Arabidopsis TOE proteins convey a photoperiodic signal to antagonize CONSTANS and regulate flowering time

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Plants flower in an appropriate season to allow sufficient vegetative development and position flower development in favorable environments. In *Arabidopsis*, *CONSTANS* (*CO*) and *FLAVIN-BINDING KELCH REPEAT F-BOX1 (FKF1)* promote flowering by inducing *FLOWER LOCUS T* (*FT*) expression in the long-day afternoon. The CO protein is present in the morning but could not activate *FT* expression due to unknown negative mechanisms, which prevent premature flowering before the day length reaches a threshold. Here, we report that TARGET OF EAT1 (TOE1) and related proteins interact with the activation domain of CO and CO-like (COL) proteins and inhibit CO activity. TOE1 binds to the *FT* promoter near the CO-binding site, and reducing *TOE* function results in a morning peak of the *FT* mRNA. In addition, TOE1 interacts with the LOV domain of FKF1 and likely interferes with the FKF1–CO interaction, resulting in partial degradation of the CO protein in the afternoon to prevent premature flowering.

[*Keywords*: photoperiod; flowering; *FT* expression; protein interaction; CONSTANS; TARGET OF EAT]

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Plants maximize their reproduction in part by regulating flowering time (the timing of the transition from vegetative to reproductive development) via integration of environmental cues with endogenous signals (Bäurle and Dean 2006; Salazar et al. 2009; Song et al. 2010). In particular, plants sense seasonal day length (photoperiod) changes through detection of lighted period and light quality, thereby effecting flowering in the appropriate season but not before (Guo et al. 1998; Yanovsky and Kay 2002; Imaizumi 2010; Song et al. 2013). Premature flowering reduces the period of vegetative development and decreases reproductive fitness and thus should be avoided. In *Arabidopsis thaliana*, flowering is promoted by long days, and a crucial photoperiodic regulator of flowering is the long-day-specific protein FLOWERING LOCUS T (FT), which serves as a mobile signal from the light-sensing leaves to the shoot apical meristem (SAM), where flower development is initiated (Corbesier et al. 2007; Jaeger and Wigge 2007; Kobayashi and Weigel 2007; Mathieu et al. 2007). *FT* transcription is directly activated by the B-box transcription factor CONSTANS (CO) in the long-day afternoon when day length reaches a certain threshold (Putterill et al. 1995; Suárez-López et al. 2001).

The CO protein is stabilized under light due to the contribution of the phytochrome A (phyA) and cryptochrome (CRY1 and CRY2) photoreceptors (Guo et al. 1998; Valverde et al. 2004) but is degraded in darkness through ubiquitin-mediated proteolysis that requires a RING finger E3 ubiquitin ligase, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) (Jang et al. 2008; Liu et al. 2008). In addition, phyB also facilitates CO degradation through interaction with a nuclear protein, PHYTOCHROME-DEPENDENT LATEFLOWERING (PHL) (Endo et al. 2013). Moreover, the CO protein is also degraded by the HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1)-mediated pathway in the late morning of long days or under cold stress (Jung et al. 2012; Lazaro et al. 2012).

The F-box containing blue-light receptor FKF1 perceives light information and, together with GIGANTEA (GI), mediates the degradation of the CYCLING DOF FACTOR (CDF) proteins in a blue-light-dependent

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manner in the long-day afternoon (Imaizumi et al. 2005; Sawa et al. 2007; Fornara et al. 2009). CDF proteins (CDF1, CDF2, CDF3, and CDF5) are transcription factors that repress *CO* transcription during the late morning (Sawa et al. 2007; Fornara et al. 2009). Consequently, the transcription of *CO* increases in the early morning and again in the afternoon (Suárez-López et al. 2001; Salazar et al. 2009). In addition, FKF1 also stabilizes the CO protein in the late afternoon of long days through direct protein interaction (Song et al. 2012, 2013). Therefore, the levels of both the *CO* transcript and CO protein oscillate with an early morning peak and an afternoon peak of long days (Song et al. 2012); however, *FT* is expressed only in the late afternoon, not in the early morning (Salazar et al. 2009). The lack of *FT* expression in the morning increases the length of day needed for flowering and helps to avoid premature flowering early in the spring; this failure to express *FT* in the presence of CO in the morning indicates that CO is inhibited by a negative mechanism involving unknown factors.

Arabidopsis flowering is also positively regulated by *microRNA172* (*miR172*), which mediates repression of members of the *APETALA2* (*AP2*) family—the *TOE* genes, including *TOE1*, *TOE2*, *TOE3*, *SCHLAFMÜTZE* (*SMZ*), and *SCHNARCHZAPFEN* (*SNZ*) (Aukerman and Sakai 2003; Chen 2004). Although *SMZ* was reported to repress *FT* expression (Mathieu et al. 2009), the mechanism of such repression is unclear. As part of a search for proteins that interact with AP2/ethylene-responsive element-binding proteins (EREBPs), we found that the TOE proteins physically interact with CO and its homologs, CO-like (COL) proteins; specifically, TOE1 interacts with the transcriptional activation domain of CO. Our genetic studies indicated that the *TOE* genes delayed flowering in a manner that partially depends on *CO*, and plants with reduced *TOE* function showed a morning peak of *FT* mRNA, suggesting that TOE1 inhibits CO activity for *FT* expression. Furthermore, TOEs interact with FKF1 and two related F-box proteins, ZEITLUPE (ZTL) and LOV KELCH PROTEIN2 (LKP2), providing a mechanism for the observed interference by TOE1 of the stabilization of CO due to FKF1 in the long-day afternoon. Therefore, we propose that TOE1 inhibits CO function via two different mechanisms to limit *FT* transcription until late afternoon of long days, thereby ensuring flowering only after the day length reaches a threshold.

Results

TOE proteins interact with the CO activation region and COLs

To probe the transcriptional regulatory network containing TOE proteins, we used TOE1 as a representative in a yeast two-hybrid (Y2H) screen for TOE-interacting proteins. Two positive clones encoded COL1 (At5g15850), suggesting that TOEs might interact with CO and COLs, which are highly similar in amino acid sequence. Thus, we tested in yeast for interactions of TOE1, TOE2, TOE3, and SNZ (also a TOE) with COL1, COL2, COL3, and COL5 and the CO C-terminal region (COC) (Fig. 1A) because of the toxicity of the full-length CO in yeast (Supplemental Fig. S1A) and found that most TOEs could interact with COC, COL1, and COL5. Furthermore, interactions of TOE1 with CO and COL1 proteins in the nuclei of plant cells were further supported by bimolecular fluorescence complementation (BiFC) assays (Fig. 1B). Moreover, we generated transgenic plants carrying both *35S::TOE1-10myc* and *35S::CO-ECFP*, and coimmunoprecipitation (co-IP) demonstrated that TOE1 interacted with CO in vivo (Fig. 1C). Therefore, TOE1 can physically interact with CO and COLs in plant cell nuclei.

To dissect the regions of the TOE1 and CO proteins important for their interaction, we investigated the interaction using truncated TOE1 or CO proteins by Y2H assays (Supplemental Fig. S1). We found that most of the truncated CO proteins could interact with TOE1 except the truncated COs that lacked the activation domain (Supplemental Fig. S1A), indicating that this domain is required for the TOE1–CO interaction. Similar experiments with truncated TOE1 proteins and COC (177–374) containing the activation domain (the full-length CO was toxic) revealed that the C-terminal region of TOE1 (294–449) was both necessary and sufficient for the interaction with CO (Supplemental Fig. S1B). Although no conserved motifs were reported previously for this region of TOE1, our sequence analysis of TOE proteins and their homologs in other plants identified a conserved motif with sequence similarity to the EAR motif known to inhibit transcription (Fig. 1D; Supplemental Fig. S1C; Kagale and Rozwadowski 2011). These results strongly support the hypothesis that the TOE1 C-terminal domain containing an EAR-like (EARL) motif interacts with the activation domain of CO (see Fig. 1E for CO and TOE1 domain structures). To probe the role of the EARL motif in protein–protein interactions and in flowering time regulation, we generated two versions of truncated TOE1: TOE1 (–EARL) (without the EARL motif) and TOE1 (1–310) (lacking the C-terminal region, including EARL). We tested the interactions of these truncated TOE1 proteins with the COC and found that TOE1 (–EARL) still could interact with the COC, but TOE1 (1–310) could not interact with the COC (Supplemental Fig. S1D). We then generated transgenic plants overexpressing TOE1 (–EARL) to test the function of the EARL motif and found that the transgenic plants flowered earlier than wild type and the *toe1-1* mutant (Supplemental Fig. S1E). Therefore, the EARL motif does not seem to affect the protein–protein interaction but is needed for repressing flowering by TOE1.

It is known that the *toe1* and *toe1 toe2* mutants flowered early, whereas overexpression of *TOE*s caused late flowering (Aukerman and Sakai 2003; Jung et al. 2007; Mathieu et al. 2009). *TOE* overexpression caused reduced *FT* expression but did not affect *CO* expression (Aukerman and Sakai 2003), suggesting that TOEs act upstream of *FT* but do not regulate *CO* mRNA level. Our results that TOE1 interacted with the transcriptional activation domain provide a potential mechanism for the inhibition of CO activity by TOE1.

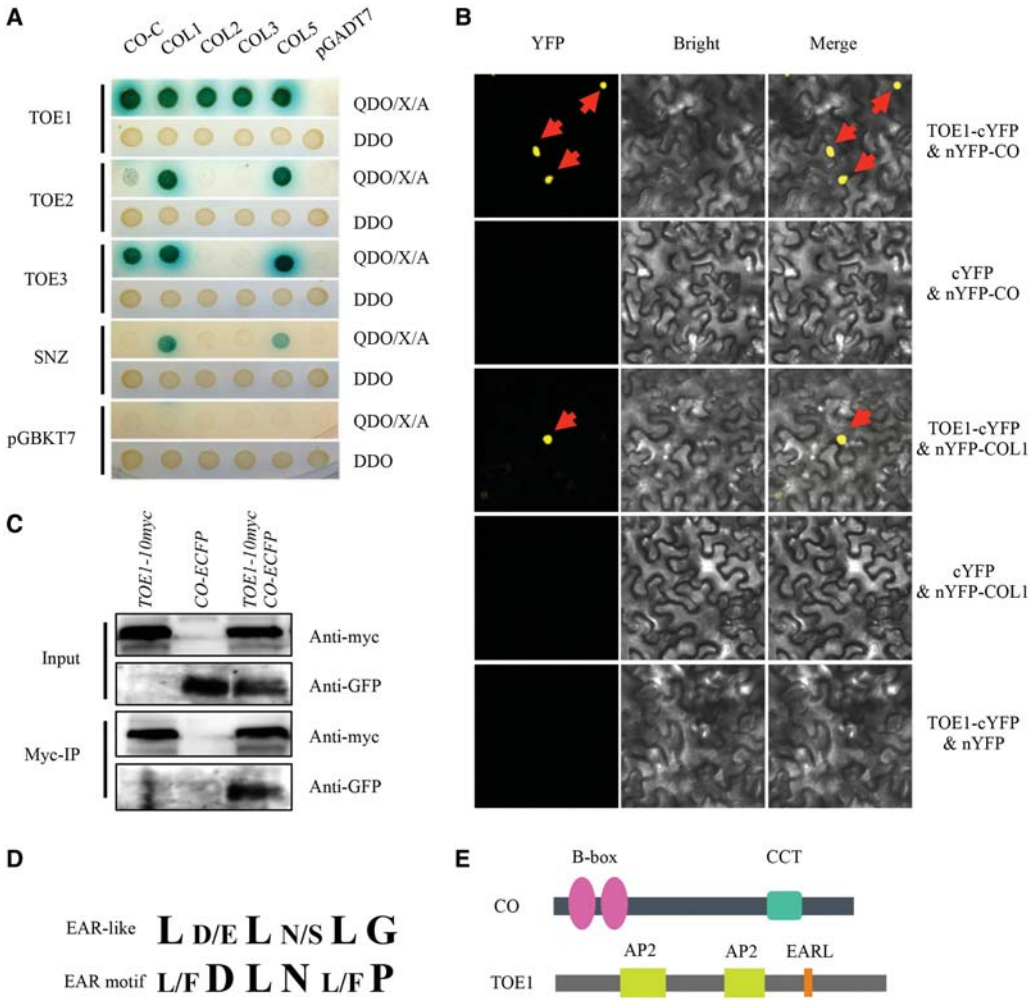

Figure 1. TOE proteins interact with CO and COLs. (*A*) Y2H analysis of the interaction between TOE1, TOE2, TOE3, SNZ, or the empty bait vector GAL4-BD with COC, COL1, COL2, COL3, COL5, or the empty prey vector GAL4-AD. Diploid yeast cells from mating contained the bait and prey plasmids and grew on SD – Trp/– Leu (DDO) medium, which selects for both plasmids, providing an estimate for mating efficiency, and SD – Trp/– Leu/– His/– Ade + X-α-gal + Aureobasidin A (AbA) (QDO/X/A) medium, which detects reporter gene expression indicative of interaction. Blue cells indicate *MEL1* expression due to protein–protein interaction in yeast. (*B*) BiFC analysis of the interaction between TOE1 and CO or COL1. (YFP) Fluorescence of yellow fluorescent protein; (Bright) bright field; (Merge) merge of YFP and Bright. Red arrows indicate YFP signal. (*C*) In vivo interaction between TOE1 and CO. Total protein extracts from *35S::TOE1-10myc* and *35S::CO-ECFP* transgenic plants were immunoprecipitated using anti-Myc agarose beads. The coimmunoprecipitated proteins were detected by anti-GFP and anti-Myc antibodies, respectively. (*D*) Consensus sequences of EAR-like (*top*) and EAR (*bottom*) motifs. (*E*) An illustration of CO and TOE1 protein domain structures.

TOE*s counteract the promotion of flowering by* CO

To investigate the functional relationship between *CO* and *TOE1* in vivo, we tested for genetic interactions between these genes. We generated double and triple mutants for *TOE*s with or without a *co* mutation and determined the day after germination (DAG) when flowering occurred (bolting). The *toe1-1*, *toe1-1 toe2-1*, and *toe1-1 toe2-1 smz-1* mutants showed progressively earlier flowering than wild type, and the *co-1* mutant was severely late-flowering (Fig. 2A,B,D), consistent with previous reports (Putterill et al. 1995); however, the *toe1-1* and *toe1-1 toe2-1* mutations failed to cause early flowering in the *co-1* mutant background (Fig. 2B–D). We also counted the rosette leaf number (RLN), which is often used as an estimate of flowering time (Amasino 2010). Consistently, the *toe1-1*, *toe1-1 toe2-1*, and *toe1-1 toe2-1 smz-1* plants produced progressively fewer rosette leaves than wild type under long days (Fig. 2E); intriguingly, the *toe1-1 co-1* and *toe1-1 toe2-1 co-1* mutants produced fewer rosette leaves than the *co-1* single mutant (Fig. 2E), suggesting that other factors, such as COLs, were affected by the *toe* mutations. Under short days, CO is considered not active, as supported by the lack of delay in flowering in the *co-1* mutant compared with wild type (Fig. 2C,F,G; Yanovsky and Kay 2002; Amasino 2010). Nevertheless, the *toe* mutations can still cause earlier flowering than wild type in a *CO*-dependent manner (Fig. 2C,F,G).

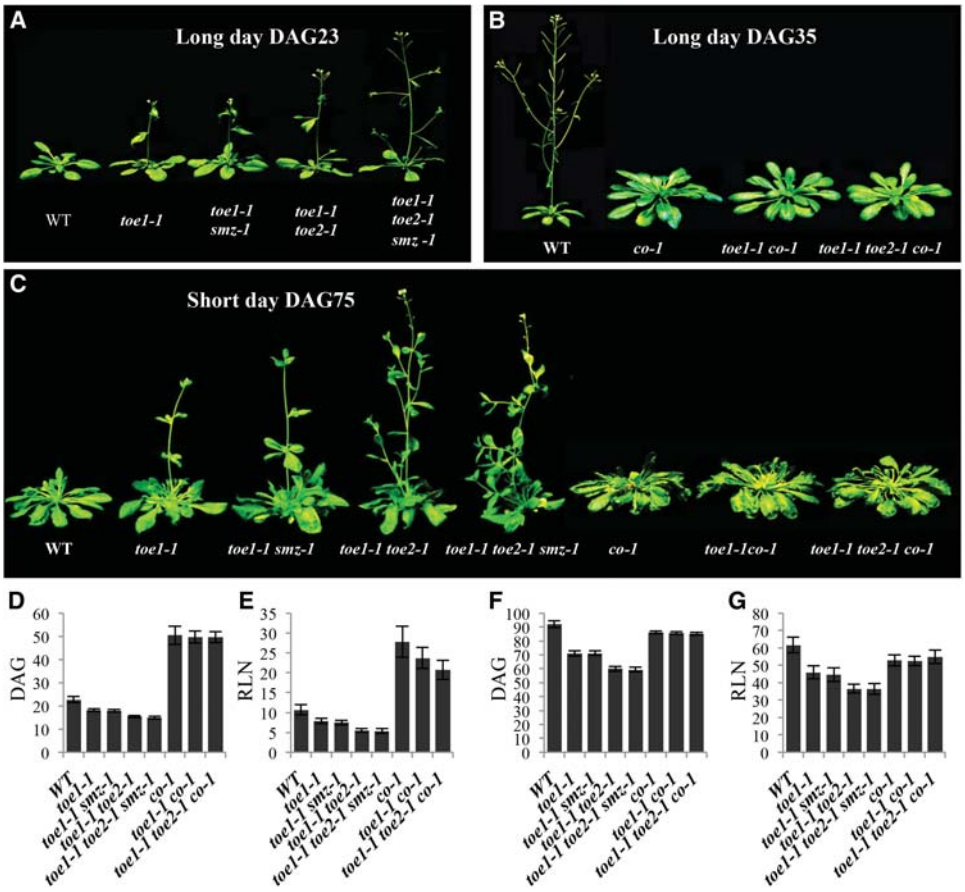

Figure 2. Flowering time of the mutants of *TOE*s and *CO*. (*A*) Wild-type (WT), *toe1-1*, *toe1-1 smz-1*, *toe1-1 toe2-1*, and *toe1-1 toe2-1 smz-1* plants at DAG23 of growth under long-day condition (16 h of light and 8 h of darkness). (*B*) Wild type, *co-1*, *toe1-1 co-1*, and *toe1-1toe2-1 co-1* at DAG35 of long-day growth. (*C*) Wild type, *toe1-1*, *toe1-1 smz-1*, *toe1-1 toe2-1*, *toe1-1 toe2-1 smz-1*, *co-1*, *toe1-1 co-1*, and *toe1-1 toe2-1 co-1* at DAG75 in short-day condition (8 h of light and 16 h of darkness). Bolting time of various genotypes as indicated in terms of DAG in long day (*D*) or short day (*F*) and RLN under long days (*E*) or short days (*G*).

Therefore, analysis with the *toe* mutations revealed that a latent *CO* function promotes flowering under short days.

To further test the antagonism between *TOE1* and *CO*, we overexpressed these genes using transgenes and found that the *35S::TOE1-10myc* and *35S::CO-ECFP* plants flowered later or earlier than normal, respectively (Supplemental Fig. S2A–C). Moreover, overexpression of *TOE1* could reduce the early flowering effect due to *CO* overexpression (Supplemental Fig. S2A–C), further supporting a negative interaction between *TOE1* and *CO*. The protein–protein interactions described above and the mutant flowering phenotypes strongly support the hypothesis that TOE proteins inhibit the transcriptional activation ability of CO on *FT*. This inhibition might be required for restricting CO function to long-day conditions, thereby allowing CO to promote flowering under long days but not short days.

We further used a dual-luciferase (Dual-LUC) system to demonstrate the regulation of *FT* by TOE1 and CO (Supplemental Fig. S2D). We found that the CO protein alone activated *FT* expression in tobacco leaves, whereas TOE1 could not. More importantly, when *TOE1* was expressed in the CO-*FT* activation system, the activation of *FT* by CO was decreased. However, the *TOE1 (–EARL)* truncation construct had no effect on the activation of *FT* by CO (Supplemental Fig. S2D), indicating that the EARL motif is required for the inhibition of *FT* activation by TOE1.

TOE*s are expressed and repress* FT *in the early morning and afternoon*

The expression of *TOE* genes decreases from juvenile to adults, opposite to the *FT* expression pattern (Jung et al. 2007); in addition, expression of *FT* but not *CO* is elevated when *TOE* function is reduced (Jung et al. 2007; Mathieu et al. 2009). Although the *FT* transcript level displays circadian periodicity with a peak in the late afternoon of long days (Suárez-López et al. 2001; Song et al. 2012), the circadian patterns of *TOE*s were unknown. To investigate this aspect of *TOE* expression, we examined the leaves of 14-d-old wild-type plants grown under long-day condition and found that the expression of *TOE1*, *SMZ*, and *SNZ* exhibited a peak at Zeitgeber time 1 (ZT1; 1 h after light was turned on) in the early morning, whereas *TOE2* expression

peaked slightly later at ZT7 (Fig. 3A; Supplemental Fig. S3A). In addition, the expression of *TOE1* and *TOE2* also displayed another peak in the afternoon, implying that the TOE proteins function in both the early morning and afternoon of long days. Furthermore, we detected *TOE1* expression under short day conditions, also with a morning peak (Fig. 3B).

Both the *CO* mRNA and the CO protein levels exhibit a peak in the morning, but the *FT* mRNA lacks such a peak (Salazar et al. 2009). Because TOEs interact with CO and are transcriptional repressors, we hypothesized that TOEs might inhibit the transcriptional activation activity of CO at the *FT* promoter in the early morning. To test this idea, we analyzed *FT* expression in 14-d-old *toe1-1 toe2-1* double mutants under long days. The *FT* expression showed a higher peak from the late afternoon to dusk (ZT13–ZT16) in the *toe1-1 toe2-1* double mutant than in wild type; furthermore, unlike wild type, the expression of *FT* in *toe1-1 toe2-1* showed a small peak in the early morning (ZT0.5–ZT1) (Fig. 3C). These results indicate that *TOE1* and *TOE2* have an inhibitory effect on *FT* expression in the early morning and afternoon.

To further test the repression of *FT* by TOEs, we generated transgenic plants carrying a *35S::miR172e* construct to increase the levels of *miR172e,* thereby reducing TOE proteins post-transcriptionally. The morning *FT* expression in the transgenic plants was higher than the wild type (Fig. 3D); moreover, the *FT* expression was also abnormally high before dawn, unlike that in the wild type and *toe1-1 toe2-1*. These results are consistent with the previous results that *miR172*-overexpressing plants showed some *FT* expression near the middle of the day (Jung et al. 2007). The increased *FT* expression in *toe1-1 toe2-1* before dawn suggested possible negative interactions between TOEs and COL proteins, as supported by our protein interaction results (Fig. 1). Therefore, the TOE proteins could repress *FT* expression in the early morning and then from the late afternoon through the night. The fact that early flowering in the *toe* mutants was dependent on *CO* function suggested that the increased *FT* expression might require *CO* too. Indeed, unlike the increased *FT* expression in various *toe* mutants at both early morning and late afternoon (ZT1 and ZT16 during long days) (Supplemental Fig. S3C), *FT* expression was not detected in *co-1*, *toe1-1 co-1*, and *toe1-1 toe2-1 co-1* mutants (Supplemental Fig. S3C), indicating that derepression of *FT* in *toe* mutants requires *CO* function. In addition, the *FT* expression in *toe* mutants under short days also exhibited a peak in the early morning (Supplemental Fig. S3B). Together with the genetic results of *TOE*s and CO under short days, *TOE*s probably also restrict the function of CO and other COLs under short days.

TOE1 binds to an AT-Rich element in the FT *promoter near the CO-binding site*

CO could bind to the CORE (CO-responsive element) of the *FT* promoter via its CCT domain (Tiwari et al.

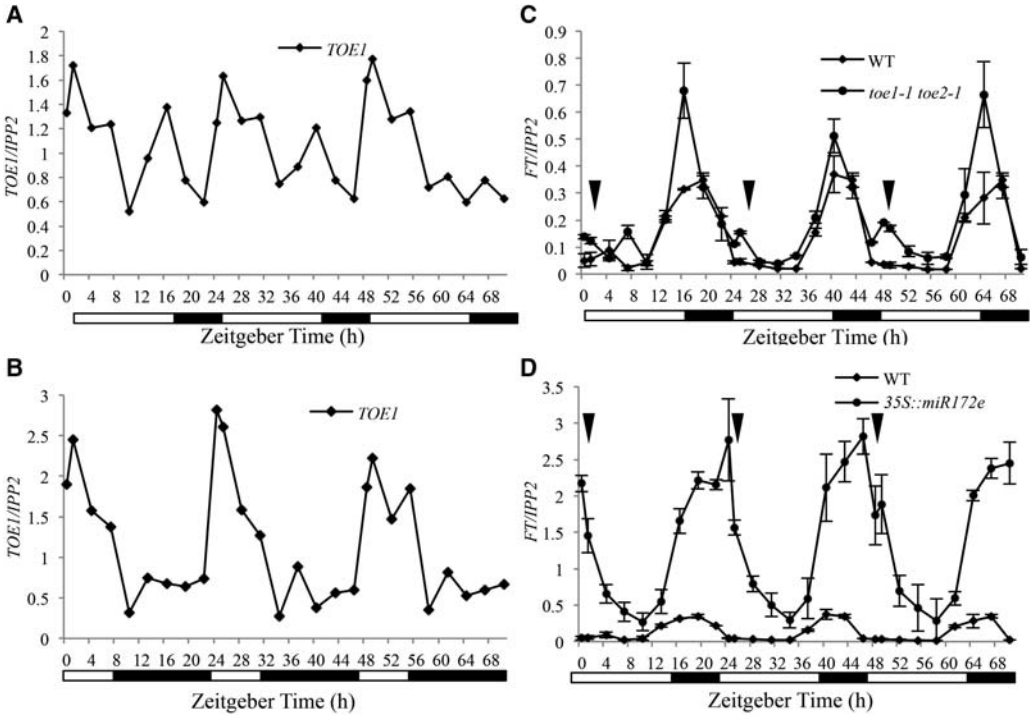

Figure 3. Temporal expression patterns of *TOE*s in wild-type plants and *FT* in *toe1-1 toe2-1* and *35S::miR172e* plants. (*A*,*B*) Expression of *TOE1* in wild type (Columbia [Col-0]) under long-day (*A*) and short-day (*B*) conditions. (*C*) Expression of *FT* in wild-type (WT; diamond) and *toe1-1 toe2-1* (circle) plants under long days. The arrowheads indicate expression peaks in *toe1-1 toe2-1* mutant plants. (*D*) Expression of *FT* in *35S::miR172e* (circle) plants under long days (wild type was same as in *C*).

2010); because TOEs belong to the AP2 family of transcription factors (Aukerman and Sakai 2003), we postulated that TOEs might also bind to the *FT* promoter. To investigate this possibility, we performed a chromatin immunoprecipitation (ChIP) analysis using *35::TOE1-10myc*-overexpressing and *35S::CO-ECFP*-overexpressing plants. We analyzed the TOE1-10myc-specific and CO-EYFP-specific enrichment of four different *FT* promoter regions (Fig. 4A) and found that both TOE1 and CO proteins were associated with regions B and D (Fig. 4B) containing CORE sequences.

As AP2 domain-containing proteins, TOEs might bind to target sites similar to those of AP2. To test this idea, we performed the electrophoretic mobility shift assay (EMSA) with DNA probes carrying AP2-binding sites, including a fragment from the *FT* promoter (Fig. 4C), and found that TOE1 protein could bind to all of these probes (Fig. 4D). In addition, the regions (B and D) enriched by the ChIP experiment contained AT-rich elements (Fig. 4C) that could bind to TOE1 in vitro. Moreover, a truncated TOE1 protein (amino acids 216–449) containing the second AP2 domain and the C-terminal region was still associated with the probes (Supplemental Fig. S4). These results indicate that the TOE1 protein could associate with the *FT* promoter in vivo and in vitro.

TOE1 interacts with FKF1 physically and acts downstream from FKF1 genetically

Our Y2H screens for TOE-interacting proteins also uncovered potential interaction with the circadian clock-related F-box protein ZTL (AT5G57360), which has two *Arabidopsis* homologs: LKP2 (LOV-KELCH PROTEIN2) and FKF1. We next tested for interactions of TOE1 with LKP2 or FKF1 and found that TOE1 also interacted with LKP2 and FKF1 in yeast (Fig. 5A). Because FKF1 is known to regulate photoperiodic flowering, we focused on the interaction between TOE1 and FKF1 and verified their interaction in vivo using BiFC and co-IP experiments (Fig. 5B,C).

To further investigate the biological effects of the TOE1–FKF1 physical interaction, we next analyzed the genetic interaction of *FKF1* and *TOE*s in regulating flowering time using double and triple mutants. The *toe1-1 fkf1-3* plants flowered later than wild type but significantly earlier than *fkf1-3*, with *toe1-1 toe2-1 fkf1-3* showing even earlier flowering (Fig. 5D,E). The RLN and bolting time statistics had similar trends (Fig. 5F–I). Next, we examined the *FT* expression at ZT1 and ZT16 and found that *FT* expression in *toe1-1 fkf1-3* and *toe1-1 toe2-1 fkf1-3* plants was higher than that in *fkf1-3* at ZT1 and ZT16 (Fig. 5J). Notably, unlike the *toe1-1 toe2-1 co-1* mutant, the significantly greater *FT* expression in the *toe1-1 toe2-1 fkf1-3* mutant than that in *fkf1-3* at ZT1 indicated that loss of TOE function in the *fkf1-3* background could still derepress *FT* expression. Therefore, these results indicated that TOEs and FKF1 act antagonistically to regulate *FT* expression.

FKF1 does not reduce the TOE1 protein level

As FKF1 is an F-box E3 ligase that regulates protein stability (Imaizumi et al. 2005; Suetsugu and Wada 2013), we hypothesize that FKF1 might influence TOE1 protein accumulation. To test this, we analyzed the diurnal TOE1 protein accumulation patterns using the *35S::TOE1-10myc* and *35S::TOE1-10myc fkf1-3* transgenic plants under long days. However, the TOE1-10MYC protein did not show an obvious increase in *fkf1-3* compared with wild type (Fig. 6A; Supplemental Fig. S5A,B). To avoid the post-transcriptional regulation by miR172, we further generated *35S::TOE1m-10myc* transgenic plants expressing an altered *TOE1* mRNA with six mismatches to miR172 (Supplemental Fig. S5C), and the results did not indicate a clear effect of *fkf1-3* on TOE1 protein levels in the *35S::TOE1-10myc* lines (Fig. 6B–D). Taken together, there was no strong evidence that FKF1 affected the TOE1 protein stability.

TOE1 *overexpression reduced CO protein accumulation*

It is known that FKF1 interacts through its LOV domain with CO and stabilizes CO in the afternoon of a long day (Song et al. 2012). Also, our results showed that TOE1 could interact with the LOV domain of FKF1, suggesting

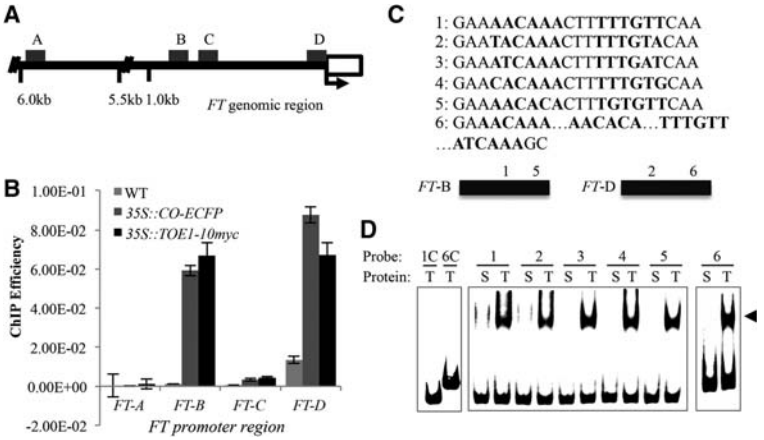

Figure 4. TOE1 protein binds to the *FT* promoter region in vivo and in vitro. (*A*) A schematic drawing of the *FT* genomic region and locations of fragments amplified in ChIP experiments. (*B*) ChIP analysis with 14-d-old plants grown under long days. Wild-type, *35S::CO-ECFP*, and *35S::TOE1-10myc* plants were harvested in ZT13–ZT16. (*C*, *top*) The core sequences of probes for binding by TOE1. Probes 1–5 are from a previous study (Dinh et al. 2012), and probe 6 is from the *FT* promoter, containing three AT-rich elements. (*B*) The sequence elements present in regions *FT*-B and *FT*-D, detected by ChIP, are indicated by the probe numbers (*below*). (*D*) Gel shift assay of TOE1 with different probes. (S) The empty vector expressing the SUMO protein; (T) the TOE1-SUMO fusion protein. The 200-fold unlabeled probes (1C and 6C) were used as competitive negative controls.

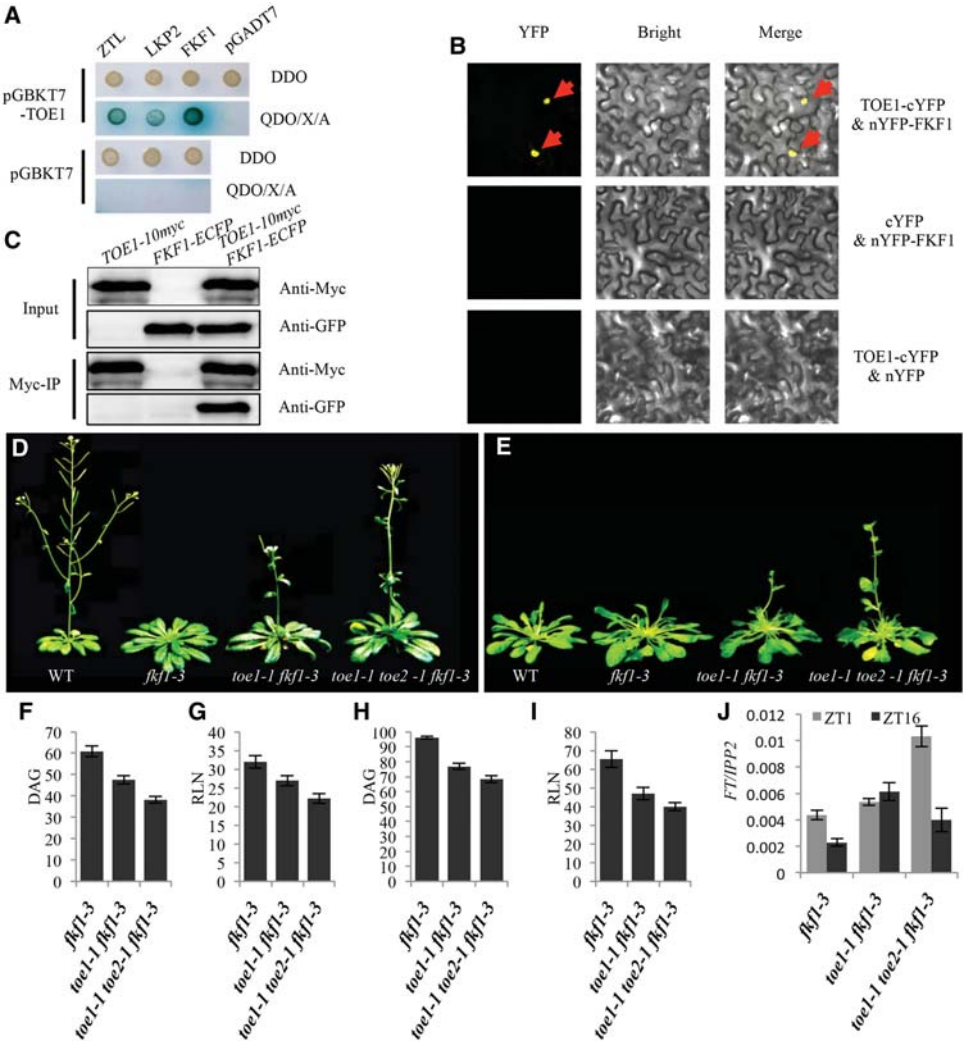

Figure 5. TOE1 interacts with ZTL, LKP2, and FKF1 physically and genetically. (*A*) Y2H analysis of the interaction between TOE1 and the LOV domains of ZTL, LKP2, and FKF1. (*B*) BiFC analysis of the interaction between TOE1 and FKF1. (*C*) In vivo interaction between TOE1 and FKF1. Total protein extracts from *35S::TOE1-10myc* and *35S::FKF1-ECFP* plants were immunoprecipitated by anti-Myc agarose beads. The coimmunoprecipitated proteins were detected by anti-GFP and anti-Myc antibodies. Wild-type, *fkf1-3*, *toe1-1 fkf1-3*, and *toe1-1 toe2-1 fkf1-3* plants at DAG52 of long-day condition (*D*) and at DAG75 under short days (*E*). Bolting time calculation of various mutants by counting DAG under long days (*F*) or short days (*H*) and RLN under long days (*G*) or short days (*I*). (*J*) *FT* expression in *fkf1-3*, *toe1-1 fkf1-3*, and *toe1-1 toe2-1 fkf1-3* plants under long days.

that TOE1 can compete with CO for interaction with FKF1. If this is true, TOE1 might cause a reduction of the CO protein level in the afternoon because of a reduction of the protection of CO by FKF1. To test this, we estimated the CO protein accumulation and found that the CO-ECFP protein level was less in the *35S::CO-ECFP 35S::TOE1-10myc* plants at ZT10–ZT13 than in the *35S::CO-ECFP* plants (Fig. 6E,F). We also detected the *TOE1* mRNA level in wild-type and *35S::TOE1-10myc* plants and found that in 10-d-old and 20-d-old plants, the *TOE1* mRNA level in the overexpression line was ~1.8-fold and ~2.7-fold of that of wild type, respectively (Supplemental Fig. S5D), suggesting that the *TOE1* mRNA generated from the *35S::TOE1-10myc* transgene was similar to or only slightly higher than that of the wild type.

Discussion

Flowering timing is regulated by complex regulatory networks that monitor the changing environment and ensure reproductive development at an optimal time (Salazar et al. 2009). A key component of the flowering regulatory network is the photoperiod pathway that controls flowering in response to seasonal changes in day length through a signaling cascade in *Arabidopsis* involving the FKF1/GI complex and the transcriptional factor CO (Imaizumi et al. 2005; Sawa et al. 2007; Song et al. 2012). One of the most important target genes of CO is the *FT* gene, which is expressed only when day length reaches a threshold and is required for the long-day-dependent acceleration of flowering in *Arabidopsis* (Suárez-López et al.

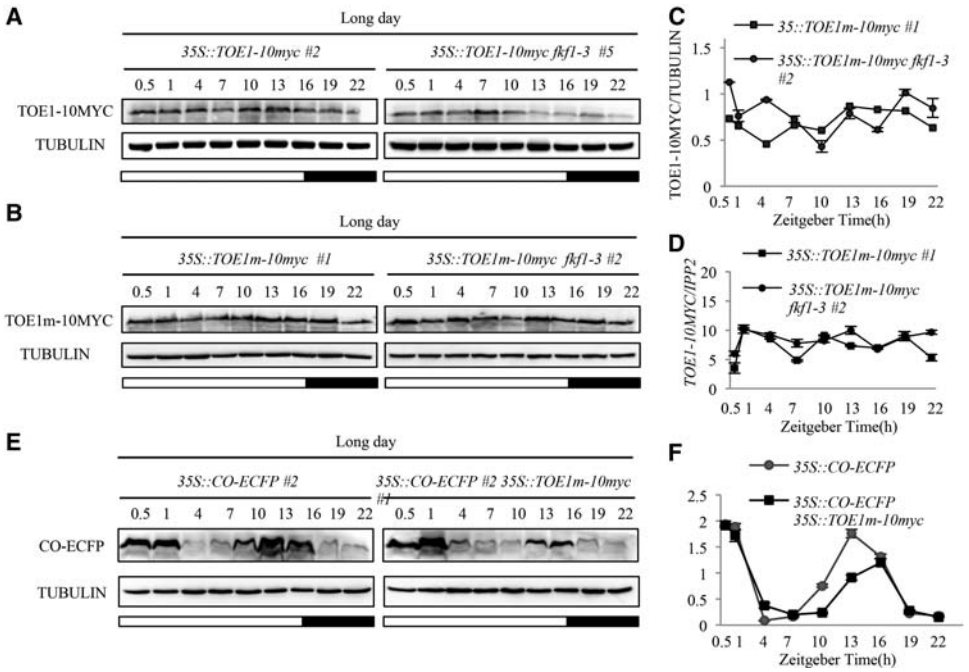

Figure 6. Analyses of TOE1 and CO protein levels. (*A*) A Western blot experiment shows that FKF1 does not cause an obvious reduction of the TOE1 protein level. Tubulin was used as a control. Plants were grown for 21 d under long days. (*B*) Similarly, FKF1 does not reduce the TOE1 protein level in *35S-TOE1m-10myc* plants. (*C*) The TOE1-Myc protein level, as estimated by the gel-pro software from Western blot results. (*D*) The *TOE1* mRNA level in the *35S::TOE1m-10myc* and *fkf1-3 35S::TOE1m-10myc* plants. (*E*) Western blot shows TOE1 influence on CO protein stability at ZT13 and ZT16. (*F*) The CO-ECFP protein level was estimated by gel-pro software from Western blot results.

2001). Precocious expression of *FT* and premature flowering would reduce vegetative development needed for full fertility and is not desirable. We showed that the repression of *FT* expression by TOE proteins through interaction with CO and related COLs and provided strong evidence that TOEs inhibit the activity of CO protein. In addition, TOE1 also influences CO protein stability by interacting with FKF1, reducing its protection of CO. Therefore, our analyses demonstrated that TOEs, as key regulators preventing premature flowering, act via two distinct and previously unknown mechanisms for negatively regulating *FT* expression and flowering: (1) a direct interaction of TOE1 and CO that likely inhibits CO activity and (2) an interaction of TOE1 with FKF1 that indirectly reduces CO protein levels.

TOE proteins repress FT *expression by binding to and inhibiting CO*

It was proposed previously that *TOE*s regulate *FT* expression in a way independent of CO because *TOE*s did not affect *CO* mRNA levels (Jung et al. 2007; Mathieu et al. 2009). However, our results demonstrated that TOEs physically interact with the CO protein, providing a mechanism for regulation of CO by TOEs in the photoperiod flowering pathway. Specifically, the TOE1 C-terminal region with a putative transcriptional repressor motif interacts with the transcription activation region of CO, thereby inhibiting CO activity. Further support for the idea that TOE1 regulates flowering by inhibiting CO is provided by genetic studies that *toe1-1 toe2-1* mutations did not cause early flowering in the *co-1* mutant background. Previously, it was reported that overexpression of *miR172a* in *co-2* led to early flowering (Jung et al. 2007), probably because the overexpression of *miR172* affected functions of COLs, some of which (COL1 and COL5) were shown to interact with TOEs in this study. The expression of *COL5* is regulated by the circadian clock and GI; in addition, *COL5* overexpression promotes flowering and activates *FT* (Hassidim et al. 2009). Meanwhile, overexpression of COL1 could also lead to early flowering (Supplemental Fig. S2E). In short, our results and previous findings support that complex interactions between members of the TOE family and those of the CO family regulate photoperiodic flowering.

TOEs prevent precocious flowering by repressing FT *expression*

It is important that long-day flowering plants do not flower before the day length is sufficiently long in order to allow enough vegetative development and promote overall fitness. The prevention of precocious flowering in *Arabidopsis* is achieved by activating *FT* expression only in the afternoon but not in the morning. The CO protein is present in both the morning and afternoon, and our results showed that TOEs are important to inhibit CO activity in the morning. In the afternoon, both CO and TOEs are

present; nevertheless, CO can activate *FT* because of the presence of *FKF1,* which is also regulated by the circadian clock (with peak expression in the afternoon) and encodes an E3 ubiquitin ligase that helps to stabilize CO (Sawa et al. 2007).

However, the positive interaction between FKF1 and CO is tempered by TOE1, which competes with CO for interaction with FKF1, as both CO and TOE1 interact with the LOV domain of FKF1. Competitive protein interaction has also been observed for other proteins important for plant light signaling. The blue-light receptor CRY1 was shown to interfere with the COP1–SPA1 interaction, which regulates the stability of a group of transcription factors and affects seedling development under light (Lian et al. 2011). Another example is the interference by PRR3 of the interaction between ZTL (a blue-light-responsive E3 ligase) and TOC1 (a key regulator of the circadian clock) and the consequent increase of the stability of TOC1 (Para et al. 2007).

Therefore, we postulate that TOE proteins inhibit CO protein activity in the morning and prevent *FT* expression. Also, our results that *FT* was expressed at a higher level in the *toe1-1 toe2-1* mutant than normal in the long-day afternoon indicate that TOEs have an afternoon function. TOEs partially counteract the positive effect of FKF1 on CO in the afternoon, delaying *FT* expression until late afternoon. In addition, the findings that *toe1-1 toe2-1* mutations increased *FT* expression in the afternoon and accelerated flowering even in the *fkf1-3* background suggest that TOE1 could inhibit CO activity in the afternoon via an FKF1-independent mechanism, perhaps via ZTL and LKP2. TOE function in both the morning and afternoon contributes to restricting *FT* expression to the late afternoon of long days, making it necessary for the day length to reach a threshold before flowering.

TOE proteins are members of the AP2 family; in addition, *AP2* also affects flowering time, possibly through regulating *SOC1* and *FT* expression (Yant et al. 2010), and the AP2 protein could bind to AT-rich elements (Dinh et al. 2012). Overexpression of *TOE1m* leads to defects of flower organs (Supplemental Fig. S2F), similar to the effect of *AP2* overexpression (Chen 2004). Moreover, our study also showed that overexpression of *AP2* leads to late flowering (Supplemental Fig. S2G), reminiscent of the phenotypes of plants with *TOE* overexpression. Therefore, AP2 and TOE1 might bind to similar DNA elements to regulate their downstream genes. The fact that CO binds to CORE sequences in the *FT* promoter (Tiwari et al. 2010; Song et al. 2012) and our ChIP results that TOE1 binds to a region near the CO-binding site support the idea that binding of both proteins to the *FT* promoter in close proximity promotes their interaction.

TOEs serve as major integrators of developmental and environmental signals to regulate flowering

Even under favorable environments, plants need to have sufficient vegetative development before the onset of reproductive development. In addition to the role in regulating the photoperiod pathway, TOEs are likely important for the repression of flowering during early vegetative development because the expression of the key negative regulator of *TOE* genes, *miR172*, increases as plants age, leading to a gradual reduction of *TOE* function from the juvenile to the adult stage (Jung et al. 2007; Mathieu et al. 2009). The idea that *TOE* genes and *miR172* together regulate age-dependent flowering is further supported by the findings that *miR172* promotes flowering when plants are old enough (Wang et al. 2009; Wu et al. 2009) and our results that overexpression of a *TOE1* cDNA with mutations relieving the inhibition of miR172 caused a greater delay of flowering in transgenic plants than overexpression of the wild-type *TOE1*.

The flowering time is also affected by temperature, another environmental signal related to seasons. In the natural diurnal condition, the temperature is low in the early morning, when *TOE* genes are highly expressed, suggesting that *TOE*s might play a role in inhibiting flowering at low temperature. In contrast, the microRNA genes *miR172a*, *miR172b*, *miR172c*, and *miR172e* show increased expression at 23°C compared with their levels at 16°C. Consequently, the miR172s target genes *TOE1, TOE2, SMZ*, and *SNZ* all show relatively high-level expression at 16°C (Lee et al. 2010). The relatively abundant TOE proteins could then repress the activity of CO and COL proteins at low temperatures, leading to the repression of *FT* expression. Although early spring is associated with both short days and low temperatures, the separate regulation of *TOE1* expression by the circadian clock and *miR172* allows early flowering if the temperature rises more quickly than usual, ahead of the timing determined by day length.

In conclusion, we investigated the molecular function and transcriptional regulatory network of TOEs at the protein level for the first time. We propose a molecular model of TOE protein function (Fig. 7). In the early morning, the CO protein is present but is inhibited by TOE proteins, leading to the lack of *FT* transcription. In the afternoon, there are at least three protein–protein interaction events: TOEs–CO, FKF1–CO, and FKF1–TOEs. FKF1 stabilizes CO protein, allowing it to activate *FT* transcription. TOE proteins could still interfere with the interaction of FKF1–CO and also repress the CO protein activity. Therefore, when *TOE* genes are mutated, *FT* is expressed with a morning peak and a level greater than normal in the afternoon. However, TOE proteins also function in short days and under environmental stresses as well as during early development. Therefore, TOE proteins serve as major integrator of developmental and environmental signaling pathways, especially the photoperiod flowering pathway, triggering flowering at an appropriate age and time.

Materials and methods

Y2H experiments

The Y2H screening was performed according to the Matchmaker Gold Y2H system user manual (Clontech) using reagents provided by the system. The full-length *TOE1* cDNA was amplified by primers BLZ1 and BLZ2 (all of the primers used in this study are

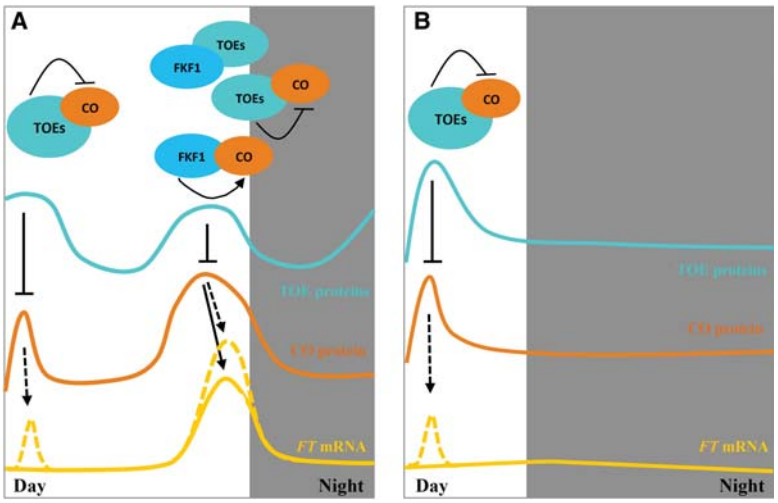

Figure 7. A model for regulation of flowering by a signaling pathway from FKF1 to *FT*. The diurnal phases of TOEs proteins, CO protein, and *FT* expression are shown, with both morning and afternoon peaks for TOEs and CO (TOE levels are higher in the morning, whereas CO is higher in the afternoon) under long days (*A*) but only a morning peak for TOEs and CO under short days (*B*). (*A*,*B*) In the early morning, TOE proteins bind to the activation region of CO and inhibit CO activity, resulting in lack of *FT* expression in both long and short days. (*A*) In the long day afternoon, FKF1 binds to and stabilizes CO, but TOEs can interfere with this interaction by binding to both FKF1 and CO. When *TOE* genes are mutated, the *FT* is expressed in the morning (dashed lines) in the long and short day (*A*,*B*) and at a higher level in the long day afternoon (*A*), resulting in early flowering.

provided in Supplemental Table S1) and cloned into the bait vector pGBKT7 (Clontech); the construct was introduced into the bait strain after verification by sequencing and tested for autoactivation and toxicity. The *TOE1* bait strain did not exhibit autoactivation of the reporter gene or toxicity and was then mixed with the *Arabidopsis* Y2H cDNA library (Clontech, $\sim 1.0 \times 10^7$ transformants) for mating. The mating cell mixture was plated on a medium (QDO/X/A) lacking His, Ade, Trp, and Leu and containing X-α-gal and Aureobasidin A (AbA) to select for cells with expression of the *HIS3*, *ADE2*, *TRP1*, *LEU2*, *MEL1*, and *AUR1-C* reporter genes. The positive colonies were transferred to and grown on fresh QDO/X/A plates and used for PCR amplification of the sequences of the prey clones.

For additional Y2H experiments to test for specific interactions, the full-length *TOE2*, *TOE3*, and *SNZ* cDNAs were amplified by primers BLZ3 to BLZ8, cloned into pGBKT7, and transformed into the Y2H Gold yeast strain. The *CO-C* (amino acids 177–347) and the full-length cDNAs of *COL1*, *COL2*, *COL3*, and *COL5* were amplified by primers BLZ9 to BLZ18, and the LOV domain of ZTL, LKP2, and FKF1 were amplified by primers BLZ19 to BLZ24; cloned into pGADT7 (prey); verified by sequencing; and transformed into the Y187 yeast strain. The two yeast strains were mixed for mating, and the mating mixture was transferred to the DDO and QDO/X/A media for selection of diploids and those with reporter gene expression, respectively. Appropriate pairs of yeast transformants were mixed and plated onto the DDO and QDO/X/A media for growth and selection.

Plant materials and growth conditions

The *toe1-1*, *toe1-1 smz-1*, *toe1-1 toe2-1*, *toe1-1 toe2-1 smz-1*, *fkf1-3*, *toe1-1 fkf1-3*, and *toe1-1 toe2-1 fkf1-3* mutants and the *35S::TOE1-10myc*, *35S::FKF1-ECFP*, *35S::CO-ECFP*, *35S::TOE1-10myc fkf1-3* transgenic plants are in the Columbia (Col-0) ecotype. The *co-1* mutant is in the Landsberg *erecta* (Ler) ecotype. To generate the *35S::TOE1-10myc* transgenic lines, the full-length *TOE1* cDNA was amplified using primers BLZ43 and BLZ44. For the *35S::CO-ECFP* transgenic plants, the full-length of *CO* cDNA was amplified using primers BLZ45 and BLZ46. For the *35S::FKF1-ECFP* transgenic plants, the full-length of *FKF1* cDNA was amplified using primers BLZ47 and BLZ48. All of the PCR fragments were cloned into the pDONOR vector (Gateway) (Nakagawa et al. 2007) and verified by sequencing. The *35S::TOE1* fusion was transferred into the pGWB20 binary vector (Gateway) using LR Clonase II enzyme mix (Invitrogen) to generate the *35S::TOE1-10myc* T-DNA construct, while *CO* and *FKF1* cDNAs were transferred into pGWB44 binary vector (Gateway) to generate the *35S::CO-ECFP* and *35S::FKF1-ECFP* T-DNA constructs, respectively. For the *35S::TOE1m-10myc* construct, we used site-directed mutagenesis PCR according to the manufacturer's protocol (Transgene. The pDONOR-TOE1 plasmid was amplified using primers BLZ49 and BLZ50. After transformation and verification of the mutated sequence, the fragment of *TOE1m* was transferred into the pGWB20 binary vector. All of the binary vectors were introduced into the wild-type plants by *Agrobacterium*-mediated transformation to generate overexpressing plants in the wild-type and *fkf1-3* backgrounds. All *Arabidopsis* plants were grown on soil in growth rooms at 22°C under full-spectrum white fluorescent light under long-day (16 h light/8 h dark) or short-day (8 h light/16 h dark) conditions. All flowering experiments were repeated twice independently, and similar results were obtained. The tobacco (*Nicotiana benthamiana*) plants used in BiFC experiments were grown on soil in a growth room at 22°C–28°C under white fluorescent light (14 h light/10 h dark) conditions.

BiFC assays

The *TOE1* cDNA was amplified using primers BLZ53 and BLZ54 and cloned into the pXY104 vector (Wang et al. 2011). The cDNAs of *CO* and *COL1* and the LOV domain of *FKF1* were amplified using primers BLZ55 to BLZ60 and cloned into the pXY106 vector. After their sequences were verified, the resulting cassettes, including the constitutive promoters and gene fusions (TOE1-cYFP, nYFP-CO, nYFP-COL1, and nYFP-FKF1-LOV), were transformed into *Agrobacterium*. For BiFC experiments, leaves of 3-wk-old tobacco (*N. benthamiana*) plants were coinfiltrated with two *Agrobacterium* strains containing the appropriate constructs for the two proteins to be tested. After 36–48 h, signals of YFP were analyzed by confocal microscopy (Zeiss).

Co-IP

Leaves of 21-d-old transgenic plants containing two fusion proteins (TOE1-10myc and CO-ECFP or FKF1-ECFP) were ground to a fine powder in liquid nitrogen and resuspended in 2× extraction buffer (100 mM Tris-HCl at pH 7.5, 300 mM NaCl, 2 mM EDTA at pH 8.0, 1% TrionX-100, 10% glycerol, 50 mM MG132, protease inhibitor cocktail). The protein suspensions

were centrifuged at 20,000*g* for 10 min, the resultant supernatant was incubated with prewashed anti-Myc agarose beads (Aogma) for 3 h at 4°C, and then the agarose beads were washed four times with the 2× extraction buffer. The immunoprecipitates were eluted with 1× SDS sample buffer (50 mM Tris-Hcl at pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 1% 2-mercaptoethanol), separated on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane (Millipore), and detected with corresponding antibodies.

Gene expression analysis

To detect *TOE*s and *FT* expression, leaves of 14-d-old plants grown under long days and 45-d-old plants under short days were used for total RNA extraction by using a Trizol-based (Sigma) method (Wang et al. 2012). For cDNA synthesis, 3 µg of total RNA was reverse-transcribed using the PrimeScript first strand cDNA synthesis kit (Transgen). The cDNA was diluted to 50 µL with water in a 1:4 ratio, and 1 µL of the diluted cDNA was used for quantitative PCR (qPCR). Primers for *FT*, *CO*, and *IPP2* were as described (Song et al. 2012), and primers for *TOE1*, *TOE2*, *SMZ*, and *SNZ* are shown in Supplemental Table S1 (primers BLZ61 to BLZ68). qPCR was performed using the following program: 120 sec at 95°C, 40 cycles of 10 sec at 95°C, and 1 min at 65°C. *IPP2* expression was used as an internal control. The *FT* expression was calculated from three independent biological experiments.

Protein extraction and Western blot analysis

Leaves were ground in liquid nitrogen and then resuspended in an extraction buffer (5% SDS, 100 mM NaCl, 10 mM 2-mercaptoethanol) and boiled for 10 min. The protein suspensions were centrifuged at 20,000*g* for 10 min, and the resultant supernatant was mixed with 1/4 vol of 5× SDS sample buffer (250 mM Tris-Hcl at pH 6.8, 10%SDS, 50% glycerol, 0.5% bromophenol blue, 5% 2-mercaptoethanol). The proteins were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Millipore). The membrane was incubated overnight with anti-Myc, anti-GFP, or anti-Tubulin (Beyont) antibodies, respectively at 4°C, and then goat anti-rabbit (for co-IP experiment) or goat anti-mouse (for Western blot) HRP-conjugated secondary antibodies (Pierce Biotechnology) were used against the primary antisera. For protein level analysis, all Western blot analyses were performed three times, and similar results were obtained; the Western blot results were measured by gel-pro software, with TUBULIN level as an internal control.

ChIP assays

Leaves of 21-d-old *Arabidopsis* plants were ground to fine powder (3 mL per ChIP) in liquid nitrogen, and then the powder was resuspended in 8 mL of M1 buffer (10 mM phosphate buffer, 0.1 M NaCl, 10 mM mercapto-ethanol, 1 M hexylene glycol). To cross-link proteins and DNA, 216 µL of formaldehyde was added to the mixture followed by incubation for 10 min at 4°C. To stop the cross-link, 543 µL of 2 M glycine was added with 5 min of incubation at 4°C. To remove debris, the cross-link reaction mixture was filtered using four layers of miracloth, and then chromatin was collected by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatants were discarded, and the pellets were resuspended in 4 mL of M2 buffer (10 mM phosphate buffer, 0.1 M NaCl, 10 mM mercapto-ethanol, 1 M hexylene glycol, 10 mM $MgCl_2$, 0.5% Triton-X) and mixed. The samples were centrifuged for 1 min, and the pellets were washed with 1 mL of M2 three times, with 1-min centrifugation in between, and finally washed once with 1 mL of M3 (10 mM phosphate buffer, 0.1 M NaCl, 10 mM mercapto-ethanol) and another centrifugation.

The nuclear pellet was resuspended with 180 µL of SDS lysis buffer (Millipore) and incubated for 10 min on ice with addition of 820 µL of ChIP dilution buffer (Millipore) followed by sonification four times. The chromatin sample was precleared with 120 µL of Protein A beads and 4 mL of solution and incubated with gentle agitation for 2–3 h in a cold room. Next, the sample was incubated with antibodies with gentle agitation overnight at 4°C. The antibody solution was mixed with 80 µL of Protein A beads in each test tube and incubated for 2 h at 4°C with shaking. The beads were collected by a brief spin and then washed with gentle agitation for 10 min at 4°C sequentially in the low-salt wash buffer (Millipore), the high-salt wash buffer (Millipore), and the LiCl wash buffer (Millipore) and twice in the TE buffer (Millipore). Next, 250 µL of elution (0.084 g of $NaHCO_3$ + 1 mL of 10% SDS + ddH_2O to 10 mL) and 20 µL of 5 M NaCl were added per 500 µL of the chromatin sample with incubation overnight at 65°C followed by the addition of 10 µL of 0. 5 M EDTA (pH 8.0), 20 µL of 1 M Tris–HCl (pH 6.5–7.9), and 1.5 µL of 18.9 mg/mL proteinase K per 500 µL of solution and incubation for 1 h at 45°C.

RNA was digested by adding 10 µL of 2 µg/µL RNase A to each tube and incubating at room temperature for 30 min. DNA was extracted by phenol/chloroform and precipitated with ethanol in the presence of glycogen and NaOAc. The pellet was resuspended in 60 µL of 10 mM Tris (pH 8). DNA was diluted twofold or fivefold, and 2–5 µL was used as template in 20 µL for qPCR (Gendrel et al. 2005). qPCR was performed using the following program: 120 sec at 95°C, 70 cycles of 10 sec at 95°C, and 1 min at 65°C. The *FT* genome region and *UBQ10* were amplified by primers BLZ69 to BLZ80.

EMSA

The full-length *TOE1* cDNAs (amplified with primers BLZ93 and BLZ94) and *CO* (primers BLZ96 and BLZ97) were cloned into a modified version of the vector pPET28a-SUMO for expressing a fusion protein to SUMO (Huang et al. 2009). *Escherichia coli* cells expressing the TOE1-SUMO and CO-SUMO fusion proteins were pelleted and then resuspended in 25 µL of buffer A (10 mM Tris-HCl at pH 7.8, 50 mM NaCl, 1 mM EDTA, 6 M urea, 1 mM PMSF). For DNA-binding experiments, 20 µL of protein extracts was combined with 80 µL of buffer B (10 mM Tris-HCl at pH 7.8, 50 mM NaCl, 1 mM EDTA, 20% glycerol, 1 mM PMSF). The DNA probes labeled with biotin were prepared by annealing pairs of complementary oligonucleotides with corresponding binding sequences (BLZ81 to BLZ92). Binding reactions contained 5 µL of protein extracts, 3 µL of 1 pg/L probe, 2 µL of 10× binding buffer (10 mM Tris-HCI at pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 50 µg/mL poly(dI-dC)–poly (dIdC),100 µg/mL BSA) and ddH_2O for a total of 20 µL. The free and bound probes were separated in a 6% PAGE gel in 0.5× TBE at 100 V for 50 min, transferred to Hybond-N membrane (GE Healthcare), and cross-linked to the membrane under UV light at 120 mJ/cm^2 for 20 sec (Feng et al. 2012).

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