Mutational Analysis of Phototropin 1 Provides Insights into the Mechanism Underlying LOV2 Signal Transmission*

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Phototropins (phot1 and phot2) are blue light-activated serine/threonine protein kinases that elicit a variety of photoreponses in plants. Light sensing by the phototropins is mediated by two flavin mononucleotide (FMN)-binding domains, designated LOV1 and LOV2, located in the N-terminal region of the protein. Exposure to light results in the formation of a covalent adduct between the FMN chromophore and a conserved cysteine residue within the LOV domain. LOV2 photoexcitation is essential for phot1 function in Arabidopsis and is necessary to activate phot1 kinase activity through light-induced structural changes within a conserved α-helix situated C-terminal to LOV2. Here we have used site-directed mutagenesis to identify additional amino acid residues that are important for phot1 activation by light. Mutagenesis of bacterially expressed LOV2 and full-length phot1 expressed in insect cells indicates that perturbation of the conserved salt bridge on the surface of LOV2 does not play a role in receptor activation. However, mutation of a conserved glutamine residue (Gln575) within LOV2, reported previously to be required to propagate structural changes at the LOV2 surface, attenuates light-induced autophosphorylation of phot1 expressed in insect cells without compromising FMN binding. These findings, in combination with double mutant analyses, indicate that Gln575 plays an important role in coupling light-driven cysteinyl adduct formation from within LOV2 to structural changes at the LOV2 surface that lead to activation of the C-terminal kinase domain.

Light is critical in shaping the growth and development of plants. The effect of light on plant morphogenesis is mediated through a variety of photoreceptors with specific spectral properties. Genetic analysis using the model plant Arabidopsis thaliana has shown that the effects of blue (390–500 nm) and UV-A light (320–390 nm) are mediated by at least two classes of blue light receptors, cryptochromes and phototropins (1–4). Phototropins function to regulate a range of photoreponses, including phototropism, stomatal opening, and chloroplast movement, all of which serve to optimize the photosynthetic efficiency of plants (2). Phototropin activity has also been implicated in regulating extension-growth responses in Arabidopsis such as cotyledon expansion (5), leaf expansion (6), and growth promotion under weak light conditions (7).

Arabidopsis contains two phototropins designated phot1 and phot2 (2). Phot1 and phot2 are flavoprotein photoreceptors whose protein structure can be divided into two segments as follows: a photosensory domain at the N terminus and a serine/threonine kinase domain at the C terminus (Fig. 1A). The N-terminal photosensory domain of the phototropins contains a repeated motif of ~110 amino acids called LOV1 and LOV2, respectively (8–10). LOV2 domains are members of the large and diverse superfamily of Per, Arnt, Sim (PAS) domains associated with cofactor binding and mediating protein interactions (11). LOV domains are more closely related to a subset of proteins within the PAS domain superfamily that are regulated by external signals such as light, oxygen, or voltage, hence the acronym LOV (8).

Purification of sufficient quantities of LOV domain-containing proteins expressed in Escherichia coli has greatly facilitated the spectral and structural analysis of these light-sensing motifs. Both LOV1 and LOV2 serve as noncovalent binding sites for the chromophore flavin mononucleotide (FMN) and form a spectral species in darkness, designated LOV447, which absorbs maximally at 447 nm (12–14). In each case, irradiation of the domain induces a unique mode of photochemistry that involves the formation of a covalent adduct between the C(4a) carbon of the flavin chromophore and a conserved cysteine residue within the LOV domain (13). Light- driven FMN-cysteinyl adduct formation occurs in the order of microseconds producing a spectral species (LOV390) that absorbs maximally at 390 nm (13–15). Formation of LOV390 is fully reversible in darkness, returning the LOV domain back to its initial ground state (LOV447) within the order of ten to hundreds of seconds (13, 14).

Although both LOV1 and LOV2 exhibit the above-mentioned photochemistry, structure-function studies have demonstrated that LOV2 photoreactivity is essential for phototropin function, whereas LOV1 photoreactivity is not (16, 17). Moreover, the photochemical reactivity of LOV2 is required for the activation of the C-terminal kinase domain, which results in receptor autophosphorylation (16). In contrast, the role of LOV1 is unclear at present, although size exclusion

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2 The abbreviations used are: LOV, light, oxygen, or voltage; PAS, Per, Arnt, Sim; FMN, flavin mononucleotide; FTIR, Fourier transform infrared.

2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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chromatography (18) in addition to small angle x-ray scattering analysis (19) of purified LOV1 suggests that this domain may play a role in receptor dimerization.

Recent structural studies indicate that the predominant role of LOV2 in regulating phototropin kinase activity may stem from its position within the phototropin molecule. Solution NMR spectroscopy has identified a conserved α-helix (designated Jα), situated C-terminal to LOV2 (Fig. 1A), that functions as a helical connector coupling LOV2 photoexcitation to kinase activation (20). Jα docks onto the surface of the LOV2-core in darkness and becomes disordered upon light-induced formation of LOV2,390. Moreover, artificial disruption of the LOV2-Jα interaction through site-directed mutagenesis results in an activation of phot1 kinase activity in the absence of light (21), demonstrating that unfolding of Jα results in activation of the C-terminal kinase domain.

Disruption of the LOV2-Jα interaction most likely arises from light-induced protein changes occurring at the surface of the LOV2-core. Crystal structures of two LOV domains from independent phototropin proteins reveal that the LOV domain adopts a typical PAS domain structure comprising five antiparallel β-sheets interconnected by two α-helices, in which the FMN chromophore is bound within a central hydrophobic pocket (22–24). However, structural knowledge has provided little information on how the signal generated upon formation of LOV2,390 is transmitted to the surface of the domain in order to bring about a disorganization of Jα that leads to an activation of phototropin kinase activity.

To date, two mechanisms of LOV2 signal transmission have been proposed. First, x-ray crystallography has revealed the presence of a conserved salt bridge at the surface of the LOV domain (22, 23). Light-driven destabilization of this surface salt bridge has been hypothesized to play a role in coupling LOV domain photoexcitation to phototropin kinase activation (25). The second mechanism involves a conserved glutamine residue within the LOV domain that interacts with the FMN chromophore via hydrogen bonding (22). This glutamine has recently been shown to be involved in propagating light-induced protein conformational changes associated with LOV2 protein fragments (26, 27), indicating that this residue may serve to transmit modifications from within the chromophore-binding pocket to protein changes at the LOV domain surface.

In this study, we have employed a mutagenesis approach to clarify the role of the aforementioned regions associated with the LOV2 domain of Arabidopsis phot1 in regulating photoreceptor kinase activation. Here, we show that disruption of the conserved salt bridge does not play a role in LOV2 signal transmission. Rather the conserved surface salt bridge appears to be important for locally stabilizing the tertiary structure of LOV2. By contrast, mutation of the conserved glutamine residue within LOV2 attenuates light-dependent autophosphorylation activity of phot1 expressed in insect cells, indicating that this residue is involved in LOV2 signal transmission. This conclusion is supported by double mutant analyses demonstrating that the conserved glutamine acts between LOV2,390 formation and displacement of the Jα-helix in the reaction mechanism associated with phot1 receptor activation.

**Experimental Procedures**

**Expression and Purification of LOV2 Fusion Proteins**—A DNA fragment encoding the LOV2 domain of Arabidopsis phot1 (residues 448–600) was PCR-amplified using the Arabidopsis PHOT1 cDNA and cloned into the pCAL-n-EK vector (Stratagene, La Jolla, CA) via EcoRI and NcoI to create an N-terminal calmodulin-binding peptide fusion. LOV2 protein was expressed and purified by calmodulin affinity chromatography as described previously (12, 13, 15) using the E. coli strain BL21(DE3)pLysS (Novagen, Madison, WI).

**Site-directed Mutagenesis**—Single amino acid substitutions were introduced using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) in accordance with the instructions of the supplier. A second round of mutagenesis was performed to generate template sequences containing two amino acid changes. All amino acid changes were verified by DNA sequencing.

**Spectral Analysis**—Fluorescence emission and excitation spectra were recorded using an LS-55 luminescence spectrometer (PerkinElmer Life Sciences). Fluorescence excitation spectra were recorded by monitoring the emission at 520 nm. Fluorescence emission spectra were obtained by using an excitation wavelength of 380 nm. Absorption spectra were measured using a Lambda 45 UV-visible spectrometer (PerkinElmer Life Sciences). Protein concentrations were determined by the Bradford protein assay (Bio-Rad) using bovine serum albumin as standard. CD spectra were recorded using a JASCO J-810 spectropolarimeter. Wild-type and mutant LOV2 proteins were analyzed in the far-UV region in a quartz cuvette of 0.02 cm path length. The average of 8 spectra was collected for each protein at 50 nm/min using a response time of 0.5 s and a bandwidth of 1 nm. Spectra were corrected following subtraction of a buffer blank spectrum, and data were expressed in units of mean residue weight ellipticity, θ (degrees cm² dmol⁻¹) (28).

**Phototropin Expression in Insect Cells**—The coding sequence of Arabidopsis PHOT1 was cloned into the EcoRI site of the baculovirus transfer vector pAChLT-A (BD Biosciences). Recombinant baculovirus encoding either wild-type Arabidopsis PHOT1 or PHOT1 carrying specific amino acid substitutions was generated using the BaculoGold™ transfection kit (BD Biosciences) in accordance with the instructions of the supplier. Recombinant baculovirus was titered by end point dilution and used to infect SF9 (Spodoptera frugiperda) insect cells. Expression of recombinant phot1 was carried out as described previously (29, 30).

**Phototropin Autophosphorylation and Western Blot Analysis**—Soluble protein extracts were prepared from insect cells as described previously (29, 30). Autophosphorylation assays were carried out under a dim red safe light as described (21). The extent of phot1 autophosphorylation was quantified with a Fujifilm FLA-5000 PhosphorImager (Fujifilm, Tokyo, Japan). All experiments were repeated at least three times, and the data presented are representative of the results obtained. Western blot analysis of soluble protein extracts from insect cells (10 μg) was performed as described (30) using a monoclonal anti-His antibody (Novagen, Madison, WI) or a purified...
polyclonal antibody raised against a peptide fragment located at the extreme C-terminal region of *Arabidopsis* phot1 (Sigma Genosys). Monoclonal anti-His antibody was used at a 1:1000 dilution, whereas the purified polyclonal anti-phot1 antibody was used at a 1:5000 dilution.

*Flavin Release Measurements*—All manipulations were carried out under a dim red safe light. For whole insect cells, dark-grown transfected S9 cultures were harvested by centrifugation at 1,000 rpm for 1 min. Cells were washed twice in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) and protein concentrations were determined using the Bradford protein assay (Bio-Rad). For individual LOV2 domains, purified proteins were incubated overnight in darkness at 4 °C to ensure photorecovery of the domain back to its ground state. The FMN chromophore was released from full-length phot1 proteins in insect cells by boiling samples in the presence of phosphorylation buffer (37.5 mM Tris, pH 7.5, 5.3 mM MgSO4, 150 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol) for 5 min. FMN was released from purified LOV2 domains by boiling in the presence of elution buffer (50 mM Tris, pH 8.0, 1 mM NaCl, 2 mM EGTA, 10 mM β-mercaptoethanol). Boiled samples were then chilled on ice for 5 min and microcentrifuged for an additional 5 min. The supernatant obtained containing released flavin was cleared using a PCR purification column (Qiagen) to remove further protein contaminants. Flavin fluorescence present in the purified supernatant was measured by fluorescence spectroscopy.

**RESULTS**

**Salt Bridge Disruption Results in a Loss of FMN Binding in Isolated LOV2**—LOV1 and LOV2 domains of higher plant phototropins contain a conserved salt bridge at the surface of the LOV-core between the αβ-helix and βC-βD loop. This ionic interaction exists between amino acid residues Glu506 and Lys547 in the LOV2 domain of *Arabidopsis* phot1 (Fig. 1B). The crystal structure of the LOV2 domain from the novel red/blue light receptor neochrome (formerly known as phy3) from the fern *Adiantum capillus-veneris* has revealed that the conserved surface salt bridge is located at the end of a series of conserved amino acids extending from the internal cavity where the primary events of LOV domain photodetection take place (25). Light-induced formation of LOV390 within the FMN cavity may therefore be communicated to the surface of the LOV-core through this conserved region of amino acids resulting in destabilization of the salt bridge. Consequently, salt bridge disruption has been proposed to serve as a signal to bring about further conformational changes within the phototropin molecule required to induce receptor autophosphorylation (25). However, there is no evidence available to date that disruption of the salt bridge plays a role in LOV domain signaling associated with phototropin autophosphorylation. Therefore, we used site-directed mutagenesis to examine the role of the conserved salt bridge in regulating phot1 activity.

Individual LOV domains expressed and purified from *E. coli* provide a rapid means to monitor the effects of point mutations on the spectral characteristics and flavin binding properties of the photosensory region. Initially, we examined the effect of disrupting the salt bridge interaction by incorporating the point mutation E506K into the LOV2 domain of *Arabidopsis* phot1. Incorporation of the E506K mutation into LOV2 was found to result in lower protein yields relative to wild type (data not shown) but produced sufficient quantities upon purification suitable for spectral characterization (Fig. 2A). The native LOV2 domain binds the chromophore FMN showing typical absorbance in both the blue and UV-A regions of the spectrum (Fig. 2A). Mutation of Glu506 to lysine results in a loss of these spectral properties indicating that disruption of the surface salt bridge in isolated LOV2 leads to a loss of chromatophore binding. This was further confirmed by fluorescence spectroscopy. As shown in Fig. 2B, the E506K variant of LOV2 exhibits a dramatic loss in fluorescence (>90%) relative to wild type. Mutation of Glu506 to either glutamine or alanine produced similar effects on FMN binding (supplemental Fig. S1).

It was important to establish whether loss of flavin binding upon disruption of the surface salt bridge in LOV2 compromised the overall secondary structural integrity of the protein. CD spectroscopy was used to compare the spectrum of the E506K variant with that of wild-type LOV2. No differences in
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**FIGURE 2. Effect of salt bridge disruption on the spectral properties of Arabidopsis phot1 LOV2.** A, SDS-PAGE analysis of wild-type LOV2 and the E506K salt bridge mutant expressed and purified from E. coli is shown on the left. Proteins (1 \(\mu\)g) were separated by 12.5% SDS-PAGE. Molecular masses of marker proteins are indicated in kilodaltons. Absorption spectra of wild-type LOV2 (solid line) and the E506K mutant (dashed line) are shown on the right. Absorption spectra were recorded using equal concentrations (0.4 mg/ml) of wild-type and mutant proteins. B, fluorescence excitation spectra (left) and fluorescence emission spectra (right) of wild-type LOV2 (solid line) and the E506K mutant (dashed line). Fluorescence spectra were recorded using equal concentrations (0.1 mg/ml) of wild-type and mutant proteins. C, CD spectra of wild-type LOV2 (solid line) and the E506K mutant (dashed line) measured in the far-ultraviolet region (190–260 nm). Equal concentrations (0.4 mg/ml) of wild-type and mutant proteins were used for CD analysis.

The far-UV CD spectra were observed (Fig. 2C) indicating that replacement of Glu\(^{506}\) with lysine does not affect the overall secondary structure of the protein. Taken together, the above findings suggest that the surface salt bridge located in LOV2 most likely plays an important role in tertiary structural conformation. Consequently, disruption of this ionic interaction, at least in the isolated LOV domain, results in a loss of chromophore binding.

**E506K Mutation Does Not Affect Phot1 Kinase Activity**—Phototropins are light receptor protein kinases that undergo autophosphorylation in response to blue light excitation (2, 4). Although our attempts to express active phot1 in *E. coli* have been unsuccessful to date, full-length phot1 can be expressed in insect cells using recombinant baculovirus (16, 29, 30). We therefore used the insect cell expression system to examine the effect of the E506K mutation on the autophosphorylation activity of *Arabidopsis* phot1.

Insect cells infected with recombinant baculovirus, encoding the *PHOT1* cDNA from *Arabidopsis*, were grown in complete darkness and harvested under dim red light. Total protein extracts were isolated and used for *in vitro* autophosphorylation analysis. As shown in Fig. 3, phot1 exhibits basal levels of autophosphorylation activity under dark conditions. Phot1 kinase activity is increased following a brief irradiation with saturating intensities of white light. Replacement of Glu\(^{506}\) in LOV2 with lysine had no effect on light-induced kinase activity of phot1 expressed in insect cells (Fig. 3, A and B). This finding is surprising, given that the photochemical reactivity of LOV2 is necessary for light-induced autophosphorylation of phot1 (16) and that the equivalent salt bridge mutation in the isolated LOV2 domain results in a substantial loss of chromophore binding (Fig. 2).

The lack of an effect of the E506K mutation on phot1 autophosphorylation in insect cells suggests that disruption of the Glu-Lys salt bridge in LOV2 might produce different consequences in the full-length receptor protein versus the isolated LOV domain. We therefore estimated the levels of flavin bound to either wild-type phot1 or the E506K variant expressed in insect cells. Baculovirus-mediated expression of phot1 in insect cells gives rise to high levels of protein (Fig. 3C), but much of the protein produced is insoluble and inactive (29). Nevertheless, in accordance with previous findings (29), insoluble protein extracts from insect cells expressing phot1 are highly fluorescent when viewed under UV light and retain FMN binding as monitored by fluorescence spectroscopy relative to control samples not expressing phot1 (supplemental Fig. S2). Indeed, the level of flavin fluorescence released from insect cells infected with wild-type baculovirus is negligible compared with that released from insect cells expressing phot1 (supplemental Fig. S2). Thus, flavin release measurements from whole insect cells provide a rapid and convenient method to assess the effect of the E506K mutation on flavin binding within the full-length photoreceptor context.

Insect cells were grown in complete darkness and harvested under dim red light to avoid light-driven LOV\(_{390}\) formation that would result in covalent attachment of the flavin chromophore to the LOV domain of the photoreceptor protein. Flavin levels released from whole cells expressing wild-type phot1 or the E506K mutant were analyzed by fluorescence spectroscopy. Surprisingly, in contrast to our mutational analysis of the individual LOV2 domain (Fig. 2), substitution of Glu\(^{506}\) to lysine in full-length phot1 resulted in only a marginal reduction (~10%) of flavin fluorescence released from whole cell extracts relative to the wild-type control (Fig. 3C). Thus, incorporation of the E506K mutation into full-length phot1 does not appear to alter flavin binding to an extent that impairs
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**FIGURE 3.** Effect of the E506K salt bridge mutation on the autophosphorylation activity of phot1 expressed in insect cells. A, autoradiograph showing light-dependent autophosphorylation activity of wild-type phot1 and the E506K mutant in soluble protein extracts prepared from insect cells. 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<sup>2</sup> upon addition of radiolabeled ATP. Western blot analysis of phot1 protein levels is shown below the autoradiograph. Soluble protein extracts prepared from insect cells expressing either wild-type phot1 or the E506K mutant of phot1 were detected using an anti-His antibody. B, autophosphorylation activity of wild-type phot1 and the E506K mutant in soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a percentage of maximal autophosphorylation activity of wild-type phot1 and the E506K mutant in soluble protein extracts prepared from insect cells. 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<sup>2</sup> upon addition of radiolabeled ATP. Western blot analysis of phot1 protein levels is shown below the autoradiograph. Soluble protein extracts prepared from insect cells expressing either wild-type phot1 or the E506K mutant of phot1 were detected using an anti-His antibody. C, estimation of flavin levels released upon denaturation of whole insect cells expressing wild-type and E506K mutant forms of phot1. SDS-PAGE analysis of wild-type phot1 and the E506K salt bridge mutant expressed in insect cells is shown on the left. Proteins (10 μg) were separated by 7.5% SDS-PAGE, and molecular masses of marker proteins are indicated in kilodaltons. The size of full-length phot1 is indicated (*). Fluorescence emission spectra of flavin levels released from equal amounts (1.5 mg of protein) of insect cells expressing wild-type phot1 (solid line) and the E506K mutant (dashed line) are shown on the right.

The spectral blue shift exhibited by the Q575L variant of LOV2 was also detectable by fluorescence spectroscopy (Fig. 4B). Furthermore, the fluorescence excitation and emission spectra obtained for the Q575L mutant indicated that this variant exhibits a dramatic reduction in fluorescence (~80%), compared with wild-type LOV2. The reduced fluorescence observed for the Q575L mutant was partly because of a loss in FMN binding; denaturation of the wild-type and mutant chromoproteins revealed that replacement of Gln<sup>575</sup> with leucine results in a 30% loss of flavin fluorescence relative to wild type (Fig. 4C). Thus, the greater difference in flavin fluorescence observed between the wild-type and mutant holoproteins (Fig. 4B) suggests that incorporation of the Q575L mutation, in addition to affecting FMN binding, also modifies the nature of the flavin environment within LOV2 that increases the degree of chromophore fluorescence quenching.

Q575L Mutation Impairs Phot1 Kinase Activity—To ascertain whether Gln<sup>575</sup> is required for phot1 autophosphorylation, we examined the effect of the Q575L mutation on the kinase activity.
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FIGURE 4. Effect of the Q575L mutation on the spectral properties of Arabidopsis phot1 LOV2. A, SDS-PAGE analysis of wild-type LOV2, and the Q575L mutant expressed and purified from E. coli is shown on the left. Proteins (10 μg) were separated by 12.5% SDS-PAGE. Molecular masses of marker proteins are indicated in kilodaltons. Absorption spectra of wild-type LOV2 (solid line) and the Q575L mutant (dashed line) are shown on the right. Absorption spectra were recorded using equal concentrations (0.5 mg/ml) of wild-type and mutant proteins. Absorption maxima of wild-type and mutant proteins (10 μg/ml) of wild-type and mutant proteins.

B, fluorescence excitation spectra (left) and fluorescence emission spectra (right) of wild-type LOV2 (solid line) and the Q575L mutant (dashed line). Fluorescence spectra were recorded using equal concentrations (0.4 mg/ml) of wild-type and mutant proteins. C, fluorescence emission spectra of flavin levels released upon denaturation of equal amounts (125 μg) of wild-type LOV2 (solid line) and the Q575L mutant (dashed line).

Residual Autophosphorylation Activity in the Q575L Mutant Is Mediated by LOV2—The conserved cysteine residue within LOV2 (Cys$^{517}$) is required for photochemical reactivity (13, 14). Mutation of Cys$^{517}$ to alanine not only results in a loss of LOV2 photochemical reactivity without affecting FMN binding (13) but also abolishes light-induced kinase activity of phot1 expressed in insect cells and transgenic Arabidopsis (16). Hence, to investigate whether the remaining level of light-induced autophosphorylation observed for the Q575L mutant could be assigned to residual LOV2 photochemical reactivity, we created a C517A/Q575L double mutant of phot1 and assayed its light-induced autophosphorylation activity using the insect cell expression system. As shown previously, replacement of Cys$^{517}$ with alanine abolishes the phot1 autophosphorylation activity (Fig. 6A). Incorporation of the C517A mutation in addition to the Q575L mutation was also found to abolish light-induced kinase activity of phot1, demonstrating that the remaining autophosphorylation activity observed in the single Q575L mutant can be attributed to residual LOV2 photochemical reactivity.

Mutation of Jα Restores Phot1 Kinase Activity in the Q575L Mutant—Mutation of Ile$^{608}$ to glutamate within the Jα-helix that immediately follows LOV2 within the phototropin apoprotein has been shown to result in the activation of phot1 autophosphorylation in the absence of light (21). The I608E mutation can therefore be used as means to artificially bypass the requirement of LOV2 photoexcitation in mediating phot1 autophosphorylation. Because our data indicate that Gln$^{575}$ plays a major role in transmission of the signal generated by the formation of LOV2$^{398}$ upon photoexcitation to activation of the C-terminal kinase domain, it should be possible to mitigate the inhibitory effect of the Q575L mutation on phot1 autophosphorylation by incorporating the I608E mutation. We therefore created a Q575L/I608E double mutant and used the insect cell expression system to determine whether the I608E mutation could restore phot1 kinase activity in the Q575L mutant background. As shown previously, replacement of Ile$^{608}$ with glutamate results in constitutive phot1 kinase activation even in the absence of light (Fig. 6B). Likewise, incorporation of the I608E mutation in addition to the Q575L mutation produces a similar effect on phot1 autophosphorylation activity. Hence, the inhibitory effect of the Q575L mutation on phot1 kinase activity can be overcome by the gain of function I608E phosphorylation response was not caused by a lack of phot1 protein (Fig. 5A).

Given that the Q575L mutation results in a partial loss of FMN binding in the isolated LOV2 domain (Fig. 4C), it was important to establish whether the reduced levels of phot1 autophosphorylation observed upon substitution of Gln$^{575}$ with leucine also stemmed from a reduction in chromophore binding to LOV2. Determination of the degree of flavin fluorescence released from whole cells expressing either wild-type phot1 or the Q575L mutant showed that the impairment of phot1 kinase activity observed for the Q575L mutant did not result from a loss in flavin binding (Fig. 5C). Thus, as proposed previously (26), Gln$^{575}$ appears to be involved in the LOV2 signal transmission pathway that acts to regulate the C-terminal kinase domain of phototropins.

![Image](372x26 to 400x38)

activity of phot1 expressed in insect cells. Replacement of Gln$^{575}$ in LOV2 with leucine severely attenuates the light-induced autophosphorylation activity of phot1 (Fig. 5, A and B) consistent with a role for this residue in coupling LOV2 photoexcitation to activation of the C-terminal kinase domain. A residual level of light-induced autophosphorylation was still detectable in the Q575L mutant indicating that this mutation does not completely abolish the photoreactivity of phot1. Western analysis also demonstrated that an impaired auto-
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**DISCUSSION**

All phototropin receptors identified to date possess two light-sensing domains at their N-terminal region, designated LOV1 and LOV2 (2). Both domains bind FMN and undergo a unique mode of photochemistry upon blue light excitation that involves the formation of a covalent adduct between the FMN chromophore and a highly conserved cysteine residue within the domain (12–14). Although both domains are photochemically active (12, 13), it is clear from recent structure-function analysis that LOV2 plays a major role in regulating phototropin receptor action (16, 17). Solution NMR spectroscopy analysis using an extended LOV2 fragment derived from oat phot1 in combination with site-directed mutagenesis of full-length *Arabidopsis* phot1 has shown that photoexcitation of LOV2 leads to the displacement of a conserved amphipathic α-helix, Jα from the surface of the LOV2-core that is required to bring about activation of the C-terminal kinase domain and in turn receptor autophosphorylation (20, 21). Hence, the predominant role of LOV2 in regulating phototropin kinase activity stems from its physical location within the photoreceptor molecule as no peptide sequences forming the Jα-helix are found associated with LOV1 (21). However, the mechanisms underlying signal transmission from the FMN chromophore to protein changes at the LOV2 surface required for Jα displacement have not yet been fully elucidated.

**Role of the Conserved Surface Salt Bridge**—All phototropin LOV domains contain a conserved salt bridge at their surface (25, 31). Formation of LOV<sub>390</sub> upon illumination is hypothesized to result in disruption of the surface salt bridge and mediate the effects of the LOV domain as a light-responsive module. The surface salt bridge is also found in LOV domains of prokaryotic sensor proteins, including YtvA from the nonphotosynthetic soil bacterium *Bacillus subtilis* (25, 31). FTIR spectroscopy studies indicate that light-driven formation of LOV<sub>390</sub> in YtvA gives rise to a conformational change in its C-terminal STAS (sulfate transporter/anti-sigma factor) domain (32). Interestingly, a Jα-like helix has also been identified in YtvA (33) suggesting that this bacterial sensor protein, and possibly others, possesses a mode of photoactivation analogous to that operative for the phototropins.

Disruption of the conserved salt bridge in the LOV2 domain of full-length *Arabidopsis* phot1 appears to result in a small loss in flavin binding but does not affect the light-induced activity of the protein (Fig. 3). Consistent with the results presented here, mutational analysis has shown that the LOV-surface salt bridge of YtvA does not appreciably contribute to the overall secondary structure of the full-length sensor protein (33). Moreover, chromophore binding is not impaired upon disruption of the LOV-surface salt bridge in full-length YtvA (33). Yet, our mutational analysis with the isolated LOV2 of *Arabidopsis* phot1 domain clearly indicates that disruption of the Glu-Lys salt bridge results in an almost complete loss of chromophore binding without compromising protein secondary structure (Fig. 2).

**FIGURE 5. Effect of the Q575L mutation on the autophosphorylation activity of phot1 expressed in insect cells.** A, autoradiograph showing light-dependent autophosphorylation activity of wild-type phot1 and the Q575L mutant in soluble protein extracts prepared from insect cells. All manipulations were carried out under dim red light. Sample preparation and experimental procedures were performed as described in Fig. 3. Western blot analysis of phot1 protein levels is shown below the autoradiograph. Soluble protein extracts prepared from insect cells expressing either wild-type phot1 or the Q575L mutant were probed with anti-phot1 antibody. B, autophosphorylation activity of wild-type phot1 and the Q575L mutant in soluble extracts prepared from insect cells. Kinase activity was quantified by phosphorimaging and expressed as a percentage of maximum activity. C, estimation of flavin levels released upon denaturation of whole insect cells expressing wild-type and Q575L mutant forms of phot1. SDS-PAGE analysis of wild-type phot1 and the Q575L mutant expressed in insect cells is shown on the left. Proteins (10 μg) were separated by 7.5% SDS-PAGE. Molecular masses of marker proteins are indicated in kilodaltons. The size of full-length phot1 is indicated (*). Fluorescence emission spectra of flavin levels released from equal protein amounts (1.5 mg of protein) of insect cells expressing wild-type phot1 (solid line) and the Q575L mutant (dashed line) is shown on the right.
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**A**

![Graph](image)

**B**

![Graph](image)

Whether this also occurs for the isolated LOV domain of YtvA is not known because Losi *et al.* (33) restricted their mutational analysis to the full-length sensor protein.

Salt bridges play an important role in protein structure and function and in many cases are involved in stabilizing the folded conformations of proteins (34). Our mutagenesis studies indicate that this is certainly the case for the isolated LOV2 domain, whereby a destabilization in LOV2 tertiary structure upon salt bridge disruption presumably accounts for the loss of flavin binding. Yet, mutagenesis of full-length phot1 shows that disruption of the LOV2-surface salt bridge does not appreciably affect protein kinase activity and has little effect on flavin binding (Fig. 3). This would therefore imply that the tertiary structure of the LOV2-core is more highly stabilized within the full-length phototropin molecule, thereby compensating for the loss of flavin binding observed in the isolated LOV2 domain upon salt bridge disruption (Fig. 2). Further comparative mutagenesis studies between isolated LOV domains and full-length sensor proteins will determine whether this is also the case for other LOV domain-containing proteins. Notably, the structure of the FAD-binding PAS domain of the *E. coli* aerotaxis sensor Aer is supported by an N-terminal cap, which precedes the Aer-PAS core (35).

**Light-induced Autophosphorylation Involves Gln$^{575}$**—Although the above findings demonstrate that the conserved salt bridge is not involved in LOV2 signal transmission to phototropin kinase activation, our site-directed mutagenesis studies indicate that residue Gln$^{575}$ within the LOV2 domain of *Arabidopsis* phot1 plays a role in bringing about light-induced receptor autophosphorylation (Fig. 5). FTIR spectroscopy (26, 27, 36, 37), CD spectroscopy (38), small angle x-ray scattering (19), and pulsed laser-induced transient gating methods (39) have shown that irradiation of LOV2-Jα protein fragments derived from several different phototropin sources results in significant structural changes at the surface of the LOV-core. In particular, FTIR spectroscopy has indicated that light-induced formation of LOV2$_{390}$ produces progressive changes that involve regions of loops, α-helices, and β-sheets (26, 27). These changes presumably include light-induced disordering of Jα from the LOV2-core that is required to couple LOV2 photoexcitation to activation of the C-terminal kinase domain of phot1 (20, 21). Indeed, LOV2 protein fragments lacking Jα are reported to exhibit minimal structural changes upon irradiation (19, 39).

Nozaki *et al.* (26) demonstrated that the light-induced structural changes associated with a LOV2-Jα protein fragment derived from *Adiantum* neochrome are considerably reduced when a conserved Gln residue within the βE-sheet is replaced with leucine. Our studies extend these observations and show that the equivalent mutation in the LOV2 domain of *Arabidopsis* phot1 significantly attenuates light-induced kinase activity without affecting flavin binding (Fig. 5). As with our salt bridge studies, incorporation of the Q575L mutation into the isolated LOV2 domain of phot1 gave rise to different consequences with respect to FMN binding (Fig. 4C), highlighting the need to be cautious when interpreting the effects of mutagenesis within an individual domain versus the full-length protein. Incorporation of the Q575L mutation was also found to attenuate the dark recovery kinetics of the bacterially expressed LOV2 domain of *Arabidopsis* phot1 (data not shown). This property has also been noted for the equivalent mutation in the LOV2 domain of *Adiantum* neochrome (27) and may partly account for the increase in chromophore fluorescence quenching observed for the Q575L / LOV2 variant of *Arabidopsis* phot1 (Fig. 4).

LOV1 domains from plant phototropins also possess the corresponding glutamine residue. Although LOV1 and LOV2 domains are structurally very similar (22–24), LOV1 photoreactivity does not appear to be essential for phototropin function (16, 17). The higher quantum efficiency of LOV2 versus that of LOV1 is also consistent with its functional importance (13, 15). Further evidence indicating that the consequence of LOV1 photocactivity is different from that of LOV2 has come from a recent comparative structural investigation of the LOV1 and LOV2 domains of *Adiantum* neochrome. FTIR spectroscopy has revealed that irradiation of LOV1 results in minimal protein structural changes (27). Small conformational changes in response to illumination have also been reported for the LOV1 domain derived from the phototropin from the unicellular...
green alga *Chlamydomonas reinhardtii* (40, 41). In fact, the structural properties observed for LOV1 photoactivation were found to be similar to those of the LOV2 domain in which the conserved glutamine was replaced with leucine (27). It will now be of importance to establish the function of the LOV1 domain present in plant phototropins and the significance of its photoreactive.

Mutation of Gln575 did not completely abolish light-induced autophosphorylation of phot1 expressed in insect cells; a residual level of activity could still be detected (Fig. 5). These findings are in accordance with the spectroscopic data of Nozaki et al. (26) showing that the LOV2 domain of *Adiantum* neochrome carrying the equivalent Gln mutation is still capable of producing minimal light-driven structural changes. Our double mutant analysis indicates that the residual level of light-induced kinase activity detected in the Q575L mutant stems from LOV2 photoactivation (Fig. 6A). Moreover, incorporation of the gain of function mutation I608E (21) is able to bypass the inhibitory effect of the Q575L mutation on phot1 kinase activity, resulting in phot1 autophosphorylation regardless of the light conditions (Fig. 6B). Phot1, even in the presence of the I608E mutation, still exhibits a greater degree of autophosphorylation upon irradiation compared with dark controls (Fig. 6B), suggesting that the I608E mutant does not fully mimic the irradiated state of the phototropin molecule and has the ability to confer a small degree of light-mediated autophosphorylation via LOV2. This property was also apparent in the Q575L/I608E double mutant of phot1 (Fig. 6B). These observations are consistent with the NMR studies of Harper et al. (21), showing that a LOV2-Jα fragment derived from oat phot1 carrying the corresponding Ile to Glu change exhibits modest structural changes upon irradiation.

**Mode of LOV2 Signal Transmission**—The double mutant analysis carried out in this study places a role for Gln575 in transmitting the signal generated upon light-induced cysteinyl adduct formation from within LOV2 to structural changes at the LOV2 surface that lead to displacement of Jα and subsequent activation of the C-terminal kinase domain of plant phototropins. This signal transmission mechanism is likely to be conserved among all phototropins given that phot2 kinase activity is also predominantly regulated by LOV2 (16). X-ray crystallography indicates that the conserved glutamine within LOV2 forms hydrogen bonds with the FMN chromophore and undergoes side chain rotation upon light-driven adduct formation (22, 23). It is therefore likely that side chain rotation of Gln575 invokes structural changes in the βE-sheet where this residue resides (Fig. 1B). Given that Jα docks onto the β-sheet strands of the LOV2-core in the dark state (20), one can envisage a structural relay mechanism whereby protein conformational changes occurring at the β-sheet surface upon LOV2 photocexcitation lead to displacement of Jα and in turn activation of the C-terminal kinase domain. Indeed, the β-sheet interface of several PAS domains has been reported to be a region important for inter- and intramolecular signaling (42). Recent evidence indicates that LOV1 may function as a site for receptor dimerization (18, 19), yet it is unclear at present whether full-length phototropin molecules form dimers. The insect system used here will be useful in assessing the dimerization status of full-length phototropins and how this contributes to photoreceptor activation.

An important question to address now is whether LOV2 acts as a dark state repressor of phototropin kinase activity or a light state activator. *In vitro* studies involving protein fragments derived from *Arabidopsis* phot2 are in accordance with LOV2 functioning as dark state inhibitor of phototropin kinase activity (43). A similar PAS-kinase domain interaction mechanism has been proposed for regulating the activities of the bacterial oxygen sensor, FixL (44), and the eukaryotic protein kinase, PAS kinase (45, 46), a regulator of mammalian glycogen synthesis (47). To date, biophysical and structural studies have been directed to regions of the phototropin molecule lacking the C-terminal kinase domain. Extending these studies to LOV2 kinase fragments and ultimately the full-length receptor proteins will provide a more detailed understanding of the photosensory transduction pathway underlying phototropin receptor activation.

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**REFERENCES**

1. Christie, J. M., and Briggs, W. R. (2001) *J. Biol. Chem.* 276, 11457–11460
2. Briggs, W. R., and Christie, J. M. (2002) *Trends Plant Sci.* 7, 204–210
3. Lin, C., and Shalitin, D. (2003) *Annu. Rev. Plant Biol.* 54, 469–496
4. Celaya, R. B., and Liscum, E. (2005) *Photochem. Photobiol.* 81, 73–80
5. Ohgishi, M., Saji, K., Okada, K., and Sakai, T. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 2223–2228
6. Sakamoto, K., and Briggs, W. R. (2002) *Plant Cell* 14, 1723–1735
7. Takemiya, A., Inoue S., Doi, M., Kinosita, T., and Shimazaki, K. (2005) *Plant Cell* 17, 1120–1127
8. Huala, E., Oeller, P. W., Liscum, E., Han, I.-S., Larsen, E., and Briggs, W. R. (1997) *Science* 278, 2121–2123
9. Jarillo, J. A., Ahmad, M., and Cashmore, A. R. (1998) *Plant Physiol.* 117, 719
10. Kagawa, T., Sakai, T., Suetsugu, N., Oikawa, K., Ishiguro, S., Kato, T., Tabata, S., Okada, K., and Wada, M. (2001) *Science* 291, 2138–2141
11. Taylor, B. L., and Zhulin, I. B. (1999) *Microbiol. Mol. Biol. Rev.* 63, 479–506
12. Christie, J. M., Salomon, M., Nozue, K., Wada, M., and Briggs, W. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 8779–8783
13. Salomon, M., Christie, J. M., Knieb, E., Lempert, U., and Briggs, W. R. (2000) *Biochemistry* 39, 9401–9410
14. Swartz, T. E., Corchnoy, S. B., Christie, J. M., Lewis, J. W., Szundi, I., Briggs, W. R., and Bogomolni, R. A. (2001) *J. Biol. Chem.* 276, 36493–36500
15. Kasahara, M., Swartz, T. E., Olney, M. O., Onodera, A., Mochizuki, N., Fukuzawa, H., Asamizu, E., Tabata, S., Kanega, H., Takan, M., Christie, I. M., Nagatani, A., and Briggs, W. R. (2002) *Plant Physiol.* 129, 762–773
16. Christie, J. M., Swartz, T. E., Bogomolni, R. A., and Briggs, W. R. (2002) *Plant J.* 22, 205–219
17. Kagawa, T., Kasahara, M., Abe, T., Yoshida, S., and Wada, M. (2004) *Plant Physiol. Cell Physiol.* 45, 416–426
18. Salomon, M., Lempert, U., and Rudiger, W. (2004) *FEBS Lett.* 572, 8–10
19. Nakasako, M., Iwata, T., Matsuoka, D., and Tokutomi, S. (2004) *Biochemistry* 43, 14881–14890
20. Harper, S. M., Neil, L. C., and Gardner, K. H. (2003) *Science* 301, 1541–1544
21. Harper, S. M., Christie, J. M., and Gardner, K. H. (2004) *Biochemistry* 43, 16184–16192
22. Crosson, S., and Moffat, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 2995–3000
Phototropin Activation by Light Involves Gln<sup>575</sup>

23. Crosson, S., and Moffat, K. (2002) *Plant Cell* **14**, 1067–1075
24. Fedorov, R., Schlichting, I., Hartmann, E., Domratcheva, T., Fuhrmann, M., and Hegemann, P. (2003) *Biophys. J.* **84**, 2474–2482
25. Crosson, S., Rajagopal, S., and Moffat, K. (2003) *Biochemistry* **42**, 2–10
26. Nozaki, D., Iwata, T., Ishikawa, T., Todo, T., Tokutomi, S., and Kandori, H. (2004) *Biochemistry* **43**, 8373–8379
27. Iwata, T., Tokutomi, S., and Kandori, H. (2005) *J. Am. Chem. Soc.* **127**, 11840–11841
28. Kelly, S. M., Jess, T. J., and Price, N. C. (2005) *Biochim. Biophys. Acta* **1751**, 119–139
29. Christie, J. M., Reymond, P., Powell, G., Bernasconi, P., Reibekas, A. A., Liscum, E., and Briggs, W. R. (1998) *Science* **282**, 1698–1701
30. Sakai, T., Kagawa, T., Kasahara, M., Swartz, T. E., Christie, J. M., Briggs, W. R., Wada, M., and Okada, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6969–6974
31. Losi, A. (2004) *Photochem. Photobiol. Sci.* **3**, 566–574
32. Bednarz, T., Losi, A., Gärtner, W., Hegemann, P., and Heberle, J. (2004) *Photochem. Photobiol. Sci.* **3**, 575–579
33. Losi, A., Ghiraldelli, E., Jansen, S., and Gärtner, W. (2005) *Photochem. Photobiol. Sci.* **81**, 1145–1152
34. Kumar, S., and Nussinov, R. (1999) *J. Mol. Biol.* **293**, 1241–1255
35. Watts, K. J., Sommer, K., Fry, S. L., Johnson, M. S., and Taylor, B. L. (2006) *J. Bacteriol.* **188**, 2154–2162
36. Swartz, T. E., Wenzel, P. J., Corchnoy, S. B., Briggs, W. R., and Bogomolni, R. A. (2002) *Biochemistry* **41**, 7182–7189
37. Iwata, T., Nozaki, D., Tokutomi, S., Kagawa, T., Wada, M., and Kandori, H. (2003) *Biochemistry* **42**, 8183–8191
38. Corchnoy, S. B., Swartz, T. E., Lewis, J. W., Szundi, I., Briggs, W. R., and Bogomolni, R. A. (2003) *J. Biol. Chem.* **278**, 724–731
39. Eitoku, T., Nakasone, Y., Matsuoka, D., Tokutomi, S., and Terazima, M. (2005) *J. Am. Chem. Soc.* **127**, 13238–13244
40. Ataka, K., Hegemann, P., and Heberle, J. (2003) *Biophys. J.* **84**, 466–474
41. Losi, A., Quest, B., and Gärtner, W. (2003) *Photochem. Photobiol. Sci.* **2**, 759–766
42. Erbel, P. J. A., Card, P. B., Karkuzu, O., Bruick, R. K., and Gardner, K. H. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 15504–15509
43. Matsuoka, D., and Tokutomi, S. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 13337–13342
44. Gong, W., Hao, B., Mansy, S. S., Gonzalez, G., Gilles-Gonzalez, M. A., and Chan, M. K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 15177–15182
45. Rutter, J., Michnoff, C. H., Harper, S. H., Gardner, K. H., and McKnight, S. L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8991–8996
46. Amezcua, C. A., Harper, S. H., Rutter, J., and Gardner, K. H. (2002) *Structure (Camb.)* **10**, 1349–1361
47. Wislon, W. A., Skurat, A. V., Probst, B., de Paoli-Roach, A., Roach, P. J., and Rutter, J. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 16596–16601