Light-induced conformational changes of LOV (Light Oxygen Voltage-sensing domain) 1 and LOV2 relative to the kinase domain and regulation of kinase activity in *Chlamydomonas* phototropin*

Koji Okajima*,b, Yusuke Aihara*, Yuki Takayama*b,d, Mihoko Nakajima*, Sachiko Kashoijiya*a,b, Takaaki Hikima*, Tomotaka Oroguchi*b,d, Amane Kobayashi*b,d, Yuki Sekiguchi*b,d, Masaki Yamamoto*b, Tomomi Suzuki*c, Akira Nagatani*c, Masayoshi Nakasako*b,d,1, and Satoru Tokutomi*a,1

aDepartment of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan, bRIKEN Harima Institute|Spring-8, 1-1-1 Kouto, Mikaduki, Sayo, Hyogo 679-5148, Japan, cDepartment of Botany, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan, dDepartment of Physics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Kanagawa 223-8522, Japan

*Running title: Structure and kinase activation of *Chlamydomonas* phototropin

1To whom corresponding should be addressed: Satoru Tokutomi, Department of Biological Sciences, Graduate School of Science, Osaka Prefecture University, Gakuen-cho 1-1, Nakaku, Sakai, Osaka 599-8531, Japan. Tel.: +81 72-254-9841; Fax: +81 72-254-9841; E-mail: toxan@b.s.osakafu-u.ac.jp. Masayoshi Nakasako, Department of Physics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Kanagawa 223-8522, Japan. Tel.: +81 45-566-1713; Fax: +81 45-566-1672; E-mail: nakasako@phys.keio.ac.jp.

Key words: phototropin, *Chlamydomonas*, solution structure, photoreaction, kinase activation

---

**Background:** The plant photoreceptor “phototropin” is a light-regulated kinase containing two photosensory domains named LOV.

**Results:** Light-induced conformational change related to the kinase activation was detected in full-length phototropin of *Chlamydomonas*

**Conclusion:** LOV1 may interact with LOV2 and modify the photosensitivity of the kinase regulation by LOV2.

**Significance:** Configuration of LOV1, LOV2 and kinase domain in a phot molecule is first demonstrated.

**SUMMARY**

Phototropin (phot)², a blue light (BL) receptor in plants, has two photoreceptive domains named LOV1 and LOV2 as well as a Ser/Thr kinase domain (KD), and acts as a BL-regulated protein kinase. A LOV domain harbors a flavin mononucleotide that undergoes a cyclic photoreaction upon BL excitation via a signaling state in which the inhibition of the kinase activity by LOV2 is negated. To understand the molecular mechanism underlying the BL-dependent activation of the kinase, the photochemistry, kinase activity and molecular structure were studied with the phot of *Chlamydomonas reinhardtii* (Cr). Full-length and LOV2-KD samples of Cr phot showed cyclic photoreaction characteristics with the activation of LOV and BL-dependent kinase. Truncation of LOV1 decreased the photosensitivity of the kinase activation, which was well-explained by the fact that the signaling state lasted for a shorter period of time compared to that of the phot. Small angle X-ray scattering (SAXS) revealed monomeric forms of the proteins in solution and detected BL-dependent conformational changes, suggesting an extension of the global molecular shapes of both samples. Constructed molecular model of full-length phot based on the SAXS data proved the arrangement of LOV1, LOV2 and KD for the first time that showed a tandem arrangement both in the dark and under BL irradiation. The models suggest that LOV1 alters its position relative to LOV2-KD under BL irradiation. This finding demonstrates that LOV1 may interact with LOV2 and modify the photosensitivity of the kinase activation through alteration of the duration of the signaling state in LOV2.

Plants sense and respond to variations in environmental conditions. Light is one of the most essential environmental signals for...
photosynthetic plants. Through evolution, plants have acquired three major light sensors: phytochrome (1), cryptochrome (2) and phototropin (3). Phototropin (phot) is a BL sensor and was determined to be a receptor for the phototropic response (4). Afterward, phot was shown to mediate chloroplast relocation (5), stomata opening (6), leaf flattering (7) and leaf photomorphogenesis (8). All of these responses serve to optimize the efficiency of photosynthetic activities. Most plants, including Arabidopsis thaliana (At), have two isoforms of phot (phot1 and phot2) that share roles depending on the light intensity (9).

The phot molecule is composed of approximately 1000 amino acid residues and has two photoreceptive domains named Light-Oxygen-Voltage (LOV) 1 and LOV2 (10) in the N-terminal region; the C-terminal half forms a Ser/Thr kinase domain (KD) (Fig. 1). LOV binds one flavin mononucleotide (FMN) noncovalently on a 5-stranded β-sheet, which is referred to as the β-scaffold in the α/β fold (11). Upon BL irradiation, the FMN in the ground state (D450) transiently forms a covalent bond with a Cys residue conserved among LOVs to form an adduct state (S390) (12) via a triplet-excited state (L660) (13). Within seconds to minutes, the S390 reverts to D450 thermally depending on the types of LOV (14) and forms a characteristic photocycle with LOV. S390 is a signaling state capable of activating the kinase (15). Of the two LOV domains, LOV2 has been shown to play a major role in kinase activation by BL through both autophosphorylation (16, 17) and substrate phosphorylation in vitro (18). Phot acts, therefore, as a light-regulated protein kinase (3). BL mediated phosphorylation of some signaling components are reported (19, 20). Recently, a Ser/Thr protein kinase, BLUS1, was found to be a substrate of phot and to mediate a primary step for phototropin signaling in stomata opening of guard cells in Arabidopsis (21).

One of the most interesting issues concerning the structure-function relation of phot is how LOV2 regulates the kinase activity. It has been postulated that an α-helix named Jα is involved in this connection. Based on the results of an NMR study with Avena sativa (As) phot1 (22), Jα, which locates next to the C-terminus of LOV2 in the linker region between LOV2 and KD (Fig. 1), is suggested to interact with the β-scaffold in the dark and to unfold and dissociate from the β-scaffold upon BL excitation. Localization of Jα onto the β-scaffold was visualized by a crystallographic study (23). At phot1 also showed structural changes in the Jα region that were detectable by SAXS (24) and Fourier transform infrared (FT-IR) spectroscopy (25). However, the molecular mechanism for the regulation of the kinase by LOV2 is still obscure. One of the main reasons for this problem is the difficulty in preparing phot samples consisting of both LOV and KD with a high enough purity and in large enough quantities for biophysical analyses. To overcome this difficulty, we have previously established a preparation system for pure LOV2-KD of At phot1 and phot2 (15, 26). The LOV2-KDs showed BL-regulated kinase activity on the N-terminal fragment of At phot1 (AtP1Nt), including the autophosphorylation sites around the LOV1 region that was created as an artificial substrate for the phot kinase (15, 26). Because the LOV2-KDs have BL regulated kinase activity, structural study of these molecules provides useful information regarding the molecular basis for the BL-dependent regulation of the kinase. In this regard, we have previously measured the SAXS of a D720N substitute of At phot2 LOV-KD and reported its molecular models, which revealed the topological organization of LOV2 and KD and its BL-induced alteration (27).

The next question is where LOV1 resides and how it interacts with LOV2 and KD in a phot molecule. Currently, the phot of the unicellular green alga Chlamydomonas reinhardtii (Cr) (28) is the only species available to answer this question. Cr phot is proposed to mediate sexual differentiation (29), expression of several photosynthetic genes (30) and size of eyespot and phototaxis (31). Cr phot is supposed to exist in a monomeric form in contrast to higher plant photos, which are proposed to be in a dimeric form in vivo (32). Furthermore, its primary structure differs from those of higher plants in the N-terminal extension and the hinge between LOV1 and LOV2 regions (Fig. 1). In spite of these differences, Cr phot is able to complement the physiological responses mediated by phot1 and phot2 in Arabidopsis (33), suggesting that the Cr phot molecule has similar machinery for the regulation of signal transduction by BL when compared to those of higher plant photos. Recently, we have established a large-scale preparation system for
highly pure full-length Cr phot and reported that the Cr phot has BL-dependent kinase activity and that the N-terminal region of LOV2 is involved in BL signaling (34).

In the present study, we investigated the photochemistry, kinase activities and molecular structures of the highly purified Cr phot and its LOV2-KD fragment. The results revealed distinct functional roles of LOV1 in the regulation of kinase activity and, for the first time, the organization of LOV1, LOV2 and KD in a full-length phot molecule. Based on these results, we discuss the regulatory mechanism of Cr phot kinase in comparison with those of higher plants.

**EXPERIMENTAL PROCEDURES**

**Sample preparation** - The vector for overexpression of full-length Cr phot (CrPFul) was described in a previous paper (34). For site-directed mutants of CrPFul, PCR-based mutageneses were performed using PrimeStar GXL DNA Polymerase (TaKaRa) with primers for C57A, C250A and D545N (Table 1). For LOV2-KD fragment of Cr phot (CrPL2K), DNA of the LOV2 + linker + kinase (192 – 749) was synthesized by PCR with primers (Table 1) and the amplified fragment was inserted into the NdeI/SalI site of the pET28a bacterial expression vector (GE Healthcare). CrPFul and CrPL2K were purified as described in the previous paper (27). The E. coli BL21 (DE3) cells overexpressed the protein, which were suspended in the purification buffer and lysed by sonication. After centrifugation (100,000 g, for 30 min), the sample was purified from the supernatant by Ni affinity (HisTrap, GE Healthcare) and size exclusion column (Superdex 200 pg, GE Healthcare) chromatography. Roughly purified sample was loaded onto an anion exchange column (MonoQ, GE Healthcare) and size exclusion column (Superdex 200 pg, GE Healthcare) chromatography. Roughly purified sample was loaded onto an anion exchange column (MonoQ, GE Healthcare). Purified sample was fractionated in the flow through. Purified sample was stored at -80°C in a buffer (20 mM Tris-HCl pH7.8, 200 mM NaCl, 10% (w/v) glycerol, 1 mM Na2EGTA) after concentration by spin column (Amicon Ultra, MILLIPORE).

**Spectroscopy** - Ultraviolet (UV) -visible absorption spectrum were recorded with a spectrophotometer (model U3100, Hitachi-hitec) equipped with a thermostat controller (model 131-0305, Hitachi hitec). Samples were excited with a combination of a BL emitting diode (LED) (LUXEON star, Lumileds Lighting, maximum emission at 465 nm) and an electronic shutter (COPAL) (15). The BL-excited spectra were recorded under the blue illumination. The reversion of S390 to D450 in the dark was monitored by the absorption changes at 450 nm at the temperatures indicated.

**Kinase assay** - CrPFul or CrPL2K was incubated with AtP1Nt as an artificial substrate in a kinase reaction buffer (20 mM Tris-HCl pH7.8, 100 mM NaCl, 10% (w/v) glycerol, 1 mM Na2EGTA, 10 mM MgCl2, 10 μM ATP containing 3.7 kBq μl⁻¹ [γ-32P] ATP) for 15 min at 20°C. The effect of blue light on phosphorylation was measured by either irradiation with a blue LED (ISL-150X150-88, CCS Inc. Japan, emission maximum at 475 nm) or mock irradiation. The intensity of the irradiation was varied with ND filters. The reaction was stopped by the addition of SDS-sample buffer followed by boiling for 3 min. Samples were run on SDS-PAGE. After Coomassie brilliant blue (CBB) staining, the gel was dried and phosphorylated bands were visualized with an imaging plate (Fuji film) and a scanner (STORM, GE Healthcare) (15). The signal intensities were quantified by Image-J software (http://rsbweb.nih.gov/ij/).

**SAXS measurement and analysis** - SAXS data of CrPL2K and CrPFul were collected at BL45XU of SPring-8 using a PILATUS3 detector (DECTRIS, Switzerland). The X-ray wavelength was tuned to 0.9000 Å, and the camera distance was approximately 2000 mm. The exposure time was 60 s, and each SAXS pattern was composed of 18 frames. Other details were described previously (27). The temperature of the sample cell was maintained at 293 K. Prior to measurements, concentrated samples were centrifuged at 30,000 g for 1 h using a Himac CP 85β ultracentrifuge (Hitachi, Japan) to remove aggregated proteins. Before SAXS measurement, the fluid dynamic diameter of CrPFul was measured by dynamic light scattering (DLS) using a Zetasizer Nano (Malvern Instruments). The decay of the time-correlation function of the back-scattered light monotonously decreased, and was well fitted by a theoretical curve calculated assuming that particles with the dimension of approximately 100 Å are monodispersively suspended. This observation indicates that CrPFul is in a monomeric form in solution.

SAXS profiles of both samples were collected in the concentration range from 1.2 to
2.3 mg mL\(^{-1}\). For each sample, SAXS was measured sequentially in the dark, under BL after pre-irradiation for 5 min, and again in the dark after the dark adaptation for more than 5 min after the measurement under BL. The fluence rate of BL at the sample position was 450 \(\mu\)M m\(^{-2}\) s\(^{-1}\) using a blue LED (27). SAXS data of hen egg white lysozyme was collected as a molecular weight reference. A small amount of radiation damage to all samples was confirmed by the stabilities of SAXS profiles, absorption spectra, and SDS-PAGE patterns after X-ray exposure.

**SAXS analysis** - The two-dimensionally recorded SAXS patterns were reduced to one-dimensional profiles after subtraction of the background scattering from the buffer solution. Profiles in the small-angle region were analyzed by Guinier’s plot (35) to obtain zero-angle scattering intensity and radius of gyration \(R_g\). Their concentration dependencies were analyzed as described previously (36), and the distance distribution function \(P(r)\) was calculated using GNOM (37). The low-resolution molecular models were restored as an assembly of small spheres with a diameter of 3.8 Å, called dummy residues, using GASBOR (38) software. GASBOR minimizes the discrepancy between experimental and calculated scattering profiles by keeping a compactly interconnected configuration of dummy residues approximating a molecular shape. The discrepancy in the observed and the calculated scattering profiles was monitored via the \(\chi^2\) value, which is defined as

\[
\chi^2 = \frac{1}{n-1} \sum_{j=1}^{n} \left[ \frac{c(S_j) I_{\text{model}}(S_j) - K I_{\text{exp}}(S_j)}{\sigma(S_j)} \right]^2
\]

where \(n\) is the number of experimental data points, \(c(S_j)\) is the scattering vector of the \(j\)th data point, \(c(S_j) I_{\text{model}}(S_j)\) is a correction factor, \(K\) is a scale factor, and \(\sigma(S_j)\) is the statistical error in the experimental scattering profile \(I_{\text{exp}}(S_j)\). \(I_{\text{model}}(S_j)\) represents the scattering profile of the predicted structural model. As GASBOR analysis does not provide a unique solution for three-dimensional structures, 20 independent calculations were performed for a targeted profile, and the obtained molecular models were aligned manually (39).

**RESULTS**

**UV-visible spectrum and photoreaction of CrPFul and CrPL2K** - The purity of the two preparations used in the experiments, CrPFul and CrPL2K (Fig. 1), was estimated at more than 95% by CBB staining of the SDS-PAGE gel (Fig. 2A). CrPFul showed the same UV-visible absorption spectrum in the dark and under BL irradiation as those reported previously (Fig. 6 in 34). Under BL irradiation, the absorption at 450 nm decreased, while the apparent \(M_w\) of a soluble protein using \(I(S=0, C=0)\) of a reference protein with a known \(M_w\). The \(S < 0.005\) Å\(^{-1}\) intensity profiles were extrapolated to the infinite dilution limit to correct for the concentration effects on the scattering profiles. The corrected profiles were merged with the \(S > 0.005\) Å\(^{-1}\) profile measured from 2.3 mg mL\(^{-1}\) solution.

The distance distribution function \(P(r)\) was calculated using GNOM (37) software. The low-resolution molecular models of P2L2K were restored as an assembly of small spheres with a diameter of 3.8 Å, called dummy residues, using GASBOR (38) software. GASBOR minimizes the discrepancy between experimental and calculated scattering profiles by keeping a compactly interconnected configuration of dummy residues approximating a molecular shape. The discrepancy in the observed and the calculated scattering profiles was monitored via the \(\chi^2\) value, which is defined as

\[
\chi^2 = \frac{1}{n-1} \sum_{j=1}^{n} \left[ \frac{c(S_j) I_{\text{model}}(S_j) - K I_{\text{exp}}(S_j)}{\sigma(S_j)} \right]^2
\]

where \(n\) is the number of experimental data points, \(c(S_j)\) is the scattering vector of the \(j\)th data point, \(c(S_j) I_{\text{model}}(S_j)\) is a correction factor, \(K\) is a scale factor, and \(\sigma(S_j)\) is the statistical error in the experimental scattering profile \(I_{\text{exp}}(S_j)\). \(I_{\text{model}}(S_j)\) represents the scattering profile of the predicted structural model. As GASBOR analysis does not provide a unique solution for three-dimensional structures, 20 independent calculations were performed for a targeted profile, and the obtained molecular models were aligned manually (39).

**RESULTS**

**UV-visible spectrum and photoreaction of CrPFul and CrPL2K** - The purity of the two preparations used in the experiments, CrPFul and CrPL2K (Fig. 1), was estimated at more than 95% by CBB staining of the SDS-PAGE gel (Fig. 2A). CrPFul showed the same UV-visible absorption spectrum in the dark and under BL irradiation as those reported previously (Fig. 2B in 34). The UV-visible absorption spectrum of CrPL2K in the D450 state exhibited peaks at 471, 445, 369, and 354 nm (Fig. 2B), which is almost the same as those of CrPFul (Fig. 6 in 34) and the truncated LOV2 of Cr phot (40). Under BL irradiation, the absorption at 450 nm decreased, while
absorption at 390 nm increased (Fig. 2B), indicating the formation of a cysteinyl-flavin adduct. The S390 of both CrPFul and CrPL2K reverted to D450 in the dark (data not shown), showing a characteristic photocycle with LOVs.

We have previously shown that the S390 in LOV2 is involved in the photosensitivity of the activation of phot kinase by BL in At phot (15); then, the dark reversion of S390 to D450 was measured. In CrPFul, the time course of the dark reversion was well approximated with a double-exponential curve. The half-lives ($t_{1/2}$) of the two components were calculated as 13.0 and 54.8 s (Fig. 3A, Table 2), which is similar to previously reported values (34). The faster and the slower components were attributed to LOV2 and LOV1, respectively (15, 40). Dark reversion of S390 to D450 in LOV2 of CrPL2K was also fitted with a double-exponential curve despite the lack of LOV1. Similar double-exponential kinetics was reported with the truncated LOV2 although the origins were unclear (40). The major (91%) and the minor (9%) components showed $t_{1/2}$ of 5.1 and 104 s, respectively (Fig. 3B, Table 2). When comparing the $t_{1/2}$ in LOV2 of CrPFul (Fig. 3A, dashed line) with that of the major component of CrPL2K, it was observed that the reversion of S390 in LOV2 is accelerated by approximately 2.5 times in the presence of LOV1. It is interesting to see if the acceleration requires the photoreaction in LOV1.

Therefore, we measured the dark reversion of S390 in LOV2 of the C57A substitute of CrPFul (CrPFul_C57A), which had lost the ability to form adducts in LOV1. The reversion was well simulated with a single-exponential curve. The $t_{1/2}$ was calculated as 10.2 s (Table 2), which is slightly shorter than that of CrPFul; however, it is 2 times longer than that of CrPL2K. This indicates that the presence of LOV1 itself is the predominant contributor to the acceleration of the dark reversion in LOV2.

In turn, we also measured the dark reversion in LOV1 of the C250A substitute of CrPFul (CrPFul_C250A), which had lost its ability to form an adduct in LOV2. The reversion in CrPFul_C250A was also well approximated with a single-exponential curve and showed the same $t_{1/2}$ as LOV1 with that of CrPFul (54.1 s) (Table 2). Hence, the dark reversion in LOV1 of CrPFul is not affected by the photoreaction in LOV2. Similar vectorial effects of the photoreactions upon the decay behavior of S390 between LOV1 and LOV2 were reported with truncated LOV1-LOV2 fragments of Cr phot (40). In addition, the effect of kinase activity on the dark reversion was measured using a D545N substitute (CrPFul_D545N), which lacks ATP-binding ability and kinase activity. CrPFul_D545N showed a double-exponential reversion profile with a $t_{1/2}$ of 13.8 and 54.1 s (Table 2), indicating that the disruption of the kinase activity does not affect the dark reversion in both of the LOVs. These results provided useful information regarding the effects of the interdomain interactions between LOV2 and LOV1 or KD on the duration of the signaling state in LOV2.

Activation of Kinase by BL in CrFul and CrPL2K - We have reported previously that CrPFul was able to phosphorylate AtP1Nt (34). We verified that the present CrPFul preparation was able to phosphorylate AtP1Nt in manner that was dependent on the kinase activity in the KD (Fig. 4A). CrPL2K was also revealed to phosphorylate AtP1Nt in a light dependent manner (Fig. 4B). The fluence response of the photoactivation of the kinase of CrPFul and CrPL2K was compared (Fig. 5); CrPFul exhibited 50% (see the legend to Fig. 5B) kinase activation at 5 μmol m$^{-2}$ s$^{-1}$, and the activation exceeded 90% at 20 μmol m$^{-2}$ s$^{-1}$. In contrast, CrPL2K showed 50% kinase activation at 10 μmol m$^{-2}$ s$^{-1}$, and the activation was saturated at 50 μmol m$^{-2}$ s$^{-1}$. Based on the BL intensity for 50% activation, CrPFul is approximately 2 times more sensitive to BL than CrPL2K in term of the photosensitivity of the kinase activation.

Strangely, CrPFul_C250A, which does not exhibit adduct formation in LOV2, also maintained BL kinase activation ability, although to a lesser extent than CrPFul (Fig. 5A). The leaky kinase activity of the C250A substitute was also observed with Cr phot by Yeast Kinase assay, however, the cause was unclear (34). Involvement of photoreaction of LOV1 in this leaky kinase activity is negated because CrPFul CrPFul_C57/250A that has both C57A and C250A substitutions showed a similar lesser degree of BL-dependent kinase activation than CrPFul to that of the CrPFul_250A (Fig. 6).

It has been shown that C250S substitute of LOV2 of Cr phot forms a reduced form of FMN instead of cysteinyl-flavin adduct upon BL irradiation. The reduced form is not reversible to the oxidized form in the dark. In contrast, C57S substitute of LOV1 forms a neutral FMN semiquinone, FMNH$^\cdot$, which is reversible to the
oxidized form (41). Formation of the reduced form has not been reported with phot of higher plants (42). One of the possible explanations for this leaky kinase activity is that BL-dependent reduction of FMN in LOV2 may induce conformational changes similar to those activate the kinase through the adduct formation in Cr phot.

Structural parameters of CrFul and CrPL2K deduced from SAXS - In the concentration range measured, the SAXS profiles of CrPFul displayed an approximately 5% increase in two S regions, $S < 0.003 \text{ Å}^{-1}$ and $0.01 < S < 0.02 \text{ Å}^{-1}$, and an approximately 3% decrease at $S \sim 0.007 \text{ Å}^{-1}$ by BL irradiation (Fig. 7), which suggests BL-induced conformational changes. Even 10 min after BL was turned off, the changes did not completely relax. This most likely reflects the delay of the global molecular changes after completion of the photocycle of the FMN chromophore. The Guinier plots (35) of all the samples were approximated by straight lines in the $S^2$ region of $1\times10^{-5} \text{ Å}^{-2} < S^2 < 4\times10^{-5} \text{ Å}^{-2}$ (Fig. 7 inset). With the exception of small variations, the calculated $C(r)(S=0, C)$ and the $R_g^2(C)$ were proportional to the concentration of CrPFul (Fig. 7B). These observations indicated that the CrPFul solutions were almost monodisperse in the measured concentration range under both dark and light conditions. Based on the $C(r)(S=0, C)$, the apparent $M_w$ of CrPFul was estimated at 70 k, which indicates that the concentrated solution consisted of a monomeric form of CrPFul. This result correlates with the monomeric form of CrPFul in the diluted solution, which was determined by size exclusion chromatography (SEC) (34). $R_g$ values at infinitely diluted conditions were 42.7 Å in the dark and 43.4 Å under BL irradiation. The maximum dimension estimated from the $P(r)$ function in the dark was 157 Å smaller than the 167 Å under BL (Fig. 7C). The changes in structural parameters regarding molecular dimensions indicate a BL-induced extension of CrPFul.

The SAXS profiles of CrPL2K under BL irradiation showed a significant increase of approximately 10% in a $S$ region of $S < 0.003 \text{ Å}^{-1}$ and decrease of approximately 3% at $S \sim 0.01 \text{ Å}^{-1}$, which demonstrates BL-induced conformational changes (data not shown). These profile changes are similar to those observed in the D720N substitute of the LOV2-KD fragment of At phot2 (AtP2L2K_D720N) (27). The Guinier plots deviated from straight lines in the $S^2$ region of $5\times10^{-6} \text{ Å}^{-2} < S^2 < 3\times10^{-5} \text{ Å}^{-2}$ because of aggregated components that were unable to be removed by ultracentrifugation. Molecular dimensions of CrPL2K were, therefore, estimated by applying the Guinier approximation to the $S^2$ region of $3\times10^{-5} \text{ Å}^{-2} < S^2 < 5\times10^{-5} \text{ Å}^{-2}$, in which region the Guinier approximation worked well with AtP2L2K_D720N (27). The $M_w$, the $R_g$ at 1.7 mg ml$^{-1}$ and the $D_{max}$ of $P(r)$ (data not shown) were estimated as 50k, 34.7 Å and 127 Å, respectively; these values were close to the values obtained for AtP2L2K_D720N (27). Under BL irradiation, the $R_g$ and the $D_{max}$ increased to 36.1 Å and 131 Å, respectively, indicating an expansion of the molecule.

Molecular models for CrPFul and CrPL2K - Low-resolution molecular models of CrPL2K in the dark and under BL irradiation were restored using the SAXS profiles of $S > 0.006 \text{ Å}^{-1}$. The restored models appeared as elongated shapes with the dimensions of $110\times40\times40 \text{ Å}^3$ (Fig. 8 left) and reproduced the experimental profiles as indicated by the small $R_g^2$-index of less than 2.0. The models of CrPL2K in the dark and under BL irradiation were similar in shape, but the model under BL is larger at approximately 10 Å greater than the model in the dark. The molecular shapes and dimensions of CrPL2K are quite similar to those of AtP2L2K_D720N (27) (Fig. 8 center and left), suggesting that the molecular structures in the LOV2-KD region do not vary between Cr and At photons.

The constructed molecular models of CrPFul in the dark appeared as an elongated shape with the dimensions of $140\times40\times40 \text{ Å}^3$ (Fig. 8 right). The molecular model of CrPFul was roughly divided into a main portion with an elongated spheroid shape and a protrusion, manually. The size and the shape of the main portion with an adjacent part of the protrusion are closely similar to those of CrPL2K (Fig. 6 center) and AtP2L2K_D720N (27) (Fig. 8 left), and the crystal structure of Cr LOV1 (43) fits well with the tip of the protrusion (Fig. 8 right). The molecular model of CrPFul under BL irradiation had nearly the same dimension as the model in the dark; however, the density assignable to LOV1 could be put at a different position relative to the KD from the position in the dark model. The differences in the molecular models suggest that the positions and orientations of the LOV2-LOV1 region and/or
LOV1 might be light dependent (Fig. 8 right).

**DISCUSSION**

The role of LOV1 in the BL-dependent kinase activation of Cr phot - Higher plant photos are thought to transmit signals downstream of the signal transduction paths via autophosphorylation (3, 16) and/or phosphorylation of some signal mediators (19-21). In this study, we used Cr phot instead of At phot due to limitations in the ability to prepare a full-length sample. The results showed that AtP1nt serves as a substrate for the kinase of Cr phot as well as At phots, suggesting that Cr phot has similar substrate recognition ability and kinase activity as At phots.

Fluence response curves showed that the photosensitivity of the BL-dependent kinase activation in Cr phot decreased to less than half when LOV1 was truncated (Fig. 5B). This suggests that LOV1 serves as an amplifier of photosensitivity. We have reported previously that the photosensitivity of the kinase activation by BL is positively correlated with the duration of S390 in LOV2 by using LOV2-KD fragments of At phot1 and phot2 with different durations and an amino acid substitution of phot1 that has a prolonged duration (15). Similarly, the fact that CrPFul has a 2 x higher photosensitivity than CrPL2K is well explained by the observation that LOV2 has a longer t1/2 in CrPFul (13.0 s) than in CrPL2K (5.1 s) (Table 2). Likewise, the only slightly shorter t1/2 of LOV2 in CrPFul_C57A than that in CrPFul (10.2 s and 13.8 s, respectively) (Table 2) may explain the finding that BL-dependent activation is almost unchanged with the C57A substitute (Fig. 4A). In contrast, small decrease of the activation was observed with the C250A substitution (Fig. 4A), in which photoreaction of LOV1 was not involved. These findings suggest that the duration of S390 in LOV2 also plays one of the key roles in activation of the kinase by BL in Cr phot as with At phot. This is shown schematically in Fig. 9A; formation of S390 by BL in LOV2 (dark blue center circle) induces a conformational change in the protein moiety. This change leads to signal transmission, i.e., activation of kinase (red outer arc).

Thus, LOV1 may alter the photosensitivity of the BL-dependent kinase activation by modifying the duration of S390 in LOV2. Because the photoreaction in LOV1 only has a small effect on the duration of S390 in LOV2, the modification may come from the structural presence of LOV1 itself rather than the photoreaction of LOV1. This is consistent with the report that the rate of the thermal back reaction from S390 in LOV2 depends on the presence of LOV1 in a Cr phot fragment consisting of LOV1 and LOV2 (40). This is depicted schematically in Fig. 9A (yellow circle and arrow).

Together with the report that Cr phot complements the phenotypes of At phots (33), the molecular mechanism of the kinase activation by LOV2 is suggested to be basically conserved between Cr phot and At phots, and Cr phot can serve as a useful molecular model to uncover this mechanism. It should be noted, however, that the observed leaky BL-dependent kinase activity of CrPFul_C250A (Fig. 4A) indicates that a currently unknown mechanism specific to Cr phot turns on the LOV2 switch to activate the kinase in a different way from the adduct formation when LOV2 lacks a reactive cysteine.

**Organization of LOV1, LOV2 and KD in a Cr phot molecule** - It has been of great interest to identify how LOV1, LOV2 and KD are configured in a phot molecule. The present SAXS studies revealed a tandem configuration of LOV1, LOV2 and KD in a photoactive full-length Cr phot molecule existing in a monomeric form in solution under both dark and BL conditions (Fig. 8). This is the first report describing the position of LOV1 relative to LOV2-KD of phot; this is shown schematically in Fig. 7B. The reconstructed tandem models suggest that LOV1 and KD are located at opposite sides of LOV2. This suggests an indirect contribution of LOV1 to the photoactivation of the kinase. This proposal is consistent with the present interpretation of the effect of LOV1 truncation on the photoactivation of the kinase that LOV2 is a main regulator of the kinase activation, while LOV1 alters its photosensitivity. This alteration is possibly achieved by modification of the duration of the signaling state in the neighbor LOV2. This modification may come from the structural interaction of LOV1 with LOV2 in which LOV1 may prolong the t1/2 of S390 in LOV2 by acting as a weight on the photoreaction, therefore, preventing the conformational change of LOV2. A similar weight effect was observed with the truncated
LOV2 of Cr phot fused with calmodulin-binding protein (44).

**BL-induced global conformation change in a Cr phot molecule** - It has been postulated that the unfolding and dissociation of Jα from the LOV core may play important roles in the photoactivation of the kinase (22, 45). Previously, based on the SAXS of LOV2-KD of At phot2, we have reported that BL induced a 13 Å shift of the LOV2 domain from the KD. We further proposed that the light-activated LOV2 domain triggers conformational changes in the linker region between LOV2 and KD, causing them to separate (25) (Fig. 8 left). Because secondary structure prediction reveals the presence of an α-helix in the linker region and the FT-IR study proposed an unfolding of an α-helix in the linker region (46), Jα may exist and unfold upon BL excitation in Cr phot. However, our previous report indicated that the amino acids around the N-terminus of LOV2 also play essential roles in the photoactivation of the kinase in Cr phot (34). Secondary structure prediction showed the presence of an α-helix consisting of 10 amino acids at that position in Cr phot. In the crystal structure of LOV2-Jα of As phot1 (23), there is a small α-helix consisting of 4 amino acids due to the truncated construct of the sample; this α-helix is called A’α. The A’α resides close to the C-terminal region of Jα. The two helices might form an intramolecular signaling module and serve cooperatively in the photoactivation of the kinase. BL-induced changes in the module may move LOV2 away from the KD and cancel the depression of the kinase activity. This is illustrated schematically in Fig. 7B. The observed differences in the molecular models of the dark and light states (Fig. 8) might reflect these conformational changes.

To date, information concerning the conformational change in a whole molecule of phot has not been available, except for the only FT-IR study of Cr phot (46). The present SAXS study presented the image of the BL-induced global conformational changes of a whole Cr phot molecule; these changes could be interpreted by the moving away and possible tilting of LOV1 relative to the LOV2-KD (Fig. 8). Because the linker region between LOV1 and LOV2 has high disorder probability, major conformational changes might take place in this linker region. A cleavage of 20 amino acids within the KD of Cr phot has been reported under high light irradiation in vivo (31); this cleavage was also observed during the preparation of Cr phot using an E. coli expression system (45) and suggests that some BL-induced conformational changes occur within the KD. This may be correlated with the helical unfolding in the activation loop of KD detected by FT-IR (45). These local changes are not included in Fig. 9B. To confirm the models presented in Fig. 9B, more structural studies, including crystallography, are required.

**The divergent role of LOV1 between Cr phot and At phots** - In Cr phot, LOV1 was shown to amplify the kinase photoactivation; however, LOV1 attenuated the photosensitivity in At phot2 (18). When we consider these opposite functions, it should be noted that despite the high homology of the amino acid sequence in LOV1 between Cr phot and higher plant phots, the lengths (see Fig. 1) and the amino acid sequences of the hinge between LOV1 and LOV2 and the N-terminal extension differ significantly between the two. Furthermore, their LOV1 domains play different roles in the formation of oligomeric structures. Crystal structures of the LOV1 of At phot1 and phot2 showed a dimeric structure contacting in the β-scaffold (47). This structure is consistent with the results of SAXS, SEC (24) and chemical cross-linking (48) studies in solution, suggesting that LOV1 act as a dimeric site. Because light-dependent but LOV1-independent dimerization of At phots in vivo was reported (32), the involvement of LOV1 in in vivo dimerization still needs further investigation. In contrast, crystal structure of the LOV1 in Cr phot indicated a monomeric form (43) that agrees with the current SAXS results on full-length Cr phot in solution and, therefore, suggests that the LOV1 of Cr phot cannot be a dimerization site. At phot was shown to phosphorylate each other in a dimeric form (32), resulting in autophosphorylation. Cr phot, however, showed less autophosphorylation activity (33), which may be explained by the reduced efficiency of the autophosphorylation in the monomeric Cr phot. If LOV1 forms a dimer, the weight effect discussed above will be altered and the modification mode of the photosensitivity of the kinase activation by LOV1 may be affected. This might explain the antagonistic effects of LOV1 upon the photosensitivity of the kinase activation between Cr and At phots.
Thus, the structural differences in the LOV1-containing regions are capable of producing the diversity of LOV1 functions, including the modification mode of the photosensitivity of the kinase activation. With all of the different oligomeric structures and the partially different roles of LOV1 of Cr phot compared to those of higher plant phots, the results provided useful information regarding the light-regulation mechanism of the kinase by LOV1 and LOV2. To determine the precise mechanism of this regulation, further structure/function studies of At phots, as well as Cr phot, are required.

In contrast to higher plant phots, Cr phot is able to transduce light signal in a different way from phosphorylation. Overexpression of a LOV1-LOV2 fragment affected the eyespot size and phototaxis in a light-dependent manner, suggesting that Cr phot transduced BL signal through protein-protein interaction (31). This may come from the different primary and quaternary structures at around LOV1 of Cr phot from those of higher plant phots discussed above.

Since LOV belongs to a subset of a PAS superfamily that act as a protein-protein interaction module in eukaryotic cellular signaling (49), it is probable that the LOV1-LOV2 of Cr phot may interact with an unidentified signaling partner(s) in a light dependent manner. Actually, two members of LOV, F-box and Kelch repeat family proteins, ZTL and FKF1, have been shown to interact with TOC1 and GI, respectively, through the LOV in a light dependent manner (50, 51). The BL-induced changes in the LOV1-LOV2 region seen in the SAXS models of Cr phot might be involved in the interaction. The light-inducible protein-protein interaction of Cr phot reminds us of the application to the light-regulated molecular switch in the cell signaling. Such approach has been attempted using LOV2-Jα construct of As phot1 (52, 53) and the interaction between FKF1 and GI (54). Further structure/function studies will provide useful information regarding the application of Cr phot to this kind of research.
REFERENCE

1. Franklin, K.A., and Quail, P.H. (2010) Phytochrome functions in Arabidopsis development. J. Exp. Bot. 61, 11-24.
2. Liu, H., Liu, B., Zhao, C., Pepper, M., and Lin, C. (2011) The action mechanisms of plant cryptochromes. Trends Plant Sci. 16, 684-691.
3. Christie, J.M. (2007) Phototropin blue-light receptors. Annu. Rev. Plant Biol. 58, 21-45.
4. Christie, J. M., Reymond, P., Powell, G.K., Bernasconi, P., Raibekas, A.A., Liscum, E., and Briggs, W.R. (1998) Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for phototropism. Science 282, 1698-1701.
5. Kagawa, T., Sakai, T., Suetsugu, N., Oikawa, K., Ishiguro, S., Kato, T., Tabata, S., Okada, K., and Wada, M. (2001) Arabidopsis NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. Science 291, 2138-2141.
6. Kinoshita, T., Doi, M., Suetsugu, N., Kagawa, T., Wada, M., and Shimazaki, K. (2001) Phot1 and phot2 mediate blue light regulation of stomatal opening. Nature 414, 656-660.
7. de Carbonnel, M., Davis, P., Roelfsema, M.R., Inoue, S., Schepens, I., Lecuit, P., Geisler, M., Shimazaki, K., Hangarter, R., and Fankhauser, C. (2010) The Arabidopsis PHYTOCHROME KINASE SUBSTRATE2 protein is a phototropin signaling element that regulates leaf flattening and leaf positioning. Plant Physiol. 152, 1391-1405.
8. Kozuka, T., Kong, S.G., Doi, M., Shimazaki, K., and Nagatani, A. (2011) Tissue-autonomous promotion of palisade cell development by phototropin 2 in Arabidopsis. Plant Cell 23, 3684-3695.
9. Sakai, T., Kagawa, T., Kasahara, M., Swartz, T.E., Christie, J.M., Briggs, W.R., Wada, M., and Okada, K. (2001) Arabidopsis nph1 and npl1: blue light receptors that mediate both phototropism and chloroplast relocation. Proc. Natl. Acad. Sci. U.S.A. 98, 6969-6974.
10. Christie, J.M., Salomon, M., Nozue, K., Wada, M., and Briggs, W.R. (1999) LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (nph1): binding sites for the chromophore flavin mononucleotide. Proc. Natl. Acad. Sci. U.S.A. 96, 8779-8783.
11. Crosson, S., and Moffat, K. (2001) Structure of a flavin-binding plant photoreceptor domain: insights into light-mediated signal transduction. Proc. Natl. Acad. Sci. U.S.A. 98, 2995-3000.
12. Salomon, M., Christie, J.M., Knieb, E., Lempert, U., and Briggs, W.R. (2000) Photochemical and mutational analysis of the FMN-binding domains of the plant blue light receptor, phototropin. Biochemistry 39, 9401-9410.
13. Swartz, T.E., Corchnoy, S.B., Christie, J.M., Lewis, J.W., Szundi, I., Briggs, W.R., and Bogomolni, R.A. (2001) The photocycle of a flavin-binding domain of the blue light photoreceptor phototropin. J. Biol. Chem. 276, 36493-36500.
14. Kasahara, M., Swartz, T.E., Olney, M. A., Onodera, A., Mochizuki, N., Fukuzawa, H., Asamizu, E., Tabata, S., Kanegae, H., Takano, M., Christie, J. M., Nagatani, A., and Briggs, W.R. (2002) Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from Arabidopsis, rice, and Chlamydomonas reinhardtii. Plant Physiol. 129, 762-773.
15. Okajima, K., Kashojiya, S., and Tokutomi S. (2012) Photosensitivity of kinase activation by blue light involves the lifetime of a cysteinyl-flavin adduct intermediate, S390, in the photoreaction cycle of the LOV2 domain in phototropin, a plant blue light receptor. J. Biol. Chem. 287, 40972-40981.
16. Christie, J.M., Swartz, T.E., Bogomolni, R.A., and Briggs, W.R. (2002) Phototropin LOV domains exhibit distinct roles in regulating photoreceptor function. Plant J. 32, 205-219.
17. Cho, H.Y., Tseng, T.S., Kaiserli, E., Sullivan, S., Christie, J.M., and Briggs, W.R. (2007) Physiological roles of the light, oxygen, or voltage domains of phototropin 1 and phototropin 2 in Arabidopsis. Plant Physiol. 143, 517-529.
18. Matsuoka, D., and Tokutomi, S. (2005) Blue light-regulated molecular switch of Ser/Thr kinase in phototropin. Proc. Natl. Acad. Sci. U.S.A. 102, 13337-13342.
19. Christie, J. M, Yang, H., Richter, G.L., Sullivan, S., Thomson, C.E., Lin, J., Titapiwatanakun, B., Ennis, M., Kaiserli, E., Lee, O.R., Adamec, J., Peer, W.A., and Murphy, A.S. (2011) Phot1 inhibition of ABCB19 primes lateral auxin fluxes in the shoot apex required for phototropism.
20. Demarsy, E., Schepens, I., Okajima, K., Hersch, M., Bergmann, S., Christie, J., Shimazaki, K., Tokutomi, S., and Fankhauser, C. (2012) Phytochrome Kinase Substrate 4 is phosphorylated by the phototropin 1 photoreceptor. *EMBO J.* **31**, 3457-3467.

21. Takemiya, K., Sugiyama, N., Fujimoto, H., Tsutsumi, T., Yamauchi, S., Hiyama, A., Tada, Y., Christie, J.M., and Shimazaki, K. (2013) Phosphorylation of BLUS1 kinase by phototropins is a primary step in stomatal opening. *Nature Comm.* **4**:2094.

22. Harper, S.M., Neil, L.C., and Gardner, K.H. (2003) Structural basis of a phototropin light switch. *Science* **301**, 1541-1544.

23. Halavaty, A. S., Moffat, K. (2007) N- and C-terminal flanking regions modulate light-induced signal transduction in the LOV2 domain of the blue light sensor phototropin 1 from *Avena sativa*. *Biochemistry* **46**, 14001-14009.

24. Nakasako, M., Iwata, T., Matsuoka, D., and Tokutomi, S. (2004) Light-induced structural changes of LOV domain-containing polypeptides from Arabidopsis phototropin 1 and 2 studied by small-angle X-ray scattering. *Biochemistry* **43**, 14881-14890.

25. Koyama, T., Iwata, T., Yamamoto, A., Sato, Y., Matsuoka, D., Tokutomi, S., and Kandori, H. (2009) Different role of the Jalpha helix in the light-induced activation of the LOV2 domains in various phototropins. *Biochemistry* **48**, 7621-7628.

26. Okajima, K., Matsuoka, D., and Tokutomi, S. (2011) LOV2-linker-kinase phosphorylates LOV1-containing N-terminal polypeptide substrate via photoreaction of LOV2 in Arabidopsis phototropin1. *FEBS Lett.* **585**, 3391-3395.

27. Takayama, Y., Nakasako, M., Okajima, K., Iwata, A., Kashojiya, S., Matsu, Y., and Tokutomi, S. (2011) Light-induced movement of the LOV2 domain in an Asp720Asn mutant LOV2-kinase fragment of Arabidopsis phototropin 2. *Biochemistry* **50**, 1174-1183.

28. Huang, K., Merkle, T., and Beck, C.F. (2002) Isolation and characterization of a *Chlamydomonas* gene that encodes a putative blue-light photoreceptor of the phototropin family. *Science* **301**, 1541-1544.

29. Huang, K., and Beck, C. F. (2003) Phototropin is the blue-light receptor that controls multiple steps in the sexual life cycle of the green alga *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6269-6274.

30. Im C.S., Eberhard, S., Huang, K., Beck, C.F., and Grossman, A.R. (2006) Phototropin involvement in the expression of genes encoding chlorophyll and carotenoid biosynthesis enzymes and LHC apoproteins in *Chlamydomonas reinhardtii*. *Plant J.* **46**, 1-16.

31. Trippens, J., Greiner, A., Schellwat, J., Neukam, M., Rottmann, T., Lu, Y., Kateriya, S., Hegemann, P., and Kreimer, G. (2012) Phototropin Influence on Eyespot Development and Regulation of Phototactic Behavior in *Chlamydomonas reinhardtii*. *Plant Cell* **24**, 4687-4702.

32. Kaiserli, E., Sullivan, S., Jones, M.A., Feeney, K.A., and Christie, J.M. (2009) Domain swapping to assess the mechanistic basis of Arabidopsis phototropin 1 receptor kinase activation and endocytosis by blue light. *Plant Cell* **21**, 3226-3244.

33. Onodera, A., Kong, S.G., Doi, M., Shimazaki, K., Christie, J.M., Mochizuki, N., and Nagatani, A. (2005) Phototropin from *Chlamydomonas reinhardtii* is functional in *Arabidopsis thaliana*. *Plant Cell Physiol.* **46**, 367-374.

34. Aihara, Y., Yamamoto, T., Okajima, K., Yamamoto, K., Suzuki, T., Tokutomi, S., Tanaka, K., and Nagatani, A. (2012) Mutations in N-terminal Flanking Region of Blue Light-sensing Light-Oxygen and Voltage 2 (LOV2) Domain Disrupt Its Repressive Activity on Kinase Domain in the *Chlamydomonas* Phototropin. *J. Biol. Chem.* **287**, 9901-9909.

35. Guinier, A., and Fournet, G. (1955) *Small-Angle Scattering of X-rays*, Wiley J., New York.

36. Svergun, D. I. (1992) Determination of the regularization parameter in indirect-transform methods using perceptual criteria. *J. Appl. Crystallogr* **25**, 495-503.

37. Svergun, D. I., Petoukhov, M. V., and Koch M. H. (2001) Determination of domain structure of proteins from X-ray solution scattering. *Biophys J.* **80**, 2946-2953.
39. Volkov, V. V., and Svergun, D. I. (2003) Uniqueness of ab initio shape determination in small-angle scattering. *J. Appl. Cryst.* **36**, 860-864.

40. Guo, H., Kottke, T., Hegemann, P., and Dick B. (2005) The phot LOV2 domain and its interaction with LOV1. *Biophys J.* **89**, 402-412.

41. Song, S.H., Dick, B., Penzkofer, A., and Hegemann, P. (2007) Photo-reduction of flavin mononucleotide to semiquinone form in LOV domain mutants of blue-light receptor phot from *Chlamydomonas reinhardtii*. *J. Photochem. Photobiol. B:* **87**, 37–48.

42. Swartz, T.E., Bogomolni, R.A., and Briggs, W.R. (2005) LOV domain photochemistry, in *Handbook of Photosensory Receptors* (Briggs, W. R., and Spudich, J. L., Eds.) pp 305-322, Wiley-VCH, Weinheim.

43. Fedorov, R., Schlichting, I., Hartmann, E., Domratcheva, T., Fuhrmann, M., and Hegemann, P. (2003) Crystal structures and molecular mechanism of a light-induced signaling switch: The Phot-LOV1 domain from *Chlamydomonas reinhardtii*. *Biophys. J.* **84**, 2474-2482.

44. Holzer, W., Penzkofer, A., Susdorf, T., Alvarez, M., Islam, Sh.D.M., and Hegemann, P. (2004) Absorption and emission spectroscopic characterisation of the LOV a maltose binding protein. *Chem. Phys.* **302**, 105–118.

45. Jones, M.A., Feeney, K.A., Kelly, S.M., Christie J.M. (2007) Mutational analysis of phototropin 1 provides insights into the mechanism underlying LOV2 signal transmission. *J. Biol. Chem.* **282**, 6405-6414.

46. Pfeifer, A., Mathes, T., Lu, Y., Hegemann, P., and Kottke, T. (2010) Blue light induces global and localized conformational changes in the kinase domain of full-length phototropin. *Biochemistry* **49**, 1024-1032.

47. Nakasako, M., Zikihara, K., Matsuoka, D., Katsura, H., and Tokutomi, S. (2008) Structural basis of the LOV1 dimerization of Arabidopsis phototropins 1 and 2. *J. Mol. Biol.* **381**, 718-733.

48. Katsura, K., Zikihara, K., Okajima, K., Yoshihara, S., and Tokutomi, S. (2009) Oligomeric structure of LOV domains in Arabidopsis phototropin. *FEBS Lett.* **583**, 526-530.

49. Cheng, P., He, Q., Yang, Y., Wang, L., and Liu, Y. (2003) Functional conservation of light, oxygen, or voltage domains in light sensing. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 5938-5943.

50. Mas, P., Kim, W.Y., Somers, D.E., and Kay, S.A. (2003) Targeted degradation of TOC1 by ZTL modulates circadian function in Arabidopsis. *Nature* **426**, 567-570.

51. Saawa, M., Nusinow, D.A., Kay, S.A., and Imaizumi, T. (2007) FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. *Science* **318**, 261-265.

52. Wu, Y.I., Frey, D., Lungu, O.I., Jaehrig, A., Schlichting, I., Kuhlman, B., and Hahn, K.M. (2009) A genetically encoded photoactivatable Rac controls the motility of living cells. *Nature* **461**, 104-108.

53. Strickland, D., Yao, X., Gawlak, G., Rosen, M.K., Gardner, K.H., and Sosnick, T.R. (2010) Rationally improving LOV domain-based photoswitches. *Nat. Methods* **7**, 623-626.

54. Yazawa, M., Sadaghiani, A.M., Hsueh, B., and Dolmetsch R.E. (2009) Induction of protein-protein interactions in live cells using light. *Nat. Biotechnol.* **27**, 941-945.
Acknowledgement - We thank Dr. Kazunori Zikihara of Osaka Prefecture University for useful discussion regarding photoreaction of phot.

FOOTNOTES

* This work was supported in part by a Grant-in-Aid for Scientific Research on Innovative Areas (No. 22120002 to A. N., No. 23120525 to M. N. and No. 22120005 to S. T.), a Grant–in-Aid for Scientific Research on Priority Areas (No. 17084002 to A. N. and No. 17084008 for S. T.), a Grant–in-Aid for Exploratory Research (No. 23657105 to S. T.) and a Grant-in-Aid for the Global COE Program "Formation of a strategic base for biodiversity and evolutionary research: from genome to ecosystem" (A06 to A. N.), from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

1 To whom corresponding should be addressed: Satoru Tokutomi, Department of Biological Sciences, Graduate School of Science, Osaka Prefecture University, Gakuen-cho 1-1, Nakaku, Osaka 599-8531, Japan. Tel.: +81 72-254-9841; Fax: +81 72-254-9841; E-mail: toxan@b.s.osakafu-u.ac.jp.

Masayoshi Nakasako, Department of Physics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Kanagawa 223-8522, Japan. Tel.: +81 45-566-1713; Fax: +81 45-566-1672; E-mail: nakasako@phys.keio.ac.jp.

2 The abbreviations used are: At, Arabidopsis thaliana; BL, blue light; As, Avena sativa; CBB, Coomassie brilliant blue; D450, ground state; FMN, flavin mononucleotide; FT-IR, Fourier transform infrared; L660, triplet exited state; LOV, light-oxygen-voltage sensing domain; NtP1, N-terminal fragment of phototropin1; PL2K, LOV2-linker-kinase of Cr phototropin; L2K, LOV2-linker-kinase of Cr phototropin; phot, phototropin; phot1, At phototropin 1; At phot2, phototropin 2; S390, adduct state; SAXS, small-angle X-ray scattering; UV, ultraviolet.

FIGURE LEGENDS

FIGURE 1. Schematics of the domain structures of Cr phot, At phot1 and phot2. Horizontal bars indicate the regions for CrPFul and CrPL2K used in this study. LOV1, LOV2, and the N-lobe and C-lobe of KD were colored in yellow, dark blue, green and red, respectively. Pink indicates Jα-helix.

FIGURE 2. (A) SDS-PAGE gel of purified CrPFul and CrPL2K stained with CBB. Arrows indicate the positions of purified proteins. (B) Absorption spectra of CrPL2K in a solution containing 20 mM Tris-HCl pH7.8, 200 mM NaCl, 10% (w/v) glycerol at 20ºC. The black and blue lines indicate the dark adopted and BL (50 μmol m^{-2} s^{-1}) irradiated samples, respectively.

FIGURE 3. Dark recovery from S390 to D450 of CrPFul (upper panel) and CrPL2K (lower panel). Red colored lines indicate calculated fitting curves of $y = 0.48\exp(-0.013x) + 0.52\exp(-0.053x) + 0.0028$ and $y = 0.906\exp(-0.135x) + 0.090\exp(-0.0067x) - 0.0005$ for CrPFul and CrPL2K, respectively. Contribution of the faster component in (A) attributable to that from LOV2 is indicated by a dashed line for comparison.

FIGURE 4. (A) Effects of amino acid substitutions on the kinase activity of CrPFul on AtP1Nt. For details on the substitutions, see the Materials and Methods and the Results. The upper and lower panels indicate the autoradiograms and the CBB staining of SDS-PAGE gels, respectively. The arrows and arrowheads indicate the positions of CrFul and its substitutes, and AtP1Nt, respectively. 0, D and L show that the SDS-PAGE samples were prepared immediately after mixing, incubation with mock or BL (50 μmol m^{-2} s^{-1}) and irradiation for 15 min, respectively, of the reaction mixtures for the phosphorylation assay at 20ºC. (B) Kinase activity of CrPL2K.

FIGURE 5. (A) Phosphorylation of AtP1Nt by CrPFul (left) and CrPFul_C57A (right) under BL at different light intensities. The upper and the lower panels indicate autoradiogram and CBB staining of SDS-PAGE gels, respectively. The samples were incubated in the phosphorylation buffer at 20ºC for 15 min under mock (dark) or BL irradiation with the indicated light intensity (μmol m^{-2} s^{-1}). Arrows
and arrowheads are the same as in Fig. 4. (B) Fluence response curves of the kinase activation by BL in CrPFul (black circles) and CrPL2K (orange triangles), respectively. The increase in the phosphorylation signal upon BL irradiation against that of the mock irradiation was quantified by phosphorimaging and is plotted as a percentage of the maximal kinase activity at 50 and 100 μmol m$^{-2}$ s$^{-1}$ for CrPFul and CrPL2K, respectively. Error bars indicate the standard deviations for three independent measurements.

**FIGURE 6.** Phosphorylation of AtP1Nt by CrPFul (left) and CrPFul_C57/250A (right) under BL at different light intensities. The upper and the lower panels indicateautoradiogram and CBB staining of SDS-PAGE gels, respectively. The samples were incubated in the phosphorylation buffer at 20°C for 15 min under mock (dark) or BL irradiation with the indicated light intensity (μmol m$^{-2}$ s$^{-1}$). Arrows and arrowheads are the same as in Fig. 4.

**FIGURE 7.** (A) SAXS profiles of CrPFul in the dark (red dots) and under BL irradiation (blue dots). The scattering intensities are plotted in a semi-logarithmic scale. The inset display the Guinier plots of CrPFul in the dark (red dots) and under BL irradiation (blue dots). The concentrations of the sample solutions are 1.75 mg mL$^{-1}$. The plots are separated vertically for clarity. The black regression lines were calculated by the least-squares method in the arrowed regions. The high-angle edges in the regions satisfy the criteria for the approximation $[SRg<(2\pi)^{-1}]$. Restored molecular models of CrPFul in the dark (red colored mesh) and under BL (blue colored mesh) are indicated at the upper right side, which is shown as density maps of dummy residues in 4×4×4 Å$^3$ cubes after the independent superimposition of individually restored models. (B) Dependence of $C/I(0,C)$ (upper) and $Rg(C)^2$ (lower) of CrPFul in the dark (red filled circle) under BL irradiation (blue filled circle) on the concentration. The regression lines for the filled circles in the dark (red line) and under BL (blue line) were calculated by the least-squares method. See the results for further explanation. (C) The $P(r)$ functions of CrPFul in the dark (red dot) and under BL irradiation (blue dots).

**FIGURE 8.** Comparison of low-resolution molecular models (mesh) of AtP2L2K_D720N, CrPL2K and CrPFul in the dark and under BL irradiation. Homology modeled Ser/Thr kinase (N-lobe: red, C-lobe; green), LOV2 for AtP2L2K_D720N (dark blue) and the crystal structure of Cr LOV1 (yellow) (43) are fitted to the density models. The orientation and positions of the kinase and the LOV2 models are fixed to those in the AtP2L2K_D720N models reported previously (27). Black bars are the references for the positions of each domain.

**FIGURE 9.** (A) Schematic explanation for the relation of the photocycle (dark blue circle) and the signaling state (red arc) is shown in the left part, in which Sa and Si denote a signal active (red full line) and inactive (red dashed line) states. In the right part, prolongation of the S390 lifetime in LOV2 by the presence of LOV1 (yellow arrow). See the results section for more details. (B) Schematic illustration explaining the molecular organization of LOV1, LOV2 and KD (S/T kinase consisting of N- and C-lobes) as well as the two helices, Jα (pink curved line) and A′ (green curved line) forming a signaling module (details, see the Discussion) in a monomeric Cr phot molecule and its BL-induced changes. Colorings of the domains are the same as in Fig. 1 and 6.
TABLE 1.

| Constructs | Primer                                                                 |
|------------|------------------------------------------------------------------------|
| C57A       | 5' -TGCTTGGGTGACAACGCCCCCCTTTCTCCAAG-3'                                 |
|            | 5' -CTTGGAGGAAGCGGCGGTGTGAGCAAGCA-3'                                   |
| C250A      | 5' -TGCTGGCGCGCGCGTCCCTGTAGAG-3'                                      |
|            | 5' -CTGCGGAGGACGCGGCGTTGCGGCCACAGA-3'                                  |
| D545N      | 5' -ACGTCCCTGCTCAGCACTTCTTGACCTGTGT-3'                                 |
|            | 5' -ACGACAGGTCGAAAGTTTGAGCTGACAGA-3'                                  |
| CrPL2K     | 5' -CGCGACCCAGCCATATGACGCGCGCCCAACGTGCCTC-3'                           |
|            | 5' -CGCAAGCTTGTCCACTAGAGTTTGTCGAGCGC-3'                                |

TABLE 2.

| Sample          | LOV1 $t_{1/2}$ (s) [S.D.] | LOV2 $t_{1/2}$ (s) [S.D.] |
|-----------------|---------------------------|---------------------------|
| CrPFul_WT       | 54.8 [0.18]               | 13.0 [0.07]               | ---                       |
| CrPL2K          | ---                       | 5.1 [0.03]               | 104.5 [4.1]              |
| CrPFul_D545N    | 54.1 [0.74]               | 13.8 [0.13]               | ---                       |
| CrPFul_C57A     | ---                       | 10.2 [0.01]               | ---                       |
| CrPFul_C250A    | 54.1 [0.29]               | ---                       | ---                       |
FIGURE 5

(A) CrPFul
BL intensity
kDa
75
50

CrPL2K
BL intensity
kDa
75
50

(B) Light dependent kinase activity (%) vs. Light intensity (μmol m⁻² s⁻¹)
FIGURE 6
FIGURE 8
Light-induced conformational changes of LOV (Light Oxygen Voltage-sensing domain) 1 and LOV2 relative to the kinase domain and regulation of kinase activity in Chlamydomonas phototropin

Koji Okajima, Yusuke Aihara, Yuki Takayama, Mihoko Nakajima, Sachiko Kashojiya, Takaaki Hikima, Tomotaka Oroguchi, Amane Kobayashi, Yuki Sekiguchi, Masaki Yamamoto, Tomomi Suzuki, Akira Nagatani, Masayoshi Nakasako and Satoru Tokutomi

J. Biol. Chem. published online November 27, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.515403

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts