# Distinct roles of FKF1, GIGANTEA, and ZEITLUPE proteins in the regulation of CONSTANS stability in *Arabidopsis* photoperiodic flowering

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**Many plants measure changes in day length to synchronize their flowering time with appropriate seasons for maximum reproductive success. In *Arabidopsis*, the day-length–dependent regulation of CONSTANS (CO) protein stability is crucial to induce *FLOWERING LOCUS T* (*FT*) expression for flowering in long days. The FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) protein binds to CO protein specifically in the long-day afternoon and stabilizes it, although the mechanism remains unknown. Here we demonstrated that the FKF1-interacting proteins GIGANTEA (GI) and ZEITLUPE (ZTL) are involved in CO stability regulation. First, our immunoprecipitation-mass spectrometry analysis of FKF1 revealed that FKF1 forms an S-phase kinase-associated protein 1 (Skp1)/Cullin(CUL)/F-box complex through interactions with *Arabidopsis* Skp1-like 1 (ASK1), ASK2, and CUL1 proteins and mainly interacts with GI protein in vivo. GI interacts with CO directly and indirectly through FKF1. Unexpectedly, the *gi* mutation increases the CO protein levels in the morning in long days. This *gi*-dependent destabilization of CO protein was cancelled by the *fkf1* mutation. These results suggest that there are other factors likely influenced by both *gi* and *fkf1* mutations that also control CO stability. We found that ZTL, which interacts with GI and FKF1, may be one such factor. ZTL also interacts with CO in vivo. The CO protein profile in the *ztl* mutant resembles that in the *gi* mutant, indicating that ZTL activity also may be changed in the *gi* mutant. Our findings suggest the presence of balanced regulation among FKF1, GI, and ZTL on CO stability regulation for the precise control of flowering time.**

photoperiodic flowering | blue-light photoreceptor | E3 ubiquitin ligase | protein degradation

Plants have evolved the ability to anticipate upcoming seasons by monitoring photoperiod (or day-length) changes and to use this information to flower at the most appropriate time (1). In *Arabidopsis thaliana*, day-length–dependent induction of the *FLOWERING LOCUS T* (*FT*) gene, which encodes a floral-inductive mobile signal, determines flowering time (2). CONSTANS (CO) transcription factor directly activates *FT* transcription in long-day (LD) conditions (3). The regulation of the *CO* gene and protein expression by the circadian clock and light is crucial for day-length–dependent *FT* induction (4–6).

The FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) protein plays a key role in maintaining the proper photoperiodic expression patterns of both *CO* mRNA and CO protein (6–8). It possesses three functional domains: LOV (light, oxygen, or voltage), F-BOX, and Kelch repeats (9). The LOV domain absorbs blue light and is important for the blue-light–dependent interaction with GIGANTEA (GI) (8). The expression profiles of *FKF1* and *GI* genes are controlled by the circadian clock, and GI protein is required for the function of FKF1 in the regulation of *CO* transcription (8, 10, 11). The FKF1–GI complex mediates the ubiquitin-dependent degradation of CYCLING DOF FACTOR (CDF) proteins that represses the transcription of *CO* and *FT* genes (6–8). In this degradation mechanism, FKF1 recognizes CDF proteins by the Kelch repeat domain, and GI is required for this degradation (8).

CO protein stability is regulated by various light-signaling mechanisms throughout the day. CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) is a RING finger E3 ubiquitin ligase, and it forms a complex with SUPPRESSOR OF PHYA-105 1 (SPA1), SPA3, and SPA4. The COP1–SPAs complex degrades CO during the night (12, 13). Phytochrome B (PHYB) is a red/far-red light photoreceptor that facilitates CO protein degradation in the morning under red-light conditions, whereas PHYA enhances CO protein abundance in the afternoon under LD and far-red-light conditions (5). Cryptochrome 1 (CRY1) and CRY2 are blue-light photoreceptors that interact with SPA1, which in turn sequesters SPA1 from COP1 and then causes enhanced CO stability under blue light (14, 15). FKF1 binds to CO through the LOV domain in a blue light-enhanced manner and stabilizes CO specifically in the LD afternoon (6).

GI has multiple roles in the photoperiodic flowering pathway. First, GI interacts with the FKF1 homologs ZEITLUPE (ZTL) and LOV KELCH PROTEIN 2 (LKP2), proteins that, together with FKF1, synergistically degrade CDF2 protein (16, 17). Additionally, GI stabilizes FKF1 and ZTL proteins (16, 17). Second, nuclear GI protein forms a complex with *FT* repressors including SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO 1 (TEM1), and TEM2 *in planta*, although the biological relevance

## Significance

**In many plants, seasonal flowering is a crucial aspect of maximizing reproductive fitness. Changes in day length (or photoperiod) provide the most reliable cue that enables plants to anticipate approaching seasonal variation in the surrounding environment. The induction of the *FLOWERING LOCUS T* (*FT*) gene controlled by the light-stabilized CONSTANS (CO) protein is the key mechanism for photoperiodic flowering in *Arabidopsis thaliana*. The CO/FT module is highly conserved in many plant species, including major crops. In *Arabidopsis*, blue-light–signaling pathways are essential for the stabilization of CO protein. Here we show a unique role of GIGANTEA in photoperiodic flowering through the interactions with two homologous proteins, FLAVIN-BINDING, KELCH REPEAT, F-BOX1 and ZEITLUPE, which antagonistically control CO stability.**

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of these complexes remains unknown (18). Third, GI indirectly induces *FT* transcription through the microRNA172 (miR172) pathway (19). The expression of miR172 is positively regulated by GI and negatively regulated by SVP. miR172 targets the transcripts of *APETALA 2* (*AP2*)-related transcription factors including *SCHLAFMÜTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*), *TARGET OF EAT1* (*TOE1*), and *TOE2* and decreases their abundance (19, 20). Unlike its function in CDF2 degradation, ZTL acts as a negative regulator in photoperiodic flowering. A *ztl* mutant flowers early in short day (SD) conditions, and *ZTL* overexpression causes a delayed flowering concomitantly with the drastic decrease in *FT* expression in LD conditions (21). It seems that ZTL captures FKF1 in the cytosol by forming a heterodimer complex in *Arabidopsis* protoplast cells (22).

Although the roles of FKF1 in photoperiodic flowering are relatively well characterized, the biochemical properties of FKF1 still remain underexplored. The expression of FKF1 protein occurs in the afternoon; this timing is crucial for the timing of CO stabilization under the same conditions, although how FKF1 stabilizes CO protein remains elusive (6, 10). Here, we demonstrate that FKF1 tightly binds to GI and forms the SCF complex through interacting with *Arabidopsis* Skp1-like 1 (ASK1), ASK2, and CULLIN1 (CUL1) in vivo. In addition, GI binds to CO and indirectly destabilizes CO in the morning. One of the potential indirect mechanisms by which GI may regulate CO protein is changing ZTL stability. ZTL destabilizes CO protein by directly interacting with the protein in *Arabidopsis*. Our results indicate that proteins of the ZTL group and GI are involved in regulating CO protein stability.

## Results

**Identification of FKF1-Interacting Proteins in Vivo.** CO stabilization that occurs in the LD afternoon is crucial for photoperiodic flowering (5). We previously demonstrated that FKF1 regulates the timing of CO stability, although the mechanisms underlying this regulation remain largely unknown (6). To elucidate molecular mechanisms that may be involved in this regulation, we aimed to identify the FKF1-containing protein machinery. To identify the in vivo FKF1 complex, we used tandem affinity purification coupled with mass spectrometry (TAP-MS). We generated *pFKF1:FKF1-3F6H/fkf1* and *35S:FKF1-3F6H*/Col transgenic lines in which 3xFLAG and 6xHistidine-tagged FKF1 (FKF1-3F6H) is expressed under the control of the *FKF1* promoter (10) in *fkf1* (9) and under the control of the cauliflower mosaic virus (CaMV) *35S* promoter in WT plants. The transgene completely complemented the *fkf1* late-flowering phenotype, and the expression patterns of FKF1-3F6H protein in the *pFKF1:FKF1-3F6H/fkf1* lines showed a diurnal oscillation with an evening peak (Fig. S1 *A* and *B*). Three biological replicate samples were prepared from 10-d-old LD-grown plants (*pFKF1:FKF1-3F6H/fkf1* and WT plants) harvested at zeitgeber time 13 (ZT13), as well as one replicate of a *35S:FKF1-3F6H*/Col plant grown in the same fashion and subjected to anti-FLAG immunoprecipitation and then to 6xHis purification (Fig. S1*C*). Proteins in the purified samples were identified by MS analysis. We used stringent criteria (a greater than twofold change in numbers of peptides between FKF1 and WT samples, $P$ values < 0.1, and no peptides recovered from WT control samples) to identify the conserved list of FKF1 complex components (Table 1, Fig. S1 *D–H*, and Table S1). After the FKF1 peptides, the peptides derived from GI protein were the second highest in number that we recovered from purified FKF1 samples. Our list also included ASK1, ASK2, and CUL1 proteins, all of which are components of the SCF complex (Table 1 and Table S1). Although FKF1 interacted with ASK1 and ASK2 in yeast (23), the interactions have never been tested in vivo. Our result strongly indicates that FKF1 forms an $SCF^{FKF1}$ complex. HEAT SHOCK PROTEIN 90 (HSP90) interacts with ZTL and stabilizes it (24). Our assay identified HSP90 as an FKF1-binding protein, suggesting that a conserved mechanism by which HSP90 regulates the stability of both ZTL and FKF1 likely exists. The list also contains GLUTAMINE SYNTHETASE 2 (GLN2) protein that functions in leaf mitochondria and chloroplasts (25) and an unknown protein that has a similarity to AAA-type ATPase (Table 1). This result suggests that FKF1 also may regulate responses other than photoperiodic flowering.

**Table 1. FKF1 and its interacting proteins identified by TAP-MS analysis**

| AGI no. | Name | No. of peptides* | | | |
|---|---|---|---|---|---|
| | | Rep.1 | Rep. 2 | Rep. 3 | Unique† |
| At1g68050 | FKF1 | 152 | 159 | 149 | 67 |
| At1g22770 | GI | 29 | 40 | 37 | 33 |
| At1g75950 | ASK1 | 20 | 22 | 23 | 11 |
| At5g42190 | ASK2 | 25 | 26 | 27 | 11 |
| At4g02570 | CUL1 | 4 | 11 | 16 | 15 |
| At5g56010 | HSP90.3 | 23 | 22 | 27 | 17 |
| At5g35630 | GLN2 | 9 | 9 | 10 | 9 |
| At4g02480 | Unknown | 7 | 17 | 17 | 14 |

Results are derived from *pFKF1:FKF1-3F6H/fkf1 #4*. No peptides from these proteins were recovered from three replicates (Rep.) of wild-type samples.
*Number of peptides identified in each MS analysis.
†Number of unique peptide sequences across all experiments (three replicates plus the data from Table S1).

MS analysis also can identify posttranslational modification of proteins. We found that FKF1 proteins (of 83% coverage of the entire peptides) bear at least two kinds of amino acid modification (Fig. S1 *D* and *I*). S198 of the FKF1 protein was phosphorylated, whereas K294 was ubiquitinated. The phosphorylation site was not conserved in either ZTL or LKP2 proteins. The Lys found to be ubiquitinated was conserved in these proteins, indicating that the Lys residue in these proteins also may be ubiquitinated. The function of these modifications is currently unknown.

**GI Physically Interacts with CO in Yeast and *in Planta*.** Our TAP-MS analysis revealed that the protein–protein interactions within the $SCF^{FKF1}$–GI complex are tight in vivo. In addition, GI associates with the same *FT* promoter region where both the FKF1 and CO proteins exist (6, 8, 18, 26). These observations prompted us to analyze the role of GI in regulating CO protein. First, we tested whether FKF1 interacts with GI in the cytosol and/or nucleus, because GI has both a cytosolic-specific interactor, such as ZTL (16), and nuclear-specific interactors, such as SVP, TEM1/2, EARLY FLOWERING 3 (ELF3), and ELF4 (18, 27, 28). If GI is involved in FKF1-dependent CO stabilization, GI should be in the FKF1 complex (at least in the nucleus). Plant lysate derived from the *pFKF1:HA-FKF1 pGI:GI-TAP/fkf1-2 gi-2* plants (8) was separated into cytosolic- and nuclear-enriched fractions. Coimmunoprecipitation (co-IP) experiments using GI-TAP showed that the FKF1–GI protein complex exists in both the cytosol and nucleus throughout the day (Fig. 1*A*).

Because CDF1 interacts with both FKF1 and GI, we next tested a potential protein–protein interaction between GI and CO using a yeast two-hybrid assay. The N-terminal deletion of CO (175–373), which interacts with LKP2 (29) and the FKF1 LOV and F-box domains (Fig. 1*B*), binds to the full length and the N-terminal region of GI (Fig. 1*B*). We then attempted to confirm the physical interactions among CO, FKF1, and GI proteins *in planta* using a tobacco transient expression system. HA-tagged GI (HA-GI) was coimmunoprecipitated with TAP-tagged CO (CO-TAP) from both cytosolic- and nuclear-enriched fractions. We found a relatively weak interaction between CO and GI in both fractions (Fig. 1*C*). In contrast, the CO–FKF1 interaction was strong. The amount

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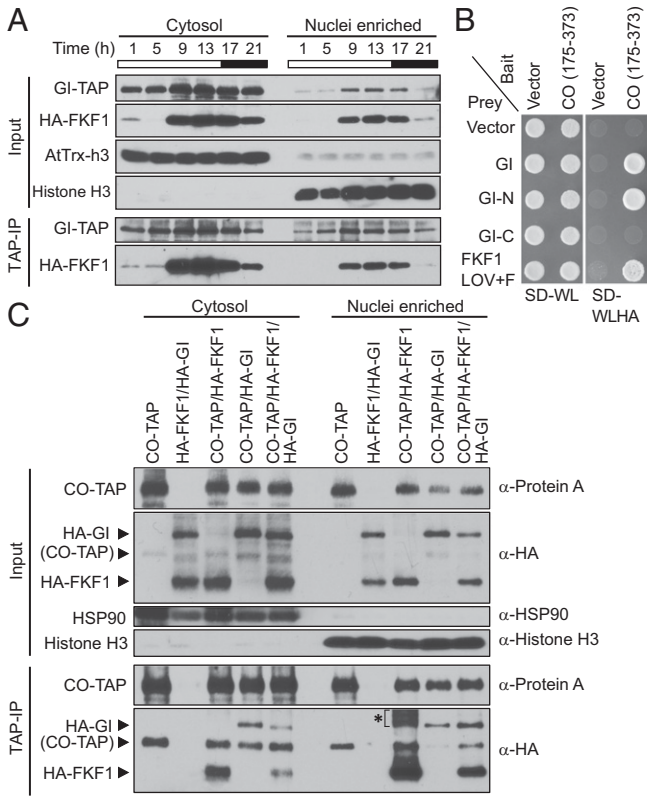


**Fig. 1.** CO, GI, and FKF1 all interact with each other. (*A*) The FKF1–GI interaction in *Arabidopsis*. *pFKF1:HA-FKF1 pGI:GI-TAP/fkf1-2 gi-2* seedlings were grown for 10 d in LD conditions. Co-IP assays were performed using cytosolic- and nuclei-enriched fractions. Time (h) indicates hours after light onset within a day; white and black areas on the time scales represent periods of light and dark, respectively. Anti–AtTrx-h3 and anti-histone H3 antibodies were used as cytosolic and nuclear markers, respectively. Similar results were observed from two biological replicates. (*B*) Yeast two-hybrid assay for the CO–GI interaction. The N terminus deletion of CO [CO (175–373)] in which the self-activation of transcription in yeast is eliminated was used as bait. Empty vectors were used for negative bait and prey controls, and the truncated FKF1, FKF1 LOV+F, was used as a positive control. All positive interactions were selected on synthetic defined media lacking tryptophan (W), leucine (L), histidine (H), and adenine (A). (*C*) Interactions among CO, FKF1, and GI in *N. benthamiana*. Anti-Protein A antibody was used to detect the Protein A-tag in CO-TAP protein. The nondenatured Protein A-tag in CO-TAP protein can bind any antibodies; therefore, anti-HA antibody can detect some CO-TAP signals. Note that the input samples that contain HA-GI show a background signal, which migrates similarly to CO-TAP. Anti-HSP90 antibody was used as a cytosolic marker. The asterisk for smear bands represents nonspecific background. Similar trends were obtained in three independent co-IP experiments.

of coimmunoprecipitated HA-FKF1 protein in the nuclear-enriched fraction was much higher than in the cytosol (Fig. 1*C*), indicating that the CO-FKF1 interaction preferentially occurs in the nucleus. Interestingly, the amount of nuclear CO–GI interaction was increased by the presence of FKF1 protein (Fig. 1*C*), indicating that GI interacts with CO directly and indirectly through binding to FKF1. These data suggest that CO, FKF1, and GI proteins exist within the same complex in the nucleus, where all three proteins regulate *FT* transcription (6, 8, 18, 26).

**GI Negatively Regulates CO Stability.** To investigate the importance of GI function on CO stability regulation genetically, we analyzed the effect of the *gi* mutation on *FT* levels, which reflect CO activity. Because FKF1 and GI proteins positively regulate the expression of *CO* and *FT* genes, and CO activity is largely reduced in the *fkf1* mutant (6, 8, 17), the *gi* mutation also might weaken CO protein activity and consequently reduce the *FT* mRNA levels. We constitutively expressed the *3HA-CO* cDNA (6) in WT and in the *gi-2* mutant, and analyzed *CO* and *FT* mRNA levels using quantitative real-time PCR (qRT-PCR) in LD conditions (Fig. 2 *A* and *B* and Fig. S2 *D* and *E*). As expected, the *FT* levels in the *35S:3HA-CO/gi-2* lines were decreased drastically compared with those in the *35S:3HA-CO* lines, even though expression levels of *CO* mRNA in these two backgrounds were similar (Fig. 2 *A* and *B* and Fig. S2 *D* and *E*). In addition, in LD conditions, the *35S:3HA-CO/gi-2* #1 line flowered significantly later than the WT plants overexpressing *CO* (Fig. S2*A*). In contrast, all *CO* overexpressors in the WT and *gi* mutant plants flowered much earlier than the WT plants in SD conditions and showed similar flowering phenotypes in these conditions (Fig. S2*B*). These data suggest that the *gi* mutation attenuates CO protein activity regulating *FT* expression specifically in LD conditions.

Given that CO protein interacts with GI (Fig. 1*C*) and that nuclear GI is responsible for the regulation of *CO* and *FT* gene expression (30), attenuated CO protein activity in the *gi* mutant

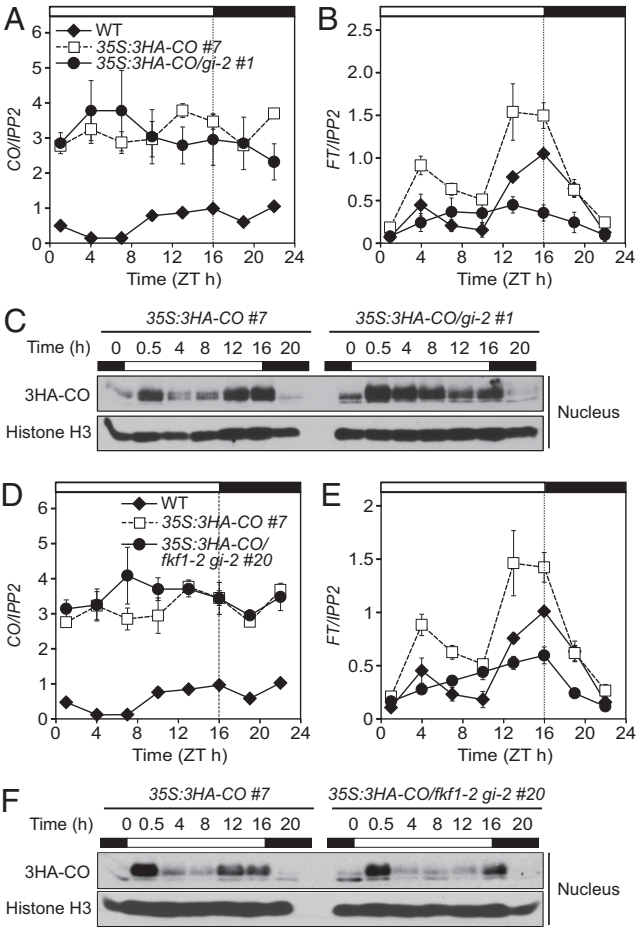


**Fig. 2.** Diurnal patterns of CO protein abundance are altered in *gi* and *fkf1 gi* mutants. (*A*, *B*, *D*, and *E*) Gene-expression analysis for *CO* (*A* and *D*) and *FT* (*B* and *E*) genes. *IPP2* was used as an internal control. All expression data normalized against *IPP2* are shown relative to the peak expression values of each gene in WT. (*C* and *F*) Immunoblot assays for daily expression profiles of CO proteins. (*A–C*) WT, *35S:3HA-CO #7*, and *35S:3HA-CO/gi-2 #1* plants were grown for 10 d in LD conditions. (*C*) The protein profiles of 3HA-CO were compared between two *CO* overexpressors in WT and the *gi* mutant background. (*D–F*) Ten-day-old WT, *35S:3HA-CO #7*, and *35S:3HA-CO/fkf1-2 gi-2 #20* seedlings grown in LD conditions were used for gene- and protein-expression analyses. The results represent means ± SEM from three biological replicates.

(Fig. 2*B* and Fig. S2*E*) could be caused by the reduction of CO protein accumulation. Therefore, we examined the daily expression profiles of CO protein in the *35S:3HA-CO/gi-2* lines. Unexpectedly, the amount of CO protein in the *gi* mutant increased in the morning but in the afternoon was similar to that in the *CO* overexpressors in the WT background (Fig. 2*C* and Fig. S2 *C* and *F–H*). Similar trends in the CO profiles were observed in both the cytosol and nucleus (Fig. S2 *F–H*). These results indicate that GI negatively regulates CO protein stability in the morning.

If GI is involved in the destabilization of CO protein, overexpression of *GI* might decrease the amount of CO protein. To test this possibility, we next generated a *CO* overexpressor in the *GI* overexpressor (*35S:GI-TAP/gi-2*) background (31). In the *35S:3HA-CO 35S:GI-TAP/gi-2 #1* line, the levels of *FT* transcript were elevated as compared with the levels in the *CO* overexpressor in the WT background (Fig. S3 *A* and *B*). However, unlike the *gi* mutation, *GI-TAP* overexpression has little effect on the daily profile of CO protein expression (Fig. S3*C*), indicating that a higher amount of GI does not change CO protein stability. Interestingly, even though CO protein profiles in the *GI-TAP* overexpressor background and the WT background were similar, *FT* expression in the *GI-TAP* overexpressor was higher than in the WT background (Fig. S3*B*), indicating that GI also induces *FT* expression independently of CO protein level. GI regulates the expression of miR172, which regulates the amount of *FT* repressor mRNAs (19). GI also directly interacts with several *FT* repressors (18). These regulations may contribute to the control of *FT* expression in these lines.

CO is the primary activator of *FT* transcription in LD conditions, and the abundance of *FT* mRNA is strongly correlated with the expression level of CO protein (3, 5, 6). Therefore, the expression of *FT* usually is a good proxy for the amount of CO protein. However, in the case of GI, because GI has other roles that also affect *FT* expression, this CO protein–*FT* relationship might not be sufficient to explain the phenotype of the *gi* mutant and *GI* overexpressor. We postulated that the discrepancy between the CO protein profiles and the amount of *FT* mRNA levels in these lines is partly the result of alterations in the gene expression of *FT* repressors. We therefore analyzed the expression of *FT* repressors, such as *FLOWERING LOCUS C* (*FLC*), *SVP*, *SMZ*, *SNZ*, *TEM1*, *TEM2*, *TOE1*, and *TOE2*. The expression of many genes tested (*FLC*, *SVP*, *SMZ*, *SNZ*, and *TEM2*) was up-regulated in the *gi* background in LD conditions (Fig. S4 *A–H*). In contrast, *FLC*, *TEM1*, *TEM2*, and *TOE2* are down-regulated in the *GI* overexpressor background (Fig. S4 *I–P*). These results indicate that the changes in mRNA levels of some *FT* repressors, in part, may account for the changes in *FT* expression in these backgrounds.

**The Function of *FKF1* in CO Stabilization Is Epistatic to *GI*.** To determine how the CO protein profile is generated in the *gi* mutant, we have tested several possibilities. Phytochromes and cryptochromes regulate CO protein stability (5). In addition, the *gi* mutation attenuates PHYB signaling (32). We thus analyzed the protein-expression profiles of PHYB and PHYA and cryptochromes in the *gi* mutant (as well as the *fkf1-2* and *fkf1-2 gi-2* mutants). No obvious differences in the levels of PHYA, PHYB, CRY1, and CRY2 proteins were observed between WT and mutant plants in LD conditions (Fig. S5). This result indicates that the changes in CO protein stability observed in the *gi* mutant (and the *fkf1* mutant) are not caused by the changes in the expression levels of these photoreceptors.

Next, we genetically assessed the relationship between *GI* and *FKF1* in regulating CO protein stability, because GI is genetically required for FKF1 to degrade CDF1 protein (8). We analyzed the CO protein profile in the *fkf1 gi* double mutant (8). As in the *gi* mutant background, *FT* transcript levels in the *35S:3HA-CO/fkf1-2 gi-2* lines were much lower than those in the *35S:3HA-CO* lines (Fig. 2 *D* and *E* and Fig. S6 *C* and *D*). The CO protein levels were noticeably reduced in the *fkf1 gi* mutant in the morning as compared with those in the *gi* mutant (Fig. 2*F* and Fig. S6 *A* and *D*). In addition, in the afternoon the amount of CO protein was lower in the *35S:3HA-CO/fkf1-2 gi-2* lines than in the *35S:3HA-CO* plants (Fig. 2*F* and Fig. S6 *A*, *D*, and *E*) and was similar to the CO protein level in *fkf1* (6). These observations indicate that the function of *FKF1* is genetically epistatic to *GI* in the regulation of CO stability.

**ZTL Binds to and Destabilizes CO Protein.** Unlike the *fkf1* mutation, the *gi* mutation by itself stabilizes CO protein in the morning. The CO protein profile in *35S:3HA-CO/fkf1-2 gi-2* resembles that in the *35S:3HA-CO/fkf1-2* line. It is also noteworthy that both GI and FKF1 proteins are expressed at the lowest levels in the morning (8). These results suggest that there might be at least another factor (which might be genetically influenced by both *gi* and *fkf1*) that also controls CO protein stability in the morning. We speculated that one such component might be ZTL, based on the following observations. First, similar to FKF1, ZTL physically interacts with GI (8, 16). ZTL is abundant in the morning, and GI directly stabilizes ZTL throughout the day (16). Second, the flowering phenotypes of *ztl* mutants and *ZTL* overexpressors genetically depend on *FKF1* function (22), in part because ZTL directly interacts with FKF1 and changes the intracellular localization of FKF1 to regulate flowering time (22). Third, together with FKF1, ZTL also is involved in the regulation of CDF2 stability for flowering time regulation (17). Fourth, ZTL interacts with CO in yeast (29). Therefore, we hypothesized that ZTL also may regulate CO protein stability and that the *gi* phenotype is caused by the combinational effects of changes in FKF1 and ZTL functions. To test whether ZTL can interact with CO in plants, we performed co-IP experiments using a tobacco transient system as well as the *35S:CO-3F6H/35S:Myc-ZTL Arabidopsis* transgenic line. CO-TAP strongly interacts with HA-ZTL in tobacco (Fig. 3*A*). As also shown in yeast, LKP2 also

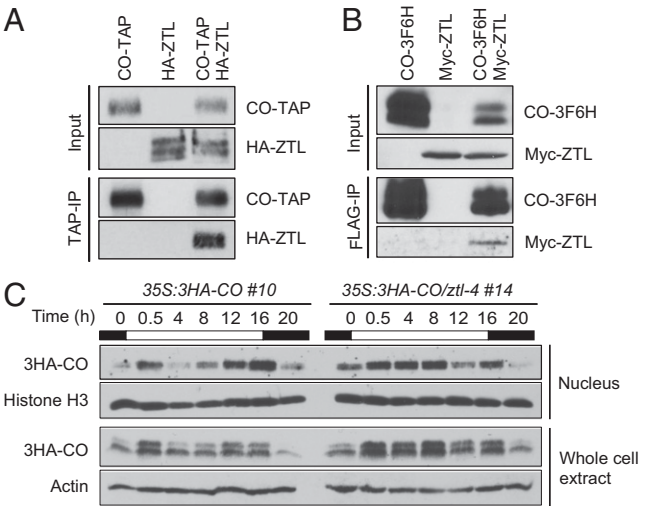


**Fig. 3.** ZTL interacts with CO and regulates its stability. (*A* and *B*) Co-IP experiments to detect CO–ZTL interaction were carried out using tobacco leaf tissues (*A*) and *35:CO-3F6H/35S:Myc-ZTL Arabidopsis* plants (*B*). Similar results were observed with three biological replicates. (*A*) *CO-TAP* and *HA-ZTL* overexpression constructs, individually or together, were infiltrated into *N. benthamiana* leaves. (*B*) LD-grown *35S:CO-3F6H #2*, *35S:Myc-ZTL*, and *35S:CO-3F6H/35S:Myc-ZTL* plants were treated with 10 μM of MG-132 8 h before sampling to minimize CO degradation. Plants were harvested in the morning (ZT 4) on day 10. (*C*) Protein profiles of 3HA-CO in the *ztl* mutant and WT backgrounds. Anti-actin and anti-histone H3 antibodies were used for loading controls and to normalizeing the quantification of CO amounts. Experiments were repeated three times independently.

interacts with CO in tobacco (Fig. S7), indicating that all three ZTL group proteins may interact directly with CO. In addition, Myc-ZTL was coimmunoprecipitated with CO-3F6H in *Arabidopsis* (Fig. 3*B*). These results verify that ZTL and CO interact in vivo.

Next, using the *35S:3HA-CO/ztl-4* line, we analyzed whether ZTL regulates *FT* expression levels and CO protein stability. *FT* mRNA level was largely reduced in the *35S:3HA-CO/ztl-4* line (Fig. S8 *A* and *B*). Notably, in LD conditions, the amount of CO protein in the morning was higher in the *35S:3HA-CO/ztl-4* line than in the WT plant overexpressing *3HA-CO* (Fig. 3*C* and Fig. S8 *C* and *D*), indicating that ZTL destabilizes CO protein in the morning. This phenotype is similar to that in *35S:3HA-CO/gi*. In contrast to the function of FKF1 in regulating CO stability, these results demonstrate that ZTL negatively controls CO stability through physical interaction. This result also clearly suggests that the stoichiometric changes of FKF1, ZTL, and GI in part regulate CO protein stability and *FT* expression.

## Discussion

**FKF1-Binding Proteins Isolated by TAP-MS Analysis.** The FKF1–GI interaction originally was identified based on the genetic interaction on flowering time regulation (8). Our current results suggest that the interaction between FKF1 and GI is strong under our experimental conditions. As is ZTL protein, GI is involved in the stabilization of FKF1 (17) and is required for FKF1 to degrade CDF1 (8). Because the FKF1–GI interaction is light dependent and GI also interacts with different FKF1 interactors, such as CDF1 (7, 8) and CO (this study), the binding of FKF1 to the GI complex seems to be the process activating FKF1 function.

The results of our TAP-MS analysis also confirmed that FKF1 forms the $SCF^{FKF1}$ complex in vivo. FKF1 contains an F-box domain (9) and degrades CDF1 in a proteasome-dependent manner (7). FKF1 also binds to ASK1, ASK2, ASK11, and ASK14 in yeast (23). We therefore previously assumed that FKF1 functions as a component of the SCF complex. It would be interesting to know whether the light-dependent interaction of FKF1 with GI also regulates the formation of the SCF complex. We also found that K294 of FKF1 is ubiquitinated. It is not known whether K294 is monoubiquitinated or polyubiquitinated and whether $SCF^{FKF1}$ by itself autoubiquitinates FKF1 or another ubiquitin ligase catalyzes the reaction. The significance of FKF1 protein modification currently remains unknown.

HSP90 also was copurified with FKF1. ZTL is an HSP90 client, and the ZTL–HSP90 interaction increases ZTL stability (24). In addition, GI and HSP90 are connected functionally and act in the same pathway for ZTL stabilization; the function of HSP90 increases ZTL stability and is affected by the presence of GI (24). Moreover, the treatment of the HSP90 inhibitor reduces the amount of FKF1 protein (24). Together with FKF1 binding to HSP90, these findings suggest that HSP90 probably is involved in the stabilization of FKF1 in a GI-dependent manner.

The list also includes GLN2 and an unknown ATPase-like protein. This result may suggest that FKF1 potentially is involved in some metabolic pathways. However, because there are no other results to confirm the connection of FKF1 to metabolic pathways, we need additional experiments to assess this potential interaction.

Our TAP-MS analysis did not detect some known FKF1 interactors, such as CO, CDFs, ZTL, and LKP2. Among those, CDF proteins most likely are degraded by FKF1 at ZT13, when the samples were harvested. One of the technical challenges of affinity purification (AP)-MS analysis is reducing false positives (33). The TAP procedures can reduce nonspecific background dramatically; therefore, it is the ideal method for AP-MS analyses (34). However, because the TAP procedure is more stringent than the single AP procedure, the chances of identifying weak or transient interactions may be reduced. In addition, performing the two sequential AP processes takes longer than the single AP process. Thus, unstable proteins, such as CO, could be degraded during the TAP procedure. We also are not always aware of when and where specific interactions occur. For instance, the time during the day when FKF1 interacts with ZTL and/or LKP2 in vivo remains unknown. Potentially, they might not form a complex at ZT13. One experimental condition that might improve the outcome of the TAP-MS assay is pretreatment of the samples with MG-132 to cease proteasome degradation processes before the samplings. That treatment may increase the chance of detecting proteins (i.e., CDF proteins) that usually are degraded rapidly by proteasome after the interaction with FKF1.

**GI Acts as a Negative Regulator in the Stability of CO.** Our yeast two-hybrid assays and co-IP experiments showed that GI directly and indirectly (through FKF1) forms a complex with CO (Fig. 1*C*), indicating that GI may regulate CO protein stability through both direct and indirect mechanisms. Immunoblot analysis revealed that the *gi* mutation caused a high abundance of CO protein in the morning, when *CO* mRNA is constitutively expressed (Fig. 2*C* and Fig. S2 *C* and *F–H*). Genetically, this result indicates that GI is a negative regulator of CO protein stability in the morning. However, because the expression level of GI protein is low in the morning (Fig. 1*A*) and *GI* overexpression had little effect on changing the CO accumulation pattern throughout the day (Fig. S3*C*), the elevated accumulation of CO protein in the morning could be an indirect effect of the *gi* mutation. In addition, the CO protein profile in *35S:3HA-CO/fkf1 gi* resembles that in *35S:3HA-CO/fkf1* (Fig. 2*F*). The interaction between GI and FKF1 by themselves simply cannot explain these phenotypes. Hence, we hypothesized that at least one more factor is involved in FKF1- and GI-dependent CO protein regulation. We proposed that the factor could be ZTL and found that the CO protein profile was altered by the *ztl* mutation (Fig. 3*C* and Fig. S8 *C* and *D*). Because the *gi* mutation strongly destabilizes ZTL protein (16), the CO protein phenotype in the *gi* mutant might be caused by very low levels of ZTL expression. ZTL also forms the $SCF^{ZTL}$ and degrades both TOC1 and PRR5 (35–37). The negative effect of ZTL on CO stability might be caused by the direct degradation of CO by ZTL. FKF1 and ZTL seem to have antagonistic roles in regulating CO protein (Fig. S9). To understand the effect of the *GI* overexpressor on CO protein stability, considering the effect of *GI* overexpression on both FKF1 and ZTL may help. *GI* overexpression causes stabilization of both FKF1 and ZTL proteins (16, 17). Stabilizing both positive and negative factors in the regulation of CO protein stability may not cause the change in the accumulation of the CO protein.

Our results clearly showed that even though CO proteins were stabilized in the morning in *gi* and *ztl* mutant backgrounds, *FT* expression was not highly induced. This finding suggests that the abundance of CO protein does not always reflect the level of *FT* expression. The CO protein accumulated in the morning in these mutants did not (or could not) participate in *FT* induction. This finding also suggests that the *gi* and *ztl* mutations repress the expression of *FT* independently of CO protein accumulation. In other words, through unknown mechanisms, GI and ZTL are involved in the mechanisms activating CO protein (e.g., changing DNA-binding affinity, binding to other transcriptional activators/basal transcriptional machinery, recruiting CO into a specific location/structure inside the nucleus, among others) to induce *FT*. Thus, our results indicate that GI, ZTL, and likely FKF1 are involved in regulating the activity of CO protein.

ZTL is a cytosolic protein, and the *ztl* mutation destabilizes GI protein (16). Nuclear-localized GI regulates *CO* and *FT* transcription (30), and GI interacts with SVP, TEM1, and TEM2, which are direct repressors of *FT*, in the nucleus. Also, the gene expression of *FT* repressors including *FLC*, *SVP*, *SMZ*, *SNZ*, *TEM1*, *TEM2*, *TOE1*, and *TOE2* is regulated by GI (Fig. S4), possibly through the regulation of miR172 for *SMZ*, *SNZ*, *TEM1*, *TEM2*, *TOE1*, and *TOE2* (19). Therefore, the loss of *GI* also changes the expression and/or activity of *FT* repressors, and this change may affect CO protein activity.

**Posttranslational Regulation of CO Protein Mediated by the ZTL/FKF1/LKP2 Family.** Our results indicate that GI influences CO stability, possibly by modulating the accumulation of ZTL/FKF1/LKP2 family members. FKF1, ZTL, and LKP2 interact with CO in yeast and *in planta* (Fig. 3 *A* and *B* and Fig. S7) (6, 29). The *lkp2* mutation enhances the early flowering phenotype of the *ztl* mutant (22). This effect suggests that LKP2 also may negatively regulate CO protein stability. In addition, both ZTL and LKP2 interact with FKF1 through the Kelch repeat domain and capture FKF1 in the cytosol (22). Therefore, ZTL and potentially LKP2 may regulate CO protein stability by direct interaction as well as by changing the intracellular localization of FKF1.

Because GI plays an important role in regulating the stability of ZTL and FKF1 (and possibly LKP2), we need to analyze the effect of the mutations in these genes more systematically to understand how the CO protein profile is regulated by FKF1, ZTL, and LKP2. Also, determining whether ZTL degrades CO directly will help us understand this mechanism better. We currently are studying the complicated relationship among these family members in CO protein regulation to understand better the molecular mechanisms involved.

Here we report evidence that both GI and ZTL are involved in regulating the stability of CO protein and show that ZTL/FKF1/LKP2 family proteins and GI form an interrelated complex mechanism to regulate CO protein stability for photoperiodic flowering. Together with the implication of GI function in developmental age- and temperature-dependent flowering regulation (18, 19, 38), the distinct roles of GI in photoperiodic flowering (this study and refs. 8 and 17) facilitate the ability of plants to increase flexibility and to adapt to ensure timely reproductive success.

## Materials and Methods

Plant materials and growth conditions, RNA isolation, and gene-expression analysis, including all primers used, protein preparation, TAP-MS experiments, and immunoblot analysis and protein quantification are given in *SI Materials and Methods*.

To test protein–protein interactions in yeast, the cDNA encoding the truncated CO peptides (amino acids 175–373), designated CO (175–373) (29), was cloned into pENTR/D-TOPO (Invitrogen) and then transferred to pASGW-attR bait vector. The constructs for FKF1 LOV+F, full-length GI, GI-N, and GI-C and protocols for yeast two-hybrid assays were described previously (8). For tobacco co-IP assays, *35S:CO-TAP* (6), *35S:HA-FKF1* (8), and *35S:HA-GI* (31) constructs were infiltrated into ∼3-wk-old *Nicotiana benthamiana* plants grown in LD conditions as described in ref. 6. To analyze the in vivo interaction, the *Myc-ZTL* construct was cloned into the pRTL2 (39) vector carrying the CaMV *35S* promoter. The *35S:Myc-ZTL* expression cassette was transferred to pPZP221 binary vector (40). Then, the pPZP221 vector harboring the *Myc-ZTL* overexpression construct and the pH7WG2 vector harboring *35S:3HA-CO* (6) were sequentially transformed with WT plants harboring *CAB2::LUC* reporter (41) to generate double-overexpression (*35S:CO-3F6H/35S:Myc-ZTL*) lines. Procedures for co-IP experiments using tobacco and *Arabidopsis* tissues were described previously (6).

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