 2000). Recent X-ray crystallography (Crosson and Moffat, 2001; Crosson and Moffat, 2002) and nuclear magnetic resonance (NMR) studies (Salomon *et al.*, 2001), involving the LOV2 domain from two different phototropins, have confirmed this photochemical reaction mechanism. Replacement of the conserved cysteine residue with alanine in either LOV1 or LOV2 abolishes the light-induced photochemical reaction without affecting the stoichiometry of FMN-binding (Salomon *et al.*, 2000). The conserved cysteine residue has been designated cysteine 39 (Cys39), after its relative amino acid position in the individually expressed LOV1 and LOV2 fusion proteins (Salomon *et al.*, 2000; Swartz *et al.*, 2001).

All phototropin homologues identified to date, including a phototropin from the green alga *Chlamydomonas rheinhardtii*, contain two FMN-binding sites, LOV1 and LOV2 (Briggs *et al.*, 2001a). Hence the occurrence of two chromophore-binding domains within the phototropin molecule has been conserved throughout the course of evolution, and probably has some functional significance. Indeed, detailed photochemical analyses have shown that the LOV domains of phot1 and phot2 exhibit different quantum efficiencies and reaction kinetics (Kasahara *et al.*, 2002; Salomon *et al.*, 2001), suggesting that LOV1 and LOV2 may have different light-sensing roles in regulating phototropin activity. Although studies of individual LOV domains have been instrumental in elucidating the primary mechanisms associated with phototropin photochemistry, recent photochemical studies have shown that bacterially expressed fusion proteins containing both LOV domains (designated LOV1 + LOV2) possess photochemical properties that more closely resemble those of full-length phot1 and phot2 expressed in insect cells (Kasahara *et al.*, 2002). Therefore the truncated LOV1 + LOV2 fusion proteins represent a more appropriate model system to study phototropin photochemistry in relation to the full-length photoreceptor proteins.

In the present study, we have used the Cys39Ala mutation to investigate the individual roles of LOV1 and LOV2 in regulating phototropin function in *Arabidopsis*. Using a LOV1 + LOV2 fusion protein, we show that LOV2 serves as the principal light-sensing domain for phot1. In addition, we demonstrate that LOV2 is required to mediate phot1 kinase activity and to elicit phot1-mediated hypocotyl curvature in response to low fluence rates of unilateral blue light. LOV1, on the other hand, plays at most a minor light-sensing role in regulating the photochemical reactivity of phot1, and is insufficient to elicit phot1-mediated hypocotyl curvature under low light conditions. We also show that phot2 photochemistry and kinase activity are principally mediated by LOV2. Thus, phototropin LOV domains appear have evolved to carry out distinct functional roles.

Results

LOVCys39Ala mutations alter the fluorescence of a bacterially expressed phot1 LOV1 + LOV2 fusion protein

To examine the role of each LOV domain in mediating phot1 photochemistry, we have used the Cys39Ala mutation to block the photochemical activity of either LOV1 or LOV2, selectively, in a bacterially expressed LOV1 + LOV2 fusion protein of *Arabidopsis* phot1. The resulting fusion proteins were designated ~~LOV1~~ + LOV2 and LOV1 + ~~LOV2~~, respectively, where the strikethrough indicates the presence of the Cys39Ala mutation.

The fluorescence excitation spectra obtained for equal concentrations of wild-type phot1 and LOVCys39Ala mutant fusion proteins are shown in Figure 1(a). In each case, the spectrum is characteristic for those obtained previously for LOV domain fusion proteins (Christie *et al.*, 1999; Kasahara *et al.*, 2002; Salomon *et al.*, 2000), with fluorescence excitation greatest between 400 and 500 nm, and a broad peak at 370 nm. Introducing the Cys39Ala mutation into the LOV2 domain of the fusion protein (LOV1 + ~~LOV2~~) results in a large increase in fluorescence emission relative to that of the wild-type fusion protein (fourfold). This increase in fluorescence reflects that replacement of Cys39 with nonpolar alanine reduces fluorescence quenching of the FMN chromophore, as well as inhibiting photochemical activity (Salomon *et al.*, 2000; Swartz *et al.*, 2001). In contrast, introducing the Cys39Ala mutation into the LOV1 domain (~~LOV1~~ + LOV2) has very little effect on the fluorescence emission of the LOV1 + LOV2 fusion protein.

Previous studies have shown that the individual LOV domains of phot1 have different quantum efficiencies (Kasahara *et al.*, 2002; Salomon *et al.*, 2001). This difference is reflected, in part, by the degree of chromophore fluorescence quenching exhibited by the two domains. LOV2 has a 10-fold higher quantum efficiency relative to LOV1, and exhibits a higher degree of chromophore fluorescence quenching (Salomon *et al.*, 2001). Therefore the more dramatic increase in fluorescence emission observed for the

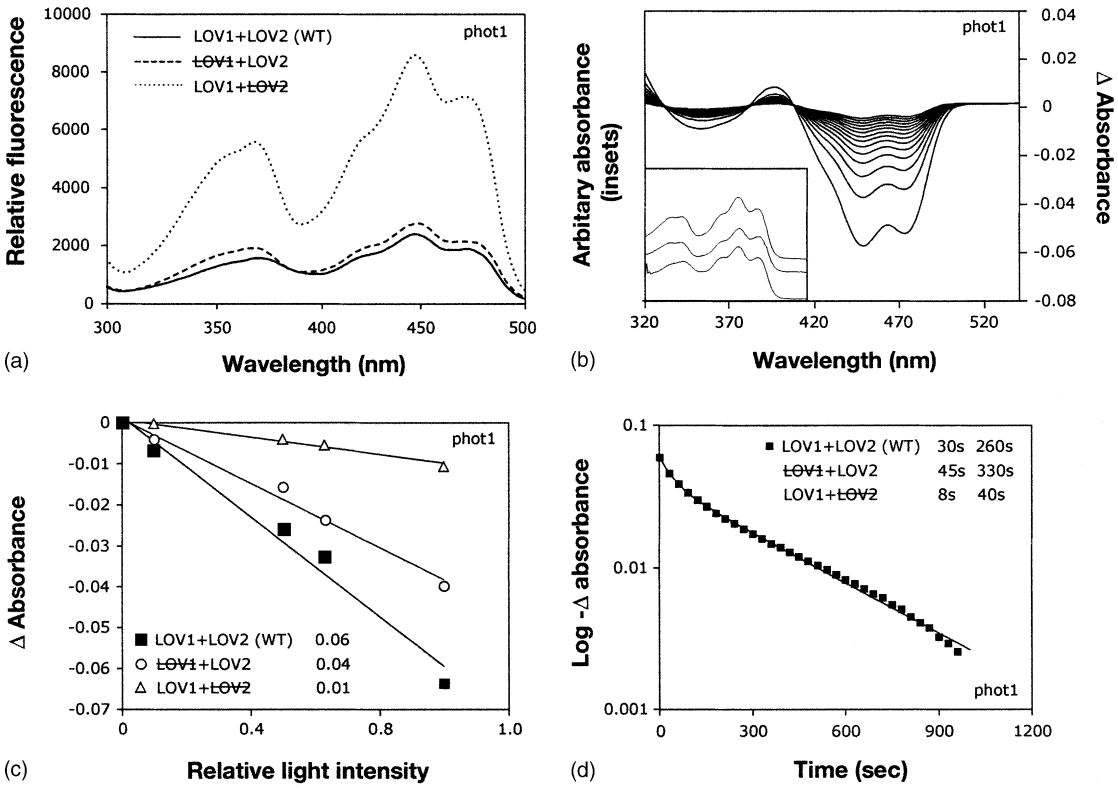

Figure 1. Photochemical and mutational analyses of the phot1 LOV1 + LOV2 fusion protein.
(a) Fluorescence excitation spectra of wild-type phot1 (WT) and LOVCys39Ala mutant fusion proteins. Mutant fusion proteins are designated ~~LOV1~~ + LOV2 and LOV1 + ~~LOV2~~, respectively, where the strikethrough indicates the presence of the Cys39Ala mutation. Fluorescence emission of equal concentrations of wild-type and mutant fusion proteins (0.3 mg ml^{-1}) was monitored at 520 nm.
(b) Light-minus-dark difference spectrum obtained for the wild-type phot1 LOV1 + LOV2 fusion protein (main panel). The difference spectrum shows dark recovery to the ground state after a light flash. Spectra were recorded at 1 min intervals following the light flash. Absorption spectra of wild-type and LOVCys39Ala mutant fusion proteins are also shown (inset). Spectra are offset for clarity and are arranged as follows: WT (bottom), ~~LOV1~~ + LOV2 (centre), and LOV1 + ~~LOV2~~ (top).
(c) Relative bleaching measurements for FMN-cysteinyl adduct formation obtained for wild-type and LOVCys39Ala mutant fusion proteins. Light-induced absorbance changes were monitored at 450 nm over a range of light intensities (see Experimental procedures for details). The slope of each line gives a measure of the relative bleaching at 450 nm obtained for each fusion protein. Relative bleaching values for each fusion protein are shown, and represent the average obtained from three independent protein preparations. In each case, values were within 10% of the mean.
(d) Dark-regeneration kinetics for the wild-type phot1 LOV1 + LOV2 fusion protein measured at 450 nm (main panel). Half-life times for dark regeneration obtained for each fusion protein are shown.

LOV1 + ~~LOV2~~ fusion protein correlates with the greater quantum efficiency reported for the individual LOV2 domain of phot1.

Photochemical activity and dark regeneration of the phot1 LOV1+ LOV2 fusion protein is predominantly mediated by LOV2

The absorption spectra of wild-type and mutant phot1 LOV1 + LOV2 fusion proteins are shown in Figure 1(b) (inset) and are offset for clarity. The absorption spectra of the LOVCys39Ala mutant fusion proteins are nearly identical to that of the wild-type fusion protein, but show key differences in the near-UV region of the spectrum. These differences probably reflect changes in the electrostatic environment of the chromophore resulting from the Cys39Ala mutation (Swartz *et al.*, 2001). However, given that both the fluorescence and absorption properties of the mutant fusion proteins are very similar to those of the wild-type fusion protein (Figure 1a,b), and do not alter flavin binding (Salomon *et al.*, 2000), it seems unlikely that the Cys39Ala mutation has any deleterious effect on protein structure.

As reported previously for the individual LOV domains of phot1 (Salomon *et al.*, 2000; Swartz *et al.*, 2001), irradiation of the LOV1 + LOV2 fusion protein with a brief pulse of light results in a rapid loss of absorption (or bleaching) in the blue region of the spectrum, and the appearance of an absorption band at around 390 nm. These light-induced absorbance changes are illustrated by the light-minus-dark difference spectrum shown in Figure 1(b) (main panel). The reaction is fully reversible in the absence of light, and

returns to the initial ground state after many minutes. The photoproduct and the initial ground state of the LOV1 + LOV2 fusion protein share three isosbestic points, at approximately 330, 375 and 410 nm, which correspond to the formation of the FMN-Cys39 adduct within each of the LOV domains (Salomon *et al.*, 2000; Swartz *et al.*, 2001). Similar light-induced absorbance changes were observed for ~~LOV1~~ + LOV2 and LOV1 + ~~LOV2~~ fusion proteins (data not shown), indicating that both LOV domains are photochemically active within the LOV1 + LOV2 fusion protein.

Figure 1(c) shows maximal bleaching (at 450 nm) for each of the fusion proteins in response to a 1 ms pulse of blue light, given at different intensities. In each case, maximal bleaching is proportional to light intensity, indicating that the blue light excitation used is not saturating. The slope of the line for each fusion protein can therefore be used as a measure of relative bleaching at 450 nm in response to blue light. The ~~LOV1~~ + LOV2 fusion protein retains a comparatively high degree of relative bleaching (0.04) as compared to the wild-type fusion protein (0.06), whereas blocking the photochemical activity of LOV2 results in a substantial decrease in relative bleaching (0.01). Hence LOV2 functions as the main light-sensing domain regulating the photochemical activity of the phot1 LOV1 + LOV2 fusion protein.

The difference in relative bleaching observed for the ~~LOV1~~ + LOV2 and LOV1 + ~~LOV2~~ fusion proteins is consistent with the difference in quantum efficiency reported for the individual LOV domains of phot1. Because LOV2 has a higher quantum efficiency than LOV1 (Kasahara *et al.*, 2002; Salomon *et al.*, 2001), the ~~LOV1~~ + LOV2 fusion protein exhibits a greater degree of bleaching than the LOV1 + ~~LOV2~~ mutant. However, the ratio of relative bleaching obtained for the LOVCys39Ala mutant fusion proteins is 4 : 1 (~~LOV1~~ + LOV2 : LOV1 + ~~LOV2~~). This differs from the 10 : 1 ratio in quantum efficiency reported for the individual LOV2 and LOV1 domains of phot1 (Kasahara *et al.*, 2002; Salomon *et al.*, 2001). These findings indicate that the LOV2 : LOV1 quantum efficiency ratio for the LOV1 + LOV2 fusion protein differs from that obtained when the domains are studied individually.

Dark-regeneration kinetics for the LOV1 + LOV2 fusion protein (measured at 450 nm) are shown in Figure 1(d). The kinetics can be resolved into two components, with half-life times of 30 and 260 sec, respectively (Figure 1d). On the contrary, dark-regeneration kinetics for the individual LOV domains of phot1 occurs over a much shorter time scale (Kasahara *et al.*, 2002; Salomon *et al.*, 2001; Swartz *et al.*, 2001). Half-life times for dark regeneration of ~~LOV1~~ + LOV2 and LOV1 + ~~LOV2~~ fusion proteins are also shown. Dark-regeneration kinetics for the ~~LOV1~~ + LOV2 fusion protein are very similar to that of the wild-type fusion protein, indicating that photochemically active LOV2 alone is sufficient to mediate the slow dark regeneration of the LOV1 + LOV2 fusion protein. On the other hand, dark-regeneration kinetics for the LOV1 + ~~LOV2~~ fusion protein more closely resemble those of the isolated LOV1 domain (Kasahara *et al.*, 2002; Salomon *et al.*, 2001). LOV2 therefore dominates the kinetics of dark regeneration, as well as the photochemical activity of the phot1 LOV1 + LOV2 fusion protein.

Insect cell expression provides a convenient system to study phot1 kinase activity

Although our efforts to express active phot1 in *E. coli* have been unsuccessful, full-length phot1 can be expressed in insect cells using recombinant baculovirus (Christie *et al.*, 1998). Previous studies have also shown that phot1 expressed in insect cells exhibits phosphorylation kinetics and photosensitivity virtually identical to that of the native plant photoreceptor (Christie *et al.*, 1998). When expressed in insect cells, phot1 binds FMN and undergoes autophosphorylation in response to blue light (Christie *et al.*, 1998). This reaction is illustrated in Figure 2(a). Insect cells infected with recombinant baculovirus, encoding the *PHOT1* cDNA from *Arabidopsis,* were grown in complete darkness and harvested under dim red light as described previously (Christie *et al.*, 1998; Sakai *et al.*, 2001). Soluble protein extracts were isolated and used for *in vitro* phosphorylation analysis. As shown in Figure 2(a), phot1 becomes heavily phosphorylated following a brief irradiation with high-intensity white light. Replacement of an essential aspartate residue with asparagine, in subdomain VII of the C-terminal kinase domain of phot1, results in a loss of light-dependent autophosphorylation.

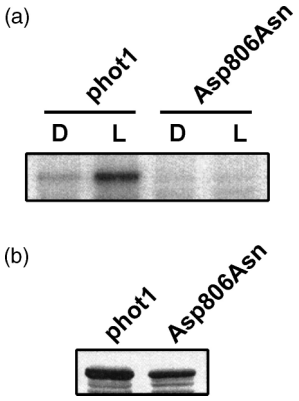

Figure 2. Autophosphorylation activity of phot1 expressed in insect cells.
(a) Autoradiograph showing light-dependent autophosphorylation in soluble protein extracts prepared from insects cells expressing phot1. No light-dependent autophosphorylation was observed for the Asp806Asn mutant of phot1. All manipulations were carried out under dim red light. Samples were given a mock irradiation, D, or irradiated with white light, L, at a total fluence of 30 000 μmol m^{-2} prior to the addition of radiolabelled ATP.
(b) Western blot analysis of phot1 protein levels. Soluble protein extracts prepared from insect cells expressing either wild-type phot1 or the Asp 806Asn mutant of phot1 were probed with antiphot1 antibody.

This aspartate is required for chelating Mg^{2+}, an ion that is necessary for phosphate transfer (Hanks and Hunter, 1995). Substitution of this aspartate with asparagine has no effect on phot1 protein levels (Figure 2b). These findings demonstrate that autophosphorylation is mediated by phot1 itself, and not some other kinase present in the insect cell extract. Thus insect cell expression provides an appropriate model system to study the effect of the LOVCys39Ala mutations on phot1 kinase activity.

LOV2 mediates the autophosphorylation activity of phot1 expressed in insect cells

Recombinant baculovirus encoding *PHOT1* cDNA carrying the LOV1Cys39Ala mutation, LOV2Cys39Ala mutation, or both, was generated to study the effect of these mutations on phot1 kinase activity in insect cells. As mentioned above, wild-type phot1 (WT) expressed in insect cells becomes heavily phosphorylated in response to a brief pulse of high-intensity white light (Figure 3a). Blocking the photochemical activity of LOV1 (~~LOV1~~ + LOV2) appears to have little, if any, effect on the level of phot1 autophosphorylation under saturating light conditions. Elimination of LOV2 activity (LOV1 + ~~LOV2~~), however, results in a severe loss of light-dependent autophosphorylation. Therefore phot1 autophosphorylation is predominantly mediated by LOV2 under saturating light conditions. These findings are consistent with the above photochemical studies, indicating that LOV2 functions as the principal light-sensing domain regulating the photochemical reactivity of the LOV1 + LOV2 fusion protein. No light-dependent autophosphorylation was detected for phot1 carrying both LOV1 and LOV2 Cys39Ala mutations (~~LOV1~~ + ~~LOV2~~). Western analysis also demonstrated that an impaired autophosphorylation response was not caused by a lack of phot1 protein (Figure 3b).

The extremely low level of autophosphorylation detected for the LOV2Cys39Ala mutant (Figure 3a) has made it difficult to determine whether this mutant retains any residual light-induced kinase activity. To investigate further the role of LOV1 in regulating phot1 kinase activity, we examined the autophosphorylation response of the LOV1-Cys39Ala mutant in more detail. Light-induced autophosphorylation of phot1 is a rapid response that reaches saturation at room temperature within several minutes in several plant species (Christie *et al.*, 1998; Palmer *et al.*, 1993; Reymond *et al.*, 1992b; Short *et al.*, 1992; Short *et al.*, 1994). Phot1 kinase activity is also dependent on the intensity of blue light used (Christie *et al.*, 1998; Reymond *et al.*, 1992b; Salomon *et al.*, 1997; Short and Briggs, 1990). We therefore investigated whether the kinetics and fluence-response requirements for blue light-induced autophosphorylation were altered in the LOV1Cys39Ala mutant. As shown in Figure 4, inactivation of LOV1 does not significantly alter either the kinetics or the fluence-response requirements for phot1 autophosphorylation, indicating that LOV1 plays little, if any, role in regulating phot1 kinase activity.

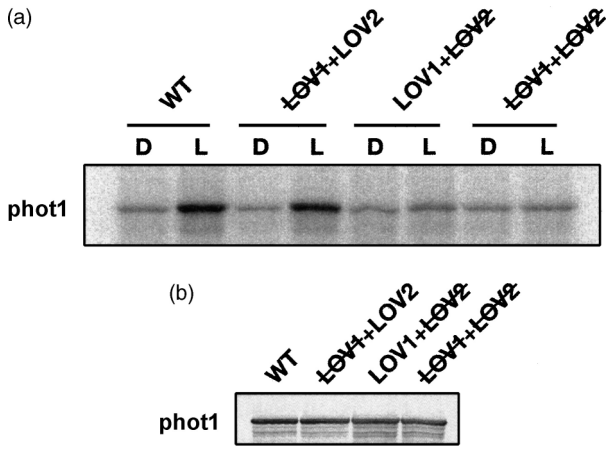

Figure 3. Autophosphorylation analysis of LOVCys39Ala mutants of phot1 expressed in insect cells.
(a) Light-dependent autophosphorylation activity of wild-type (WT) and LOVCys39Ala mutants of phot1 in soluble protein extracts prepared from insects cells. LOVCys39Ala mutants of phot1 are represented by a strike-through in the corresponding LOV domain. Sample preparation and experimental procedures were performed as described in Figure 2.
(b) Western blot analysis of phot1 protein levels. Soluble protein extracts prepared from insect cells expressing either wild-type phot1 or LOVCys39 Ala mutants of phot1 were probed with antiphot1 antibody.

LOV2 is essential for phot1-mediated hypocotyl phototropism in transgenic Arabidopsis

In addition to the insect cell expression system, we have used the *phot1* null mutant, *phot1-5* (formerly *nph1-5*, Huala *et al.*, 1997; Liscum and Briggs, 1995), to examine the effect of the LOVCys39Ala mutations on phot1-mediated phototropic responsiveness. Stable transformation of this mutant with the *PHOT1* gene from *Arabidopsis* restores wild-type levels of phototropic curvature to low fluence rates of unilateral blue light ($<1\,\mu mol\,m^{-2}\,sec^{-1}$) (Huala *et al.*, 1997). We have transformed *phot1-5* with *PHOT1* cDNA carrying the LOV1Cys39Ala mutation, the LOV2Cys39Ala mutation, or both, driven by the constitutively expressed cauliflower mosaic virus *35S* promoter. Transformants carrying wild-type *PHOT1* cDNA driven by the *35S* promoter were also generated as a positive control. For each construct, approximately 50 kanamycin-resistant T_1 plants were selected and self-fertilized. The resulting T_2 lines were analysed for phot1 expression by Western blotting using specific polyclonal phot1 antisera (Christie *et al.*, 1998). The phototropic responses of at least five independent phot1-expressing lines were examined for each construct. The data shown in Figure 5 are from individual lines, but are representative of the results obtained.

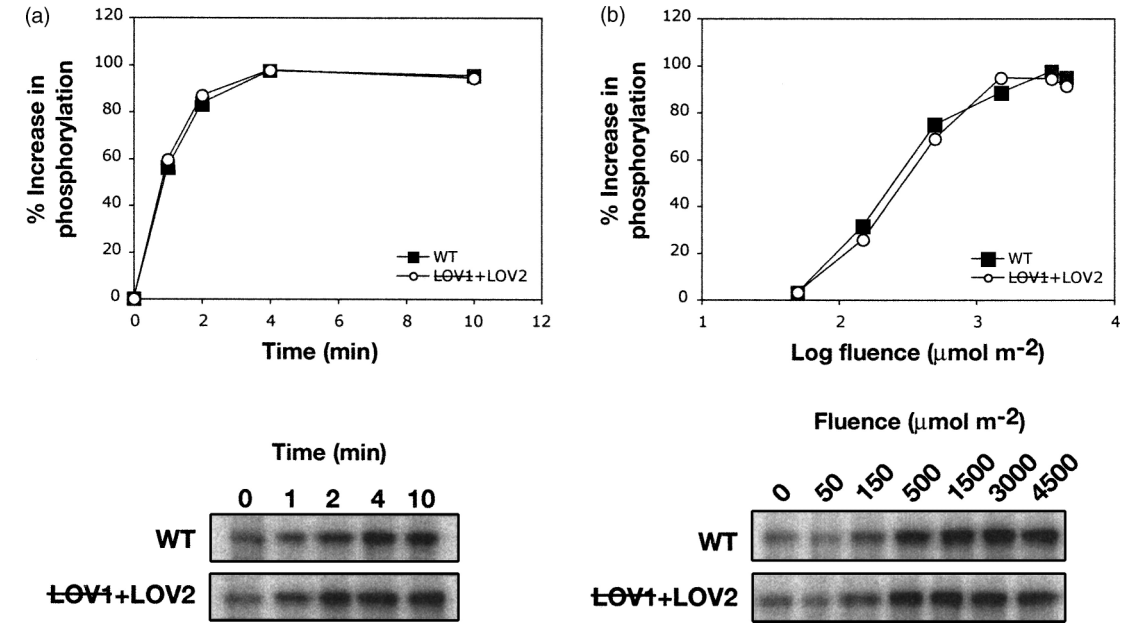

Figure 4. Photochemical and biochemical analysis of the LOV1Cys39Ala mutant of phot1 expressed in insect cells.
(a) Kinetics for blue light-induced autophosphorylation obtained for wild-type phot1 (WT) and the LOV1Cys39Ala mutant of phot1 (~~LOV1~~ + LOV2) in soluble protein extracts prepared from insect cells. Sample preparation and experimental procedures were performed as described in Figure 2. Samples were treated with blue light at a total fluence of 3300 μmol m^{-2} as described.
(b) Blue light fluence-response requirements for autophosphorylation of wild-type phot1 (WT) or the LOV1Cys39Ala mutant of phot1 (~~LOV1~~ + LOV2) in soluble protein extracts prepared from insect cells. In each case, all values are relative to dark controls and represent the average of three independent experiments. All values were within 10% of the mean. Autoradiographs from individual experiments are also shown.

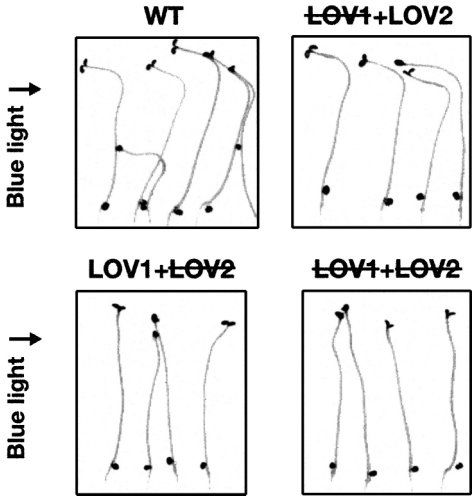

Figure 5. Functional analysis of LOVCys39Ala mutants of phot1 expressed in the *phot1-5* mutant of *Arabidopsis*.
The phototropic response of *phot1-5* transformants expressing either wild-type phot1 (WT) or LOVCys39Ala mutants of phot1 is shown. LOVCys39Ala mutations in phot1 mutant proteins expressed in *Arabidopsis* are represented by a strikethrough in the corresponding LOV domain. Plants were grown in the dark for 3 days and transferred to unilateral blue light (1 μmol m^{-2} sec^{-1}) for 24 h before imaging.

Transgenic *Arabidopsis* seedlings, expressing phot1 carrying both LOV1Cys39Ala and LOV2Cys39Ala mutations (~~LOV1~~ + ~~LOV2~~), do not complement the phot1 null mutant phenotype. Similarly, plants expressing phot1 with LOV2 inactivated (LOV1 + ~~LOV2~~) fail to show hypocotyl phototropism, indicating that LOV1, on its own, is insufficient to restore hypocotyl curvature to low fluence rates of unilateral blue light. In contrast, plants expressing phot1 with LOV1 inactivated (~~LOV1~~ + LOV2) exhibit hypocotyl curvature similar to that mediated by the wild-type photoreceptor (WT). Hence, photochemically active LOV2 appears to be essential and sufficient for regulating phot1-mediated phototropism in *Arabidopsis* under low light conditions.

LOV2 mediates autophosphorylation activity of phot1 in transgenic Arabidopsis

phot1 is a highly hydrophilic protein (Huala *et al.*, 1997), but it is found associated with the plasma membrane in *Arabidopsis* (Sakamoto and Briggs, 2002) and other plant species (Briggs *et al.*, 2001b). Although the exact nature of its association with the plasma membrane is unknown, it is likely that phot1 undergoes post-translational modification and/or binds a protein cofactor to facilitate membrane interaction. Indeed, biochemical studies have shown that phot1 interacts with additional proteins that form a plasma membrane-associated photoreceptor complex (Motchoulski and Liscum, 1999; Warpeha and Briggs, 1993).

Given the above results, it was important to examine autophosphorylation activity of phot1 in membrane

preparations isolated from the *35S::PHOT1* transgenic lines described above. Microsomal membranes were isolated from dark-grown seedlings under dim red light and used for *in vitro* phosphorylation analysis. As found for phot1 expressed in insect cells (Figure 3), phot1 autophosphorylation in microsomal membranes is predominantly mediated by LOV2, whereas LOV1 appears to mediate little, if any, autophosphorylation response (Figure 6a, upper arrow). No light-induced phot1 kinase activity was detected in membrane extracts isolated from transgenic seedlings carrying phot1 with both LOV1Cys39Ala and LOV2Cys39Ala mutations.

The level of phot1 protein expressed by the *35S* promoter (Figure 6b) appears to be considerably lower than that driven by the native *PHOT1* promoter in wild-type seedlings (data not shown). While the reason for this discrepancy is unknown, the reduced level of phot1 protein in the transgenic lines allowed us to detect a second protein that undergoes light-dependent autophosphorylation in microsomal membranes (Figure 6a, lower arrow). *PHOT2* is expressed at very low levels in dark-grown *Arabidopsis* seedlings, increasing upon exposure to UV-A, blue, red and white light (Jarillo *et al.*, 2001b; Kagawa *et al.*, 2001). The size of the second phosphoprotein is consistent with that of phot2. This light-dependent autophosphorylation reaction is absent in membranes isolated from a *phot1-phot2* double mutant (Figure 6c); hence we attribute the additional light-induced autophosphorylation response to the activity of phot2, indicating that phot2, like phot1, is membrane-associated in dark-grown *Arabidopsis* seedlings.

LOVCys39Ala mutations increase the fluorescence of a bacterially expressed phot2 LOV1 + LOV2 fusion protein

Taken together, the above results indicate that LOV2 plays an important role in regulating phot1 activity under the conditions examined. As the exact role of LOV1 remains unknown, it was of interest to establish whether the LOV1 and LOV2 domains of phot2 exhibit similar properties. Once again, we used the Cys39Ala mutation to investigate the individual roles of LOV1 and LOV2 in regulating the photochemical reactivity of a bacterially expressed *Arabidopsis* phot2 LOV1 + LOV2 fusion protein. As before, the resulting mutant fusion proteins were designated ~~LOV1~~ + LOV2 and LOV1 + ~~LOV2~~, respectively, where the strikethrough indicates the presence of the Cys39Ala mutation.

The fluorescence excitation spectra obtained for equal concentrations of wild-type and mutant phot2 LOV1 + LOV2 fusion proteins are shown in Figure 7(a). In each case, the spectrum is similar to that obtained for the phot1 fusion protein (Figure 1a). As found for the phot1 fusion protein, introducing the Cys39Ala mutation into LOV2 of the phot2 fusion protein (LOV1 + ~~LOV2~~) results in an increase in fluorescence emission relative to that of the wild-type fusion protein (>twofold). Introducing the same mutation into the LOV1 domain of the phot2 fusion protein (~~LOV1~~ + LOV2)

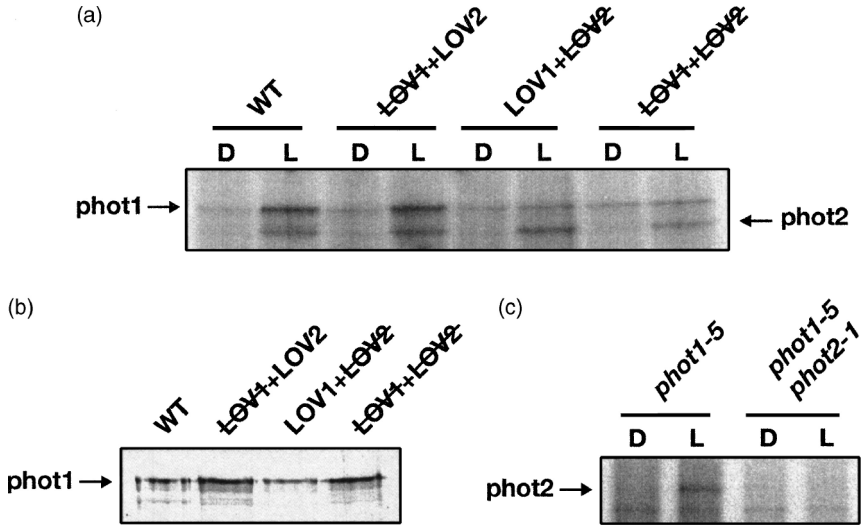

Figure 6. Autophosphorylation analysis of the LOVCys39Ala mutants of phot1 expressed in the *phot1-5* mutant of *Arabidopsis*.
(a) Light-dependent autophosphorylation activity in membranes isolated from transgenic *Arabidopsis* expressing either wild-type (WT) or LOVCys39Ala mutants of phot1 driven by the *35S* promoter (upper arrow). LOVCys39Ala mutations in phot1 mutant proteins expressed in *Arabidopsis* are represented by a strikethrough in the corresponding LOV domain. Sample preparation and experimental procedures were performed as described for Figure 2. The lower arrow indicates the presence of a second light-dependent autophosphorylation reaction.
(b) Western blot analysis of phot1 protein levels. Membranes prepared from transgenic *Arabidopsis* expressing wild-type or LOVC39Ala mutants of phot1 were probed with antiphot1 antibody. (c) Light-dependent autophosphorylation activity in microsomal membranes isolated from the *phot1-5* mutant and a *phot1phot2* double mutant. The autophosphorylation reaction observed in *phot1-5* is absent in the *phot1phot2* double mutant.

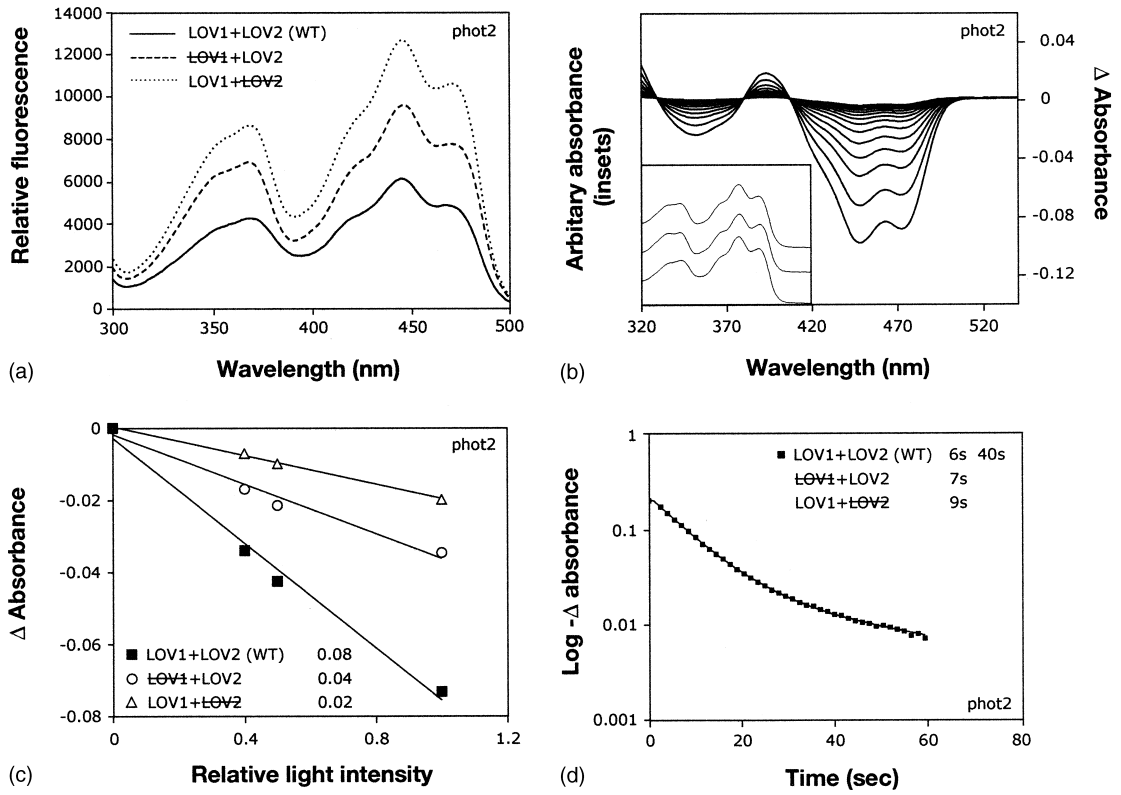

Figure 7. Photochemical and mutational analyses of the phot2 LOV1 + LOV2 fusion protein.
(a) Fluorescence excitation spectra of phot2 wild-type (WT) and LOVCys39Ala mutant fusion proteins. Mutant fusion proteins are designated ~~LOV1~~ + LOV2 and LOV1 + ~~LOV2~~, respectively, where the strikethrough indicates the presence of the Cys39Ala mutation. The fluorescence emission of equal concentrations of wild-type and mutant fusion proteins (0.3 mg ml^{-1}) were monitored at 520 nm.
(b) Light-minus-dark difference spectrum obtained for the wild-type phot2 LOV1 + LOV2 fusion protein (main panel). The difference spectrum shows dark recovery to the ground state after a light flash. Spectra were recorded at 2 sec intervals following the light flash. Absorption spectra of phot2 wild-type and LOVCys39Ala mutant fusion proteins are also shown (inset). Spectra are offset for clarity and are arranged as follows: WT (bottom), ~~LOV1~~ + LOV2 (centre), and LOV1 + ~~LOV2~~ (top).
(c) Relative bleaching measurements for FMN–cysteinyl adduct formation obtained for phot2 wild-type and LOVCys39Ala mutant fusion proteins. Light-induced absorbance changes were monitored at 450 nm over a range of light intensities (see Experimental procedures for details). Relative bleaching values for each fusion protein are shown and represent the average obtained from three independent protein preparations. In each case, all values were within 10% of the mean.
(d) Dark-regeneration kinetics for the wild-type phot2 LOV1 + LOV2 fusion protein measured at 450 nm (main panel). Half-life times for dark regeneration obtained for each fusion protein are shown.

also results in an increase in fluorescence emission relative to that of the wild-type fusion protein (1.6-fold). This differs from the effect found for phot1, where the LOV1Cys39Ala mutation has very little effect on the fluorescence emission of phot1 LOV1 + LOV2 fusion protein (Figure 1a). However, Kasahara *et al.* (2002) have recently shown that the LOV1 domains of phot1 and phot2 exhibit different quantum efficiencies. The LOV1 domain of phot2 has an almost fourfold higher quantum efficiency than that of the LOV1 domain of phot1. This difference in quantum efficiency correlates to the different degree of chromophore fluorescence quenching observed for the LOV1 domains of phot1 and phot2 (Figures 1a and 7a). The greater increase in fluorescence emission observed for phot2 ~~LOV1~~ + LOV2, as compared to the similar mutant in phot1, is consistent with the higher quantum efficiency reported for the individual LOV1 domain of phot2 (Kasahara *et al.*, 2002).

Photochemical activity of the phot2 LOV1 + LOV2 fusion protein is predominantly mediated by LOV2

The absorption spectra for wild-type phot2 and LOVcy39Ala mutant fusion proteins are shown in Figure 7(b) (inset) and are offset for clarity. As found for phot1, the absorption spectra of the phot2 LOV1 + LOV2 mutant fusion proteins are nearly identical to that of the wild-type phot2 fusion protein. Again, like the LOVCys39Ala mutant fusion proteins of phot1, the phot2 LOV1 + LOV2 mutant fusion proteins show key differences in the near-UV region of the spectrum, which probably reflect changes in the chromophore environment resulting from the Cys39Ala mutation.

The photochemical activity of the phot2 LOV1 + LOV2 fusion protein (Figure 7b, main panel) is very similar to that of the phot1 fusion protein (Figure 1b), except that it

recovers in the dark over a period of many seconds rather than minutes. Similar light-induced absorbance changes were observed for the phot2 LOV1 + LOV2 mutant fusion proteins (data not shown). Figure 7(c) shows maximal bleaching (at 450 nm) for each of the phot2 fusion proteins in response to a pulse of blue light at different intensities. Again, the slope of the line for each fusion protein gives a measure of relative bleaching in response to blue light. The phot2 ~~LOV1~~ + LOV2 fusion protein retains a comparatively high degree of relative bleaching (0.04) compared to that of the wild-type fusion protein (0.08), whereas blocking the photochemical activity of LOV2 results in a fourfold decrease in relative bleaching (0.02). Thus, as found for phot1 fusion protein, photochemical activity of phot2 LOV1 + LOV2 is predominantly mediated by LOV2.

The difference in relative bleaching observed for the phot2 ~~LOV1~~ + LOV2 and LOV1 + ~~LOV2~~ fusion proteins also correlates with the difference in quantum efficiency reported for the individual LOV domains of phot2. As the LOV2 domain of phot2 has a twofold higher quantum efficiency than that of LOV1 (Kasahara *et al.*, 2002), the ~~LOV1~~ + LOV2 fusion protein exhibits a greater degree of bleaching than the LOV1 + ~~LOV2~~ mutant. The ratio of relative bleaching obtained for the phot2 LOVCys39Ala mutant fusion proteins is 2 : 1 (~~LOV1~~ + LOV2 : LOV1 + ~~LOV2~~). This is consistent with the 2 : 1 ratio in quantum efficiency reported for the individual LOV2 and LOV1 domains of phot2 (Kasahara *et al.*, 2002).

Dark-regeneration kinetics for the phot2 LOV1 + LOV2 fusion protein (measured at 450 nm) are shown in Figure 7(d). Like the phot1 fusion protein, dark-regeneration kinetics for phot2 LOV1 + LOV2 can be resolved into two components, with half-life times of 6 and 40 sec, respectively. However, the dark-regeneration kinetics for the phot2 LOV1 + LOV2 fusion protein are approximately 10-fold faster than those of the phot1 fusion protein (Figure 1d). Half-life times for dark regeneration of the phot2 ~~LOV1~~ + LOV2 and LOV1 + ~~LOV2~~ fusion proteins are shown (Figure 7d). Inactivation of either LOV1 or LOV2 appears to result in a loss of the second component. Nevertheless, we are unable to conclude from these data whether one particular domain dominates the dark regeneration of the phot2 LOV1 + LOV2 fusion protein, as was found for the phot1 fusion protein.

Phot2 kinase activity in insect cells is predominantly mediated by LOV2

Phot2, like phot1, undergoes light-dependent autophosphorylation when expressed in insect cells (Sakai *et al.*, 2001) and reaches saturation within several minutes (data not shown). Therefore we used the insect cell expression system once again to examine the effect of the LOVCys39Ala mutations on phot2 kinase activity. As reported previously (Sakai *et al.*, 2001), wild-type phot2 (WT) expressed in insect cells becomes heavily phosphorylated in response to a brief irradiation with high intensity white light (Figure 8a). Blocking the photochemical activity of LOV1 (~~LOV1~~ + LOV2) appears to have little, if any, effect on the level of phot2 autophosphorylation under saturating light conditions. In contrast, inactivation of LOV2 (LOV1 + ~~LOV2~~) results in a severe loss of light-dependent autophosphorylation. Thus, as found for phot1, phot2 autophosphorylation is predominantly mediated by LOV2 under these conditions. These findings are consistent with the above observations that LOV2 functions as the principal light-sensing domain in regulating the photochemical activity of the phot2 LOV1 + LOV2 fusion protein. Even so, the LOV1 domain of phot2 does appear to mediate a small level of light-induced autophosphorylation. No light-dependent autophosphorylation was detected for phot2 carrying both LOV1Cys39Ala and LOV2Cys39Ala mutations (~~LOV1~~ + ~~LOV2~~). Western analysis of wild-type and mutant phot2 protein levels is shown in Figure 8(b).

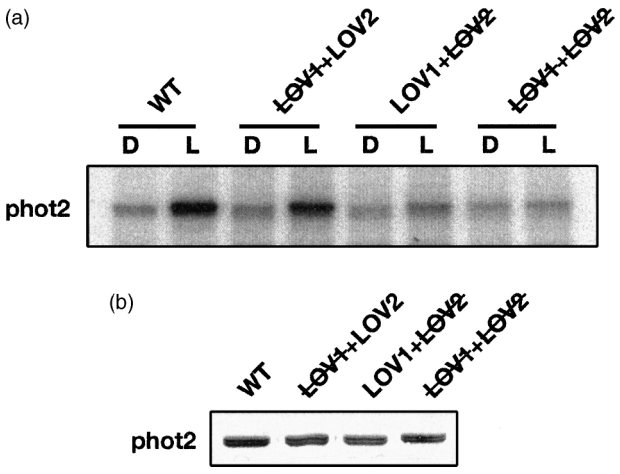

Figure 8. Autophosphorylation analysis of the phot2 LOVCys39Ala mutants expressed in insect cells.
(a) Light-dependent autophosphorylation of wild-type (WT) and LOVC39Ala mutants of phot2 in soluble protein extracts prepared from insect cells. Cys39Ala mutants of phot1 are represented by a strikethrough in the corresponding LOV domain. Sample preparation and experimental procedure were performed as described for Figure 2.
(b) Western blot analysis of phot2 protein levels. Soluble protein extracts prepared from insect cells expressing either wild-type phot2 or LOVC39Ala mutants of phot2 were probed with anti-His antibody.

Discussion

Cys39Ala mutations offer a means to study LOV domain function

Phototropin LOV domains operate through an initial photochemistry that involves the formation of a covalent adduct

between the FMN chromophore and a highly conserved cysteine residue (Cys39) (Briggs and Christie, 2002; Christie and Briggs, 2001). Cys39 is part of the highly conserved motif NCRFLQ, found in all phototropin LOV domains (Briggs *et al.*, 2001a; Christie and Briggs, 2001). The NCRFLQ motif, in addition to other conserved flavin-binding residues, is also present in non-phototropin proteins that contain a single LOV/PAS domain (Crosson and Moffat, 2001; Crosson and Moffat, 2002). A number of these proteins are involved in the regulation of circadian rhythms. These include ZTL, FKF1 and LKP2 from *Arabidopsis* (Jarillo *et al.*, 2001a; Kiyosue and Wada, 2000; Nelson *et al.*, 2000; Somers *et al.*, 2000), and WC-1 and VIVID from the filamentous fungus, *Neurospora crassa* (Ballario *et al.*, 1996; Crosthwaite *et al.*, 1997; Heintzen *et al.*, 2001).

Proteins containing single LOV/PAS domains have also been identified in prokaryotes, including a putative sensor histidine kinase from *Caulobacter crescentus* (Crosson and Moffat, 2002) and YtvA, a protein from *Bacillus subtilis* reported to function as a positive regulator of the general stress transcription factor, σ^B (Akbar *et al.*, 2001). Losi *et al.* (2002) have recently shown that YtvA, expressed in *E. coli*, binds a flavin chromophore and exhibits blue light-dependent photochemistry analogous to that of the phototropin LOV domains. Therefore the presence of LOV domain-containing photoreceptors in bacteria (Losi *et al.*, 2002), as well as green algae (Holzer *et al.*, 2002; Kasahara *et al.*, 2002), suggest that this light-driven molecular switch has been conserved throughout evolution. It will now be of interest to establish whether the other LOV/PAS domain-containing proteins described above bind a flavin cofactor and play a direct role in photoreception. The Cys39Ala mutation described here may prove useful in determining whether the conserved cysteine of each these LOV/PAS domains is essential for regulating protein function, as is the case for the phototropins.

LOV domains function to regulate phototropin kinase activity

It has been hypothesized that light sensing by the LOV domains of phototropin results in a protein conformational change, which in turn leads to activation of the C-terminal kinase (Briggs and Christie, 2002; Christie and Briggs, 2001; Salomon et al., 2001). Indeed, recent NMR studies suggest that light-induced FMN-cysteinyl adduct formation results in a conformational change of the LOV2 apoprotein (Salomon et al., 2001). Similarly, activation of the bacterial PAS domain-like photoreceptor, PYP, has been reported to involve a light-driven protein conformational change (Lee et al., 2001; Xie et al., 2001).

Our present findings are consistent with the hypothesis that the N-terminal region, including LOV1 and LOV2, acts to regulate phototropin kinase activity. Simultaneous inactivation of both LOV1 and LOV2 results in a loss of light-induced autophosphorylation of phot1 and phot2 (Figures 3, 6 and 8). Moreover, phot1 carrying both LOV1Cys39Ala and LOV2Cys39Ala mutations fails to restore phototropic responsiveness in the phot1 mutant, phot1-5 (Figure 5). Thus the N-terminal region, including LOV1 and LOV2, must somehow act to repress phototropin kinase activity in the absence of light. On irradiation, light sensing by the LOV domains unlocks this repression to activate the C-terminal kinase domain. A similar PAS/kinase domain interaction mechanism has been proposed for regulating the activities of the bacterial oxygen sensor, FixL (Gong et al., 1998), and the novel eukaryotic protein kinase, PAS kinase (Rutter et al., 2001). At least for phot1, biochemical evidence suggests that such a process might involve intermolecular communication between distinct phototropin molecules (Reymond et al., 1992a). However, given that phot1 and phot2 exhibit light-dependent kinase activity when expressed in insect cells (Figures 3 and 8), it seems unlikely that this light-driven process involves any additional protein factor(s).

Light sensing by phot1 is predominantly mediated by LOV2

All phototropin blue light receptors identified to date possess not one, but two FMN-binding domains (Briggs *et al.*, 2001a). Here we demonstrate that LOV2 plays a prominent role in regulating the autophosphorylation activity of phot1 expressed in both insect cells and transgenic *Arabidopsis* (Figures 3 and 6), and is required to elicit phot1-mediated hypocotyl curvature to low fluence rates of unilateral blue light (Figure 5). In addition, photochemical analyses of the bacterially expressed phot1 LOV1 + LOV2 fusion protein demonstrate that LOV2 functions as the principal light-sensing domain for phot1 (Figure 1). LOV2 not only constitutes the main photosensitive component of phot1, but is sufficient to bring about the slow dark-regeneration kinetics observed for the phot1 LOV1 + LOV2 fusion protein. Moreover, the slow dark-regeneration kinetics observed for the phot1 LOV1 + LOV2 fusion protein are not observed when the separate LOV domains of phot1 are mixed together prior to assay (data not shown), suggesting that the intervening amino acid sequence between LOV1 and LOV2 is required to bring about this property.

The cause of the slow dark-regeneration process observed for the phot1 LOV1 + LOV2 fusion protein is currently unknown. However, similar dark-regeneration kinetics have been reported for full-length phot1 expressed in insect cells (Kasahara *et al.*, 2002). Further biochemical analysis of the phot1 LOV1 + LOV2 fusion protein will help to determine the nature of this slow recovery process. One possibility is that the reversal of phot1 photochemistry is rate-limited by protein conformational change(s). Such protein change(s)

may be associated with regulating the activity of the C-terminal kinase domain. Indeed, autophosphorylation of phot1 *in vitro* has been shown to possess a memory for a light pulse when subsequently transferred to darkness (Christie *et al.*, 1998; Palmer *et al.*, 1993; Salomon *et al.*, 1996; Short *et al.*, 1992). The time course for this response is very similar to the dark-regeneration kinetics observed for phot1. The dark-regeneration kinetics for phot1 also correlate with the recovery of phototropic sensitivity following a saturating pulse of light (Briggs *et al.*, 2001b). Therefore, dark regeneration of phot1 is likely to be associated with restoring the photosensory system to the ground state.

Phot2 kinase activity is detectable in the phot1 mutant

phot2, like phot1, undergoes light-dependent autophosphorylation when expressed in insect cells (Sakai *et al.*, 2001; Figure 8). In the present study, we have also shown that phot2 kinase activity can be detected in microsomal membranes isolated from dark-grown seedlings of the *phot1-5* null mutant (Figure 6c). Whether the high light intensities used here for *in vitro* phosphorylation are necessary to observe phot2 kinase activity in the *phot1-5* mutant requires further investigation. Nevertheless, the level of phot2 activity detected in the absence of phot1 is not sufficient to mediate phototropic curvature to low fluence rates of unilateral blue light (Huala *et al.*, 1997; Liscum and Briggs, 1995). In addition, phot2-mediated hypocotyl phototropism probably requires RPT2, a protein proposed to function as an adaptor or scaffold that brings signalling components together (Liscum and Stowe-Evans, 2000; Sakai *et al.*, 2000). *RPT2* gene expression, like *PHOT2*, is induced by light in dark-grown *Arabidopsis* seedlings (Sakai *et al.*, 2000), and appears to be under the control of phytochrome A (Tepperman *et al.*, 2001). Therefore phot2 appears to mediate phototropism only at high light intensities (Sakai *et al.*, 2001). In contrast, phot1-induced hypocotyl curvature requires the adaptor protein, NPH3 (Motchoulski and Liscum, 1999), and occurs in response to both low and high light intensities (Sakai *et al.*, 2001).

One reason why we failed to detect phot2 kinase activity in previous studies could be that the high level of phot1 autophosphorylation typically found in wild-type seedlings masks the small phosphorylation signal detected for phot2. The level of phot1 protein expressed from the *35S* promoter (Figure 6c) is reduced relative to the expression level driven by the native *PHOT1* promoter (data not shown). Hence, phot2 kinase activity is detectable in each of the *35S::PHOT1* transgenic lines generated (Figure 6a). It is currently unknown why phot1 protein levels are reduced in the *35S::PHOT1* transgenic lines. However, reduced levels of functional phot1 do not appear to impair phototropic responsiveness to low fluence rates of unilateral blue light (Figure 5).

LOV1 and LOV2 also exhibit distinct roles in regulating phot2 kinase activity

phot2, like phot1, appears to operate through a mechanism by which LOV2 acts as the principal light-sensing domain. Photochemical analyses of the bacterially expressed phot2 LOV1 + LOV2 fusion protein demonstrate that LOV2 plays a major role in light detection for phot2 (Figure 7). Similarly, LOV2 plays a prominent role in regulating phot2 kinase activity in insect cells (Figure 8). Thus, as found for phot1, phot2 activity is predominantly mediated by LOV2. Yet our studies show that the LOV1 domain of phot2 is able to mediate a small degree of light-dependent autophosphorylation (Figure 8a). This is not as apparent for phot1, but correlates with the higher quantum efficiency reported for the LOV1 domain of phot2 (Kasahara *et al.*, 2002). It will now be important to establish whether the LOV1 and LOV2 domains of phot2 mediate autophosphorylation on the same or different amino acid residues.

At present, it is unclear whether light-induced autophosphorylation plays a role in receptor signalling and/or is involved in some other function, say, receptor desensitization. Mutant alleles carrying single amino acid substitutions in the kinase domain have been identified for both phot1 and phot2, indicating that kinase activity is essential for signalling (Huala *et al.*, 1997; Kagawa *et al.*, 2001). At least for phot1, autophosphorylation occurs on multiple serine residues (Short *et al.*, 1994). Mapping the sites of autophosphorylation and mutagenesis of these sites will provide information on the role of autophosphorylation in phototropin signalling.

While LOV1 + LOV2 fusion proteins of phot1 and phot2 exhibit similar degrees of relative bleaching in response to blue light (Figures 1c and 7c), their kinetics for dark regeneration differ significantly. The dark-regeneration kinetics observed for the phot2 LOV1 + LOV2 fusion protein are 10-fold faster than those of the phot1 fusion protein (Figures 1c and 7c). Given that full-length phot1 and phot2 expressed in insect cells also show this difference (Kasahara *et al.*, 2002), it seems likely that it serves some functional importance. For instance, the rapid recovery of phot2 would be expected to yield steady-state levels of photoproduct much lower than those of phot1 at a given light intensity. As a result, higher light levels would be required to drive phot2 to the same photostationary equilibrium as that of phot1. Therefore, the different dark-regeneration kinetics observed for phot1 and phot2 may partially account for the difference in fluence requirements observed between phot1 and phot2 in regulating phototropism and light-induced chloroplast movement in *Arabidopsis* (Sakai *et al.*, 2001).

Implications for distinct roles of LOV1 and LOV2 in regulating phototropin function

Collective alignment of LOV1 or LOV2 domains from different phototropins shows that each domain is highly conserved between diverse plant species (65–92% amino acid sequence identity within LOV1 or LOV2; Christie *et al.*, 1999). Sequence identity between LOV1 and LOV2, either intramolecularly or intermolecularly, is significantly lower (≈40%; Christie *et al.*, 1999). Thus LOV1 and LOV2 domains can be separated into two groups based on their amino acid sequence identity. The LOV1 and LOV2 domains from several different phototropins have also been shown to exhibit distinct photochemical properties (Kasahara *et al.*, 2002), suggesting that LOV1 and LOV2 have unique functional roles. This conclusion is supported by the results presented here, whereby LOV2 acts as the principal light-sensing domain regulating phot1 and phot2 kinase activity (Figures 3, 6 and 8). At least for phot1, LOV1 appears to mediate little photochemical reactivity in comparison to LOV2 (Figure 1). In addition, LOV1 is insufficient to restore phot1-mediated hypocotyl curvature to low fluence rates of blue light in the *phot1-5* null mutant (Figure 5). Moreover, inactivation of LOV1 has no effect on the kinetics and fluence-response requirements for blue light-induced autophosphorylation of phot1 expressed in insect cells (Figure 4). Thus, LOV1 and LOV2 domains of plant phototropins appear to carry out different functions.

While our findings indicate that LOV2 plays an important role in regulating phototropin activity, the exact role of LOV1 remains unclear. The LOV1 domain of oat phot1 has been reported to self-dimerize, whereas the LOV2 domain does not (M. Salomon, personal communication). Similarly, the LOV domain of WC-1 from *Neurospora* has been shown to homodimerize *in vitro* (Ballario *et al.*, 1998). LOV1 may therefore play a role in receptor dimerization. If so, receptor dimerization may be affected by light and, in turn, control the sensitivity of a phototropin-associated signalling complex (Liscum and Stowe-Evans, 2000). Dissociation of such a photoreceptor complex may function to desensitize the system. Indeed, recent localization studies have shown that a fraction of phot1 rapidly dissociates from the plasma membrane in dark-grown *Arabidopsis* seedlings following blue light irradiation (Sakamoto and Briggs, 2002).

A detailed analysis of independent homozygous lines carrying wild-type phot1 or the LOV1Cys39Ala mutant is now required to assess the possible role of LOV1 in regulating phot1-mediated curvature to low fluence rates of unilateral blue light. Any qualitative and/or quantitative physiological differences observed between these two lines will provide insights into the role of LOV1 in regulating phot1-mediated hypocotyl curvature under low light conditions. We were unable to assess whether the LOV2-Cys39Ala mutant of phot1 is able to mediate hypocotyl phototropism under high light conditions owing to the presence of phot2 in these transgenic lines. Transformants expressing either phot1 or phot2 carrying the LOV1Cys-39Ala or LOV2Cys39Ala mutations are currently being generated in the *phot1phot2* double mutant, to avoid the partial overlap in function between phot1 and phot2 (Kinoshita *et al.*, 2002; Sakai *et al.*, 2001). Given our present findings, it is possible that LOV1 activity may be involved in regulating a response(s) other than phototropism. The *phot1phot2* transgenics will provide further information regarding the role of LOV1 and LOV2 in regulating phot1 and phot2 function, not only for phototropism, but also for other phototropin-mediated responses such as light-induced chloroplast movement (Kagawa *et al.*, 2001 Jarillo *et al.*, 2001b; Sakai *et al.*, 2001); stomatal opening (Kinoshita *et al.*, 2002); and the rapid inhibition of hypocotyl growth (Folta and Spalding, 2001).

Experimental procedures

Expression and purification of LOV domain fusion proteins

LOV domain fusion proteins were expressed in *E. coli* and purified by calmodulin affinity chromatography as described previously (Christie *et al.*, 1999; Kasahara *et al.*, 2002; Salomon *et al.*, 2000). The *E. coli* strain used was BL21(DE3)pLysS (Novagen, Madison, WI, USA).

Site-directed mutagenesis

Single amino acid mutations were introduced by using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) in accordance with the supplier's instructions. Two rounds of mutagenesis were performed to generate sequences carrying both LOV1 and LOV2 Cys39Ala mutations. All amino acid changes were verified by DNA sequencing.

Spectral analysis

Fluorescence excitation spectra of LOV domain fusion proteins were recorded using a Photon Technology International Alphascan spectrofluorometer (Photon Technology International, South Brunswick, NJ, USA). Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA) using BSA as standard. Absorption spectra and light-minus-dark difference spectra were collected on a Hewlett Packard 8452 A diode array spectrometer as described previously (Swartz *et al.*, 2001). In each case, the optical path length was 1 cm. Dark-regeneration kinetics were obtained by measuring the recovery of absorption at 450 nm following a pulse of blue light. Exponential fitting was performed using SIGMA PLOT (SPSS, Richmond, CA, USA). The blue light source for light-minus-dark difference spectra was provided by a white light camera strobe flash (1 ms, ≈100 mJ pulse), filtered through Corning Glass filters 3–73 and 4–96, and a Corning 100 nm band pass filter with maximum transmission at 400 nm (Swartz *et al.*, 2001). Control of data acquisition and flash were automated with software written in LABVIEW (National Instruments, Austin, TX, USA). Temperature was not controlled but

was measured to be 20 ± 2°C. Relative bleaching measurements at 450 nm for each LOV domain fusion protein were obtained by reducing the light intensity of the camera strobe flash. This reduction was achieved by placing neutral density filters (Thermo-oriel, Stratford, CT, USA) in front of the actinic light path. A 2 × 10 mm cuvette was used for the measurements of relative bleaching, with the actinic light directed along the 2 mm path (90° to the measuring beam). In all cases, sample concentration was adjusted to 0.2 OD at 450 nm. The entire sample was irradiated in all spectral measurements. The same geometry was used for all relative bleaching measurements.

Phototropin expression in insect cells

Recombinant baculovirus encoding either *Arabidopsis PHOT1* or *PHOT2* carrying specific amino acid mutations was generated using the BaculoGold Transfection Kit (BD Biosciences Pharmingen, Palo Alto, CA, USA) in accordance with the supplier's instructions. Recombinant baculovirus was titred by end-point dilution and 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).

Construction of plant transformation vectors

35S::PHOT1 transformation vectors were constructed using the binary expression vector pEZR(K)-LC (P. Eiken, G.-J. de Boer and D.W. Ehrhardt, unpublished results). This vector was created for cloning of cDNA sequences to the C-terminus of green fluorescent protein (GFP). The *GFP* coding sequence was removed using *Hin*dIII and *Sal*I. *35S::PHOT1* transformation vectors were constructed as follows: using the *PHOT1* cDNA of *Arabidopsis* inserted into the baculovirus transfer pAcHLT-A as a template (Christie *et al.*, 1998), the N-terminal region of phot1, including the first 192 amino acids fused to a 6XHis affinity tag, was synthesized as a DNA fragment using PCR and specific DNA primers. The resulting DNA fragment was then cloned into the binary expression vector following the removal of GFP. The cloning vector pBluescript (Stratagene), carrying the *PHOT1* cDNA of *Arabidopsis* (Huala *et al.*, 1997), was used to generate the LOVCys39Ala mutants by site-directed mutagenesis. Each mutated cDNA sequence was then subcloned into the transformation vector using *Sal*I and *Bam*HI such that a full-length *PHOT1* cDNA clone including an N-terminal 6XHis affinity tag was generated.

Transformation of Arabidopsis

35S::PHOT1 constructs were transformed into the phot1 null mutant, *phot1-5* (formerly *nph1-5*; Huala *et al.*, 1997; Liscum and Briggs, 1995) with *Agrobacterium* by floral dipping (Clough and Bent, 1998). The *Agrobacterium* strain used was GV3101 (pMP90). Kanamycin-resistant plants were selected on 0.5 × Murashige and Skoog (MS) agar. All analyses were carried out using independent transformants from the T_2 generation.

In vitro phosphorylation and Western blot analysis

Soluble protein extracts were prepared from insect cells as described (Christie *et al.*, 1998; Sakai *et al.*, 2001). Microsomal membranes were prepared from 3-day-old, dark-grown *Arabidopsis* seedlings as described (Liscum and Briggs, 1995). *In vitro* autophosphorylation analysis of soluble protein extracts from insect cells (10 μg) and microsomal membranes from *Arabidopsis* (20 μg) were carried out in the absence of Triton X-100, as described previously (Sakai *et al.*, 2001). The blue light source used for the study of kinetics and fluence-response requirements for blue light-induced autophosphorylation are described elsewhere (Liscum and Briggs, 1995). All experiments were repeated at least three times and the data shown, unless otherwise stated, are representative of the results obtained. Western blot analysis of soluble protein extracts from insect cells (10 μg) and microsomal membranes from *Arabidopsis* (20 μg) was performed as described (Sakai *et al.*, 2001) using antiphot1 antibody (Christie *et al.*, 1998) or anti-His antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Measurement of phototropic curvature

Hypocotyl curvatures were assayed as described (Lascève *et al.*, 1999). Three-day-old dark-grown seedlings of *Arabidopsis* were illuminated with unilateral blue light (1 $\mu mol\, m^{-2}\, sec^{-1}$) for 24 h before imaging.

Acknowledgements

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