The F-box protein FKF1 inhibits dimerization of COP1 in the control of photoperiodic flowering

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In Arabidopsis thaliana, CONSTANS (CO) plays an essential role in the regulation of photoperiodic flowering under long-day conditions. CO protein is stable only in the afternoon of long days, when it induces the expression of FLOWERING LOCUS T (FT), which promotes flowering. The blue-light photoreceptor FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) interacts with CO and stabilizes it by an unknown mechanism. Here, we provide genetic and biochemical evidence that FKF1 inhibits CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1)-dependent CO degradation. Light-activated FKF1 has no apparent effect on COP1 stability but can interact with and negatively regulate COP1. We show that FKF1 can inhibit COP1 homodimerization. Mutation of the coiled-coil domain in COP1, which prevents dimer formation, impairs COP1 function in coordinating flowering time. Based on these results, we propose a model whereby the light- and day length-dependent interaction between FKF1 and COP1 controls CO stability to regulate flowering time.
Most flowering plants bloom in response to seasonal changes in environmental factors such as day length and temperature. In the model dicot plant Arabidopsis thaliana, flowering time is mainly regulated by the photoperiodic, autonomous, gibberellin, and vernalization pathways1. These signaling pathways converge to induce expression of the flowering gene FLOWERING LOCUS T (FT), which encodes a mobile protein that can induce the shoot apical meristem to make the transition from vegetative to reproductive development2,3. In the photoperiodic pathway, CONSTANS (CO) has a major role in inducing FT transcription, although other regulators also independently affect FT expression4-6. CO encodes a zinc finger-type transcription factor containing two B-boxes and a CO, CO-LIKE, and TOC1 (CCT) domain7. CO directly binds to the FT promoter and activates its transcription7. Levels of CO mRNA are regulated in a circadian manner: the CO mRNA is abundant during the daytime under long-day (LD) conditions and during the nighttime under short-day (SD) conditions8,9. However, FT transcription, controlled by CO, differs remarkably between LD and SD conditions because light signals tightly regulate CO at the posttranslational level. Elucidation of these regulatory mechanisms showed that FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) and CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) control CO stability10-12.

FKF1 is a key component of the SKP1/CUL1/F-box (SCF)-type E3 ligase complex and has three domains (LOV, F-box, and KELCH-repeat). FKF1 functions as a blue-light receptor in which the LOV domain binds to a flavin mononucleotide chromophore13. In photoperiodic flowering, FKF1 positively regulates CO at both the transcriptional and posttranslational levels11,12. In the morning, CYCLING DOF FACTOR (CDF) transcription factors (CDF1, CDF2, CDF3, and CDF5) repress the expression of CO and FT. In the afternoon of LD, FKF1 is expressed and light-activated FKF1 interacts with GIGANTEA (GI) to degrade CDFs; this induces transcription of CO and FT, leading to early flowering.

FKF1 interacts with COP1 in vivo. The genetic analysis indicated that FKF1 and COP1 act in the same pathway of photoperiodic flowering. Since both CO and FKF1 function as E3-ubiquitin ligases in proteasome-mediated proteolysis of their target proteins13,21, we examined whether FKF1 directly regulates CO. We first tested whether FKF1 and COP1 can physically interact and found that FKF1 interacts with COP1 in yeast two-hybrid assays, primarily via the RING domain of CO (Supplementary Fig. 1). This interaction between FKF1 and COP1 was also observed in planta by co-immunoprecipitation (Co-IP) following transient expression in Nicotiana benthamiana20 and by bimolecular fluorescence complementation (BiFC) assays in onion epidermal cells (Fig. 1c, d). Next, to map the interacting domains of FKF1 and COP1, we separated COP1 into three domains: (1) RING, (2) CC, and (3) WD40-repeat16. COP1 partitions between the nucleus and cytoplasm in a light-dependent manner17 and forms homodimers through the CC domain; the homo-dimerization is required for CO function and subcellular localization18,19. COP1 associates with SUPPRESSOR OF PHOTOCYTOCHROME A 1 (SPA1) to form a (COP1)2(SPA1)4 tetramer. Homo- and hetero-dimerization of COP1 have important roles in COP1 function19,20. The (COP1)2(SPA1)4 tetramer is involved in poly-ubiquitination and destabilization of CO in the dark21. In addition to CO, COP1 promotes destabilization of several nuclear proteins involved in flowering time and photomorphogenesis22-24.

Thus, the E3 ubiquitin ligases FKF1 and COP1 play critical roles in controlling photoperiodic flowering by directly regulating CO stability10,12,15, as FKF1 stabilizes CO in the light and COP1 destabilizes CO in the dark. Other important regulators also affect the stability of CO: (i) in the morning, HOS1 and photochrome B (phyB) decrease CO stability14,24,25, (ii) in the afternoon of LD, phyA, cryptochrome 2 (cry2), and FKF1 increase CO stability10,12,26,27, and (iii) in darkness, COP1 mediates degradation of CO15.

Here, we provide evidence that FKF1 acts as an upstream negative regulator of COP1. FKF1 and COP1 regulate CO stability and photoperiodic flowering. FKF1 can interact with COP1 and reduce COP1 activity in a day-length-dependent manner. We suggest that posttranslational control of CO stability, mediated by negative regulation of COP1 by FKF1, promotes early flowering in LD.

Results
FKF1 negatively regulates COP1 in photoperiodic flowering. To investigate whether COP1 and FKF1 act in the same genetic pathway of flowering-time regulation, we generated cop1-4 fkf1-t double mutants in which cop1-4, a weak mutant allele, carries a premature stop codon at the 283rd amino acid, and fkf1-t (SALK_059480) is a T-DNA insertion mutant28,29. Since cop1-4 gi-1 mutants flowered as late as gi-1 in both LD and SD25, we speculated that cop1-4 fkf1-t mutants would flower as late as fkf1-t in both photoperiods. However, cop1-4 fkf1-t flowered as early as cop1-4 mutants in both LD and SD (Fig. 1a, b; Supplementary Table 1). Furthermore, we found that the FKF1-overexpressing plants, such as 35S::FKF1 #3 and 35S::FKF1 #18, showed an early-flowering phenotype compared with wild type (WT, Col-0), and 35S::Myb::FKF1 #5/col-1 plants also flowered earlier than WT but similar to cop1-1 in LD. In SD, both 35S::Myb::FKF1 #3 and 35S::FKF1 #18 flowered earlier than WT, and 35S::Myb::FKF1 #3/cop1-1 flowers as early as cop1-4. These data indicate that cop1 is epistatic to fkf1, and FKF1 inhibits COP1 mainly in LD. Considering these results, we concluded that FKF1 functions as an upstream negative regulator of COP1 in the floral induction pathway.

CO is stabilized in the cop1 mutant independently of FKF1. Previous work reported that both FKF1 and COP1 E3-ubiquitin ligases interact with CO to control its function in LD-dependent early flowering antagonistically12,15. FKF1 and COP1 increase and decrease the stability of CO, respectively, because CO levels decrease in fkf1-2 mutants and increase in cop1-4 mutants. Thus, we tested whether the presence or absence of FKF1 activity affects CO stability in the cop1-4 background (Fig. 2a, b). We first analyzed CO levels at ZT15 (1 h before darkness) when FKF1 expression and activity are the highest in LD. In cop1-4 fkf1-t
mutants, CO accumulated to levels similar to those in cop1-4, but CO was not detected in either WT or the fkf1-t mutant (Fig. 2a). Next, we analyzed CO levels every 4 h during the course of a day in LD, and found that CO levels were not altered in the cop1-4 background regardless of FKF1 genotype. CO levels were nearly constant during day and night in cop1-4, cop1-4 fkf1-t, and 35S::Myc-FKF1/cop1-4 plants, but CO was hardly detectable in WT, fkf1-t, or 35S::Myc-FKF1 #3 plants (Fig. 2b).

Finally, we analyzed the relative abundance of CO mRNA in different mutant backgrounds (Fig. 2c, d). CO mRNA levels decreased in fkf1-t, and increased in 35S::Myc-FKF1, consistent with the observation that FKF1 functions to degrade CDF1, a negative regulator of CO transcription. Interestingly, despite high accumulation of CO, the CO mRNA levels were lower in the cop1 mutant background, including in cop1-4, cop1-4 fkf1-t, and 35S::Myc-FKF1 #3/cop1-4 (Fig. 2c). However, it did not appear that high accumulation of CO negatively affected CO transcription, because native CO mRNA levels in 35S::CO-GFP were similar to those of WT (Fig. 2d). Taking these observations and those of a previous study together, we suggest that FKF1 increases CO stability by reducing COP1 function in the late afternoon of LD to induce flowering.

FKF1 does not affect COP1 stability. To examine whether FKF1 negatively regulates the stability of COP1, because FKF1 has E3-ubiquitin ligase activity, we generated an anti-COP1 polyclonal antibody, and analyzed COP1 levels in fkf1-t and 35S::FKF1 #18 plants over the course of a day. Unexpectedly, we found that steady-state levels of COP1 persisted in both fkf1-t and 35S::FKF1 #18 compared with WT (Fig. 3; Supplementary Fig. 4), indicating that FKF1 does not destabilize COP1. Similarly, we found that FKF1 levels were not significantly altered in cop1-4 or 35S::TAP-COP1 plants (Supplementary Fig. 5). These results indicate that the FKF1–COP1 interaction does not affect the stability of either protein.

FKF1 can inhibit COP1 homo-dimerization. Since FKF1 does not affect the stability of COP1, we assumed that the FKF1–COP1 interaction decreases COP1 activity. COP1 interacts with SPA1 to form a (COP1)2(SPA1)2 tetramer, and homo- and hetero-dimerization of COP1 is important for its biological function. Therefore, we speculated that the FKF1–COP1 interaction prevents COP1–COP1 dimerization, the COP1–SPA1 interaction, or both, thus decreasing COP1 activity and increasing CO stability in the late afternoon of LD. To test these possibilities, we performed Co-IP assays in N. benthamiana. We found that COP1 dimerization occurred under both light and dark conditions in the absence of FKF1. Surprisingly, FKF1 overexpression diminished COP1 dimerization in the light but not in the dark (Fig. 4a). In the light, COP1 dimerization was severely decreased.
and, instead of forming homodimers, COP1 interacted with FKF1. In the dark, COP1 dimerization occurred normally and COP1 did not interact with FKF1. Next, we tested whether the in vivo interaction of FKF1 with COP1 depends on light. For this, we used Co-IP assays with 35S::FKF1 #18 plants, which revealed that the in vivo interaction requires light (Fig. 4b).

We further used yeast three-hybrid assays to test whether FKF1 inhibits COP1–COP1 homo-dimerization and/or COP1–SPA1

**Fig. 2** CO accumulates to high levels in the cop1-4 background regardless of FKF1. **a** CO accumulation at ZT15 in 10-day-old seedlings under LD. **b** CO accumulation in various plants. Plants were grown for 10 days under LD and harvested every 4 h over the course of a day. Nuclear protein-enriched fractions were immunoblotted using an anti-CO antibody (α-CO) to measure CO levels and an anti-H3 antibody (α-H3) for a loading control. Data are means ± s.d. from at least three biological repeats. **c** Relative abundance of CO mRNA during a day in 10-day-old seedlings. **d** Native CO mRNA levels during a day in 35S::CO-GFP transgenic plants. To detect the native CO mRNA, a specific primer was designed from the 3′-UTR region of the CO mRNA sequence. **c, d** For RT-qPCR, the relative expression level of each gene was normalized to the mRNA level of ACTIN (AT3G18780) as a loading control. Data are means ± s.d. from three biological replicates.

**Fig. 3** The FKF1–COP1 interaction does not affect COP1 stability. The diurnal patterns of COP1 levels are shown in wild type (Col-0), fkh1-t, and 35S::FKF1 #18 under LD. The 10-day-old seedlings were harvested every 4 h over the course of a day. Total protein (70 μg) extracted from each sample was immunoblotted to measure the COP1 levels using an anti-COP1 polyclonal antibody. The intensity of each COP1 band (‘∗’, lower band) was normalized to the non-specific band (‘∗∗’, upper band) in each lane. Quantitative COP1 data were exported from ImageJ (https://imagej.nih.gov/ij/index.html). Data are means ± s. d. from five immunoblot replicates.
hetero-dimerization. In these assays, FKF1 transcription was controlled by the Met-repressible pMET25 promoter. We analyzed the inhibition of COP1 homo-dimerization by FKF1 under blue light or in darkness, and found that FKF1 inhibits COP1 homo-dimerization more under blue light than in darkness (Fig. 4c; Supplementary Fig. 6). COP1 homo-dimerization was completely inhibited by FKF1 under blue light and was reduced in methionine-deficient conditions in the dark, based on both yeast colony survival and β-galactosidase activity. Moreover, we found that FKF1 does not inhibit the COP1–SPA1 interaction, regardless of light conditions. These results strongly suggest that in yeast, FKF1-mediated inhibition of COP1 dimerization is promoted by blue light, although some of the activity remains in darkness. Taken together, these results suggest that in Arabidopsis, blue-light-activated FKF1 can interact with and attenuate COP1 homo-dimerization.

FKF1 partially inhibits COP1 during hypocotyl elongation. The COP1 E3-ubiquitin ligase mediates degradation of specific target proteins, including HY5, HYH, LAFl, HFR1, BBX4/STO, BBX4/COL3, and BBX22/LZF1/SSTH3 transcription factors, all of which are involved in light signaling and photomorphogenesis in Arabidopsis.18,21 Therefore, we further examined whether FKF1 also negatively affects COP1 function in hypocotyl elongation (Supplementary Fig. 7a; Supplementary Table 2). The hypocotyls of fkf1-t seedlings were as long as those of WT, regardless of day-length conditions (LD/SD/constant darkness; DD). Interestingly, hypocotyls of both 35S::FKF1 #18 and 35S::Myc-FKF1 #3 were significantly shorter than those of WT and fkf1-t in SD and slightly shorter in LD, but this was not statistically significant, and they were as long as WT in DD. These results suggest that FKF1 overexpression negatively regulates COP1 in hypocotyl elongation only in SD.

Next, we analyzed HY5 levels in 35S::Myc-FKF1 #3 and fkf1-t plants (Supplementary Fig. 7b), since HY5 is one of major regulators of hypocotyl elongation, although other COP1 target proteins are also involved in this process.21 HY5 stability in WT depends on the light period, as HY5 is more stable in LD than in SD, and not detected in DD. However, we could not find any evidence that HY5 becomes more stable in 35S::Myc-FKF1 #3 in SD. Thus, we concluded that FKF1 negatively affects COP1 function in hypocotyl growth in SD when FKF1 is constitutively overexpressed, and this is seemingly not related to the regulation of HY5 stability.

COP1 mutants that are unable to dimerize do not promote flowering. COP1 forms a homodimer and/or a heterodimer with SPA1 through the CC domain and finally forms a (COP1)(2)(COP1)(2) tetramer for its functional activity.18–20 When COP1 dimerization is prevented, it is not functional in photomorphogenesis. To examine the effect of COP1 dimerization on flowering, we prepared mutated cDNAs using WT (Col-0) COP1 (COP1WT), and the mutant versions COP1L105A and COP1L170A which were previously reported to undergo normal or poor dimer formation, respectively.19 (Fig. 5a). First, we tested the binding between COP1 and mutated COP1, and FKF1 and mutated COP1 in N. benthamiana. The COP1WT–COP1L105A Co-IP signal was nearly the same as that of COP1WT–COP1WT, while that of COP1WT–COP1L170A was much weaker consistent with a prior publication.19 This indicates that COP1 homo-dimerization

Fig. 4 FKF1 inhibits COP1 homo-dimerization in a light-dependent manner. a FKF1 requires light to interact with COP1 and inhibit COP1 dimerization. In the absence of FKF1, COP1 dimerization occurs in both light and dark conditions. In the presence of FKF1, FKF1 interacts with COP1 only under light in N. benthamiana. Two days after co-infiltration, tobacco leaves were harvested under light (L) and dark (D) in LD. b Light-dependent interaction between FKF1 and COP1 in 35S::FKF1 #18. The 10-day-old seedlings were harvested in the light period (L) and dark period (D) in LD. * indicates COP1 and ** indicates non-specific bands derived from an anti-COP1 antibody. FKF1 was detected by an anti-FKF1 antibody. COP1 homo-dimerization was detected by an anti-COP1 antibody. FKF1 was detected by an anti-FKF1 antibody. FKF1 partially inhibits COP1 during hypocotyl elongation in methionine-deficient conditions in the dark, based on both yeast colony survival and β-galactosidase activity. Moreover, we found that FKF1 does not inhibit the COP1–SPA1 interaction, regardless of light conditions. These results strongly suggest that in yeast, FKF1-mediated inhibition of COP1 dimerization is promoted by blue light, although some of the activity remains in darkness. Taken together, these results suggest that in Arabidopsis, blue-light-activated FKF1 can interact with and attenuate COP1 homo-dimerization.
Fig. 5 COP1 variants that are unable to homo-dimerize are non-functional. **a** Mutation sites in the CLS motif of COP1. **b** COP1L170A forms dimers poorly when compared with COP1WT or COP1L105A in *N. benthamiana*. **c** FKF1 interacts more weakly with COP1L170A (poor dimer formation) than COP1L105A (normal dimer formation) in *N. benthamiana*. **d** Various transgenic plants at bolting under LD and SD. Scale bars, 2 cm. **e** Flowering times of the plants in **d**. The number of rosette leaves at bolting represents the flowering time of each genotype. Data are means ± s.d. of 20 plants. **f** CO accumulation of each genotype in **d**. Ten-day-old plants were harvested at ZT15 (1 h before dark) in LD. **g** Hypocotyl elongation of plants in **d** grown for 5 days in complete darkness. Scale bars, 1 cm. **h** Hypocotyl length of transgenic plants in darkness. Data are means ± s.d. of 20 plants. **i** HY5 accumulation in various transgenic plants.
requires the L170 residue of COP1 (Fig. 5b; Supplementary Fig. 8). Similarly, FKF1 also interacted with COP1WT and COP1L105A much more strongly than with COP1L170A (Fig. 5c).

To examine the effect of these alterations on COP1 function in flowering time, we also generated transgenic plants carrying the 35S::COP1WT-GFP, 35S::COP1L105A-GFP, and 35S::COP1L170A-GFP constructs in the cop1-4 background. Both 35S::COP1WT-GFP and 35S::COP1L105A-GFP transgenes were able to fully rescue the early-flowering phenotype of cop1-4, whereas 35S::COP1L170A-GFP failed to delay flowering in SD (Fig. 5d, e; Supplementary Table 3). Moreover, the degree of COP1 dimerization was inversely proportional to CO levels in the late afternoon of LD. The plants carrying 35S::COP1L170A-GFP/cop1-4 had much higher CO levels than the 35S::COP1WT-GFP/cop1-4 and 35S::COP1L105A-GFP/cop1-4 plants (Fig. 5f). 35S::COP1L170A-GFP/cop1-4 plants flower early, similar to cop1-4. These results show that the L170A variant that is unable to dimerize not only is non-functional in regulation of seed color as previously reported but also in regulation of flowering time.

Finally, we examined whether the COP1 variants differed in their ability to destabilize HY5 for hypocotyl elongation in the dark (Fig. 5g, h; Supplementary Fig. 9, Supplementary Table 4). The COP1WT-GFP and COP1L105A-GFP fusion proteins, which form dimers normally, complemented the short-hypocotyl and cotyledon-expansion phenotypes of the cop1-4 mutant. However, COP1L170A-GFP, which forms dimers poorly, did not rescue the defect. In cop1-4 mutants, HY5 was almost completely degraded in darkness by either COP1WT-GFP or COP1L105A-GFP, but not by COP1L170A-GFP (Fig. 5i). Although we cannot rule out that these effects may be due to some other effect of the L170A mutation, these results are consistent with the level of COP1 dimerization correlating with the level of its functional activity in the timing of flowering and photomorphogenesis.

**Discussion**

For successful reproduction, most flowering plants bloom in a certain season, which they recognize mainly by sensing changes in temperature and day length. In *Arabidopsis*, CO is a key positive regulator of *FT* transcription in an LD-dependent manner, although *FT* expression is finely controlled by many regulators in other flowering pathways. FKF1 and COP1 are direct positive and negative regulators, respectively, of the stability of CO. Here, we demonstrate a direct link between FKF1 and COP1, in which FKF1 negatively regulates COP1 by the posttranslational regulation of CO. First, FKF1 genetically acts as an upstream negative regulator of COP1, as the late-flowering phenotype of the *fkf1* mutation is not present in the *cop1* background (Fig. 1a, b; Supplementary Table 1). Second, neither FKF1 overexpression nor *fkf1* mutation alters CO abundance in the *cop1* background (Fig. 3). Third, FKF1 strongly interacts with COP1 in the presence of light (Figs. 1c–e and 4a, b). Fourth, COP1 mutant variants that are unable to dimerize are unable to function in flowering as well as photomorphogenesis (Fig. 5).

Finally, the interaction between FKF1 and COP1 can inhibit COP1 homo-dimerization in a light-dependent manner (Figs. 4a, c). In summary, but, our findings show that the two important regulatory pathways for photoperiodic flowering of *Arabidopsis*, the FKF1–CO and COP1–CO pathways, that have previously been thought to act independently can act in the same pathway to regulate CO stability (Fig. 6).

*FT* is rhythmically expressed, with a peak at the end of day (around ZT16) only in LD, a few hours after the first peak of CO expression. CO12,15. Here, we demonstrate a direct link between FKF1 and COP1. The FKF1–CO1 interaction inhibits COP1 homo-dimerization, which produces different effects from that of the COP1L170A mutation: FKF1 and COP1L170A mutation both inhibit COP1 homo-dimerization, but FKF1 does not inhibit the COP1–SPA1 interaction (Fig. 4).

Homo-dimerization of COP1 occurs through the CC domain18,19 and SPA1 also binds to the CC domain of COP11,20,21. The molecular weight of a COP1 tetramer (COP1)2(SPA1)2 is approximately 440 kDa, but this tetramer is present in several multi-complexes much larger than 440 kDa in vivo. In fact, FKF1 function (inhibiting COP1 homo-dimerization) produces different effects from that of the COP1L170A mutation: FKF1 and COP1L170A mutation both inhibit COP1 homo-dimerization, but FKF1 does not inhibit the COP1–SPA1 interaction (Fig. 4).

Some photoreceptors, such as PHYs and CRYs, inhibit COP1 function although it is not clearly understood how they inhibit COP1 activity. It has been reported that photo-excited CRY2 interacts...
with SPA1 and enhances the CRY2–COP1 interaction, resulting in suppression of COP1 activity and CO degradation for early flowering. It is possible that FKFI1 and CRY2 work together to inhibit the formation of COP1 complexes, in which CRY2 inhibits COP1 homo-dimerization, and FKFI1 inhibits COP1 activity. The formation of COP1 (COP1–SPA1) is partially inhibited in the late afternoon of LD. We suggest that a specific COP1 complex is destabilized by light-activated FKFI1 and/or CRY2 thus stabilizing CO in a light-dependent manner. These two light-dependent regulatory mechanisms could have an important role in the regulation of COP1 complex formation for photoperiodic flowering, but this remains to be determined.

There are many possible mechanisms to explain how FKFI1 inhibits COP1 activity. We provide evidence that FKFI1 may negatively regulate COP1 activity by inhibiting its dimerization, but other regulatory mechanisms may exist. First, FKFI1 may compete with other E2-ubiquitin conjugating enzymes (such as AtUBC32) because FKFI1 binds to the RING domain of COP1 (Fig. 1d). Many E2 enzymes bind to the RING domain of RING-type E3 ligases and this interaction plays an important role in E3 activity. Studies of the relationship between FKFI1 and other E3 enzymes are needed to understand the regulatory mechanisms of E3-ubiquitin ligases. Second, FKFI1 may be involved in the nuclear exclusion of COP1. We analyzed COP1 protein accumulation in nuclear and cytoplasmic fractions from Col-0, fkf1-1, and 35S::FKFI1, and found that FKFI1 alone is not involved in the light/dark-induced movement of COP1 (Supplementary Fig. 10). Instead, we further found that all ZTL family members (ZTL, LKP2, and FKFI1) interact with COP1 (Supplementary Fig. 11), suggesting that the ZTL family may also be related to COP1 function throughout development. The functions of ZTL family members in other COP1-mediated regulatory mechanisms during growth and development remain to be determined.

Finally, experimental observations and mathematical modeling indicated that COP1 function is repressed in the light by a photoreceptor-related inhibitor termed “I”59. Here, we demonstrate that the blue-light receptor FKFI1 is a strong candidate among the hypothesized inhibitors, because FKFI1 interacts with and inhibits COP1 homo-dimerization in a light-dependent manner. It seems that FKFI1-mediated regulation of both CDF1 stability and COP1 activity are required to regulate light-dependent and internal rhythm-dependent control of protein expression. Based on these findings, we propose a new model involving an FKFI1–COP1–CO cascade (Fig. 6); the inhibition of COP1 homo-dimerization by light-activated FKFI1 stabilizes CO in the afternoon of LD, resulting in early flowering. In SD, however, FKFI1 expression mainly occurs after dusk and an inactive form of FKFI1 cannot interact with COP1, resulting in high levels of COP1 homodimers that can degrade CO completely, preventing FT transcription, which leads to late flowering. This FKFI1–COP1–CO regulatory cascade could be another layer in previously suggested models of the FKFI1–GI–CDF1–CO pathway.

Methods

Plant materials and growth conditions. All A. thaliana plant materials including WT, mutants, and transgenic plants were in the Columbia (Col-0) ecotype; cop1-4, fi1-1 (SALK_059480), fi1-2, fi1-2co, t 2-1, 4 plants, and 35S::TAP-COP1/cop1-6 mutant plants were used in this study. We generated the cop1-4 fi1-1 double mutant by crossing cop1-4 and fi1-1, 35S::FKFI1, 35S::MYC-FKFI1, and 35S::MYC-FKFI1 #3/cop1-4 (crossing 35S::MYC-FKFI1 #3 and cop1-4). Also, we generated COP1 variants as 35S::COP1WT-GFP/cop1-4, 35S::COP1L170A-GFP/cop1-4, and 35S::COP1L170A-GFP/cop1-4 transgenic plants. To generate COP1 variants (COP1L170A and COP1L170D), the COP1 WT cDNA was sequenced using the QuikChange Site-Directed Mutagenesis Kit using a Cloning system. To generate 35S::FKFI1, 35S::Myc-FKFI1 #3/cop1-4 plants, the 35S::FKFI1, 35S::Myc-FKFI1 vector for the expression of 35S::Myc-FKFI1 was used using the QuikChange II Site-Directed Mutagenesis kit with a Flp-in expression system. The expression of 35S::COP1 WT, 35S::COP1L170A-GFP/cop1-4, and 35S::COP1L170D-GFP constructs was transformed into Agrobacterium tumefaciens strain GV3101 by electroporation. The transformed Agrobacterium strain was cultured overnight at 30 °C in 3 mL YEP media containing antibiotics and subcultured into 50 mL YEP for overnight growth at 30 °C. The agrobacterium strain was cultured overnight at 30 °C in 3 mL YEP media containing antibiotics and subcultured into 50 mL YEP for overnight growth at 30 °C. The cultured cells were harvested by centrifugation and resuspended in infiltration buffer (100 mM MES, 100 mM MgCl2, 100 μM acetosyringone) to ODM 1.0. Each transformed Agrobacterium strain was mixed and infiltrated into the N. benthamiana leaves. The infiltrated tissues were harvested at the indicated time points after 48-h incubation in LD.

Transient co-expression by co-infiltration in tobacco leaves. The cDNAs of COP1WT, COP1L170A, and COP1L170D were amplified from the first-strand cDNA of WT plants using gene-specific primers (5′-ATGGCCAGGAAA-CATCCG-3′ and 5′-TTAAGCCGAGTCTTGGC-3′), and cloned into the pPCR8/GW/TOPO vector (Invitrogen). The FKFI1 cDNA was cloned into the pB7WG2 binary vector (Invitrogen). After the FKFI1 cDNA was confirmed by sequencing, the FKFI1 cDNA was subcloned into the pEarleyGate 203 binary vector using the LR clonase II (Invitrogen). For the FKFI1-overexpressing transgenic plants, the 35S::Myc-FKFI1 vector was transformed into WT plants. To generate 35S::Myc-FKFI1 #3/cop1-4 plants, the 35S::Myc-FKFI1 #3 plant was crossed with the cop1-4 mutant. The cop1-4 allele was selected by DCAPS and was checked in MS medium including hygromycin to select 35S::Myc-FKFI1 homozygous plants in the F2 seeds. To generate COP1 variants (35S::COP1WT-GFP/cop1-4, 35S::COP1L170A-GFP/cop1-4, and 35S::COP1L170D-GFP/cop1-4), the COP1 cDNA was amplified from first-strand cDNA of WT and 35S::Myc-FKFI1 over-expressing transgenic plants in the pDONR221 vector (Invitrogen) and introduced into the pMDC85 vector for the expression of COP1-GFP by the Gateway cloning system. To generate COP1 variants, COP1L170A/GFP, and COP1L170D/GFP constructs were transformed into cop1-4 mutant plants. Plants were grown on Murashige–Skoog (MS) phytoaerugin media containing 1% sucrose and 2 mM MES (pH 5.7) buffer or on soil in the growth chambers at constant 22 °C under cool white fluorescent light (100 μmol m/s2) under LD (16-h light/day) or SD (6-h, 9-h, or 10-h light/day).

Yeast two- and three-hybrid assays. Yeast two-hybrid assays were performed using the Matchmaker GAL4 two-hybrid system (Clontech). The full and partial COIs were digested into the pGADT7 and pGBT9 vectors of prey and bait, respectively. The full and partial (RING, aa 1–104; CC, aa 121–213; WD40, aa 371–675) cDNAs of COP1 were cloned into the pGBK vector (as baits). FKFI1 was cloned into the pGAD vector (as prey) with full and partial cDNAs of LOV (aa 1–174; LOV of V.f.-box: aa 1–283; F-box of KELCH: aa 174–618; KELCH: aa 283–618). The yeast two-hybrid experiments were performed using the Matchmaker GAL4 two-hybrid system (Clontech) in yeast strain AH109/Str-Met repressor. These vectors were used to generate the yeast strain AH109. Yeast transformation was performed according to the Yeast Handbook (Clontech). The colonies were used for yeast cell
growth assay, and a liquid assay using chlorophenol red-β-d-galactosidase (CPRG) was used to measure β-galactosidase activity.

**BiFC assays.** To examine the in vivo interaction, full-length cDNAs of COP1 and FKF1 were cloned into the BiFC Gateway vectors.46 Each cDNA was cloned into the pCRR/GW/TOPO vector (Invitrogen). After cDNA sequence confirmation, they were subcloned into the BiFC plasmid sets pSAT5-DEST-EYFP(175-end)-C1 (pS3130), pSAT(A)-DEST-eYFP(175-end)-N1 (pS3132), and pSAT(A)-DEST-eYFP-LN1 (747-174)-N1 (pS3135) and pSAT(A)-DEST-eYFP-LN1 (174-74)-C1 (pS3136). Each pair of recombinant plasmids encoding nEYFP or eYFP fusion proteins was co-barcoded into onion epidermal cell layers with a DNA particle delivery system (Biologic PDS-1000/He, Bio-Rad), and incubated with 50 μM MG132 in MS phytoagmar media for 16 h at 22 °C under continuous light, followed by image analysis using confocal laser scanning microscopy (LMS710, Carl Zeiss, Germany).

**Immunoblot and Co-IP.** To detect CO protein, seedlings were grown in MS agar media under LD and SD for 10 days, and were harvested at each time point. Nuclear protein was isolated using the Plant Nuclei Isolation/Extraction Kit (E.Z.N.A., Omega) following the manufacturer’s instructions, separated by 12% SDS-PAGE, and immunoblotted with anti-HY5 antibody (Agrisera, AS12-3097, 1:500 dilution). For Co-IP assays, total protein extracts were prepared from Arabidopsis plants in 8 h dark) and SD (9 h light: 15 h dark). When the primary injection was done, the plants were grown on soil at 22 °C for 2 weeks, and after the fourth injection, blood was gathered and the serum was available from the corresponding author upon request.

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**Data availability.** The authors declare that all data supporting the findings of this study are included in the manuscript and Supplementary Information files or are available from the corresponding author upon request.
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