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Original Paper

Pleiotropic phenotype of transgenic *Arabidopsis* plants that produce the LOV domain of LOV KELCH PROTEIN2 (LKP2)

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Abstract LOV KELCH PROTEIN2 (LKP2) is a blue-light receptor protein composed of three functional domains: a light, oxygen, or voltage (LOV) domain, an F-box motif (F), and Kelch repeats. LKP2 is postulated to be a component of an SCF complex and function in ubiquitination of proteins that control the circadian clock and photoperiodic flowering. Transgenic *Arabidopsis* plants that produce LOV, F, or a combination of LOV and F fused to green fluorescent protein (named GL, GF, and GLF, respectively) were produced using constructs containing the *Cauliflower mosaic virus 35S* promoter. Under continuous white light, the circadian rhythms of control and GF plants were similar, whereas those of GL and GLF plants were shorter. Under continuous red light, the hypocotyl lengths of control and GF seedlings were similar, whereas that of GL seedlings was longer. Late flowering and down-regulation of *CONSTANS* and *FLOWERING LOCUS T* were observed in GL and GLF plants compared to GF and control plants under long-day conditions. These results suggest that the previously reported pleiotropic phenotype of LKP2-overproducing plants, which show altered circadian rhythm, hypocotyl elongation, and photoperiodic flowering, is not only due to the promotion of ubiquitination and subsequent degradation of substrate proteins of the SCF^{LKP2} complex but may also be due to the functional disruption of regulatory proteins that interact with LKP2 LOV.

Key words: *Arabidopsis*, circadian rhythm, flowering time, hypocotyl elongation, LOV KELCH PROTEIN2 (LKP2).

Plants perceive light not only as the energy source for photosynthesis but also as an environmental stimulus that controls plant growth and development. Multiple photoreceptors including phytochromes, cryptochromes, and phototropins perceive environmental light signals in *Arabidopsis* (Kendrick and Kronenberg 1994; Nagatani 2010). In addition to these photoreceptors, members of the FLAVIN-BINDING KELCH REPEAT F-BOX1 (FKF1)/LOV KELCH PROTEIN2 (LKP2)/ZEITLUPE (ZTL) family are blue-light photoreceptors and are involved in regulation of the circadian clock, hypocotyl elongation, and flowering time (Jarillo et al. 2001; Kiyosue and Wada 2000; Miyazaki et al. 2011; Nelson et al. 2000; Schultz et al. 2001; Somers et al. 2000; Takase et al. 2011). *Arabidopsis* FKF1/LKP2/ZTL family proteins have three functional domains—the light, oxygen, or voltage (LOV) domain, F-box motif, and Kelch repeat—and function in ubiquitination of target proteins as components of the SKP1-Cullin-Rbx1-F-box protein (SCF) E3 ligase complex (Demarsy and Fankhauser 2009). The SCF^{ZTL} complex regulates the circadian clock by ubiquitin-dependent degradation of two circadian clock regulators, TIMING OF CAB EXPRESSION1 (TOC1) and PSEUDO-RESPONSE REGULATOR5 (PRR5) (Kiba et al. 2007; Kim et al. 2007). The SCF^{FKF1} complex regulates flowering time under long-day (LD) conditions by ubiquitin-dependent degradation of CYCLING DOF FACTOR (CDF) proteins, which are repressors of *CONSTANS* (*CO*) expression (Imaizumi et al. 2003, 2005; Sawa et al. 2007).

The LOV domains of FKF1/LKP2/ZTL family members are involved in blue-light perception by binding flavin mononucleotide, which functions as a chromophore, and by interaction with several regulatory proteins. The FKF1 LOV domain forms homodimers (Zikihara et al. 2006) and functions in the regulation of blue-light-dependent ubiquitination of CDF1 (Imaizumi et al. 2005). The degradation of ubiquitinated CDFs by

Abbreviations: ACT2, ACTIN2; 3AT, 3-amino-1*H*-1,2,4-triazole; CDF, CYCLING DOF FACTOR; CO, CONSTANS; FKF1, FLAVIN-BINDING KELCH REPEAT F-BOX1; FT, FLOWERING LOCUS T; GFP, green fluorescent protein; GI, GIGANTEA; LD, long-day; LKP2, LOV KELCH PROTEIN2; LOV, light, oxygen, or voltage; PRR5, PSEUDO-RESPONSE REGULATOR5; SCF, SKP1-Cullin-Rbx1-F-box protein; T-DNA, transfer DNA; TOC1, TIMING OF CAB EXPRESSION1; ZT, Zeitgeber time; ZTL, ZEITLUPE.

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26S proteasomes leads to the activation of *CO* expression, which triggers the expression of *FLOWERING LOCUS T* (*FT*), a florigen gene (Imaizumi et al. 2005). The interaction of FKF1 with GIGANTEA (GI) through FKF1 LOV is required for this activation of *CO* expression (Sawa et al. 2007). FKF1 also interacts with CO through its LOV domain in a blue-light-enhanced manner; this interaction stabilizes CO in the LD afternoon (Song et al. 2014).

ZTL interacts with TOC1 and PRR5 through its LOV domain (Kiba et al. 2007; Más et al. 2003). The ZTL LOV domain also interacts with GI. This interaction is blue-light dependent and stabilizes ZTL protein (Kim et al. 2007). Production of ZTL LOV in transgenic *Arabidopsis* causes period lengthening under red- or blue-light conditions, similar to *ztl* loss-of-function mutations, and a decreased level of endogenous ZTL protein compared to that in the control (Kim et al. 2013; Somers et al. 2000). These results suggest that production of ZTL LOV reduces endogenous ZTL protein stability by interfering with the interaction between endogenous ZTL and GI, and that the reduction in ZTL levels leads to period lengthening. Furthermore, production of ZTL LOV results in elongated hypocotyls under red- or blue-light conditions and in delayed flowering under LD conditions, similar to overproduction of ZTL (Kiyosue and Wada 2000; Somers et al. 2004). These phenotypes are postulated to be caused by the inhibition of GI functions in nuclei due to enhancement of cytosolic GI distribution caused by the ZTL LOV–GI complex (Kim et al. 2013).

LKP2 shows high amino acid sequence similarity to ZTL, and the tissue-specific expression pattern of *LKP2* overlaps largely with that of *ZTL* (Kiyosue and Wada 2000; Yasuhara et al. 2004). *LKP2*-overexpressing transgenic plants phenocopy *ZTL*-overexpressing transgenic plants: both exhibit circadian rhythm defects under continuous light or in the dark, elongated hypocotyls under continuous white or red light, and delayed flowering under LD conditions compared to control plants (Nelson et al. 2000; Schultz et al. 2001). Transfer DNA (T-DNA) insertion mutants of *lkp2* are phenotypically similar to wild-type plants (Baudry et al. 2010; Takase et al. 2011), while *ztl* T-DNA insertion mutants show slower circadian rhythms under continuous red or blue light or in the dark, shorter hypocotyls under continuous red light, and earlier flowering under non-inductive photoperiodic conditions compared to control plants (Somers et al. 2004; Takase et al. 2011). The phenotypic similarity of *lkp2* plants with wild-type plants is postulated to be due to the very low expression of *LKP2* relative to that of *ZTL* (Baudry et al. 2010; Michael et al. 2008; Mockler et al. 2007). Introduction of *LKP2* cDNA controlled by the *ZTL* promoter complemented the long-period phenotype of a *ztl* mutant (Baudry et al. 2010). These results suggest that LKP2 possesses ZTL-like functions with respect to regulation of the circadian clock, hypocotyl elongation and flowering time in *Arabidopsis*.

Similar to the overexpression of *LKP2*, production of green fluorescent protein (GFP)-LKP2 in *Arabidopsis* also causes arrhythmicity of the circadian clock, lengthens hypocotyls in continuous white light, and delays flowering under LD conditions (Miyazaki et al. 2011). In this study, we analyzed the effects of production of the GFP-tagged LOV and F-box of LKP2 on the circadian clock, hypocotyl elongation, and flowering time regulation in *Arabidopsis*, and discussed a LOV-mediated function of LKP2.

Materials and methods

Plant materials and growth conditions

All plant material was generated using the Columbia accession of *Arabidopsis thaliana*. Hypocotyl length was measured as described previously (Takase et al. 2004). *Arabidopsis* seeds were surface sterilized and placed on GM medium (Valvekens et al. 1988) supplemented with 0.8% agar. Plates were kept at 4°C for 7 days and then transferred to continuous white light at 22°C for 8 h to induce germination. After induction of germination, the plates were transferred to monochromatic light or to darkness. Blue, red, or far-red light was generated by light-emitting diodes at 450, 660, or 750 nm, respectively (NK Systems, Tokyo, Japan). Fluence rates were measured with a radiometer (model LI-189; LI-COR, Inc., Lincoln, NE, USA). Hypocotyl length was measured with Scion Image software (Scion Corp., Frederick, MD, USA). To determine the flowering time, *Arabidopsis* seeds were sown on vermiculite in pots. Pots were cold-treated at 4°C for 3 days and then transferred to LD conditions (16-h light/8-h dark) at 22°C. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA).

Vector construction and production of transgenic plants

Three constructs encoding the LKP2 LOV domain, F-box motif, and a region that includes both the LOV domain and F-box motif were generated. PCR primers are listed in Table S1. The PCR template was cDNA from *A. thaliana* accession Columbia. Each PCR fragment was subcloned into pCR4-TOPO (Invitrogen, Carlsbad, CA, USA). After the entire inserts were sequenced, they were excised with *Bgl*II and *Bam*HI and subcloned into the *Bgl*II site of pCR4-TOPO containing the S65T GFP coding sequence (Niwa et al. 1999). The resulting vectors were then digested with *Bam*HI, and all fragments (encoding S65T GFP and LKP2 domains) were subcloned into the *Bam*HI site of the pBE2113 binary vector (Mitsuhara et al. 1996). The resulting vectors were introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating (Figurski and Helinski 1979) and subsequently into *Arabidopsis*

by the floral-dip method (Clough and Bent 1998). Homogygous T_4 progeny lines were obtained and used for the experiments.

Immunoblot analysis

Protein extraction from rosette leaves and immunoblot analysis were performed as described previously (Takase et al. 2011). Immunodetection was performed using an anti-GFP antibody (Nakalai Tesque Inc., Kyoto, Japan) and an ECL Advance system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Chemiluminescence signals were captured using a light-capture system (model AE-6792; ATTO, Tokyo, Japan).

Fluorescence microscopy

GFP signals from stomatal guard cells in leaf epidermal peels were observed using an IX71 microscope with a standard GFP filter (Olympus, Tokyo, Japan). The images were captured using MetaMorph imaging software (Universal Imaging Corporation, West Chester, PA, USA). A transgenic *Arabidopsis* plant expressing GFP with a nuclear localization signal of SV40 at the N-terminus (NLS-GFP) (Chiu et al. 1996; van der Krol and Chua 1991) was used as a control for nuclear localization.

Bioluminescence analysis

To measure circadian rhythms, plants from homozygous lines of *CAB2:LUC* and *CCR2:LUC* (seeds provided by Dr. S.A. Kay, University of California, San Diego) were crossed to plants from homozygous lines of GL, GF, GLF and GFP-producing plants. The resulting F_1 seedlings were grown on GM agar plates at 22°C under 12-h light/12-h dark cycles for 4 days and then transferred to continuous white light. An automated luminometer was used to monitor luciferase activity (Okamoto et al. 2005a), and circadian period length in the individual transgenic plants was estimated by visual identification of peaks and troughs followed by analysis with a rhythm-analysis program (Okamoto et al. 2005b).

Quantitative RT-PCR

Plates were incubated at 22°C under 16-h light/8-h dark for 10 days, and then seedlings were harvested every 4 h over a 24-h period. Total RNA from the whole seedlings was extracted and 500 ng was used to synthesize cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as previously described by Takase et al. (2011). Quantitative RT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and an ABI PRISM 7000 sequence detection system (Applied Biosystems). The expression levels of *ACTIN2* (*ACT2*) were used to normalize expression of the target genes between samples. The primer pairs used in the PCR analysis are listed in Table S1.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed as reported previously (Yasuhara et al. 2004). The Columbia version of full-length cDNA for *GI* was obtained by RT-PCR with ReverTra Dash (Toyobo, Osaka, Japan) and *GI* primers listed in Table S1,

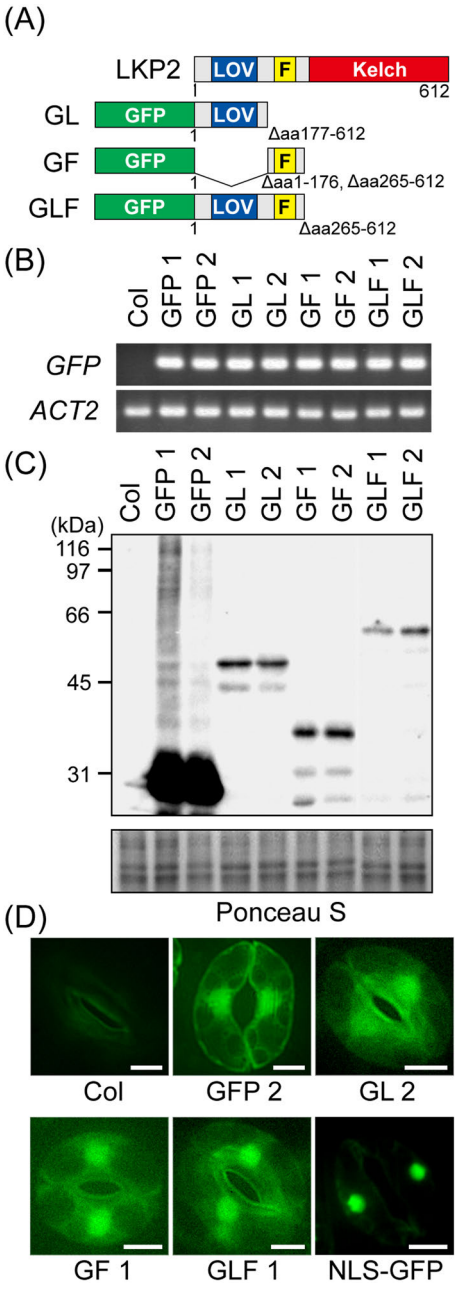

Figure 1. Structure of the GL, GF, and GLF proteins and their production in transgenic plants. (A) Schematic diagram of the GL (containing the LKP2 LOV domain), GF (containing the LKP2 F-box motif), and GLF (containing the LKP2 LOV and F-box) proteins used in this study. All of the domains were produced as C-terminal fusions to S65T green fluorescent protein (GFP). Amino acid positions correspond to those in the native LKP2 protein sequence. (B) Transgene expression in *Arabidopsis thaliana*. *GFP* primers were used to detect transgene expression by RT-PCR. Actin (*ACT2*) primers were used as the control. (C) Accumulation of GFP, GL, GF, and GLF proteins in transgenic plants. These proteins were detected by immunoblotting (top panel) using an anti-GFP antibody. Ponceau S staining of the membrane (bottom panel) was used as the loading control. (D) Subcellular localization of GFP, GL, GF, and GLF proteins in stomatal guard cells of the transgenic plants. Each panel shows a representative GFP image from the transgenic plants and non-transgenic Columbia (Col) plants. An NLS-GFP plant (containing a nuclear localization signal fused to GFP) was used as a control for the nuclear localization. Scale bars=20 μm.

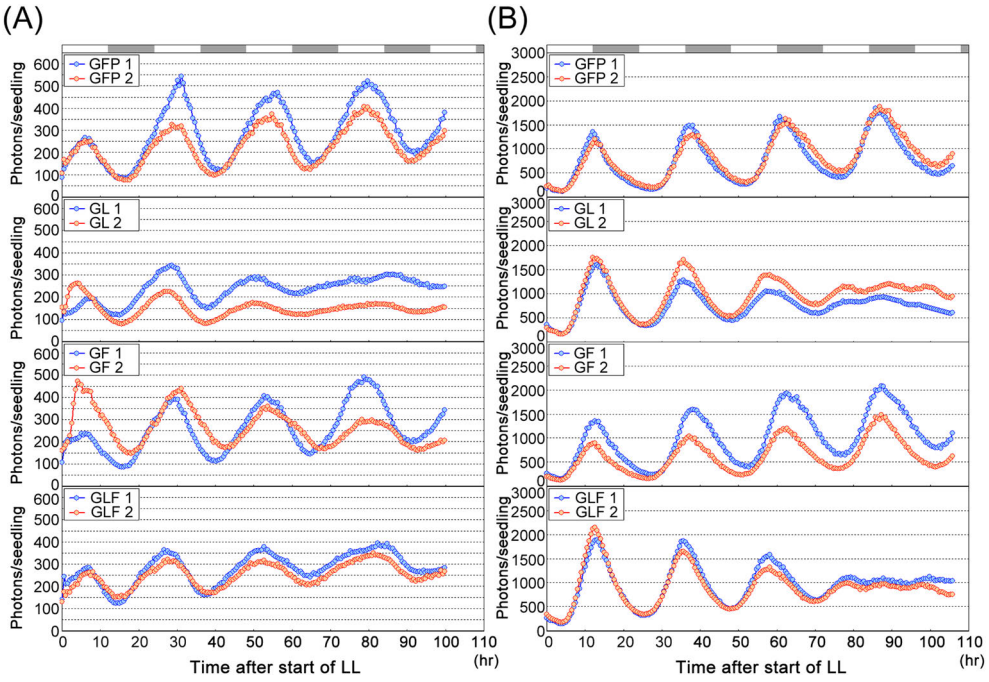

Figure 2. Circadian rhythms of GFP, GL, GF, and GLF transgenic plants under continuous white-light conditions. Graphs show luciferase activity driven by the *CAB2* (A) or *CCR2* (B) promoter under continuous white light (LL) conditions. All transgenic plants were entrained in 12-h light/12-h dark for 4 days and then transferred to LL conditions. White and gray boxes indicate subjective day and subjective night, respectively.

sequenced for verification, and then cloned into the *Bam*HI site of pGBKT7 (Clontech, Palo Alto, CA, USA), which encodes the GAL4 DNA-binding domain (bait). Fragments encoding the LOV, F-box, and Kelch repeat of LKP2 were cloned individually into pGADT7 (Clontech), which encodes the GAL4 activation domain (prey). AH109 yeast cells (Clontech) were used for the assay. Yeast transformants were grown on synthetic complete (SD) medium that lacked leucine (L) and tryptophan (W). Transformants were assayed on SD medium that lacked adenine (A), histidine (H), L, and W but was supplemented with 3-amino-1*H*-1,2,4-triazole (3AT) at 15 mM to repress the basal activity of the HIS3 reporter gene. Yeast containing two plasmids from Clontech, one plasmid encoding amino acids 72–390 of murine p53 protein (GenBank accession K01700) and the GAL4 DNA-binding domain and the other plasmid encoding the SV40 large T antigen (GenBank locus SV4CG) and the GAL4 activation domain, was used as the positive control. Interaction between GI and the LKP2 domain encoded by the prey vector was indicated by colony growth on the SD-AHLW + 3AT medium.

Results

Construction of GL, GF, and GLF transgenic plants

Three fusion genes that encode the LKP2 LOV domain (GL), the F-box motif (GF), or a combination of the LOV domain and F-box motif (GLF) attached to the C-terminus of GFP were constructed (Figure 1A) and expressed in transgenic *Arabidopsis* plants under the control of the *Cauliflower mosaic virus 35S* promoter. Two homozygous lines were examined for each construct (e.g., for GL, the lines are designated GL 1 and GL 2). The expression of each transgene was confirmed by RT-PCR (Figure 1B), and the accumulation of GL, GF, and GLF proteins of the predicted sizes was detected by immunoblotting (Figure 1C). The subcellular localization of these proteins in the transgenic plants was analyzed microscopically (Figure 1D). Fluorescence signals for GL, GF, and GLF were detected in the nucleus and cytoplasm of stomatal guard cells, while those for GFP fused with a nuclear localization signal at the N-terminus (NLS-GFP) were detected only in the nucleus.

Circadian rhythm phenotypes of transgenic plants

CAB2:LUC and *CCR2:LUC* reporters were used to measure the circadian rhythms of the transgenic plants (Figure 2 and Table S2). Both GL and GLF plants had shorter circadian rhythms (e.g., GL 1: 21.3 h, GL 2: 22.9 h; GLF 1: 23.3 h, and GLF 2: 22.7 h for *CAB2:LUC*) than control plants (e.g., GFP 1: 24.2 h and GFP 2: 24.3 h for *CAB2:LUC*) under continuous white light conditions, while the period lengths of GF plants were similar to those of control plants (e.g., GF 1: 24.5 h and GF 2: 24.5 h for *CAB2:LUC*). A decrease in amplitude over time was obvious in GL plants for both reporters and in GLF plants for the *CCR2:LUC* reporter.

Hypocotyl elongation of transgenic plants

Hypocotyl lengths were measured under continuous red (cR), blue (cB), and far-red (cFR) light and in darkness

(Figure 3 and Table S3). GFP fused to LKP2 (GLFK; Miyazaki et al. 2011) was used as a positive control. The GL, GF, and GLF seedlings and one GLFK seedling did not show significant differences in hypocotyl length in darkness compared to control (GFP) seedlings. Both GLFK lines showed longer hypocotyls under cR (0.052–1.005 W m^{-2}), cB (0.052–0.225 W m^{-2}) and cFR (0.103 W m^{-2}) than control seedlings; in particular, the GLFK hypocotyls were longer than those of any other lines under cR at 0.052 and 0.204 W m^{-2}. Both GL lines showed longer hypocotyls under cR at 0.099 and 1.005 W m^{-2} than did control seedlings. By contrast, the hypocotyl lengths of GF or GLF seedlings (at least one line per light treatment) were not significantly different from those of control seedlings under any continuous light condition examined.

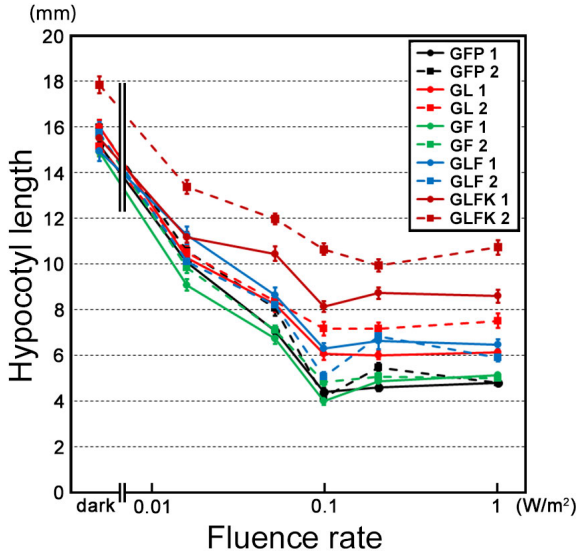

Figure 3. Hypocotyl length of GFP, GL, GF, GLF, and GFP-LKP2 (GLFK) transgenic plants under continuous red (cR) light. Seedlings were grown for 5 days under cR with various fluence rates (W m^{-2}). Each point represents the mean hypocotyl length of approximately 40 seedlings. Error bars represent standard error of the mean.

Flowering phenotypes of transgenic plants under LD conditions

Flowering times were measured under LD conditions (Figure 4). GF plants bolted after 1 month and did not show significant differences in bolting time compared with control plants. The rosette leaf numbers at bolting of GF and control plants were also not significantly different. By contrast, GL and GLF plants bolted later and had more rosette leaves at bolting than the control plants.

The expression levels of four flowering-time genes that function in the photoperiodic pathway (*FT*, *CO*, *CDF1*, and *GI*) were examined in the transgenic plants grown under LD conditions (Figure 5). Both *FT* and *CO* expression patterns in GF and control plants were similar, while those in GL and GLF plants were different. Specifically, the peak of *CO* expression at Zeitgeber time (ZT)12 and the peak of *FT* expression at ZT16, both of which were observed in GF and control plants, were absent in GL and GLF plants. By contrast, the gene expression patterns of *CDF1* and *GI* were similar among the four genotypes. These results suggest that the late flowering of GL and GLF plants is due to the down-regulation of *CO* at dusk and subsequent down-regulation of *FT*.

Interaction of LKP2 LOV with GI

Interaction of ZTL LOV with GI is postulated as a reason for the pleiotropic phenotype of ZTL LOV-producing plants (Kim et al. 2013). Though interaction between LKP2 and GI has been demonstrated *in vitro* and in yeast (Kim et al. 2007), the binding site in LKP2 has not previously been reported. Therefore, two-hybrid

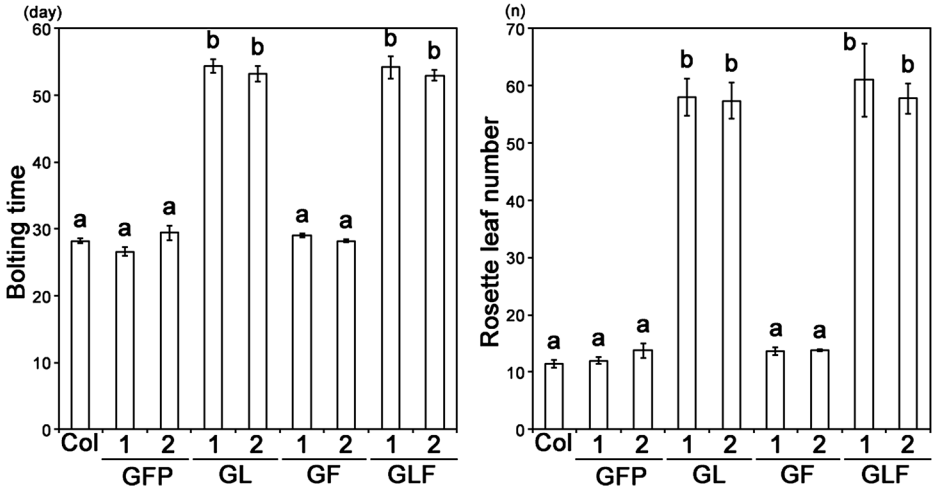

Figure 4. Flowering time of GFP, GL, GF, and GLF transgenic plants under LD conditions. Graphs show bolting time (left panel) and rosette leaf number at bolting (right panel) in transgenic plants under 16-h light/8-h dark conditions. Values are means and standard errors (n=5). Different letters indicate statistical differences detected by one-way analysis of variance (ANOVA) and Tukey–Kramer test (p<0.05).

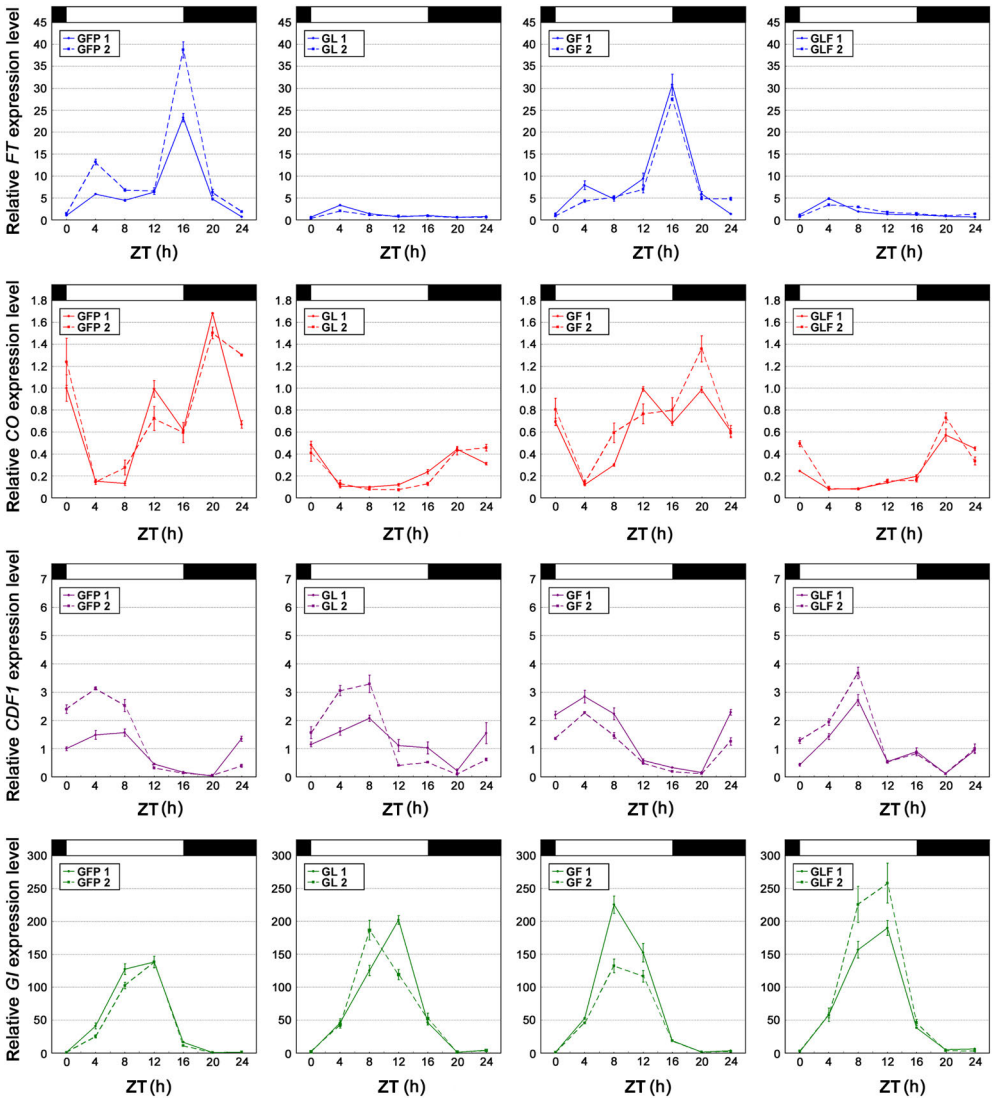

Figure 5. Expression of flowering-time genes in GFP, GL, GF, and GLF transgenic plants under 16-h light/8-h dark conditions. Relative expression levels of the flowering-time genes *FT*, *CO*, *CDF1* and *GI* were normalized to *ACT2* expression. Light and dark periods are indicated by white and black boxes, respectively. Values are means and standard error (n=3).

interaction of LKP2 LOV with GI was examined in yeast (Figure 6). GI interacted with LKP2 LOV, but not with the LKP2 F-box or Kelch repeat region.

Discussion

A fusion protein consisting of GFP and LKP2 was previously shown to be localized in the nucleus and cytoplasm of *Arabidopsis* cells (Takase et al. 2011). Consistently, the GL, GF, and GLF proteins were also localized in the nucleus and cytoplasm (Figure 1D), suggesting that the differences in phenotype among GL, GF, and GLF plants were not due to the subcellular localization of these three proteins.

Kim et al. (2013) showed that production of LOV or LOV-F of ZTL caused circadian rhythm lengthening under red- or blue light. This long-period phenotype is postulated to be due to instability of ZTL caused by interaction between ZTL LOV and GI. In contrast to ZTL LOV-producing plants, both GL and GLF plants showed short-period phenotypes under continuous white light compared to the control (Figure 2 and Table S2). The contrasting effects of ZTL LOV and LKP2 LOV in clock regulation could be due to differences between some of their interacting proteins and/or due differing affinities to interacting proteins, even though ZTL LOV and LKP2 LOV interact with overlapping sets of proteins. Both ZTL and LKP2 bind to GI (Kim et al. 2007), and both ZTL LOV and LKP2 LOV interact with two core clock proteins, TOC1 and PRR5 (Baudry et al. 2010; Yasuhara et al. 2004). The short-period phenotypes in both GL and GLF plants are phenocopies of *gi*, *toc1*, and *prr5* mutants (Mizoguchi et al. 2005; Nakamichi et al. 2005; Somers et al. 1998). Therefore, production of LKP2 LOV

might disrupt the function of clock components that can interact with LKP2 LOV and subsequently result in short circadian rhythm.

The elongated hypocotyl of GL plants is a phenocopy of that seen in LKP2 overproducers (Miyazaki et al. 2011). Hypocotyl elongation is enhanced under cR and cB by ZTL LOV production (Kim et al. 2013). As for LKP2 LOV, enhancement was obvious under cR but not under cB (Figure 3 and Table S3), though one GL line showed a significant difference from control seedlings under cB (0.102 W m^{-2} [GL 2] and 0.225 W m^{-2} [GL 1]; Table S3). Therefore, the effect of LKP2 LOV or ZTL LOV production on hypocotyl length seems to be fundamentally similar. The lower level of GLF protein accumulation in GLF seedlings compared with that of GL protein in GL seedlings might be a reason for the absence of obvious enhancement of hypocotyl elongation in GLF seedlings (Figure 1C).

Under LD conditions, production of LKP2 LOV delayed flowering and down-regulated *CO* and *FT* expression compared to control plants (Figures 4 and 5). Overproduction of LKP2 or production of LKP2 Kelch also delayed flowering and down-regulated *CO* and *FT* expression (Takase et al. 2011). LKP2 and LKP2 Kelch are postulated to capture FKF1 in the cytosol, which probably disturbs the ubiquitination and subsequent degradation of CDFs, which are repressors of *CO* and *FT* expression (Takase et al. 2011). Because LKP2 LOV cannot capture FKF1 in the cytosol (Takase et al. 2011), the mechanism for late flowering by LKP2 LOV production seems to be different from that by LKP2 Kelch. Thus, the late flowering caused by LKP2 overproduction is probably caused not only by capture of FKF1 in the cytosol but also by a LOV-mediated mechanism.

ZTL LOV facilitates cytosolic retention of GI protein by interacting with it (Kim et al. 2013). GI binds to FKF1 in the nucleus in a blue-light-dependent manner, and the GI–FKF1 complex induces expression of *CO* through ubiquitination of CDF1 (Sawa et al. 2007). Both LKP2 and LKP2 LOV can interact with GI (Figure 6; Kim et al. 2007). Given the effects of ZTL LOV production on cytosolic retention of GI (Kim et al. 2013), it is possible that the interaction between LKP2 LOV and GI enhances cytosolic GI distribution, which interferes with the formation of the GI–FKF1 complex in the nucleus and results in delayed flowering under LD conditions. This hypothesis is consistent with down-regulation of *CO* expression in both GL and GLF plants (Figure 4). GI and FKF1/LKP2/ZTL family proteins are postulated to form interrelated complexes that regulate CO stability for photoperiodic flowering (Song et al. 2014). Instability of CO could be another reason for late flowering of GL and GLF plants, in addition to the down-regulation of *CO*.

In this paper, we have shown that production of LKP2 LOV in transgenic *Arabidopsis* results in short circadian rhythm, elongated hypocotyl, and late flowering. This result provides additional evidence for the LOV-mediated functions of FKF1/LKP2/ZTL family proteins in plant growth and development.

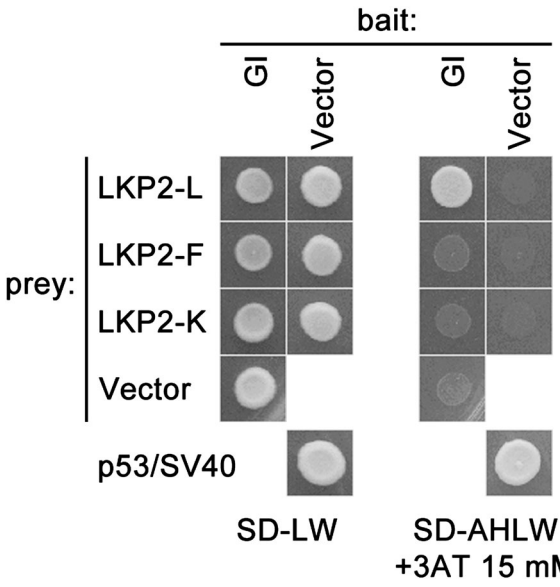

Figure 6. Yeast two-hybrid interactions between GI and three LKP2 fragments: LOV (LKP2-L), F-box (LKP2-F), and Kelch repeat (LKP2-K). Each panel in the "GI" columns shows yeast AH109 cells containing a GAL4 DNA-binding domain fusion of GI and either a GAL4 activation domain fusion of the indicated LKP2 fragment or the empty GAL4 activation domain expression vector, pGADT7 ("Vector"). The bait vector was a GAL4 DNA-binding domain expression vector, pGBKT7. Colonies were grown on SD agar medium lacking leucine and tryptophan (SD-LW; left) or lacking adenine, histidine, leucine, and tryptophan (SD-AHLW; right). The positive control (p53/SV40) contained one plasmid encoding amino acids 72–390 of murine p53 protein and the GAL4 DNA-binding domain and another plasmid encoding the SV40 large T antigen and the GAL4 activation domain.

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Table S1. PCR primers used in this study.

Table S2. Circadian rhythm of the GFP, GL, GF, and GLF plants under continuous light conditions. Average circadian periods with their standard errors of the mean were calculated from the data shown in Figure 3 by visual inspection of peaks and troughs analyzed with a rhythm-analysis program (Okamoto et al. 2005b).

Table S3. Comparison of hypocotyl lengths of GFP, GL, GF, GLF, and GFP-LKP2 plants under various light conditions. Seedlings were grown for 5 days under monochromatic light with various fluence rates (W m^{-2}). Each point represents the mean hypocotyl length of approximately 40 seedlings. Different letters indicate statistical differences detected by one-way analysis of variance (ANOVA) and the Tukey–Kramer test ($p < 0.05$).

Construction of transgenic plants	
Bam HI-GFPN	5′-GGATCCATGGTGAGCAAGGGCGAGG-3′
GFPC-*Bam* HI	5′-GGATCCTTAAGATCTCTTGTACAGCTCGTCCATGC-3′
Bgl II-LKP2[L]N	5′-AGATCTATGCAAAATCAAATGGAGT-3′
LKP2[L]C-*Bam* HI	5′-GGATCCTTAGGGCCCAGGCCTTCTAGGAATTT-3′
Bgl II-LKP2[F]N	5′-AGATCTATATCTCGCTCATTTACTTCT-3′
LKP2[F]C-*Bam* HI	5′-GGATCCTTACCTTTTTGCACCGGGAACAC-3′
Quantitative RT-PCR	
Actin2/8F	5′-GGTAACATTGTGCTCAGTGGTGG-3′
Actin2/8R	5′-AACGACCTTAATCTTCATGCTGC-3′
FT/253	5′-TATCTCCATTGGTTGGTGACTG-3′
FT/318	5′-GGGACTTGGATTTTCGTAACAC-3′
CO/19	5′-AACGACATAGGTAGTGGAGAGAACAAC-3′
CO/89	5′-GCAGAATCTGCATGGCAATACA-3′
CDF1/F	5′-AAGCTCTTTGGTATGAAAATTCCTTT-3′
CDF1/R	5′-GTTCCTGTCTTGTTTTGGTTCTTTTCTTC-3′
GI/3177	5′-GGTCGACGGTTTATCCAATCTA-3′
GI/3342	5′-CGGACTATTCATTCCGTTCTTC-3′
Yeast two-hybrid	
GI/F	5′-AGGATCCGTATGGCTAGTTCATCTTCATCT-3′
GI/R	5′-AGGATCCTTATTGGGACAAGGATATAGTAC-3′

Table S1. PCR primers used in this study.

	CAB2:LUC	CCR2:LUC
GFP 1	24.23±0.06	24.56±0.29
GFP 2	24.31±0.46	24.52±0.38
GL 1	21.34±0.17	21.68±0.65
GL 2	22.90±0.47	21.63±0.51
GF 1	24.46±0.40	24.70±0.16
GF 2	24.46±0.48	24.47±0.41
GLF 1	23.26±0.67	21.95±0.35
GLF 2	22.72±0.88	21.91±0.42

Table S2. Circadian rhythm of the GFP, GL, GF, and GLF plants under continuous light conditions. Average circadian periods with their standard errors of the mean were calculated from the data shown in Figure 3 by visual inspection of peaks and troughs analyzed with a rhythm-analyzing program (Okamoto et al.

Dark				
	n	AVE	S.E.	
GFP 1	45	15.2	0.3	a
GFP 2	42	16.0	0.3	a
GL 1	43	16.0	0.3	a
GL 2	32	15.1	0.4	a
GF 1	48	14.9	0.2	a
GF 2	51	15.7	0.3	a
GLF 1	38	14.9	0.4	a
GLF 2	38	15.7	0.5	a
GLFK 1	42	15.5	0.4	a
GLFK 2	51	17.8	0.4	b

cR	0.016				0.052				0.099				0.204				1.005			
	n	AVE	S.E.		n	AVE	S.E.		n	AVE	S.E.		n	AVE	S.E.		n	AVE	S.E.	
GFP 1	50	10.1	0.3	ab	52	7.1	0.2	ab	48	4.4	0.2	a	53	4.6	0.2	a	40	4.8	0.1	a
GFP 2	47	10.6	0.2	b	41	8.1	0.3	abc	51	4.2	0.1	a	45	5.5	0.2	abc	54	4.8	0.1	a
GL 1	39	10.3	0.3	ab	39	8.3	0.3	bc	43	6.1	0.2	b	41	6.0	0.2	bcd	35	6.1	0.3	bc
GL 2	47	10.5	0.3	ab	45	8.4	0.3	bc	39	7.2	0.3	cd	47	7.2	0.3	d	43	7.5	0.3	de
GF 1	62	9.1	0.2	a	61	6.7	0.2	a	50	4.0	0.2	a	42	4.9	0.3	ab	60	5.1	0.1	ab
GF 2	51	9.8	0.3	ab	51	7.1	0.2	ab	50	4.8	0.2	a	47	5.1	0.2	ab	49	5.0	0.1	ab
GLF 1	37	11.3	0.4	b	44	8.6	0.3	c	44	6.3	0.3	c	30	6.6	0.4	cd	42	6.5	0.2	cd
GLF 2	47	10.1	0.3	ab	38	8.2	0.2	bc	37	5.1	0.2	ab	60	6.8	0.2	d	37	5.9	0.2	abc
GLFK 1	64	11.2	0.2	b	53	10.5	0.3	d	64	8.1	0.2	d	68	8.7	0.2	e	60	8.6	0.3	e
GLFK 2	47	13.4	0.3	c	46	12.0	0.2	e	46	10.7	0.3	e	43	9.9	0.3	e	50	10.7	0.3	f

cB	0.020				0.052				0.102				0.225				1.030			
	n	AVE	S.E.		n	AVE	S.E.		n	AVE	S.E.		n	AVE	S.E.		n	AVE	S.E.	
GFP 1	47	12.3	0.3	ab	49	9.1	0.2	abc	49	6.3	0.2	ab	50	4.4	0.2	abc	52	2.7	0.1	ab
GFP 2	45	11.9	0.2	ab	47	9.0	0.3	abc	41	6.3	0.2	ab	43	4.6	0.2	abcd	48	2.4	0.1	ab
GL 1	55	12.8	0.2	bc	47	9.9	0.3	bcd	35	6.9	0.3	bc	37	6.0	0.3	ef	42	2.8	0.2	ab
GL 2	44	11.9	0.3	ab	38	10.0	0.3	cd	46	7.9	0.3	cd	39	5.6	0.3	def	47	2.6	0.1	ab
GF 1	69	11.0	0.2	a	59	8.0	0.2	a	74	5.4	0.2	a	69	3.7	0.1	a	62	2.4	0.1	a
GF 2	51	12.6	0.2	bc	50	9.3	0.3	abc	51	7.1	0.2	bc	48	4.9	0.2	bcde	48	2.4	0.1	ab
GLF 1	49	11.6	0.3	ab	39	9.1	0.3	abc	41	7.8	0.2	cd	39	5.4	0.2	cde	45	2.8	0.1	ab
GLF 2	42	12.2	0.3	ab	46	8.5	0.2	ab	37	6.6	0.3	abc	37	4.2	0.2	ab	49	2.5	0.2	ab
GLFK 1	60	12.2	0.2	ab	78	10.9	0.2	de	43	8.8	0.3	de	68	6.6	0.2	f	44	3.2	0.2	b
GLFK 2	49	13.6	0.2	c	51	11.8	0.3	e	39	10.4	0.2	e	46	7.4	0.3	g	54	3.0	0.2	ab

cFR	0.060				0.075				0.103				0.222			
	n	AVE	S.E.		n	AVE	S.E.		n	AVE	S.E.		n	AVE	S.E.	
GFP 1	50	11.8	0.3	b	51	10.7	0.3	bc	47	6.9	0.2	a	52	2.5	0.1	ab
GFP 2	47	12.6	0.3	ab	51	10.7	0.3	bc	48	7.5	0.2	ab	42	4.2	0.2	e
GL 1	43	12.0	0.4	ab	41	10.7	0.4	bc	45	8.7	0.2	bc	39	4.3	0.2	e
GL 2	47	11.5	0.4	ab	45	11.0	0.4	bc	42	8.9	0.3	c	37	3.1	0.1	abc
GF 1	74	10.4	0.2	a	72	8.9	0.2	a	45	6.5	0.2	a	78	3.2	0.1	bc
GF 2	50	12.3	0.3	b	53	11.6	0.2	cd	50	7.0	0.2	a	48	2.5	0.1	ab
GLF 1	41	11.4	0.3	ab	40	10.1	0.4	ab	47	7.6	0.2	ab	46	3.4	0.1	c
GLF 2	36	11.3	0.4	ab	46	10.4	0.3	bc	41	8.7	0.3	bc	43	2.4	0.1	a
GLFK 1	49	11.8	0.3	ab	52	11.2	0.2	bcd	43	9.9	0.3	c	52	4.1	0.2	de
GLFK 2	49	14.3	0.3	c	50	12.6	0.3	d	49	11.4	0.3	d	46	4.3	0.2	e

Table S3. Comparison of hypocotyl lengths of GFP, GL, GF, GLF, and GFP-LKP2 plants under various light conditions. Seedlings were grown for 5 days under monochromatic light with various fluence rates (W m−2). Each point represents the mean hypocotyl length of approximately 40 seedlings. Different letters indicate statistical differences detected by one-way analysis of variance (ANOVA) and the Tukey–Kramer test ($p<0.05$).