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LOV2-linker-kinase phosphorylates LOV1-containing N-terminal polypeptide substrate via photoreaction of LOV2 in Arabidopsis phototropin1

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ABSTRACT

Phototropin is a blue light receptor in plants and is thought to be a light-regulated protein kinase. Previously, we defined the role of the photoreceptive domains, LOV1 and 2, in the light activation of the kinase in Arabidopsis phototropin2 (phot2) [1]. In this study, photoregulation of the kinase in phototropin1 (phot1) was studied using LOV2-linker-kinase polypeptide. We designed a new substrate consisting of the N-terminal part of the phot1 with autophosphorylation sites. The LOV2-linker-kinase had the same spectroscopic properties as those of the LOV2 core and phosphorylated the substrate in a light-dependent manner. Amino acid substitution experiments proved that the phosphorylation comes from the activation of the kinase via photoreaction of LOV2.

Structured summary of protein interactions:
AtPhot1-LOV2 phosphorylates **AtPhot1-Nt** by protein kinase assay (View Interaction: 1, 2, 3)

1. Introduction

Phototropin (phot) [2] is a blue light (BL) receptor in plants that was first identified as a receptor for phototropic responses [3]. Since its identification, phototropin has been found to mediate chloroplast relocation [4], stomatal opening [5], and leaf expansion [6] to optimise the efficiency of photosynthesis. Most plants have two isoforms, named phototropin1 (phot1) and phototropin2 (phot2). In *Arabidopsis thaliana* (*At*), phot1 and phot2 have overlapping functions in BL responses, depending on the light intensity [7].

Phot molecules have 2 photoreceptive domains, named LOV1 and LOV2 (LOV, light-oxygen-voltage sensing) in the N-terminal half, each of which binds a flavin mononucleotide (FMN) non-covalently as a chromophore. The C-terminal half forms a Ser/Thr kinase domain (KD) connected to LOV2 with a linker (L) region containing a so-called Jα-helix [8] (Fig. 1A). The FMN in the LOV domain shows unique cyclic photoreactions upon BL excitation of the ground state (D_{450}). Excited FMN forms an adduct with a conserved Cys in the LOV domains (S_{390}) via intersystem crossing to a triplet excited state [9,10]. Phot is a BL-regulated protein kinase, and the adduct state is thought to be the signalling state responsible for kinase activation. The covalent bond breaks spontaneously, and FMN returns to D_{450} with decay half-times ($t_{1/2}$) on the order of seconds to minutes [11], which may inactivate the kinase.

Both in vivo [12] and in vitro [1] studies have revealed that LOV2 acts as a main switch of Atphot kinases that play an inhibitory role [13]. BL may cancel the inhibition by causing conformational changes in LOV2 that propagate to the KD, possibly through the L region. In order to understand the molecular mechanism underlying BL regulation of the phot kinases, appropriate assay systems are required. Previously, we have described a new in vitro phosphorylation assay system in which casein was used as an artificial substrate to define the role of the various *A. thaliana* phot2 (Atphot2) domains in kinase activation by BL [1]. In the present study, we examined the BL regulation of *A. thaliana* phot1 (Atphot1) kinase using a polypeptide consisting of LOV2-L-KD (Fig. 1A), which was expected to show kinase activation by BL in accordance with our previous results [1]. As a substrate for the kinase assay, we designed a new construct consisting of the N-terminal part of Atphot1 based on the previously reported autophosphorylation sites (Nt in Fig. 1A) [14,15]. The LOV2-L-KD of Atphot1 was found to be able to phosphorylate the N-terminal polypeptide substrate through photoreaction of LOV2. These results revealed that the kinase and the substrate provide a powerful tool to elucidate the

Abbreviations: At, *Arabidopsis thaliana*; BL, blue light; CBB, Coomassie brilliant blue; D_{450}, ground state of phototropin; FMN, flavin mononucleotide; GFP, Green Fluorescent Protein; GST, glutathione *S*-transferase; KD, kinase domain; L, linker; LOV, light-oxygen-voltage sensing; phot, phototropin; phot1, phototropin1; phot2, phototropin2; Nt, N-terminal polypeptide; S_{390}, adduct state of phototropin; $t_{1/2}$, half decay time; SAXS, small-angle X-ray scattering.

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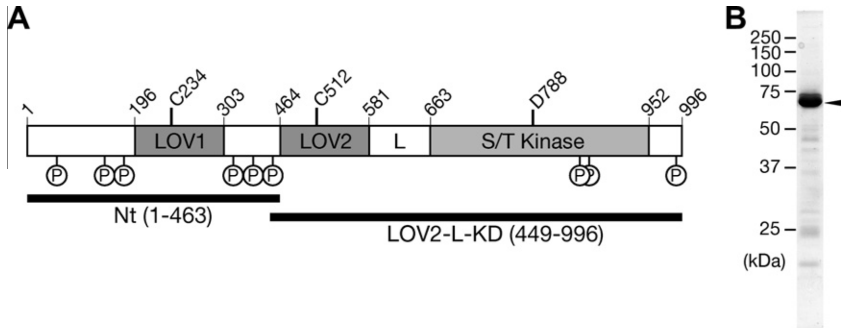

Fig. 1. (A) A schematic drawing for the domain structure of *Arabidopsis* phot1. The two horizontal bars correspond to the areas for the N-terminal substrate (Nt) and the LOV2-L-KD used in the study. The circled Ps indicate the reported autophosphorylation sites [14]. (B) 12.5% SDS–PAGE gel pattern of H-LOV2-L-KD sample stained with CBB. Filled triangles indicate the position of the intact sample.

molecular mechanisms underlying the blue-light regulation of the phot kinases.

2. Materials and methods

2.1. Construction of expression vectors

DNA of full-length Atphot1 and the Atphot1 LOV2-L-KD (449–996 aa) region (Fig. 1) were synthesised with the following PCR and oligonucleotide primers: for full-length *At*phot1, 5′-GAAA-GAATTCATGGAACCAACAGAAAAACC-3′ and 5′-GTTTGAATTCTCAAA AAACATTTGTTTGCA-3′, and for *At*phot1 LOV2-L-KD, 5′-GAAAGA ATTCGAGAGTGTGGATGATAAA-3′ and 5′-GTTTGAATTCTCAAAAAA-CATTTGTTTGCA-3′ using Atphot1 cDNA as a template. Amplified DNA was isolated, digested, and cloned into a pGEX4T1 or a pET28a bacterial expression vector (Amersham Pharmacia Biosciences) as a translational fusion with glutathione *S*-transferase (GST) or an N-terminal His6-tag, respectively. The amino-acid substitutions (Cys512Ala and Asp788Ala) were introduced using a Quick Change site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions, and the mutagenesis was verified by DNA sequencing with a CEQ2000XL DNA analysis system (Beckman Coulter). The N-terminal region construct spanned the N-terminus to the C-terminal end of the junction between LOV1 and LOV2 (1–463) of Atphot1 (Nt, Fig. 1). A stop codon was introduced into the pGEX-full-length Atphot1 using the following primers: 5′-GA AATGAGAAAGGGTtaaGATCTAGCTACTAC-3′ and 5′-GTAGTAGCTA-GATCttaACCCTTTCTCATTTC-3′.

2.2. Expression and purification of recombinant proteins

For the GST-fusion polypeptides, the *Escherichia coli* JM109 strain was transformed with each expression plasmid and was grown at 37 °C in LB medium containing 50 μg ml^{-1} ampicillin for 4 h and was incubated with 0.5 mM isopropyl β-D-thiogalactopyranoside for 24 h at 20 °C in the dark. Bacteria were collected by centrifugation and re-suspended in an extraction buffer containing 50 mM Tris–HCl (pH 7.8), 100 mM NaCl, 1 mM EGTA, 10% glycerol, and 1 mM phenylmethylsulphonyl fluoride. The cells were lysed by sonication and centrifuged (100,000*g* for 30 min, 4 °C). The supernatant was mixed with a glutathione-Sepharose 4B resin (GE Healthcare). The resin was washed with the extraction buffer containing 5 mg ml^{-1} casein and 5 mM ATP to remove chaperones. The GST-fusion polypeptides were eluted with 10 mM reduced glutathione in the extraction buffer. For the GST-LOV2-L-KD (G-LOV2-L-KD) preparation, the eluted polypeptide was purified with size-exclusion column chromatography (Sephacryl S-200, GE Healthcare). The GST-tag free LOV2-L-KD was prepared with thrombin digestion, which leaves 5 extraneous amino-acid residues (Gly-Ser-Pro-Glu-Phe) at the N-terminus. Thrombin and the cleaved GST-tag were removed with Benzamidine-Sepharose 4B and Ni-affinity columns. GST-Nt (G-Nt) was used without removing the GST tag.

For His-tagged LOV2-L-KD (H-LOV2-L-KD), the *E. coli* strain BL21 (DE3) was transformed with the expression plasmid and was grown at 37 °C in LB medium containing 30 μg ml^{-1} kanamycin for 6 h, and the strain was incubated with 0.02 mM isopropyl β-D-thiogalactopyranoside for 24 h at 20 °C in the dark. Cells were collected by centrifugation and were resuspended in an extraction buffer containing 20 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 10% glycerol, and 1 mM phenylmethylsulphonyl fluoride. The cells were lysed by sonication, and the supernatant was mixed with resin (Ni-Sepharose High Performance, GE). The resin was washed with the extraction buffer containing 30 mM imidazole. H-LOV2-L-KD was eluted with buffer containing 500 mM imidazole. The eluted polypeptide was purified with size-exclusion column chromatography (Superdex 200 pg, GE). All of the purifications were carried out at 0–4 °C under a dim red light, and the purified samples were stored at −80 °C until use. The purity of the preparations was estimated from Coomassie Brilliant Blue (CBB) staining after SDS–PAGE, is more than 95% pure for H-LOV2-L-KD (Fig. 1B), and is approximately 90% pure with LOV2-L-KD. For G-LOV2-L-KD, see the legend to Fig. 3.

2.3. Phosphorylation assay

H-LOV2-L-KD or G-LOV2-L-KD polypeptides were incubated with G-Nt substrates at 30 °C in a kinase reaction buffer containing 20 mM Tris–HCl (pH 7.8), 100 mM NaCl, 1 mM Na_2EGTA, and 10% glycerol that contained 10 mM $MgCl_2$, 20 μM ATP and 37 kBq of [γ-^{32}P] ATP. The effect of BL on phosphorylation was measured either by irradiation with a blue LED illuminator (ISL-150X150-88, CCS Inc., Japan, λ_{max} at 475 nm) or by mock irradiation. Adding concentrated SDS–PAGE sample buffer followed by boiling for 3 min terminated the reaction. All of the procedures were performed under dim red light. Next, the samples were run on SDS–PAGE, and the molecular masses of the bands were estimated by CBB staining. Phosphorylation of the bands was visualised with imaging plates (Fujifilm) and a STORM scanner (GE Healthcare).

2.4. Immunoblot analysis

H-LOV2-L-KD and a crude extract from the 3-day-old etiolated tissues of *Arabidopsis* line *gl1* [14] were run on SDS–PAGE and were electro-transferred to a PVDF membrane. Blotted proteins were incubated with an anti-Atphot1 phosphoserine 851 polyclonal