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REGULAR PAPER

Blue light diminishes interaction of PAS/LOV proteins, putative blue light receptors in *Arabidopsis thaliana*, with their interacting partners

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Abstract The light, oxygen, or voltage (LOV) domain that belongs to the Per-ARNT-Sim (PAS) domain superfamily is a blue light sensory module. The *Arabidopsis thaliana PAS/LOV PROTEIN* (*PLP*) gene encodes three putative blue light receptor proteins, PLPA, PLPB, and PLPC, because of its mRNA splicing variation. PLPA and PLPB each contain one PAS domain at the N-terminal region and one LOV domain at the C-terminal region, while the LOV domain is truncated in PLPC. RNA gel blot analysis showed that PLP mRNA was markedly expressed after exposure to salt or dehydration stress. Yeast two-hybrid screening led to the isolation of VITAMIN C DEFECTIVE 2 (VTC2), VTC2-LIKE (VTC2L), and BEL1-LIKE HOMEODOMAIN 10 proteins (BLH10A and BLH10B) as PLP-interacting proteins. The molecular interaction of PLPA with VTC2L, BLH10A or BLH10B, and that of PLPB with VTC2L were diminished when yeasts were grown under blue light illumination. Furthermore, the possible binding of flavin chromophore to PLPA and PLPB was demonstrated. These results imply that the LOV domain of PLPA and PLPB functions as a blue light sensor, and suggest the applicability of these interactions to blue light-dependent switching in transcriptional regulation in yeast or other organisms.

Keywords Blue light · LOV · PAS/LOV protein · Protein–protein interaction · Yeast two-hybrid system

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Introduction

Plants utilize environmental cues for growth and development, a prime example of which is light, which has much influence on the physiology and life cycle of plants. Plants utilize light not only as an energy source in photosynthesis but also as a key signal for growth and developmental processes, such as germination, phototropism, chloroplast relocation, stomatal opening, circadian clock adjustment, and vegetative–reproductive phase shift. In this regard, plants have developed various light receptors and downstream signaling processes (Gyula et al. 2003).

In the past decade, understanding of blue light signaling has been improved by genetic analysis of a model plant, *Arabidopsis thaliana* (L.) Heynh. Two blue light receptor proteins, phototropin 1 (phot1) and phototropin 2 (phot2), were shown to be responsible for blue light induction of chloroplast movement, tropism, and stomatal opening (Huala et al. 1997; Christie et al. 1998; Kagawa et al. 2001; Kinoshita et al. 2001; Jarillo et al. 2001; Sakai et al. 2001). phot1 and phot2 are encoded by the *PHOT1* and *PHOT2* genes, respectively, and both receptor proteins consist of two light, oxygen, or voltage (LOV) domains and one Ser/Thr protein kinase domain. The LOV domain is postulated to be the blue light-sensing module of phototropins (Christie et al. 1999). As a member of the Per-ARNT-Sim (PAS) domain superfamily, the LOV domain binds flavin mononucleotide (FMN). The absorption of blue light by FMN induces covalent adduct formation between FMN and a conserved cysteine residue of the LOV domain. This adduct dissociates in the dark. Therefore, it is hypothesized that this photo cycle of the LOV domain regulates the activity of the protein kinase domain to initiate blue light signal transduction by phototropins (Salomon et al. 2001).

In *A. thaliana*, other than *PHOT1* and *PHOT2*, four genes that encode LOV domain-containing proteins have been reported. Three of them encode ADO/FKF/LKP/ZTL family proteins: ADO1/LKP1/ZTL, ADO2/LKP2, and ADO3/FKF1 (Kiyosue and Wada 2000; Nelson et al. 2000; Somers et al. 2000; Schultz et al. 2001). These proteins consist of three functional regions: LOV domain, F-box motif, and kelch repeat. The F-box motif binds to Skp1 to form the SCF (Skp1-cullin-F-box-Rbx1) complex, which is an E3 protein–ubiquitin ligase of the ubiquitin–proteasome system for protein degradation. The kelch repeat is postulated to be a protein–protein interacting domain and a substrate-recognizing domain of the F-box proteins. LKP1/ZTL regulates the circadian clock (Somers et al. 2000), while FKF1 is involved in the induction of flowering under long-day conditions (Nelson et al. 2000; Imaizumi et al. 2003). The LOV domains of these three proteins were shown to bind FMN and to form covalent adducts by blue light irradiation (Imaizumi et al. 2003). Therefore, they are postulated to be photoreceptors. The fourth gene (*At2g02710*) encodes PAS/LOV protein (PLP). PLP has a PAS domain at its N-terminal region and an LOV domain at its C-terminal region. The PAS domain at the N-terminal region of PLP is different from the LOV domain because it does not contain the conserved cysteine residue that forms a covalent adduct with FMN on blue light irradiation (Crosson et al. 2003). However, its physiological function, biochemical activity, and interacting partners remain unknown.

The LOV domain also functions in protein–protein interaction. PHOT1 interacts with NPH3 and RPT2, and LKP1/ZTL and LKP2 interact with TOC1 and PRR5 at their LOV domains (Motchoulski and Liscum 1999; Sakai et al. 2000; Más et al. 2003; Yasuhara et al. 2004; Inada et al. 2004). However, the direct relationship between blue light and these interactions has not been reported so far. In contrast, the LOV domain of phot2 was shown to interact with its protein kinase domain in the absence of blue light to inhibit the activity of the kinase (Matsuoka and Tokutomi 2005). Blue light irradiation induces conformational change of the LOV domain to promote protein kinase activity in phot2 (Salomon et al. 2001). Therefore, for LOV-domain-containing blue light receptors, blue light-dependent modulation of protein–protein interaction via the LOV domain is postulated to be the trigger for signal transduction.

In this study, we isolated PLP-interacting proteins, VTC2, VTC2L, BLH10A, and BLH10B, with the yeast two-hybrid system, and showed that the interactions of PLPA with VTC2L, BLH10A, and BLH10B, and that of PLPB with VTC2L were significantly diminished under blue light illumination, but not red, far-red, or green light illumination.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. Columbia accession was grown on germination medium (GM) containing 0.8% agar under long-day conditions of 16 h light and 8 h dark at 22°C (Valvekens et al. 1988; Kiyosue et al. 1996). Stress treatments (cold, dry, NaCl, and ABA) were performed according to Yamaguchi-Shinozaki and Shinozaki (1994) using three-week-old plants for 0, 1, 2, 5, 10, and 24 h.

RNA gel blot analysis

Total RNA was isolated from whole rosette plants of *A. thaliana* harvested before or after the stress treatment described above, separated on agarose gel containing formamide, and transferred on to a Nylon membrane, as previously reported (Kiyosue et al. 1996). A digoxigenin-11-dUTP (DIG-dUTP)-labeled RNA probe prepared from the full-length coding region of PLPB was used for hybridization according to the manufacturer's instructions (Roche Diagnostics, Germany). Chemiluminescence signals were generated with a CDP-Star visualization kit (GE Healthcare UK, Buckinghamshire, UK) and detected with a light capture system (Model AE-6955: ATTO, Japan).

DNA sequence analysis

Plasmid DNA templates for sequencing were prepared and DNA sequences were determined as described previously (Yasuhara et al. 2004). GENETYX (Software Development, Tokyo, Japan) and Sequencher (Gene Codes, Ann Arbor, MI, USA) software systems were used for DNA sequence analysis.

Plasmid construction

PLPA cDNA (AB038798) was isolated by screening cDNA libraries (CD4-13, CD4-14, CD4-15, and CD4-16) (Kieber et al. 1993) using a PCR fragment for the LOV domain that had been amplified by PCR from T20F6 BAC DNA. *PLPA* cDNA was PCR amplified with primers containing a BamHI site, subcloned into pCR2TOPO (Invitrogen, Carlsbad, CA, USA), digested with BamHI, ligated in frame to pGBK-T7 vector, and sequenced for verification.

Full-length cDNAs of *PLPB (AT2G02710)*, *VTC2 (AT4G26850)*, *VTC2L (AT5G55120)*, and *BLH10A (AT1G19700)* were obtained from RIKEN BRC. *BLH10B* cDNA was amplified by RT-PCR. Each gene was amplified by PCR to attach appropriate restriction enzyme cleavage sites, subcloned into pGEM-T vector (Promega, Madison, WI, USA), sequenced entirely, and inserted into pGADT7. The following primers were used for PCR: 5′-AGAGTCTG TATGTTGAAAATCAAAAGAGTT-3′ and 5′-AGAGCTC TCACTGAAGGACAAGGCACTCGG-3′ for *VTC2*; 5′-AG GATCCGTATGTTGTTGAAGATCAAAAGA-3′ and 5′-A GGATCCTCAATTAGAGACAGCCTCTTCTT-3′ for *VTC2L*; 5′-AGGATCCGTATGGCAGTTTATTACACAA GT-3′ for *BLH10A and BLH10B* forward primers; 5′-AGG ATCCCTAACAATCTGAATTTGTTCCTT-3′ for *BLH 10A* reverse primer; and 5′-AGGATCCTCACACAAC AAAGTCGTGTAAGT-3′ for *BLH10B* reverse primer.

Yeast two-hybrid system

Construction of the cDNA library and subsequent two-hybrid screening were performed according to the manufacturer's instructions (Matchmaker Library Construction and Screening Kit, Clontech, Palo Alto, CA, USA), using RNA isolated from rosette leaves of *A. thaliana* plants that had been exposed to salt or dehydration stress for 10 h. The two-hybrid assay was performed as described elsewhere (Yasuhara et al. 2004). To test the effect of various light conditions, yeast colonies were cultured under blue, green, red, and far-red light illumination at an intensity of 100 μmol m^{-2} s^{-1} (LED System, Sanyo, Osaka, Japan).

Immunoblot analysis

For immunoblot analysis, yeast cells were grown on SD-Leu, Trp agar medium for 4 days at 30°C in the dark or under blue light illumination at appropriate intensities. A similar number of cells were harvested and frozen in liquid nitrogen. The cells were resuspended in 400 μL extraction buffer (40 mmol L^{-1} Tris–HCl, pH 6.8, 8 mol L^{-1} urea, 5% SDS, 0.1 mmol L^{-1} EDTA, 0.4 mg mL^{-1} bromophenol blue, 0.8% 2-mercaptoethanol, 6.2 μg mL^{-1} pepstatin A, 1.86 μmol L^{-1} leupeptin, 9.0 mmol L^{-1} benzamidine, 23.0 μg mL^{-1} aprotinin, and 0.77 mg mL^{-1} phenylmethylsulfonyl fluoride), and homogenized. Crude cell extract (12 μL) was loaded on to each lane and proteins were separated by SDS–PAGE and electro-transferred on to PVDF membranes. Protein blots were incubated with anti-c-Myc or anti-hemagglutinin (HA) antibody and detected with ECL (GE Healthcare UK).

Preparation of PLPA, PLPB, and LOV domains

Full-length coding regions of PLPA and PLPB were cloned into a pGEX4T3 expression vector (GE Healthcare UK) as a fusion protein with GST. The following primers were used for PCR: PLP forward: 5′-AGGATCCATGTCCTTA ACGAAATCTTCAGA-3′, reverse: 5′-AGTCGACTTAGC ATGTCACCATCAATGAGC-3′. PLPA and PLPB proteins with Gly–Ser at the N-terminus were prepared using the *Escherichia coli* expression (*E. coli*) system under dim red light illumination, as described elsewhere (Zikihara et al. 2006) except that the induction was performed with 0.2 mmol L^{-1} isopropyl-*β*-D-thiogalactopyranoside at 18°C and that the purification by gel-filtration column chromatography was omitted. The purified proteins were concentrated by ultrafiltration with a Microcon YM-10 instrument (Millipore), and stored at 4°C until use.

UV–visible absorption spectroscopy

UV–visible absorption spectra of the sample solutions were acquired with a UV-3310 spectrophotometer (Hitachi, Tokyo, Japan) at room temperature (25°C). Irradiation of sample solutions in a cell fitted into the cell holder of the spectrophotometer was performed with an array of blue-

light-emitting diodes (λ_{max} = 468 nm, 100 μmol m^{-2} s^{-1}) for 30 s.

Results

Isolation of PLP-interacting proteins

There are three PLP splice variants in the annotation database of The Arabidopsis Information Resource (TAIR), which we named *PLPA*, *PLPB*, and *PLPC*. *PLPA* encodes a protein of 397 amino acids while *PLPB* encodes a protein of 399 amino acids where two amino acids (Ser and Asn) are inserted into the LOV domain. *PLPC* encodes a protein of 358 amino acids and a frame shift causes deletion of 46 C-terminal amino acids and addition of the last seven amino acids (Fig. 1). Therefore, the LOV domain of PLPC lacks the last β-structure (βE) (Crosson et al. 2003). A cDNA library of 6.3×10^5 clones was screened by means of the yeast two-hybrid system with PLPA as bait. To construct the cDNA library, a mixture of RNAs prepared from rosette leaves of *A. thaliana* plants that had been subjected to salt stress and dehydration stress was used, because *PLP* mRNA was markedly expressed in plants exposed to those stress treatments (Fig. 2). As a result, five clones were isolated for PLP-interacting proteins. Three of them were partial clones for VITAMIN C DEFECTIVE 2 (VTC2, Jander et al. 2002), and two others were those for VTC2 paralog (VTC2L) and BEL1-LIKE HOMEODOMAIN 10 (BLH10) (Hackbusch et al. 2005). Full-length cDNAs for VTC2 and VTC2L were obtained from RIKEN BRC. BLH10 has two splice variants that were named BLH10A and BLH10B. BLH10A lacks its entire homeodomain, while the homeodomain of BLH10B exists at its C-terminal region. cDNA of BLH10A was obtained from RIKEN BRC while that of BLH10B was obtained by RT-PCR. Interactions of PLPA and PLPB with the full-length proteins of VTC2, VTC2L, BLH10A, and BLH10B were also examined using the yeast two-hybrid system (Fig. 3). PLPA interacted with all four proteins, while PLPB interacted with VTC2 and VTC2L but not with BLH10A or BLH10B.

Blue light diminishes interaction of PLPA and PLPB with their interacting proteins in yeast

Since PLPA and PLPB have an LOV domain, they may function as blue light receptors. The LOV domain of PHOT2 binds its C-terminal protein kinase domain in the absence of blue light (Matsuoka and Tokutomi 2005). Therefore, the effect of light illumination on the interaction of PLPA and PLPB with their interacting proteins was examined. Interactions of PLPA with VTC2L, BLH10A, and BLH10B, and that of PLPB with VTC2L were diminished or not detected under blue light illumination at 100 μmol m^{-2} s^{-1}, while those interactions were detected in the dark or under illumination with other light (red, far-red, and green) (Fig. 4). By contrast, interactions of PLPA and PLPB with VTC2 were detected regardless of light conditions.

Next, the effect of blue light intensity on the two-hybrid interaction was examined (Fig. 5). The interaction of PLPA with VTC2L was weakened at 100 μmol m^{-2} s^{-1} or higher intensity, while that of PLPB with VTC2L was undetectable at that intensity. The interaction of PLPA with BLH10A was undetectable above 50 μmol m^{-2} s^{-1}, while that of PLPA with BLH10B was weakened above that intensity. The interaction of PLPA or PLPB with VTC2 was not affected even at 190 μmol m^{-2} s^{-1}.

To exclude the possibility that PLPs or PLP-interacting proteins were unstable under blue light illumination, the

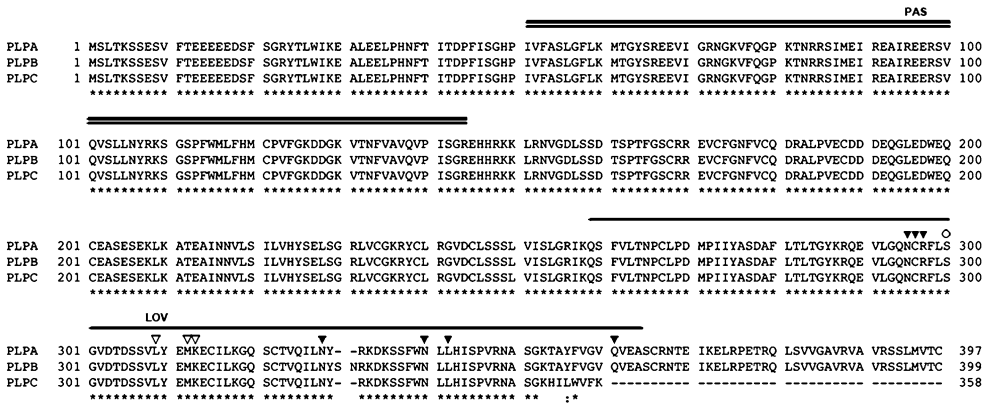

Fig. 1 Alignment of deduced amino acid sequences of PLPA, PLPB, and PLPC. Alignment was analyzed with ClustalW. Amino acids that interact with flavin in phy3 LOV2 are indicated with *closed triangles* (conserved), *open triangles* (similar), and *open circles* (not conserved) (Crosson et al. 2003). *Asterisks* indicate identical amino acids among the three proteins. The PAS domain is indicated with a *double line* and the LOV domain is indicated with a *line* above amino acid sequences

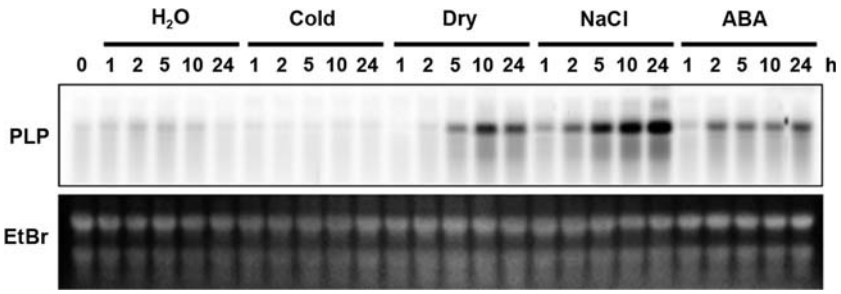

Fig. 2 RNA gel blot analysis of PLP. *Arabidopsis thaliana* plants were grown under a 16 h light and 8 h dark cycle for 3 weeks, followed by exposure to cold stress (*Cold*), dehydration stress (*Dry*), salt stress (*NaCl*), or ABA for 0, 1, 2, 5, 10, and 24 h. As control, plants were transferred to water (H_2O). Total RNA was then isolated, separated on agarose gel (4 μg total RNA/lane), and visualized by staining with ethidium bromide (*lower panel*). The blot was hybridized with the DIG-labeled RNA probe of PLPB and subjected to detection using chemiluminescent substrate (*upper panel*)

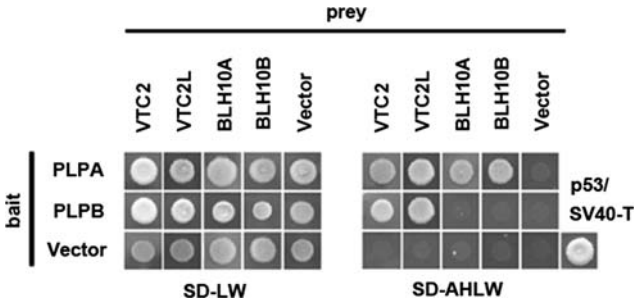

Fig. 3 PLP-interacting proteins isolated by the yeast two-hybrid system. A single colony of yeast AH109 harboring a GAL4 DNA-binding domain fusion of PLPA or PLPB (*bait*) and a GAL4 activation domain fusion of VTC2, VTC2L, BLH10A, or BLH10B (*prey*) was grown on SD–Leu, Trp (SD–LW) or SD–Ade, His, Leu, Trp (SD–AHLW) agar medium at 30°C for 4 days

expression of these proteins was detected by immunoblot analysis (Fig. 6). Both PLPA and PLPB were detected with anti-c-Myc antibody, and PLP-interacting proteins were detected with anti-HA antibody in extracts from yeast cells grown in the dark. When yeasts were grown under blue light at the intensity that affected the two-hybrid interactions, no significant difference in protein expression was detected between the presence and absence of blue light. These results indicate that blue light in itself specifically diminishes the interactions of PLPA or PLPB with PLP-interacting protein(s).

Binding of flavin chromophore and photoreactivity of PLPA and PLPB

To determine if PLPs bind the flavin chromophore and show photochemical reaction, recombinant PLPA and PLPB were prepared using an *E. coli* system. Figure 7 shows the UV–visible absorption spectra of PLPA (a) and PLPB (b) in solution. Both spectra show an absorption peak at around 450 nm, although the peak in the PLPA spectrum is extremely small. The absorption may be ascribed to oxidized flavin; in particular, the minor peak appearing at 370 nm in the PLPB spectrum supports this notion (Claiborne 1986). However, these spectra lack vibrational structures in the main peak, which are characteristic of flavin chromophore in proteins, such as the LOV domains of phototropins and FKF1 in the dark state (Salomon et al. 2000; Zikihara et al. 2006). Furthermore, the spectra did not exhibit any photoreactivity (data not shown). These observations suggest that the absorption peaks originate in free flavin in solution and not in that bound to the proteins. However, since free flavin could be eliminated during the GST-glutathione region purification procedure, the remaining flavin is concluded to be bound to the proteins at least in the crude extracts from the *E. coli* system. During the purification procedure, e.g., thrombin cut of GST-tag, bound flavin chromophore was dissociated from the apoprotein. From the absorption spectrum, the ratio of flavin to apoprotein was estimated to be ca. 1% in the PLPB solution.

Binding of flavin chromophore and photoreactivity of LOV domains

Next, we examined whether the LOV domains of PLPA and PLPB bind flavin chromophore and show photoreactivity. The LOV domains of PLPA, PLPB, LKP2 (Yasuhara et al. 2004), and ZTL (Más et al. 2003), and the PAS domains of PLP were prepared also as GST-fusion proteins. Since the results of the full-length preparation suggest the dissociation of flavin chromophore during the thrombin cut, absorption spectra were measured without this purification procedure. Surprisingly, however, no peak corresponding to the absorption of both free and bound flavin was found in either PLPA-LOV or PLPB-LOV domain solution (see Fig. S1a in electronic supplementary material). Both domains were associated with large amounts of molecular chaperones, particularly GroEL, compared with the preparation of

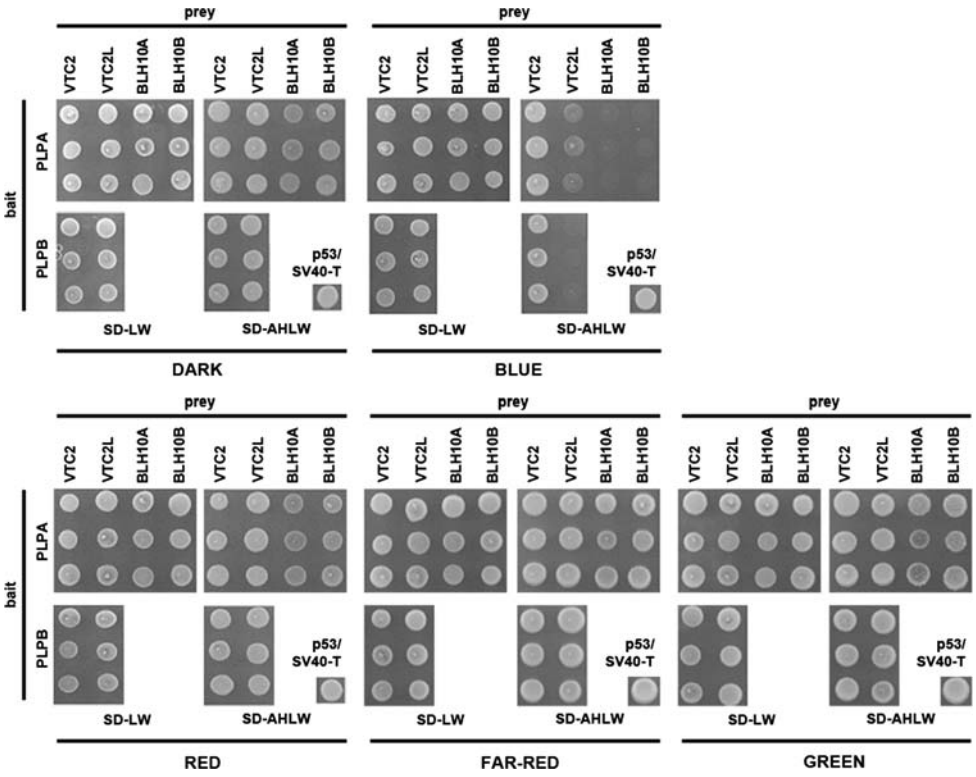

Fig. 4 Effect of light on protein interaction of PLP. A single colony of yeast AH109 harboring a GAL4 DNA-binding domain fusion of PLPA or PLPB (*bait*) and a GAL4 activation domain fusion of VTC2, VTC2L, BLH10A, or BLH10B (*prey*) was grown on SD–Leu, Trp (SD–LW) or SD–Ade, His, Leu, Trp (SD–AHLW) agar medium at 20°C for 7 days in the dark or under illumination with various types of light (*BLUE*, *RED*, *FAR-RED*, or *GREEN*) at the intensity of 100 μmol m^{-2} s^{-1}

LKP2-LOV or ZTL-LOV domain (Fig. S2 in electronic supplementary material) that binds photoreactive flavin (Fig. S1a, b in electronic supplementary material). These findings suggest that the LOV domains of both PLPA and PLPB are folded improperly in the protein moiety, and this may explain the weak binding of flavin to these LOV domains. The possibility that the PAS domain binds flavin was excluded, based on the absorption spectra (Fig. S1a, b in electronic supplementary material).

Discussion

PLP-interacting proteins

In this study, we isolated VTC2, VTC2L, BLH10A, and BLH10B as PLP-interacting proteins with the yeast two-hybrid system. *VTC2* was originally isolated as the gene for *vitamin C defective2* (*vtc2*) mutant (Jander et al. 2002). The *vtc2* mutant was isolated in a screening for increased sensitivity to ozone, and showed a low level of vitamin C (Conklin et al. 2000). Recently, VTC2 was shown to be L-galactose-1-phosphate-hexose-1-phosphate guanyltransferase, an enzyme functioning in the L-galactose pathway for vitamin C (L-ascorbic acid) biosynthesis (Laing et al. 2007; Linster et al. 2007). VTC2L is a paralog of VTC2, although its function remains unknown. BLH10 is a member of 3-aa loop extension (TALE) homeodomain proteins including BEL1/BELL (Hackbusch et al. 2005). BEL1/BELL functions in ovule development. Although the physiological function of BLH10 is not known, it was shown to interact with other BLH family proteins, BLH1, BLH3, and BLH4, and is hypothesized to have a close functional association with these proteins (Hackbusch et al. 2005). BLH1 and BLH3 were shown to form heterodimers with the TALE-HD protein SHOOT MERISTEMLESS (STM), which is a transcription factor required for the initiation and maintenance of the shoot apical meristem. This heterodimer formation was necessary for the nuclear transport of STM (Cole et al. 2006). The interaction of BLH10 with PLPA may affect the formation of these homo- or heterodimers. The fact that BLH10A, which lacks the C-terminal homeodomain, and BLH10B interacted with PLPA demonstrates the importance of the N-terminal region of BLH10B for the interaction with PLPA.

Blue light diminishes PLP–protein interaction

As expected from the finding that both PLPA and PLPB have an LOV domain, blue light affected the interactions of PLPA with VTC2L, BLH10A, and BLH10B, and that of PLPB with VTC2L. Two models of this effect are speculated. One is that the blue light-dependent conformational change of the LOV domain directly affects the state of interaction of LOV with LOV-interacting protein. The

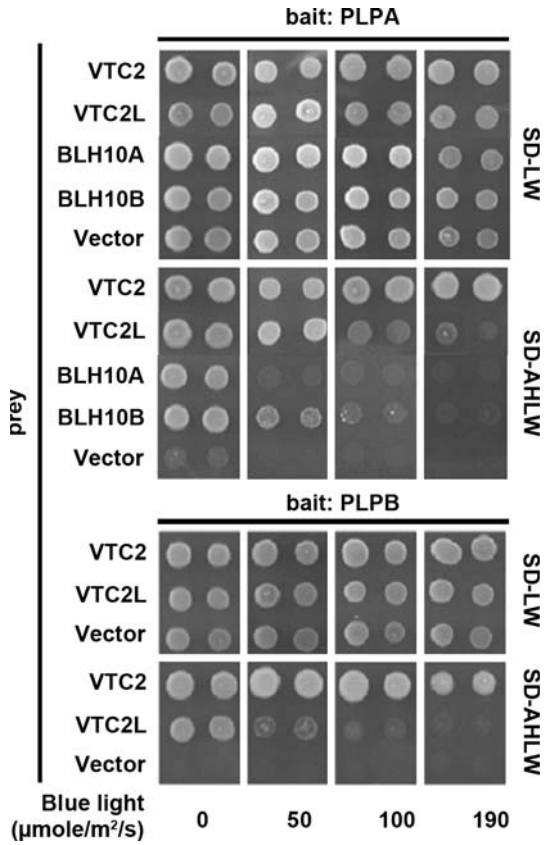

Fig. 5 Effect of blue light intensity on protein interaction of PLPA and PLPB. A single colony of yeast AH109 harboring a GAL4 DNA-binding domain fusion of PLPA or PLPB (*bait*) and a GAL4 activation domain fusion of VTC2, VTC2L, BLH10A, or BLH10B (*prey*) was grown on SD–Leu, Trp (SD–LW) or SD–Ade, His, Leu, Trp (SD–AHLW) agar medium at 30°C for 4 days in the dark or under blue light illumination at various intensities (50, 100, or 190 μmol m^{-2} s^{-1})

other is that the blue light-dependent conformational change of the LOV domain affects its intramolecular binding state, and this indirectly affects the interaction of LOV-containing protein with its partner protein by masking or unmasking the interacting region.

High intensities of blue light may be harmful to yeast cells. However, the reduction of the two-hybrid interaction of PLP with PLP-interacting proteins under blue light illumination probably does not reflect the general inhibitory effect of blue light on yeast growth, because yeast cells cultivated on medium without selection for two-hybrid interaction (SD–LW) and those harboring the positive control plasmid (p53-SV40-T) grew well under blue light illumination (Figs. 4, 5).

The LOV domain of PLPA is highly similar to those of phototropins. Among eleven amino acids that interact with flavin in phy3 LOV2, seven, including an important cysteine residue that forms a covalent adduct with FMN, are conserved and three are similar in the LOV domains of PLPA and PLPB (Crosson et al. 2003), although the LOV domain of PLPB has two amino acid insertions compared with the LOV domains of phototropins and PLPA (Fig. 1). Therefore, the LOV domains of PLPA and PLPB seem to be photochemically functional. Indeed, recombinant PLPA and PLPB showed possible binding of flavin chromophore (Fig. 7). Blue light illumination is known to induce the formation of a covalent adduct between an LOV domain and FMN. This is postulated to lead to the conformational change of LOV domain-containing proteins, such as phototropins and FKF (Salomon et al. 2001; Imaizumi et al. 2003). Therefore, it is important future work to examine whether adduct formation of the LOV domains of PLPs can occur in response to blue light illumination.

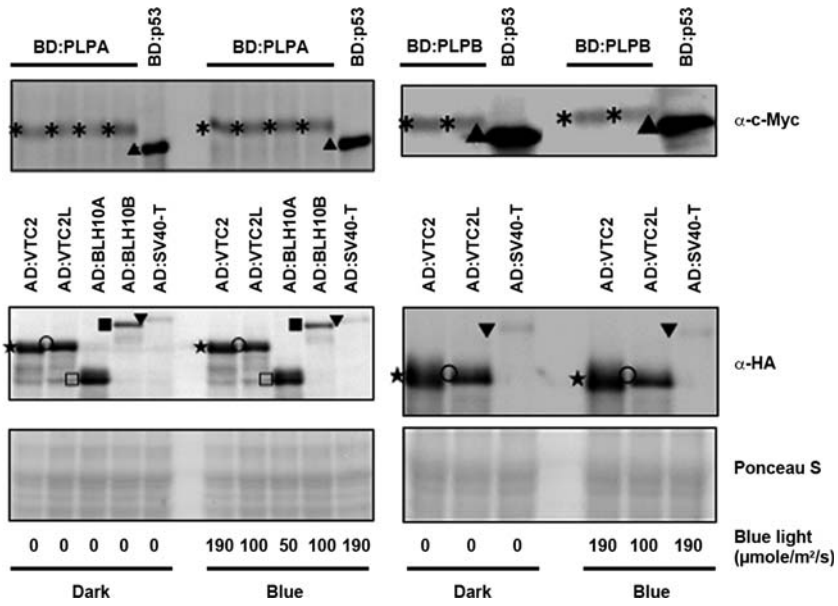

Fig. 6 Effect of blue light on bait and prey protein expression in yeast cells. Yeast colonies used in Fig. 5 were grown under indicated light conditions, harvested, and subjected to protein extraction. Crude extracts were separated by SDS-PAGE and transferred on to PVDF membranes. PLPA and PLPB were detected with anti-c-Myc antibody and VTC2, VTC2L, BLH10A, and BLH10B were detected with anti-HA antibody. *Asterisks* for PLPA or PLPB, *closed stars* for VTC2, *open circles* for VTC2L, *open squares* for BLH10A, *closed squares* for BLH10B, and *closed triangles* for p53 and SV40-T controls, are used as indicators

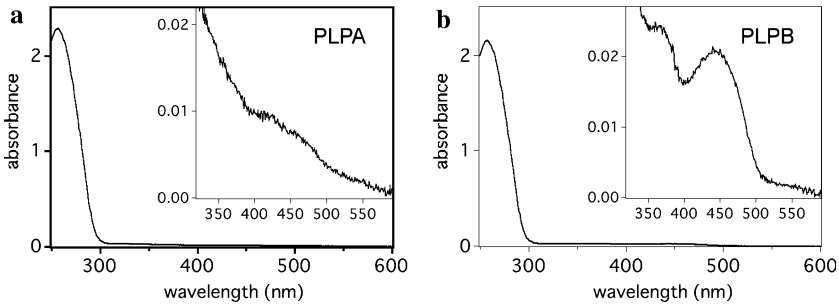

Fig. 7 Absorption spectra of PLPA (**a**) and PLPB (**b**) in phosphate-buffered saline (PBS, 8.0 mmol L^{-1} Na_2HPO_4, 1.5 mmol L^{-1} KH_2PO_4, 140 mmol L^{-1} NaCl, and 2.7 mmol L^{-1} KCl, pH 7.4). *Insets* are magnifications of absorption spectra in the wavelength range 330–580 nm

We could not detect the effect of blue light on the interaction of PLPA and PLPB with VTC2. It is possible that blue light of intensities higher than 190 μmol m^{-2} s^{-1} is necessary to detect the effect of blue light on their interaction.

The interaction of PLPB with VTC2L was greatly reduced by illumination with blue light at 50 μmol m^{-2} s^{-1}, while that of PLPA with VTC2L was unaffected at the same blue light intensity. Since the core region of the LOV domain of PLPB has two amino acid insertions between the βC structure and conserved Lys, which form a salt bridge (Crosson et al. 2003), these insertions may be one reason for the difference.

To confirm the interaction of PLP with PLP-interacting proteins, experiments other than the yeast two-hybrid system, such as the pull-down assay, would be necessary. However, it was technically impossible for us to examine the effect of blue light on the interaction of PLP with PLP-interacting proteins with the pull-down assay, because we were unable to purify the required amount of photoactive FMN-bound PLP (haloprotein) from *E. coli* cells. Therefore, the expression of photoactive PLP in another system is important future work. One experiment that could be performed is immunoprecipitation analysis in planta. Very recently, Kim et al. (2007) reported the blue light-dependent interaction of GI (GIGANTEA) with ZTL using the co-immunoprecipitation technique in *Nicotiana benthamiana* Domin cells. They showed the effect of a missense mutation in a conserved cysteine residue of the LOV domain, which is required for covalent adduct formation with flavin chromophore, and proposed that the enhancement of the interaction of GI with ZTL is a consequence of covalent adduct formation between the cysteine residue of the LOV domain and FMN in response to blue light. This kind of experiment is also important future work to analyze the function and protein–protein interaction of PLPs in planta.

Binding of flavin chromophore to full-length PLPA and PLPB

The present results indicated the possible binding of flavin chromophore to full-length PLPA and PLPB in vivo, although its photoreactivity was not proved. Flavin in the GST-free protein fractions existed in the free form; however, the experiment showed that flavin had been bound to the protein at least until affinity purification. These results suggest that flavin may bind to the LOV domain in *A. thaliana*. In comparison with other LOV proteins, the LOV domains in PLPs seem to be unable to assume the proper conformation, thus inducing the easy dissociation of flavin chromophore. In general, the expression of LOV domain itself using the *E. coli* system is relatively easy and produces stable holoproteins, compared with that of full-length LOV protein (Christie et al. 1999). However, this is not the case with PLP. To realize proper binding of flavin to the apoproteins of PLP, the N-terminal region, including the PAS domain and the LOV domain, might be necessary.

Through this study, we found that the interactions of PLPA and PLPB with some of their interacting proteins were changed in response to blue light. This points to the possibility that PLPs are new blue light receptors in plants. In addition, the finding that PLP mRNA was markedly expressed after exposure to salt or dehydration stress suggests that PLP plays a role in the response against these stresses in plants. To test these possibilities, functional analysis of PLPs is necessary. On the other hand, the biotechnological application of blue light regulated PLP–protein interaction is expected. For example, when the reporter gene of the two-hybrid system using PLPA or PLPB and their interacting proteins were substituted with the gene of interest, the gene expression would be regulated by blue light. As two-hybrid systems are available not only in yeast but also in mammalian and plant cells, PLPs and their interacting proteins could be useful tools for the regulation of gene expression in eukaryotic cells. It is also possible to regulate gene expression by pinpoint irradiation of blue light on a cell, tissue, or organ of interest.

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