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Title:

Higher plants use LOV to perceive blue light.

Short title:

LOV domain photoreceptors in plants

Authors:

Emilie Demarsy and Christian Fankhauser

Address:

Center for Integrative Genomics, University of Lausanne, Genopode Building, CH-1015 Lausanne, Switzerland.

Email: emilie.demarsy@unil.ch, christian.fankhauser@unil.ch

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Summary

Higher plants use several classes of blue light receptors to modulate a wide variety of physiological responses. Among them, both the phototropins and members of the Zeitlupe (ZTL) family use LOV (Light Oxygen Voltage) photosensory domains. In Arabidopsis, those families comprise phot1, phot2 and ZTL, LOV Kelch Protein 2 (LKP2) and Flavin-binding Kelch F-box1 (FKF1). It has now been convincingly shown that blue-light induced autophosphorylation of the phot1 kinase domain is an essential step in signal transduction. Recent experiments also shed light on the partially distinct photosensory specificities of phot1 and phot2. Phototropin signaling branches rapidly following photoreceptor activation to mediate distinct responses such as chloroplast movements or phototropism. Light activation of the LOV domain in ZTL family members modulates their capacity to interact with GIGANTEA (GI) and their ubiquitin E3 ligase activity. A complex between GI and FKF1 is required to trigger the degradation of a repressor of *CO* (*CONSTANS*) expression and thus modulates flowering time. In contrast, light-regulated complex formation between ZTL and GI appears to limit the capacity of ZTL to degrade its targets, which are part of the circadian oscillator.

Introduction

As sessile organisms, plants must utilize cues from the ever-changing environment to regulate their development. Plants have evolved a sophisticated network of signaling pathways that integrate information about light quality, quantity, duration and direction [1-3]. At least four types of photoreceptors with each family composed of several members elicit a huge diversity of physiological responses. Phytochromes primarily respond to red and far-red light, whereas cryptochromes, phototropins and

Zeitlupe/FKF1/LKP2 families mediate blue-light-induced responses [1-3]. Blue-light photoreceptors trigger rapid and reversible responses (e.g. stomatal opening, chloroplast movements), long term and irreversible responses (e.g. seedling de-etiolation, flowering) and entrainment of the circadian clock. Comprehensive reviews present how plants perceive and respond to blue light [1,2]. We will focus on recent advances in our understanding of LOV-domain-mediated light responses in higher plants. LOV-domains bound to Flavin MonoNucleotide (FMN) constitute blue light sensors, they are associated with a large variety of output domains [1].

Phototropin structure

Phot1 and phot2 harbor an N-terminal photosensory domain composed of two LOV domains (LOV1 and LOV2) and a C-terminal Ser/Thr kinase domain belonging to the AGC family (cAMP-dependent protein kinase, cGMP-dependent protein kinase, phospholipid-dependent protein kinase C) (Figure 1). In the dark each LOV domain non-covalently binds FMN. Blue light triggers covalent binding of the FMN chromophore to an invariant Cystein residue within each LOV domain. This leads to a conformational change of the protein and results in enhanced kinase activity [1,4]. This reaction is dark reversible allowing a rapid switch for phototropin activation. Among the numerous LOV-domain photoreceptors present in plants and prokaryotes, the phototropins are the only ones harboring two LOV domains [5]. LOV1 and LOV2 have distinct properties and functions [1,4]. LOV1 may be a dimerization domain [6,7], it may attenuate the light-regulated kinase activity of phot2 [8] and it was proposed to slow the dark-recovery process of LOV2 [9]. The reduced light sensitivity of plants expressing a truncated phot1

consisting of LOV2 and the kinase domain could result from an absence of dimerization or faster dark recovery of LOV2 [10]. However, point mutants blocking LOV-domain photochemistry showed that LOV1 photochemistry is essentially dispensable for phot1 while for phot2 it slightly contributes to light activation of the photoreceptor [11]. These studies also demonstrate the predominant role of LOV2 for light activation of both phot1 and phot2 [11]. *In vitro* the LOV2 domain binds to the kinase domain of phot2 and inhibits its activity in the dark while this function of LOV2 is inhibited by blue light [8]. An amphipatic α-helix called the Jα-helix connects the LOV2 domain with the kinase domain (Figure 1). In the dark this α-helix is structured and docked on the LOV2 surface. Upon light excitation, the helix becomes unfolded and liberates the kinase domain from the inhibitory effect of LOV2 [12-14]. Homology modeling studies suggest that this mechanism is analogous to inhibition of PKA by the regulatory subunit PKI (Protein Kinase Inhibitor) [4]. Finally, expression of the isolated kinase domain of phot2 in Arabidopsis leads to constitutive phototropin responses, confirming that the photosensory domain of phot2 inhibits kinase activity in darkness [15]. Given that the proposed dimerization domain (LOV1) is lacking in this construct further investigations are needed to determine whether dimerization is required for phototropin activity [15]. Thus while the function of LOV2 is clearly established, the role of LOV1 remains unclear.

Phototropin kinase activity and signal transduction

Little is known about the targets of phototropin kinase activity. *In vitro* both phot1 and phot2 autophosphorylate and phot2 also phosphorylates casein [1,8]. However, no direct substrate for kinase activity *in planta* has been identified so far except the phototropins

themselves [1]. *In vivo* mapping of phot1 phosphorylation sites provides new insights into the physiological relevance of phototropin phosphorylation [10,16]. Four phosphorylation sites were identified upstream of the LOV2 domain, similar regions of phot1 are phosphorylated *in vitro* [10]. A second study identified two additional sites in the photosensory domain, and three phosphorylation sites in the kinase domain of phot1 [16] (Figure 1). The characterization of point mutants showed that autophosphorylation of Ser851 (but none of the other residues) is necessary to trigger phototropin-dependent responses [16]. This demonstrates for the first time that phosphorylation of phot1 is an essential step of signaling [16]. This residue of the kinase-domain activation loop is well conserved among members of the PKA family [4,16]. Phosphorylation of the equivalent site in PKA enhances kinase activity and substrate recognition [4].

The physiological importance of the other phosphorylation sites remains unclear. Blue-light-dependant phosphorylation of two sites between the LOV domains is necessary for binding of a 14-3-3 protein, known to regulate ATPase-H+ activity in stomatal guard cells [16,17]. The importance of this interaction needs further evaluation because a phot1 phosphorylation mutant disrupted for 14-3-3 binding remains functional for stomatal opening and proton pump activation [16]. A second study also suggests that the phosphorylation sites in the photosensory domain of phot1 may not be essential for activity *in planta* given that a construct consisting only of LOV2 and the kinase domain is fully functional but with reduced light sensitivity [10]. Although no phosphorylation sites of phot2 have been identified expression of the kinase domain leads to constitutive

phototropin activity [15]. Altogether, these data suggest that phototropin phosphorylation in the light-sensing domain is not essential to activate downstream events.

Post-translational modification appears to modulate the subcellular localization of these photoreceptors [15,18,19]. In the dark, both phot1 and phot2 are tightly associated with the plasma membrane [18,19]. How this is achieved remains to be elucidated, but the kinase domain of phot2 is sufficient for this localization [15,19]. Blue light triggers the relocalization of a fraction of phot1 to the cytoplasm, whereas a pool of phot2 relocalizes to the Golgi [18,19]. Interestingly, phot2 kinase activity seems to regulate internalization [15,19]. Establishing the functional significance of these relocalizations is an important task.

Phototropins trigger numerous responses resulting in physiological adaptations required to optimize photosynthetic activity (Figure 2). An important question is how phot1 and phot2 selectively control these processes. Some of these responses are coordinately controlled by both phototropins (phototropism, leaf flattening, stomatal opening, chloroplast accumulation response) while others specifically depend on phot1 or phot2 (e.g. chloroplast avoidance response) (Figure 2) [1,20]. Phot1 predominates under low light while phot2 mediates mainly high-light responses [1]. Light-induced expression and the faster dark-recovery associated with phot2 are consistent with a predominant function of phot2 in high light [1,11]. In agreement with the distinct dark-recovery rates, swapping experiments between the photosensory and kinase domains of phot1 and phot2 indicate that the N-terminal domain of phot1 enhances phot2 photosensitivity [21]. Interestingly,

the C-terminal region of phot1 also contributes to the enhancement of photosensitivity [21]. Given that photoactivation is controlled by the interaction between LOV2 and the kinase domain [4,8], it is possible that phot1 and phot2 display different affinities for this interaction. Another possibility is that phot1 and phot2 kinase domains differ in substrate recognition. Indeed, phot2 but not phot1 can use casein as a substrate *in vitro* [4,8]. The distinct subcellular localization of the two phototropins, which is conferred by their kinase domain, may also explain functional differences [21].

Genetic analyses suggest that signaling downstream of the phototropins branches rapidly. Some elements are selectively required for phot1 or phot2 responses [1,20]. Moreover, even signaling elements acting downstream of a single phototropin appear to be required for a subset of phototropin responses. NPH3, RPT2 and PKS1 directly interact with phot1 suggesting that they act early in the pathway [22-24]. However none of them is required for all the phototropin responses that were tested [22-24]. For example, NPH3 is required for phot1 and phot2-mediated phototropism, phot1-mediated leaf positioning but dispensable for chloroplast positioning and stomatal opening [22,25] (Figure 1). Interestingly, NPH3 undergoes a blue-light induced dephosphorylation that depends on phot1 but not phot2 [24]. A second example illustrating rapid branching in phot1 signaling comes from the analysis of Ca^{2+} requirements for phot1 responses. Within seconds of blue light irradiation, phot1 mediates an increase in cytoplasmic Ca^{2+} concentration [26]. This Ca^{2+} response is required for phot1-dependent inhibition of hypocotyl elongation, but not for phototropism [26]. Phot1 negatively regulates the expression of an inositol polyphosphate 5-phosphatase gene, which modulates inhibition

of hypocotyl growth via Ca^{2+} concentration regulation. Unfortunately, it is unknown whether this protein is also required for phototropism [27]. Growth responses such as phototropism require auxin and brassinosteroids [28-31] while there is no evidence suggesting that these hormones are involved in the control of chloroplast movements. For chloroplast movement responses the nucleus is not even required [20]. In contrast, gene expression changes are essential for phototropism [27,28] and presumably occur following the asymmetric distribution of auxin that is required for directional growth [28]. Such changes in gene expression are hard to record by whole plant analysis because they occur in specific cells [28,32]. Rapid branching following photoreceptor activation may also be explained by the presence of distinct phototropin pools (depending on the cell type) in combination with different signaling elements.

Zeitlupe family

In Arabidopsis a second LOV-domain photoreceptor family comprised of ZTL, FKF1 and LKP2 modulates the circadian clock and the photoperiod-dependent flowering pathway [1,5,33]. This review will not cover our knowledge of circadian biology and of the regulation of flowering by the photoperiod (for recent reviews please consult [34-36]), but briefly describe the mode of action of this novel class of blue light photoreceptors.

Members of this family possess an N-terminal LOV domain followed by an F-box and six Kelch repeats, which suggests that they may act in light-regulated protein

degradation, reviewed in [1,33] (Figure 1). The LOV domain of these proteins binds an FMN chromophore and displays analogous photochemical properties to phototropin LOV domains [37,38]. However the absence of dark recovery suggests that ZTL family members probably mediate non-reversible light responses [37,38]. F-box proteins are typically components of SCF-type (Skp1, Culin, F-box) ubiquitin E3 ligases [39]. Several studies have confirmed that ZTL/FKF/LKP2 can form SCF-type complexes with ASK proteins, CUL1 and RBX1 strongly supporting the notion that these proteins are involved in ubiquitin-mediated protein turnover [40,41]. Interestingly, these interactions seem to occur in a light-independent way and do not require the LOV domain.

FKF1 promotes the expression of *CO* a central element of day-length-regulated flowering [34,36,38]. The regulation of *CO* occurs both at the transcriptional and post-translational level involving multiple photoreceptors from distinct families [34,36,42,43]. *CO* transcription is regulated by day length while the protein is unstable in the dark and stabilized during the day [34,36]. FKF1 regulates the stability of Cycling Dof Factor 1 (CDF1), which directly represses *CO* expression [44]. The LOV domain of FKF1 interacts with a plant-specific protein called GIGANTEA (GI), which is another positive regulator of *CO* expression [45]. This interaction occurs specifically in blue light and depends on photoexcitation of the LOV domain [45]. GI also directly interacts with CDF1, but it is only when a complex between GI, FKF1 and CDF1 is formed that CDF1 gets degraded [45] (Figure 3). The combination of circadian expression of *GI* and *FKF1* together with the light-regulated interaction between their gene products enables expression of *CO* during the late afternoon in long day-grown plants [45] (Figure 3).

ZTL also interacts with GI specifically upon photo-excitation [46]. Formation of this complex stabilizes ZTL indicating that ZTL is a blue light photoreceptor facilitating its own stability. *GI* expression is clock controlled, thus this light-regulated interaction explains diurnal-regulated accumulation of ZTL protein despite constitutive RNA expression [33,46]. This complex post-translational mechanism of ZTL accumulation is required to establish high amplitude rhythms of the TOC1 protein (Timing Of Cab expression 1), which is a target of SCF^{ZTL} [40,46]. TOC1 is one of five PRRs (Pseudo Response Regulator) present in Arabidopsis. All five of them control the pace of the circadian oscillator but ZTL exclusively controls the stability of TOC1 and PRR5 [46-48]. These recent studies also suggest that the ZTL-GI interaction controls the degradation of SCF^{ZTL} targets by restricting substrate binding to ZTL [46-48] (Figure 3). The light-regulated interaction between GI and members of the ZTL family thus has distinct outcomes depending on the photoreceptor. For FKF1 the complex is required for the degradation of its target while for ZTL, GI-binding limits the access to SCF^{ZTL} substrates. It will be interesting to see whether LKP2 also binds GI and what the consequences of this interaction are.

Conclusions and Perspective

The LOV domains of phototropins and members of the ZTL family modulate the activity of distinct signaling domains. Moreover, while the LOV domains of phototropins have rapid photocycles, the LOV domains of ZTL family members hardly dark revert at all. Those distinct photochemical cycles correlate with the rapid responses controlled by the phototropins (e.g. chloroplast movements) versus the slow responses triggered by FKF1,

ZTL and LKP2 (e.g. induction of flowering). Light activation of phot1 kinase activity and the resulting autophosphorylation in the kinase domain activation loop (S851) are essential features of signaling. By analogy with PKA, autophosphorylation of S851 may result in enhanced kinase activity and substrate recognition. The functional consequences of light-induced kinase activity, the identification of phototropin substrates and the substrate specificity of phot1 and phot2 are important questions to be answered. Recent studies reveal an intriguing analogy between the role of phot1 and NPH3 in phototropism and the role of the AGC kinase pinoid and an NPH3-related protein in auxin signaling or transport [49,50]. Given that phototropin signaling modulates auxin transport it will be interesting to study this connection further. The LOV domain of ZTL and FKF1 modulates their capacity to interact with GI. For reasons that are currently not understood the formation of this complex has distinct consequences for ZTL and FKF1 activity. We have presented the physiological outputs of ZTL and FKF1 separately, however ZTL also influences flowering time by affecting circadian-controlled gene expression. A recent study showing that TOC1 and PRR5 regulate *CDF1* expression illustrates this point [51]. Future studies are required to address the mode of action and precise function of LKP2 and of another putative LOV photoreceptor present in Arabidopsis, which only consists of a LOV and a PAS domain [52].

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Figure legends

Figure 1: Domain organization of a phototropin and a Zeitlupe-type photoreceptor. In both classes of photoreceptors the approximately 110 amino acid long LOV domain bound to an FMN molecule functions as the blue light sensor. Proposed functions of each domain are indicated below.

a) Phototropins harbor two FMN-binding LOV domains in their N-terminal part (LOV1 and LOV2) and a Serine/Threonine Kinase Domain in the C terminal part (KD). The Jα-

helix (Jα) connects LOV2 and KD. Regions of phot1 phosphorylation are indicated with arrows.

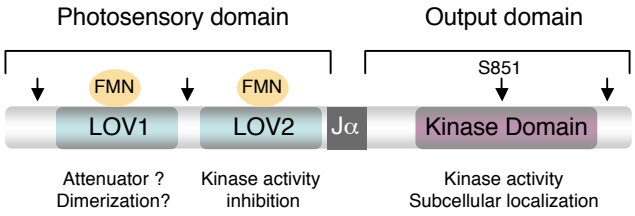
b) Zeitlupe family photoreceptors harbor one LOV domain at the N-terminus, followed by an F-Box motif and six Kelch repeats (KELCH) in the C-terminal region. By analogy with other proteins the KELCH repeats may serve as protein-protein interaction domains. Consistent with this idea mutations in the ZTL KELCH repeats interfere with TOC1 binding, however *in vitro* the isolated LOV domain of ZTL is sufficient for TOC1 binding [40,41].

Figure 2 : Phot1 and phot2 mediate distinct and overlapping physiological responses. Physiological responses are mediated specifically by phot1 (green), by phot2 (pink) or both phot1 and phot2 (dark blue). The response and the phototropin involved also depend on the fluence rate of blue light (Low or High Blue Light). For example light-dependent nuclear positioning is induced upon high blue light activation of phot2 [53]. Signaling components (NPH3 and RPT2) are involved in a subset of phot1 pathways (green) or phot1 and phot2 pathways (blue). Several signaling components, in particular those involved in chloroplast relocation, are not depicted in this figure.

Figure 3: A simplified model for the light-regulation of ZTL and FKF1 activity. Both FKF1 and ZTL specifically form a complex with GI upon blue light activation. In the light (L) the GI-ZTL complex stabilizes ZTL but apparently restricts the access of ZTL to its targets TOC1 and PRR5 (for simplicity we only indicate TOC1 in this figure), thus TOC1 and PRR5 are mostly degraded by ZTL in darkness (D). The FKF1-GI-CDF1

complex forms on *CO* chromatin specifically upon blue light activation leading to derepression of *CO* expression in the late afternoon.

Phototropins



Zeitlupe

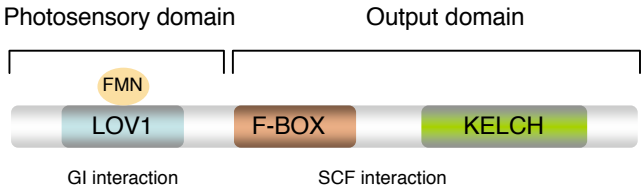


Figure 1

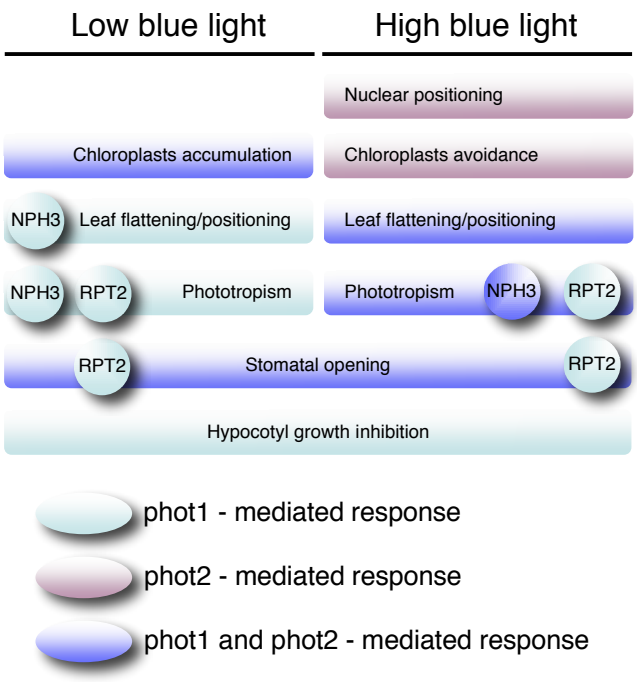

Low blue light
High blue light
Nuclear positioning
Chloroplasts accumulation
Chloroplasts avoidance
NPH3
Leaf flattening/positioning
Leaf flattening/positioning
NPH3
RPT2
Phototropism
Phototropism
NPH3
RPT2
RPT2
Stomatal opening
RPT2
Hypocotyl growth inhibition
phot1 - mediated response
phot2 - mediated response
phot1 and phot2 - mediated response

Figure 2

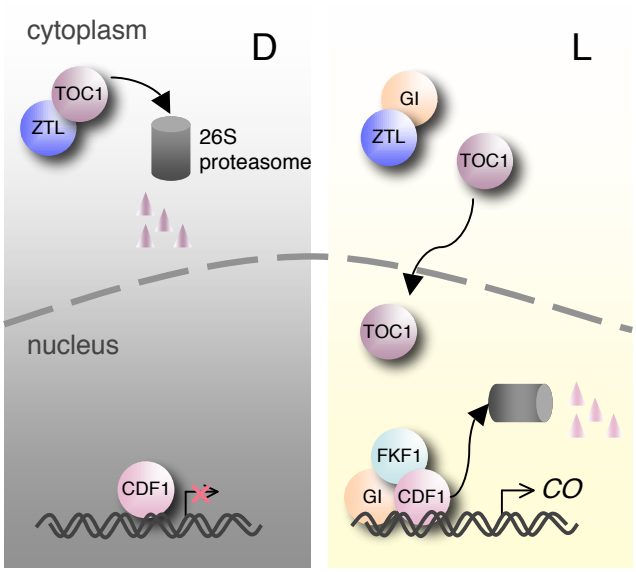

cytoplasm
D
L
TOC1
ZTL
26S
proteasome
GI
ZTL
TOC1
TOC1
nucleus
FKF1
GI
CDF1
CO
CDF1

Figure 3