Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray diffraction anaylsis of the LOV1 domains of phototropin 1 and 2 from *Arabidopsis thaliana*

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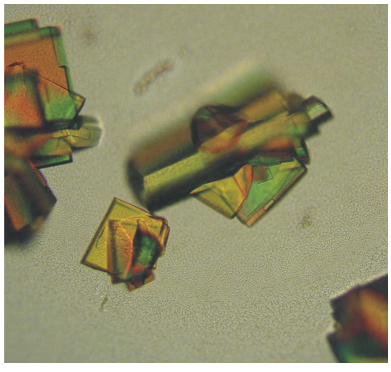
Received 11 March 2008
Accepted 23 May 2008

Phototropin is a blue-light receptor protein in plants that is responsible for phototropic responses, stomata opening and photo-induced relocation of chloroplasts. Higher plants such as *Arabidopsis thaliana* have two isoforms of phototropin: phototropin 1 and phototropin 2. Both isoforms comprise a tandem pair of blue-light-absorbing light–oxygen–voltage domains named LOV1 and LOV2 in the N-terminal half and a serine/threonine kinase domain in the C-terminal half. The LOV1 domain is thought to function as a dimerization site. In the present study, recombinant LOV1 domains of *A. thaliana* phototropin 1 and phototropin 2 were crystallized. The crystal of the LOV1 domain of phototropin 1 belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 61.2$, $b = 64.9$, $c = 70.8$ Å, and diffracted X-rays to a resolution of 2.1 Å. The crystal of the LOV1 domain of phototropin 2 belonged to space group $P2_1$, with unit-cell parameters $a = 32.5$, $b = 66.5$, $c = 56.7$ Å, $\beta = 92.4^\circ$, and diffracted X-rays to beyond 2.0 Å resolution. In both crystals, two LOV1 domains occupied the crystallographic asymmetric unit.

1. Introduction

Phototropin (phot; Christie, 2007), a blue-light receptor protein in plants, is responsible for phototropic responses (Christie *et al.*, 1998), chloroplast relocation (Kagawa *et al.*, 2001; Jarillo *et al.*, 2001) and stomata opening (Kinoshita *et al.*, 2001). These responses are necessary to maximize the efficiency of photosynthesis. In higher plants such as *Arabidopsis thaliana*, two isoforms of phototropin, phototropin 1 (phot1) and phototropin 2 (phot2), are known (Briggs & Christie, 2002; Fig. 1) and share roles in phototropic responses. While phot1 senses blue light in a broad intensity range, phot2 acts in a high-intensity region (Christie, 2007).

A. thaliana phot1 and phot2 comprise 996 and 915 amino-acid residues, respectively. They share 86% amino-acid sequence homology (Fig. 2). Both phots have two light–oxygen–voltage (LOV) domains denoted LOV1 and LOV2 in the N-terminal half and a C-terminal serine/threonine kinase domain (Fig. 1; Christie, 2007). The LOV domains belong to a subset of the Per-Arnt-Sim superfamily and are known to be important modules in cellular signalling in all kingdoms of life (Taylor & Zhulin, 1999). Either of the LOV domains, which comprise about 130 amino-acid residues, noncova-



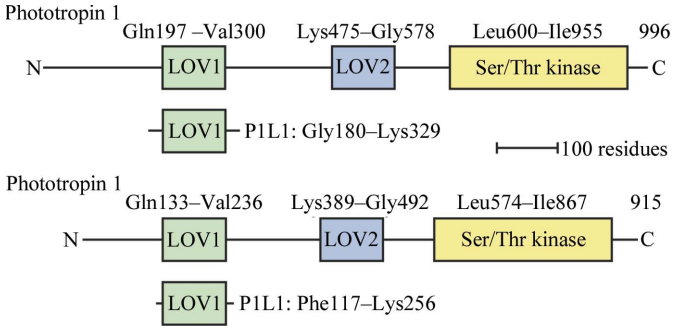


Figure 1
A schematic illustration of the organization of functional domains in phot1 and phot2. The three functional domains are shown by coloured boxes. The LOV1 domains targeted by the present study are shown separately.

lently binds one flavin mononucleotide (FMN) molecule as a chromophore (Christie *et al.*, 1999). Upon light irradiation, a LOV domain undergoes a photoreaction cycle characterized by the formation of a covalent adduct between a conserved cysteine residue and the FMN (Salomon *et al.*, 2000; Swartz *et al.*, 2001; Iwata *et al.* (2002). The covalent bond is thought to be a molecular switch that transmits structural changes in the LOV to the kinase domain. In particular, the LOV2 domain is known to act as a modulator that induces light-dependent phosphorylation of the kinase domain (Christie *et al.*, 2002). The LOV1 domain controls the sensitivity of light regulation by LOV2 (Matsuoka & Tokutomi, 2005; Matsuoka *et al.*, 2007) and is thought to act as a dimerization site in either isoform, as determined by small-angle X-ray scattering (SAXS; Nakasako *et al.*, 2004) and size-exclusion gel chromatography (Salomon *et al.*, 2004).

The structures of LOV2 domains are known for phots from some higher plants (Crosson & Moffat, 2001; Harper *et al.*, 2003; Halavaty & Moffat, 2007). In contrast, for LOV1 only the crystal structure of the *Chlamydomonas reinhardtii* phot LOV1 domain is known (Fedorov *et al.*, 2003). In the present study, we crystallized the LOV1 domains of phot1 (residues Gly180–Lys329) and phot2 (Phe117–Lys265) from *A. thaliana* (Figs. 1 and 2) in order to understand the structural basis of the dimerization. The domains contain the minimal functional unit for the photoreaction cycle and display monodisperse characteristics in solution, as revealed by an SAXS study (Nakasako *et al.*, 2004).

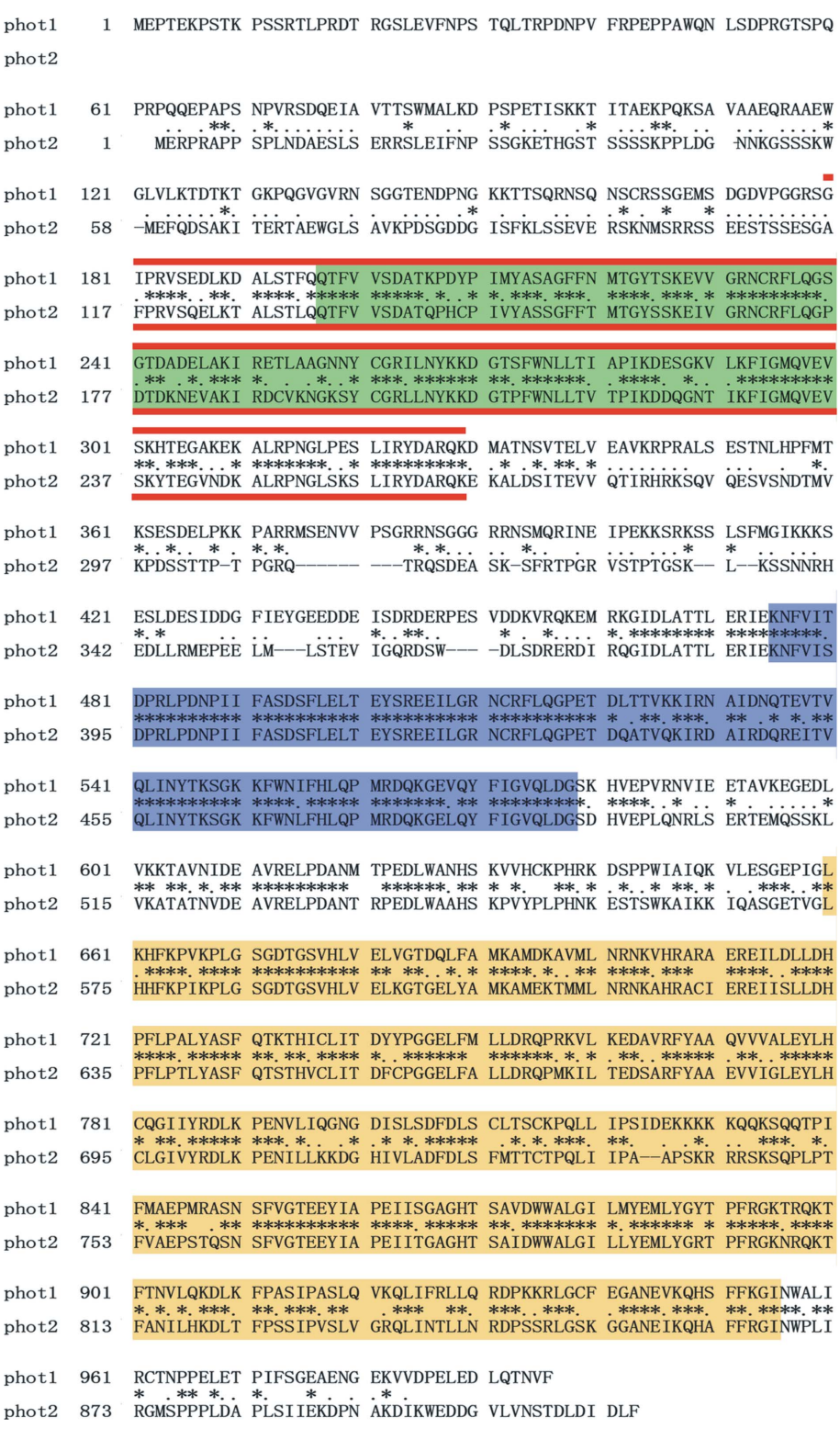


Figure 2
An alignment of the sequences of phot1 (NP_001030814) and phot2 (NP_851212). The background colours correspond to those of the functional domains in Fig. 1. The amino-acid residues indicated by '*' and '.' are identical and homologous, respectively, for the isoforms. The residue numbers are shown on the left. The regions indicated by thick red lines were overexpressed as fusion proteins with GST and used in this study.

2. Methods and results

2.1. Purification of recombinant LOV1 domains

The LOV1 domains of phot1 and phot2 from *A. thaliana* were overexpressed as fusion proteins with glutathione *S*-transferase (GST) using *Escherichia coli* strain JM109 with the expression vector pGEX-4T-1 (Amersham Biosience, Sweden) coding for the cloned LOV1 gene corresponding to Gly180–Lys329 in phot1 or Phe117–Lys265 in phot2. The N-terminal end of LOV1 was connected by a linker sequence (Gly-Ser-Pro-Glu-Phe) to the C-terminal end of GST. The *E. coli* JM109 strain was grown at 310 K in L-broth medium supplemented with ampicillin at 50 µg ml^{-1} until the culture reached an OD_{600} of 0.3 and was then incubated further for more than 20 h in the dark at 293 K in the presence of 0.1 m*M* isopropyl *β*-D-1-thiogalactopyranoside in order to express the LOV1 domains.

The purification procedures were carried out at 273–277 K under dim red light. The harvested cells were suspended in phosphate-buffered saline (PBS) containing

1 m*M* phenylmethylsulfonyl fluoride and disrupted by sonication. After centrifugation, the supernatant (20 ml) was mixed with glutathione-Sepharose 4B (Amersham Biosience, Sweden) suspended in 4 ml PBS solution. The solution was kept in the dark for 3 h at 277 K. The glutathione-Sepharose 4B beads adsorbing the GST-LOV1 fusion proteins were washed several times with PBS. The fusion proteins were eluted with a solution containing 10 m*M* reduced glutathione, 50 m*M* Tris–HCl, 100 m*M* NaCl and 1 m*M* EGTA pH 7.5. In the next stage, the GST-LOV1 fusion protein was digested by thrombin at the linker sequence. For the digestion, 4 ml of the eluted solution mixed with 200 µl thrombin solution was incubated for more than 20 h in the dark at 293 K. The digestion reaction was terminated by adding benzamidine to a concentration of 10 m*M*.

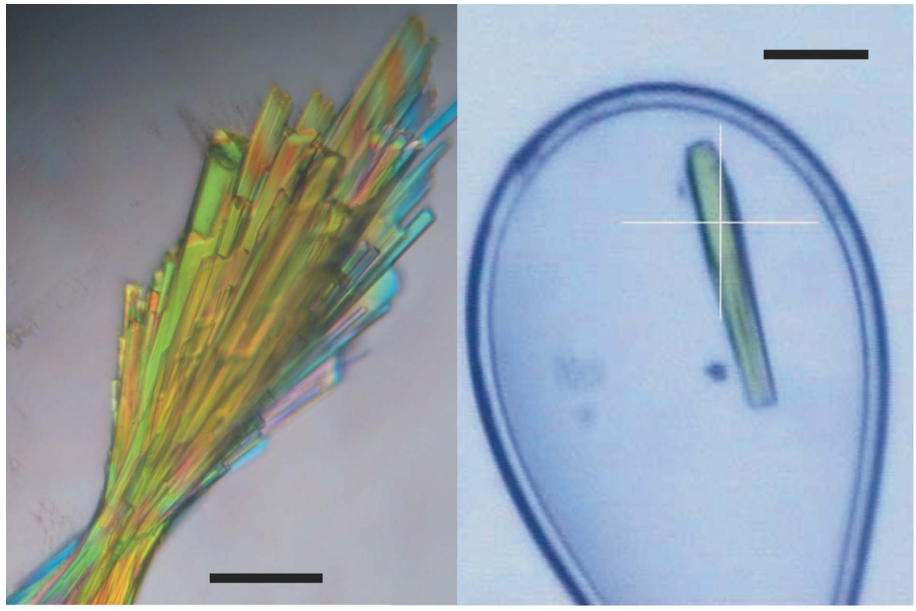
The LOV1 domains were further purified by gel chromatography with Sephacryl S-100 HR (Amersham Biosience, Sweden) and a buffer solution containing 100 m*M* NaCl, 25 m*M* Tris–HCl and 1 m*M* Na_2EDTA pH 7.8. The eluted protein solution showed single bands on Coomassie Brilliant Blue staining after SDS–PAGE. The molecular mass of the purified LOV1 domains and the purity of the sample solutions were also examined using an AXIMA-QIT time-of-flight mass spectrometer (Shimadzu, Japan). The purified LOV1 domains were concentrated to 10 mg ml^{-1} by ultrafiltration for subsequent crystallization.

2.2. Crystallization and X-ray diffraction experiments of the phot1 LOV1 domain

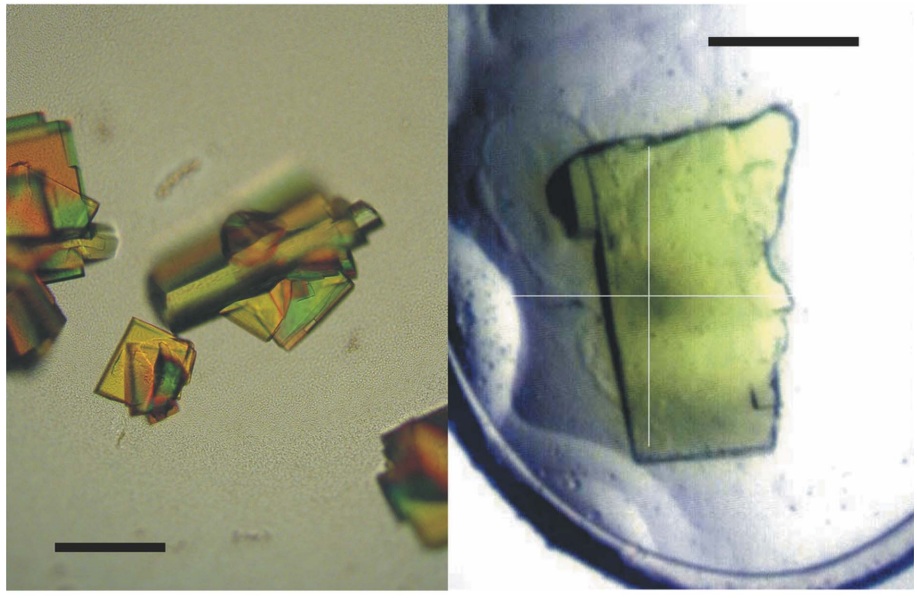
Crystallization of the phot1 LOV1 domain was carried out by the hanging-drop vapour-diffusion method at 293 K. 2 µl phot1 LOV1 solution mixed with 2 µl precipitant solution was equilibrated against 1 ml precipitant solution. Crystallization was set up under dim red light and the crystallization trays were wrapped in aluminium foil and kept in the dark.

Crystal Screens I and II (Hampton Research, USA) were used in the initial survey of crystallization conditions. Phot1 LOV1 crystals appeared as bundles of thin needles using a precipitant solution containing 2.0–2.2 *M* ammonium sulfate pH 7.9–8.5 (Fig. 3*a*). Prior to flash-cooling, crystals picked up from a drop were transferred into a dialysis cell and dialyzed for 12 h against a cryo-buffer containing 2.2 *M* ammonium sulfate, 22%(*w*/*v*) glycerol, 0.1 *M* sodium acetate and 0.1 *M* Tris pH 8.3. After dialysis, a crystal was carefully broken off from a bundle using a thin needle and flash-cooled in a cold nitrogen-gas stream produced by a cryocooling device (Rigaku, Japan). Crystals were screened in our laboratory by the oscillation method using Cu $K\alpha$ radiation (λ = 1.5418 Å) from an Ultrax-18 X-ray generator (Rigaku, Japan) operated at 45 kV and 90 mA with Pt-coated double-focusing optics (Rigaku, Japan) and an R-AXIS IV detector (Rigaku, Japan).

Finally, diffraction data for crystal structure analysis were collected to a resolution of 2.1 Å on BL44B2 (Adachi *et al.*, 2001) of SPring-8. A typical diffraction pattern from a phot1 LOV1 crystal (Fig. 3*a*) is shown in Fig. 4(*a*). Because the crystals were fragile, we used cryo-loops with far larger sizes than the maximum dimensions of the crystals in order to minimize mechanical damage from crushing the crystals with the loops (Fig. 3*a*). Indexing, integration of diffraction intensities, scaling and post-refinement procedures were carried out using the *HKL*-2000 suite (Otwinowski & Minor, 1997). The experimental conditions and the statistics of the collected diffraction data



(*a*)



(*b*)

Figure 3
Photographs of crystals of phot1 LOV1 (*a*) and phot2 LOV1 domains (*b*). The scale bars are 0.1 mm in length. The photographs on the left are of crystals after dialysis against cryo-buffers. Those on the right show crystals in loops during diffraction data collection at 110 K. The loop used for picking up the phot1 LOV1 crystal in the cryogenic experiment had a diameter of 500 µm and a fibre diameter of 20 µm. In the case of the phot2 LOV1 crystal, a loop with a diameter of about 700 µm and a fibre diameter of 20 µm was used.

Table 1
Experimental conditions and statistics of the collected diffraction data.

Values in parentheses are for the last shell.

Crystal	Phot1 LOV1	Phot2 LOV1
Temperature (K)	110	110
Crystal dimensions (μm)	300 × 30 × 20	250 × 100 × 40
Wavelength (Å)	1.000	1.0000
Beam size (μm)	Diameter, 200	Vertical, 20; horizontal, 30
Detector	ADSC Quantum 210	ADSC Quantum 315
Sample-to-detector distance (mm)	190	250
Oscillation (°)	0.6	1
Exposure time (s)	10	1
No. of plates	720	180
Space group	$P2_12_12_1$	$P2_1$
Unit-cell parameters		
a (Å)	61.15	32.52
b (Å)	64.92	66.51
c (Å)	70.81	56.69
β (°)	90.00	92.42
Molecules in ASU	2	2
V_M (Å^3 Da^{-1})	2.05	1.78
Solvent content (%)	40.6	30.8
Resolution (Å)	50.00–2.10 (2.17–2.10)	15.00–2.00 (2.05–2.00)
No. of reflections	236044	53650
No. of unique reflections	16967	15677
Completeness† (%)	99.9 (99.2)	99.5 (99.2)
$\langle I/\sigma(I)\rangle$	41.8 (4.8)	14.7 (6.6)
R_{merge}‡	0.057 (0.352)	0.066 (0.136)

† Percentage of unique reflections measured compared with the theoretical prediction. ‡ $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl.

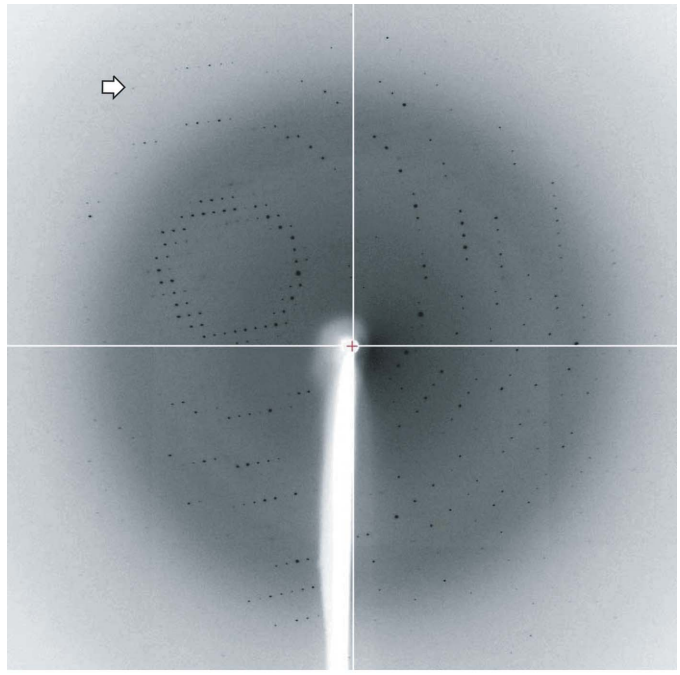
are shown in Table 1. According to the acceptable range of partial specific volumes (V_M) for crystals of soluble proteins (Matthews, 1968), the possible number of phot1 LOV1 domains occupying the crystallographic asymmetric unit is two.

2.3. Crystallization and X-ray diffraction experiments of the phot2 LOV1 domain

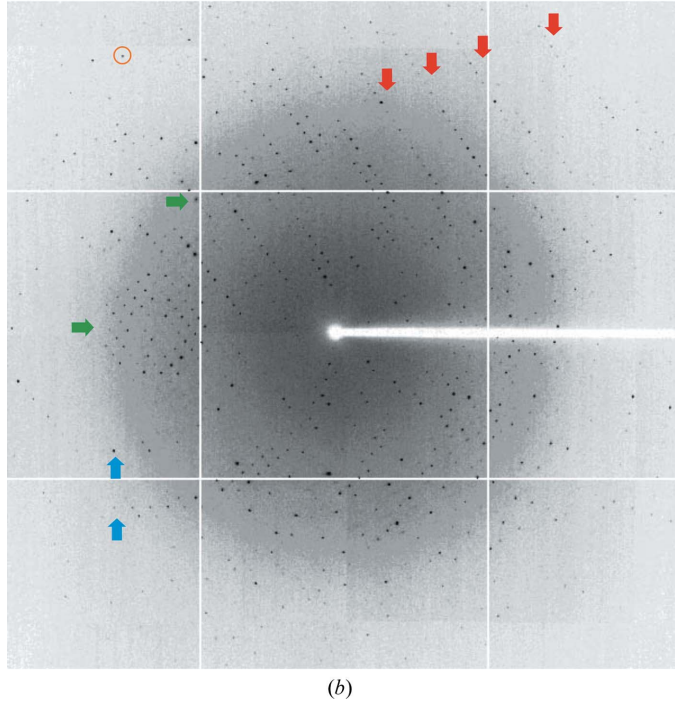
Crystallization of the phot2 LOV1 domain was carried out as described for the crystallization of the phot1 LOV1 domain. Crystals of the phot2 LOV1 domain appeared as considerably stacked thin plates using a precipitant solution containing 26–36%(*w*/*v*) polyethylene glycol 4000 (PEG 4000), 0.1 *M* Tris–HCl pH 8.0–8.6. In order to improve the crystal growth and quality, we attempted crystallization in the presence of small amounts of organic solvents using Additive Screen (Hampton Research, USA). Crystal growth was improved slightly (Fig. 3*b*) using the PEG 4000 precipitant solution containing 3–6%(*w*/*v*) 1,4-dioxane. For cryogenic X-ray diffraction experiments, crystals consisting of stacked plates were transferred from the drops into dialysis cells and dialyzed against a cryo-buffer containing 35%(*w*/*v*) PEG 4000, 0.1 *M* Tris, 6%(*w*/*v*) 1,4-dioxane and 15%(*w*/*v*) glycerol pH 8.0.

The quality of the crystals was examined in our laboratory. Even when the stacking of crystals was minimal, the shape of each Bragg spot in the diffraction patterns displayed severe streaking, making the spots unsuitable for the integration of diffraction intensities. The shapes of the diffraction spots indicated that there were still several thin plate crystals in the volume irradiated by the ∼200 μm X-ray beam available at our laboratory.

In order to collect diffraction data from small volumes of the stacked crystals, we used the X-ray microbeam available at BL41XU of SPring-8 (Kawamoto *et al.*, 2001). The experimental conditions are shown in Table 1. Small volumes of crystals, which are likely to be composed of several plates oriented differently with respect to each other, were irradiated by the microbeam. Of 40 different crystals tested, only two yielded diffraction patterns that could be processed using the *HKL*-2000 suite. Figs. 3(*b*) and 4(*b*) show a photograph of one of the two crystals and a diffraction pattern from the crystal, respectively. The diffraction pattern shown in Fig. 4(*b*) was interpreted as a superimposition of diffraction patterns from four crystals



(*a*)



(*b*)

Figure 4
Oscillation X-ray diffraction patterns from phot1 LOV1 (*a*) and phot2 LOV1 (*b*) crystals. The exposure conditions are shown in Table 1. The Bragg spacing of the diffraction spot indicated by an arrow in (*a*) is 2.26 Å and that indicated by an orange circle in (*b*) is 2.06 Å. The coloured arrows in (*b*) indicate the diffraction spots from four different crystals. When the spots indicated by the green arrows were magnified, they showed two peaks in the intensity profile.

occupying the irradiated volume. The diffraction spots indicated by the green arrows in Fig. 4(*b*) came from two crystals that were in nearly the same orientation with respect to the X-ray beam. The diffraction spots indicated by the red and cyan arrows came from two crystals that were oriented differently with respect to the incident X-ray beam. For the indexing and integration of diffraction patterns, we carefully inspected the patterns and selected Bragg spots from a targeted crystal. The autoindexing and integration protocols in *HKL*-2000 then successfully worked throughout the diffraction patterns collected. The diffraction intensity data collected from the crystal indicated by red arrows in Fig. 4(*b*) gave the best statistics and resolution, and were used for subsequent structure analysis (Table 1). The V_M value calculated suggested that two phot2 LOV1 domains occupied the crystallographic asymmetric unit.

2.4. Preliminary results from molecular-replacement analyses

Crystal structure analyses for both LOV1 domains are currently under way by the molecular-replacement method using the LOV1 domain of phot from *C. reinhardtii* (Fedorov *et al.*, 2003; PDB code 1n9l) as a search model. From the preliminary calculation of the rotation and the translation functions using the *CNS* suite (Brünger *et al.*, 1998), it was confirmed that two LOV1 domains occupy a crystallographic asymmetric unit in the crystals of both phot1 and phot2. The preliminary structure model for either LOV1 domain gives a crystallographic *R* factor of better than 0.46 for reflections with Bragg spacing 15.0–3.0 Å. Refined crystal structures may reveal the mode and function of the homodimerization of LOV1 in phototropins.

This work was supported by Grants-in-Aid from the MEXT Japan to MN (Nos. 15076210 and 19204042) and ST (Nos. 131392051 and 17084008), and from JSPS Japan to ST (No. 19370070). The X-ray diffraction experiments for P2L1 were carried out under the approval of the organizing committee of SPring-8 (Proposal Nos. 2006A1414 and 2006B1388).

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