LOV Domain-Containing F-Box Proteins: Light-Dependent Protein Degradation Modules in *Arabidopsis*

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ABSTRACT **Plants constantly survey the surrounding environment using several sets of photoreceptors. They can sense changes in the quantity (=intensity) and quality (=wavelength) of light and use this information to adjust their physiological responses, growth, and developmental patterns. In addition to the classical photoreceptors, such as phytochromes, cryptochromes, and phototropins, ZEITLUPE (ZTL), FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1), and LOV KELCH PROTEIN 2 (LKP2) proteins have been recently identified as blue-light photoreceptors that are important for regulation of the circadian clock and photoperiodic flowering. The ZTL/FKF1/LKP2 protein family possesses a unique combination of domains: a blue-light-absorbing LOV (Light, Oxygen, or Voltage) domain along with domains involved in protein degradation. Here, we summarize recent advances in our understanding of the function of the *Arabidopsis* ZTL/FKF1/LKP2 proteins. We summarize the distinct photochemical properties of their LOV domains and discuss the molecular mechanisms by which the ZTL/FKF1/LKP2 proteins regulate the circadian clock and photoperiodic flowering by controlling blue-light-dependent protein degradation.**

Key words: blue light; LOV domain; ZTL; FKF1; LKP2; photoperiodic flowering; circadian clock; *Arabidopsis*.

INTRODUCTION

Plants have developed many sensor mechanisms to monitor various changes in the surrounding environment. One of the most crucial environmental factors for plants is light. Plants use light not only as a primary energy source for photosynthesis, but also as a way to judge changes in their surroundings. Most plants possess multiple sets of photoreceptors that cover a broad range of wavelengths (=colors) as well as intensities of light. These photoreceptors enable plants to accurately survey ambient light conditions (Chen et al., 2004) and adjust their development, morphology, and metabolic rates to the specific environment in which they live. Plants have acquired three major classes of photoreceptor molecules: a red-/far-red-light-reversible photoreceptor called phytochrome (Franklin and Quail, 2010), and two types of blue-light photoreceptors, cryptochrome (Chaves et al., 2011) and phototropin (Christie, 2007). Phytochrome and cryptochrome signals coordinate to regulate various developmental processes throughout the plant's life, such as seed germination, hypocotyl elongation, greening, and flowering (Chen et al., 2004). In contrast, phototropin signals mainly control directional movement (Christie, 2007).

In *Arabidopsis thaliana*, there are two phototropins, phot1 and phot2, that share partially overlapping functions. Both phototropins regulate phototropism (Sakai et al., 2001), chloroplast relocation movement (Kagawa et al., 2001; Sakai et al., 2001), light-induced stomatal opening (Kinoshita et al., 2001), cotyledon and leaf expansion (Sakamoto and Briggs, 2002; Takemiya et al., 2005), and hypocotyl growth (Folta and Spalding, 2001). Phototropins have two photosensory domains called Light, Oxygen, or Voltage (LOV) domains in the N-terminal half and a Ser/Thr kinase at the C-terminal half (Christie et al., 1998). Through the LOV domains, blue light regulates the kinase activity of phototropins and blue-light-dependent phosphorylation is an important initial process of phototropin signaling (Pedmale and Liscum, 2007; Christie et al., 2011). In addition to the phototropins, *Arabidopsis* has two additional types of protein that possess LOV domains (Figure 1A). One such family comprises three proteins that each possess a single LOV domain: ZEITLUPE (ZTL), FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1), and LOV KELCH PROTEIN 2 (LKP2) (Nelson et al., 2000; Somers et al., 2000; Schultz

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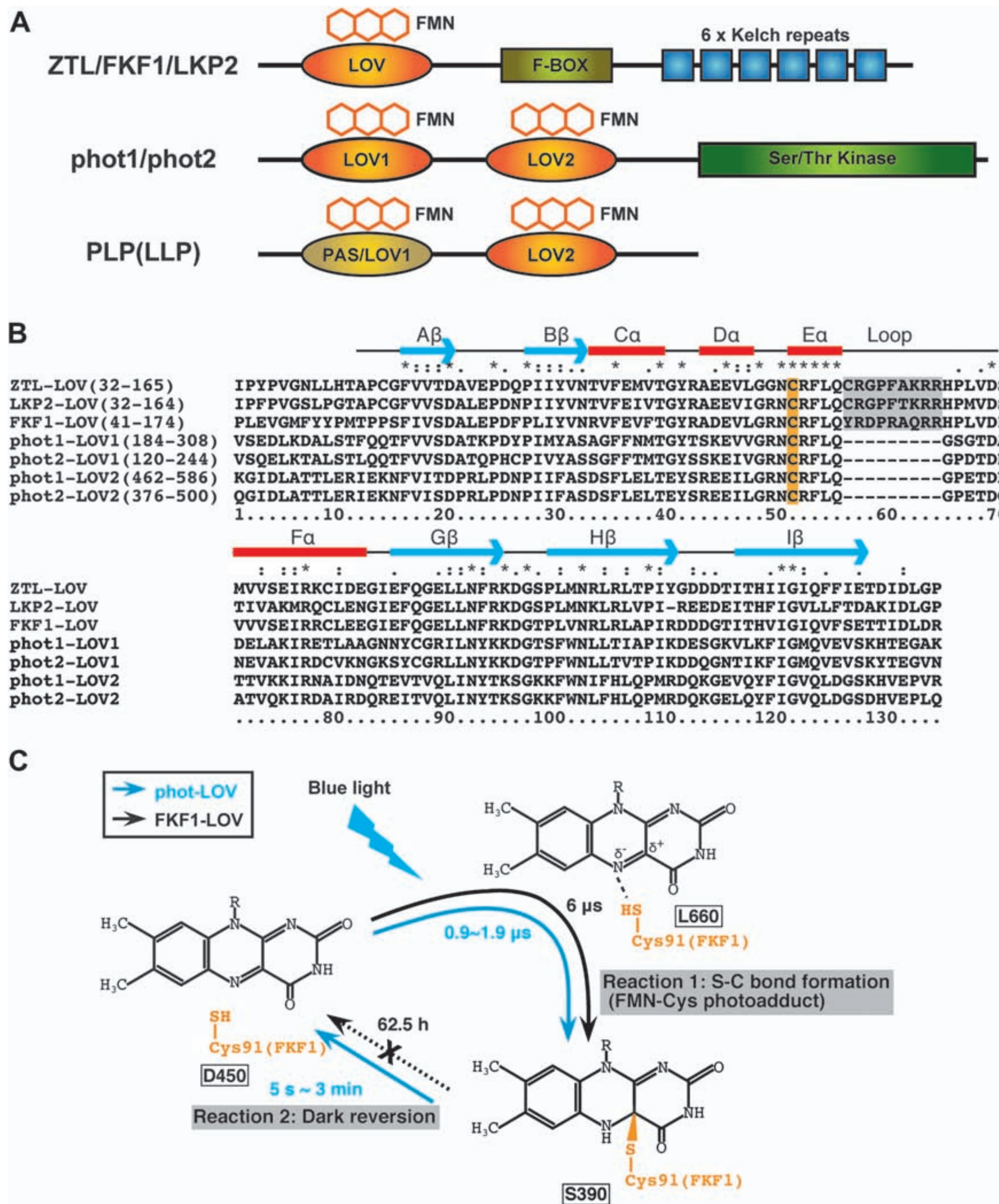

Figure 1. The Domain Structures of ZTL/FKF1/LKP2 and Related Proteins, Amino Acid Sequence Alignments of LOV Domains, and Photochemical Properties of Phototropin and FKF1 LOV Domains.

(A) Schematic illustration of functional domains of ZTL/FKF1/LKP2 family proteins, phototropin proteins, and PAS/LOV proteins. LOV domain bound to an FMN molecule functions as a blue-light-sensing domain. The ZTL/FKF1/LKP2 family proteins possess one LOV domain at the N-terminus region followed by an F-box domain and six Kelch repeats in the C-terminal region. The phototropins contain two FMN-binding LOV domains in their N-terminal region (LOV1 and LOV2) and a serine/threonine kinase domain at the C-terminus. The PAS/LOV proteins (PLP or also called LLP) contain two LOV domains; however, the cysteine residue essential for the cysteinyl adduct formation within the first LOV domain is not always conserved in some plant species, including *Arabidopsis* (Kasahara et al., 2010), indicating that there is no light-induced photocycle of the LOV domain. The LOV2 domains of PLP proteins usually contain the conserved cysteine and show the blue-light-induced photocycle *in vitro*.

(B) Sequence alignment of several LOV domains. The alignment includes *Arabidopsis thaliana* ZTL, LKP2, FKF1, phot1-LOV1, phot1-LOV2, phot2-LOV1, and phot2-LOV2. Identical and similar amino acids are marked on top of the alignment by asterisks (*) and (: or .), respectively. The conserved cysteines for FMN binding are highlighted in orange. The characteristic loop regions of ZTL/FKF1/LKP2 LOV are gray-shaded. The predicted secondary structure elements are shown on top of the alignment. Arrows and boxes indicate β-strands and α-helices, respectively.

et al., 2001) (Figure 1A). The ZTL/FKF1/LKP2 proteins play roles in the circadian clock and photoperiodic flowering (Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001). The other type of protein is referred to as PAS/LOV protein (PLP) (also called LOV/LOV protein (LLP)), which contains two LOV domains, although the physiological function of PLP is largely unknown (Ogura et al., 2008; Kasahara et al., 2010) (Figure 1A).

Based on amino acid sequence similarities between the ZTL/FKF1/LKP2 LOV domains and the phototropin LOV domains, it was proposed that the ZTL/FKF1/LKP2 proteins may be blue-light photoreceptors as well (Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001). Since then, we have acquired ample photochemical evidence of the blue-light-absorbing properties and blue-light-specific functions of the ZTL/FKF1/LKP2 LOV domains. It is now clear that the ZTL/FKF1/LKP2 proteins fulfill the following criteria for being photoreceptors: (1) they possess chromophores required for light absorption, (2) light absorption induces functional changes at the molecular level, and (3) they regulate light-dependent responses.

All ZTL, FKF1, and LKP2 proteins possess two additional functional domains: an F-box domain and a Kelch repeat domain (Figure 1A). The combination of these domains suggests that they are involved in protein stability regulation (Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001). Recent results indicate that these proteins function as E3 ubiquitin ligases and mediate proteasome-dependent protein degradation in a light-dependent manner (Más et al., 2003; Imaizumi et al., 2005; Kim et al., 2007; Sawa et al., 2007). They are involved in regulation of the circadian clock and day-length-dependent flowering by controlling accumulation of key regulator proteins in the clock and flowering pathway (Más et al., 2003; Kiba et al., 2007; Fornara et al., 2009). Here, we summarize recent advances in our understanding of the molecular properties of the LOV domains of ZTL, FKF1, and LKP2 proteins and discuss the mechanisms by which these three proteins control the photoperiodic flowering pathway and the circadian clock in a light-dependent manner.

STRUCTURE AND PHOTOCHEMICAL PROPERTIES OF THE LIGHT-SENSING FKF1 LOV DOMAIN

The crystal structures and photochemical properties of the LOV domains have been well characterized using phototropin LOV domains (Christie, 2007; Matsuoka et al., 2007; Tokutomi et al., 2008). Although there are fewer papers describing the ZTL/FKF1/LKP2 LOV domains, our knowledge of the photochemical reactions of the ZTL/FKF1/LKP2 LOV domains and potential structural changes has been expanding, mainly using the FKF1 LOV domain (Imaizumi et al., 2003; Nakasako et al., 2005; Zikihara et al., 2006; Kikuchi et al., 2009; Nakasone et al., 2010). The FKF1 LOV domain has features conserved in all LOV domains as well as properties unique from the phot-LOV domains. This unique property of the LOV domain may be beneficial for the light-dependent degradation of target proteins, since FKF1 functions as an E3 ubiquitin ligase.

The LOV domain is a small photo-sensing module that belongs to a subclass of the Per-ARNT-Sim (PAS) domain super-family with members that possess diverse functions as versatile sensors (Taylor and Zhulin, 1999). In addition to the recent discovery of the blue-light-activated LOV-domain-containing histidine-kinases in various prokaryotes (Swartz et al., 2007), LOV domains are found in blue-light-sensing proteins of organisms ranging from archaea to eukaryotes. In plants, the LOV domains generally non-covalently bind a flavin cofactor, flavin mononucleotide (FMN), as a chromophore (Taylor and Zhulin, 1999). The core of the LOV domain consists of four α-helixes and five β-sheets. According to recently proposed nomenclature (Harper et al., 2003), the following secondary structural elements for the LOV domain core have been assigned: Aβ-Bβ-Cα-Dα-Eα-Fα-Gβ-Hβ-Iβ (Figure 1B).

When blue light is perceived by the phototropin LOV domain, it undergoes a unique photochemical reaction cycle (Figure 1C) (Kasahara et al., 2002). The ground state of the FMN in the LOV domains, called D_{450}, shows an absorption spectrum typical of flavin with an absorption maximum of around 450 nm. The D_{450} is elevated to a singlet-excited state by blue light and then inter-converted to a triplet-excited state, called L_{660} (Figure 1C). A stable adduct then forms between the FMN chromophore and a cysteine conserved within the LOV domains localized in the Eα helix (Figure 1B), converting the LOV domain to the S_{390} state that has an absorption maximum of around 390 nm (Reaction 1, Figure 1C) (Salomon et al., 2000; Swartz et al., 2001). This is the so-called FMN–Cys photoadduct, and it occurs in 0.9–1.9 μs—almost instantly—after the FMN absorbs light energy. Reversion of S_{390} back to D_{450} also occurs rapidly, with time constants from several seconds to a few minutes, depending on the LOV domain (Reaction 2, Figure 1C) (Kasahara et al., 2002). Rapid reversion to D_{450} is surprising, because it means that the covalent bond between cysteine and FMN is easily broken in the phototropin LOV domain, requiring relatively higher energy to do so. Thus, FMN in the phototropin LOV domain can be repeatedly activated by blue-light exposure.

Recombinant FKF1, ZTL, and LKP2 LOV domains all bind the FMN chromophore and undergo a light-induced photochemical reaction similar to that of the phototropin LOV domain *in*

(C) Schematic representation of LOV-domain photochemistry. In darkness, the FMN chromophore is non-covalently bound in the LOV domain (L450). Light triggers the production of a reactive triplet-state flavin (L660) that leads to formation of a covalent bond between the FMN and a conserved cysteine residue in the LOV domain (S390). The photoreaction process of the phot-LOV protein (right blue arrow circuit) is fully reversible in the dark. In contrast, the FKF1–LOV protein (a black dotted arrow) shows a slow dark reversion rate. The dark reversion rate (half-lives; $t_{1/2}$) of each protein is shown.

vitro (Salomon et al., 2000; Corchnoy et al., 2003; Imaizumi et al., 2003). The absorption spectrum of the FKF1 LOV domain is also similar, showing a typical flavoprotein spectrum with a peak maximum at 450 nm. After light irradiation, absorption of around 450 nm decreases, while absorption of around 390nm increases, indicating formation of the S_{390} state. Recently, FMN–Cys adduct formation of FKF1 LOV polypeptides was characterized using the pulsed laser-induced transient grating (TG) method and the D_{450} to S_{390} change took place with a time constant of 6 μs (Nakasone et al., 2010). This is slow relative to phot1–LOV2 and phot2–LOV2 (1.9 and 0.9 μs, respectively) (Figure 1C) (Eitoku et al., 2005; Nakasone et al., 2006). Substitution of the conserved cysteine to alanine (C91A) abolished FMN–Cys photoadduct formation, although binding of FMN to the FKF1 LOV domain was unaffected. Absorption of D_{450} was similar to that of the non-mutated LOV domain (Imaizumi et al., 2003).

The FKF1 and phototropin LOV domains differ most profoundly in their rates of dark reversion. In contrast to the phototropin LOV domains, the FKF1 LOV domain does not show appreciable dark recovery in short-term experimental conditions (Imaizumi et al., 2003) (Figure 1C). The FKF1–LOV polypeptides revert from S_{390} to the D_{450} ground state with a half-life of 62.5 h at room temperature (Zikihara et al., 2006). This extremely slow dark recovery may be caused by an additional 9-amino acid insertion, which forms a loop structure between the Eα helix near the conserved cysteine residue and the Fα helix (Figure 1B). This insertion is only found in the slow dark-recovery class of LOV domains (Zikihara et al., 2006). Dark-recovery rates using an FKF1–LOV domain lacking the loop region (FKF1–LOV–NL) revealed that losing the loop region accelerates dark recovery up to approximately threefold (20.9 h) (Zikihara et al., 2006). Further, conformational change between dark and light conditions was detected for FKF1–LOV but not for FKF1–LOV–NL (Nakasone et al., 2010). This observation suggests that the loop region of FKF1–LOV is important for conformational changes. However, dark reversion of FKF1–LOV–NL is still significantly slower than that of the phototropin LOV domains, indicating that conformational differences of other residues surrounding the chromophore also affect the stability of the light-adapted state. A series of site-directed mutagenesis studies in LOV domains from several organisms supports this notion (Christie et al., 2007; Yamamoto et al., 2008; Jentzsch et al., 2009; Zoltowski et al., 2009). Most FKF1 protein synthesized within a day disappears by the end of the day (Imaizumi et al., 2003). It seems then that, once a conformational change of FKF1 is triggered by blue-light absorption, FKF1 remains in its light-activated form until it is degraded. Of course, we cannot exclude the possibility that the full length of FKF1 has a different dark-recovery rate *in vivo*.

Phototropins have two tandemly aligned LOV domains. *In vitro*, LOV1 homodimerizes regardless of light conditions (Figure 2A) (Salomon et al., 2004; Eitoku et al., 2007), indicating that the LOV1 domain functions as a dimerization site. The LOV2 domain interacts with its neighboring α-helix linker region (Jα) in the

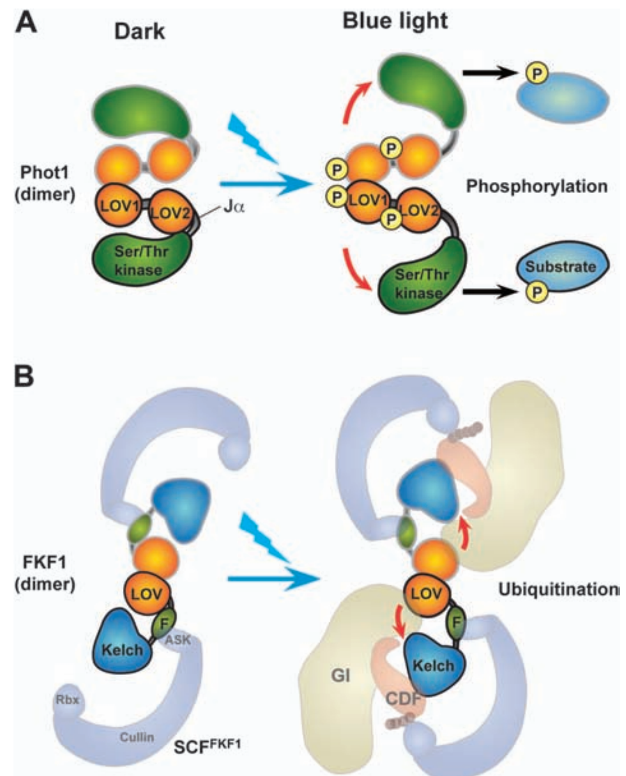

Figure 2. Tentative Structure Changes of FKF1 Triggered by Blue-Light Absorption.

(A) Proposed structural changes of phot1. Two phot1 molecules dimerize through their LOV1 domains. The LOV2 domain is important for light-dependent function. Once phot1 absorbs blue light, the Jα-helix located adjacent to the LOV2 domain detaches from the LOV2 domain. This structural change is thought to activate the C-terminal kinase. The kinase phosphorylates N-terminal regions as well as substrate proteins to send phosphorylation signals.

(B) FKF1 also forms at least a homodimer with anti-parallel configuration *in vitro*. In the dark, FKF1 may be incorporated in the SCF^{FKF1} complex (which consists of FKF1 F-box protein, ASK, Cullin, and Rbx). After blue-light exposure, FKF1 interacts with GI through the LOV domain. Light may cause a similar structural change to phot1 to enable FKF1 to bind to GI. GI also binds to CDF1, a substrate of FKF1 for ubiquitination-dependent protein degradation. FKF1 binds to CDF1 through the Kelch repeat domain and is involved in protein degradation.

dark but dissociates upon illumination and plays a key role in regulating C-terminal kinase activity (Figure 2A) (Eitoku et al., 2005, 2007; Nakasone et al., 2007). Functionally, LOV2 is more important for photoreceptor function than LOV1 in plants (Christie et al., 2002). The FKF1 LOV domain forms a stable dimer with an anti-parallel configuration regardless of the light conditions (Figure 2B) (Nakasako et al., 2005; Nakasone et al., 2010). Since FKF1 has only one LOV domain, the FKF1 LOV domain seems to have two functions. One role is interdomain interactions including homo- or hetero-dimerization among ZTL/FKF1/LKP2 members (Yasuhara et al., 2004), and the other is signal transduction activity caused by blue-light-induced conformational changes around the loop region (Figures 1 and 2B).

OTHER DOMAIN STRUCTURES IN ZTL/FKF1/LKP2 PROTEINS

In addition to the LOV domain, ZTL/FKF1/LKP2 proteins possess two other functional domains: F-box and Kelch repeat. An F-box protein is a component of the SKP–Cullin–Rbx–F-box (SCF) complex (Figure 2B). The F-box motif of the ZTL family of proteins interacts with *Arabidopsis* SKP1-like (ASK) proteins, indicating the formation of SCF E3 ubiquitin ligases *in vivo* (Han et al., 2004; Yasuhara et al., 2004). The Kelch repeat domain forms a β-propeller structure and functions as a protein–protein interacting domain that binds substrates for ubiquitin-mediated protein degradation (Andrade et al., 2001). These domain structures clearly indicate that the ZTL/FKF1/LKP2 proteins mediate ubiquitin-dependent protein degradation, possibly in a light-dependent manner. Since these proteins share high degrees of amino-acid sequence homologies (70–80% identities throughout the entire protein) (Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001), it was predicted that these proteins may have overlapping functions and may even degrade the same target proteins. Based on loss-of-function mutant phenotypes, at least ZTL and FKF1 play different roles in the circadian clock and photoperiodic flowering regulation (Nelson et al., 2000; Somers et al., 2000). These results indicate that ZTL and FKF1 must have different targets for degradation as well. In the following sections, we summarize our current understanding of the molecular roles of light-absorbing domains and the protein degradation function of the ZTL/FKF1/LKP2 protein family in the regulation of photoperiodic flowering and circadian clock oscillation.

FUNCTIONS OF ZTL/FKF1/LKP2 PROTEINS IN THE REGULATION OF PHOTOPERIODIC FLOWERING

Plants use photoperiod-sensing mechanisms in order to control timing of seasonal flowering to maximize their reproductive success. In *Arabidopsis*, expression of the *FLOWERING LOCUS T* (*FT*) gene regulated by CONSTANS (CO) protein is a crucial aspect of photoperiodic flowering (Suárez-López et al., 2001; Valverde et al., 2004; Abe et al., 2005; Wigge et al., 2005; Sawa et al., 2007). Mutations in *CO* and *FT* genes cause a strong delay in flowering under inductive long-day (LD) conditions, whereas overexpression of *CO* and *FT* strongly accelerates flowering regardless of day length (Samach et al., 2000). The *CO*/*FT* module is highly conserved not only in LD plants such as *Arabidopsis*, wheat, and barley, but also in short-day (SD) plants such as rice (Song et al., 2010). Therefore, studies of the regulation of the *Arabidopsis CO*/*FT* module may contribute to our understanding of the general mechanism of seasonal flowering in plants.

All three LOV-containing F-box proteins (ZTL, FKF1, and LKP2) are involved in the control of flowering time through the regulation of the *CO*/*FT* module (Imaizumi et al., 2003; Somers et al., 2004; Takase et al., 2011). Three functional domains in the ZTL/FKF1/LKP2 proteins are important for their roles in flowering and the circadian clock—especially light-activated LOV domains, which determine characteristic features of the proteins (Kim et al., 2007; Sawa et al., 2007). The function of FKF1 in flowering regulation is the most characterized among the three F-box members. A mutation in the *FKF1* gene strongly delays flowering under LD conditions (Nelson et al., 2000; Imaizumi et al., 2003). The FKF1 Kelch repeat domain interacts with CYCLING DOF FACTOR (CDF) transcriptional repressors for poly-ubiquitination-dependent degradation (Imaizumi et al., 2005) (Figure 3A). The CDF1 protein represses transcription of the *CO* gene by direct binding to Dof binding sites in the *CO* promoter (Imaizumi et al., 2005). GIGANTEA (GI) is a large nuclear protein and a positive regulator of *CO* and *FT* gene expression (Fowler et al., 1999; Huq et al., 2000). GI also physically interacts with CDF1 on the *CO* promoter (Sawa et al., 2007). When the FKF1 LOV domain absorbs blue light, FKF1 interacts with GI through its LOV domain to form a protein complex in the late afternoon under LD conditions (Sawa et al., 2007). Then, FKF1 in the FKF1–GI complex degrades the CDF1 protein on the *CO* promoter, resulting in activation of *CO* transcription at the end of the day (Figure 3A). In contrast, the timing of FKF1 and GI expression is out of phase under SD conditions and FKF1 is mainly expressed in the dark. Thus, little of the FKF1–GI complex forms in light under SD conditions causing a low abundance of *CO* mRNA during the day (Sawa et al., 2007). CO protein is stabilized at the end of the day in LD by phytochrome A and cryptochrome photoreceptor signaling. This time- and day-length-dependent stabilization of CO is thought to be important for *FT* induction in LD (Valverde et al., 2004). Therefore, an alignment of *CO* expression with the timing of CO protein stabilization is a crucial component of this pathway. These findings establish the importance of FKF1 in a day-length measurement mechanism through regulation of the timing of daytime *CO* expression.

In addition to *CO* transcriptional regulation, a recent study predicts another role for FKF1 in photoperiodic flowering. Using a computational model for the photoperiodic gene circuit, Salazar et al. (2009) predicted that FKF1 may control *FT* expression in addition to the role of *CO* transcriptional activation (Salazar et al., 2009). In their simulation model, the current molecular mechanism by which FKF1 induces *FT* through activation of *CO* transcription cannot entirely explain the low levels of *FT* mRNA in *fkf1* mutant plants; therefore, FKF1 may influence *FT* expression directly. This prediction is supported by the evidence that FKF1 associates with *FT* chromatin *in vivo* (Sawa and Kay, 2011). GI interacts with the *FT* repressors (SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO (TEM) 1, and TEM2), which directly bind to the *FT* promoter regions where GI exists (Sawa and Kay, 2011). Although FKF1 and GI bind to similar *FT* promoter regions, FKF1 may not be involved in target degradation of these proteins, since no interaction between FKF1 and these *FT* repressors was observed (Sawa and Kay, 2011). This suggests that GI has an additional role in the activation of *FT* expression through complexes with the *FT* repressors, although the functions of the complexes remain to be revealed. The

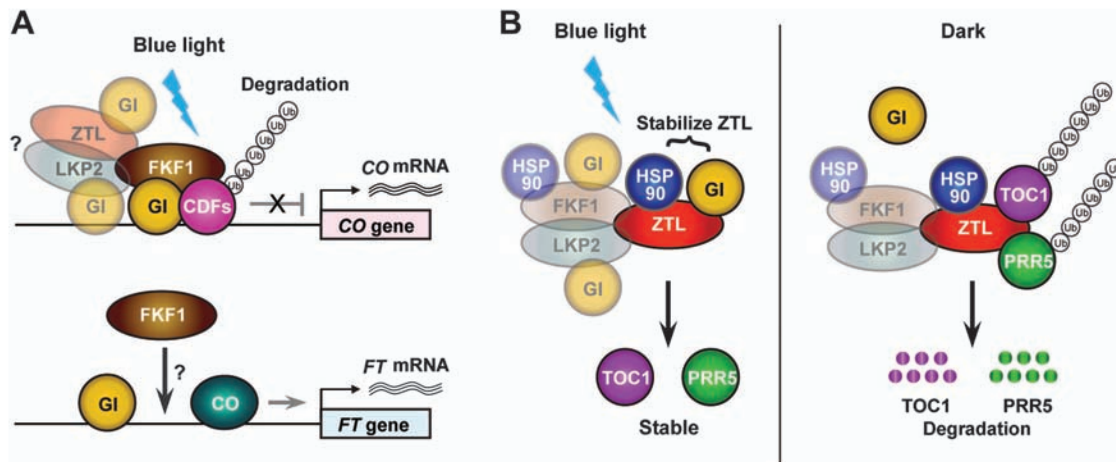

Figure 3. Light-Mediated Proteolysis Controlled by ZTL, FKF1, and LKP2 in the Regulation of Flowering Time and the Circadian Clock.

(A) The function of FKF1 in the control of flowering time. GI forms protein complexes with CDFs on the *CO* promoter. In the afternoon, FKF1 is recruited to the *CO* promoter region through blue-light-dependent interaction with GI. FKF1 degrades CDFs, facilitating expression of *CO*. Since CDF2 is stabilized further in the *ztl fkf1 lkp2* triple mutants than in the *fkf1* mutants, the ZTL–GI and LKP2–GI complexes may also contribute to the degradation of some CDF proteins. CO and GI bind to the *FT* promoter and activate *FT* transcription, which induces flowering. FKF1 may also bind to the *FT* promoter; however, the function of this interaction is unknown.

(B) The function of ZTL in regulation of the circadian clock. Under blue light, ZTL interacts with GI. ZTL also forms a protein complex with HSP90. The interaction of ZTL with GI and HSP90 stabilizes ZTL protein. The ZTL–GI complex formation may sequester ZTL from interaction with TOC1 or PRR5. This enables TOC1 and PRR5 proteins to accumulate in the late afternoon. The FKF1–GI and LKP2–GI complex may also have a similar role (left side). In the dark, ZTL (FKF1 and LKP2) interact with TOC1 and PRR5 proteins and degrade them through a proteasome pathway (right side).

potential role of FKF1 in *FT* expression, if any, has yet to be addressed (Figure 3A).

The ZTL and LKP2 proteins are also involved in control of flowering time and *CO* expression; however, unlike FKF1, ZTL and LKP2 do not seem to be simple positive regulators of *CO*. A *ztl* mutant shows a weak early-flowering phenotype under SD conditions (Somers et al., 2004; Takase et al., 2011). An *lkp2* mutation enhances the phenotype of the *ztl* mutant under both LD and SD conditions, even though the *lkp2* mutant by itself has little effect on the regulation of flowering (Takase et al., 2011). Introducing *ztl* and *lkp2* mutations into an *fkf1* background further reduces the *CO* expression level (Fornara et al., 2009). One explanation for the *ztl fkf1 lkp2* triple mutant phenotype is that FKF1, ZTL, and LKP2 are all involved in protein stability regulation of the *CO* repressor, CDF2, and possibly other CDFs (Figure 3A). Introduction of *ztl* and *lkp2* mutations to the *fkf1* mutant further increases the abundance of CDF2 protein, which may lead to lower levels of *CO* transcript in the mutants (Fornara et al., 2009).

Interestingly, overexpression of *ZTL* or *LKP2* also downregulates *CO* transcription, which results in a late-flowering phenotype similar to the *ztl fkf1 lkp2* triple mutant under LD conditions (Schultz et al., 2001; Somers et al., 2004). The severely depressed *CO* expression levels in *ZTL* and *LKP2* overexpressors also resemble that of the *gi* mutant. Since ZTL and LKP2 are mainly expressed in the cytosol (Kim et al., 2007; Takase et al., 2011), one possible explanation for the flowering phenotype of the *ZTL*/*LKP2* overexpressor is that overexpression of ZTL or LKP2 may sequester GI in the cytosol by forming a ZTL(/LKP2)–GI complex. This may reduce the amount of GI–FKF1 complex in the nucleus, causing stabilization of the CDF *CO* repressors.

A second possibility is that overexpression of ZTL and LKP2 may enhance degradation of their substrates, TIMING OF CAB EXPRESSION 1 (TOC1) and PSEUDO RESPONSE REGULATOR 5 (PRR5). Both TOC1 and PRR5 are core clock components and the degradation of these clock proteins by the ZTL family is an important regulation in the circadian clock (Más et al., 2003; Kiba et al., 2007) (see details in the next section). Both TOC1 and PRR5 levels might be very low in the *ZTL*/*LKP2* overexpressors. Coincidentally, the *toc1 prr5* double mutant flowering phenotype resembles the late-flowering phenotypes of the *ZTL* and *LKP2* overexpressors (Ito et al., 2008). In addition, both TOC1 and PRR5 indirectly affect expression of *CO* (Yanovsky and Kay, 2001; Nakamichi et al., 2007) and overexpression of *PRR5* represses *CDF1* transcription (Nakamichi et al., 2007), indicating that ZTL/LKP2 may regulate *CO* transcription through the functions of TOC1 and/or PRR5. Interestingly, each *toc1* and *prr5* single mutant phenotype (the early- and late-flowering phenotypes, respectively) is different from the *ZTL*/*LKP2* overexpressor phenotypes (Nakamichi et al., 2007; Niwa et al., 2007). This suggests that both TOC1 and PRR5 levels should be low in the *ZTL*/*LKP2* overexpressors in order to explain their flowering phenotype by TOC1 and PRR5 flowering function.

Recently, Takase et al. (2011) reported yet another possible explanation for the *ZTL* and *LKP2* overexpression phenotype. The Kelch repeat domains in ZTL and LKP2 interact with FKF1

in yeast and *in vitro* (Takase et al., 2011). The authors showed that ZTL and LKP2 exclude FKF1 from the nucleus in *Arabidopsis* protoplast. In addition, overexpression of the LKP2 Kelch repeats is sufficient to reduce *CO* and *FT* expression under LD conditions, leading to late flowering. Since repression of *CO* expression in *fkf1* mutants is less severe than that in *ZTL* and *LKP2* overexpressors, it is difficult to explain the phenotype of the overexpressors by this mechanism alone. Thus, several mechanisms may regulate *CO* expression in *ZTL* and *LKP2* overexpressors.

Since loss of function of the entire *ZTL* family and overexpression of *ZTL* and *LKP2* both cause *CO* mRNA levels to be low throughout the day, the mechanisms of ZTL and LKP2-dependent *CO* regulation could be indirect and achieved by several different mechanisms. These results indicate that specific stoichiometries of ZTL, LKP2, FKF1, and possibly GI may be important for balancing the proper ratio for the formation of each complex, each of which has a different function, and regulating this balance may be crucial for achieving the proper expression of *CO*.

REGULATION OF THE CIRCADIAN CLOCK BY ZTL/FKF1/LKP2 PROTEINS

Similarly to photoperiodic flowering regulation, all ZTL/FKF1/LKP2 proteins are involved in the regulation of the circadian clock in *Arabidopsis* as well. However, among these, protein turnover of clock components mediated by ZTL is a principal mechanism for ZTL/FKF1/LKP2-dependent progress of the circadian clock. The *ztl* mutants exhibit a longer period phenotype under constant light conditions (Somers et al., 2000) and this long-period phenotype is mainly caused by the increased stability of the TOC1 core clock protein (Más et al., 2003) (Figure 3B). The ZTL protein also targets the PRR5 core clock protein for proteasome-dependent degradation through SCF^{ZTL} (Kiba et al., 2007) (Figure 3B). The ZTL LOV domain plays a crucial role in the degradation of TOC1 and PRR5 proteins (Más et al., 2003; Kiba et al., 2007). Despite amino acid sequence similarities with TOC1 and PRR5, the other PRRs (PRR3, PRR7, and PRR9), all of which are involved in circadian clock progression, are not targets of ZTL for degradation (Fujiwara et al., 2008). As with FKF1, blue light absorbed by the ZTL LOV domain enables ZTL to form a protein complex with GI during the day (Kim et al., 2007). As GI protein abundance robustly oscillates with the peaks in the afternoon, the ZTL–GI complex reaches its maximum quantity in the afternoon (Kim et al., 2007). This interaction stabilizes ZTL protein; therefore, ZTL protein is highly stable in the afternoon (Kim et al., 2007). Fujiwara et al. (2008) have proposed a possible mechanism in which the light-dependent ZTL–GI interaction separates ZTL protein from TOC1 and PRR5 and consequently protects both TOC1 and PRR5 from ZTL-dependent degradation from the active SCF^{ZTL} complex in the afternoon (Fujiwara et al., 2008).

In addition to the GI-dependent stabilization of ZTL, Kim et al. (2011) reported another mechanism by which HEAT SHOCK PROTEIN 90 (HSP90) affects ZTL protein stability. HSP90 functions as a molecular chaperone and binds to ZTL to facilitate maturation of the ZTL protein (Kim et al., 2011). This interaction affects TOC1, PRR5, and ZTL stabilities in a way that is not light-dependent. Reduction in HSP90 activity by geldanamycin (HSP90 inhibitor) treatment reduces ZTL accumulation and simultaneously increases TOC1 and PRR5 levels (Kim et al., 2011). It seems that GI and HSP90 function in the same ZTL-stabilization mechanism, because reduced HSP90 activity does not further decrease ZTL levels in the *gi* mutants. In addition, FKF1 protein levels are reduced when HSP90 activity is lower, indicating that the same mechanism may stabilize FKF1 and possibly LKP2 (Figure 3B).

Unlike the *ztl* mutants, *lkp2* and *fkf1* single mutants do not exhibit an obvious long-period clock phenotype (Baudry et al., 2010). Using all possible combinations of *ztl*, *fkf1*, *lkp2* double and triple mutants, Baudry et al. (2010) described the overlapping roles of FKF1 and LKP2 with ZTL in the circadian clock. The *fkf1* mutation enhances the longer period phenotype of the *ztl* mutant when these two mutations are combined. In addition, compared to the *ztl* single mutant, adding the *lkp2* and *fkf1* mutations to the *ztl* mutant background reduces expression of morning clock genes, such as *LATE ELONGATED HYPOCOTYL* (*LHY*) and *PRR9* (Baudry et al., 2010). This is most likely due to increased levels of TOC1 and PRR5 stability. Indeed, significant stabilization of PRR5 and TOC1 proteins in the *ztl lkp2*, *ztl fkf1* double, and *ztl lkp2 fkf1* triple mutants was reported (Baudry et al., 2010; Wang et al., 2010). These data indicate that, together with ZTL, both LKP2 and FKF1 may also contribute to the ubiquitin-dependent degradation of TOC1 and PRR5 (Figure 3B). It is noteworthy that the *ZTL* promoter-driven *ZTL* and *LKP2*, but not *FKF1*, rescued the *ztl* circadian phenotype (Baudry et al., 2010), indicating that the molecular mechanisms by which ZTL and FKF1 regulate the stabilities of the circadian clocks might not be simply comparable.

FUTURE PERSPECTIVES

Knowledge of the molecular function of ZTL/FKF1/LKP2 proteins has accumulated in the past decade. In the area of photochemistry, only the FKF1 LOV domain has been analyzed during recent years. The ZTL LOV and LKP2 LOV domains should be analyzed as well. It will be interesting to determine whether various combinations of LOV dimers have similar photochemical properties, or whether unique pairings serve unique functions. Although the crystal structures of the LOV, F-box, and Kelch repeat domains within the ZTL/FKF1/LKP2 protein family are known, we do not understand how these domains are spatially localized within a molecule and whether there are intramolecular interactions between domains. Analysis of the blue-light-induced photocycle using the ZTL/FKF1/LKP2 recombinant proteins containing not only the LOV domain but also other domains may facilitate further understanding of the molecular properties of the ZTL/FKF1/LKP2 protein family.

The ZTL/FKF1/LKP2 proteins seem to possess both overlapping and contrasting roles in the circadian clock and

photoperiodic flowering. The contributions of these three proteins to either regulation of period length or degradation of CDF proteins may be partially explained by the absolute amount (=copy number) of protein (Fornara et al., 2009; Baudry et al., 2010). However FKF1 protein cannot rescue the *ztl* long-period phenotype and the flowering phenotype of *ZTL* overexpressors is similar to that of *fkf1* mutants (Somers et al., 2004; Baudry et al., 2010). These results clearly indicate that ZTL and FKF1 possess distinct functions that are not interchangeable. In addition, FKF1, LKP2, and ZTL proteins all interact with GI through their LOV domains *in planta* (Kim et al., 2007). It will be exciting to determine how affinities between each light-activated LOV domain and GI differ, and whether these affinities contribute to their unique functions in regulation of photoperiodic flowering or/and the circadian clock.

In addition, our knowledge of the light-dependent protein–protein interaction facilitated the creation of an *in vivo* molecular light switch. Blue-light-dependent FKF1–GI interaction was recently utilized in an optogenetic application to regulate gene expression and protein subcellular localization in a light-dependent manner in mammalian cells (Yazawa et al., 2009). This relatively long-lasting molecular switch mediated by the stable FKF1–GI interaction could be a useful tool in cell biology and other fields, in contrast to other, more transient, photoreceptor-based tools (Moglich and Moffat, 2010).

During the last decade, we have learned a lot about the molecular nature and functions of the newly identified light-regulated ZTL, FKF1, and LKP2 proteins. Since the spatial expression patterns of ZTL, FKF1, and LKP2 largely overlap (Kiyosue and Wada, 2000; Nelson et al., 2000; Yasuhara et al., 2004) and their LOV domains form homo- and heterodimers (Takase et al., 2011), the next challenge could be to figure out the potential functional differences of different dimer combinations and whether certain dimers are preferentially formed under certain conditions or in a specific pathway. In addition, it is likely that accessory proteins besides GI and components of the SCF machinery exist that affect their function *in vivo*. Moreover, detailed analyses of the intercellular distribution of these proteins *in vivo* would aide deciphering of the role of each protein. Finally, to accurately understand the function of the ZTL/FKF1/LKP2 proteins, we need to describe when and where these proteins and their complexes are formed at the whole plant level as well as specific tissues.

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