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

Bailong Zhang,1,2 Liang Wang,1 Liping Zeng,1,2 Chao Zhang,1 and Hong Ma1,2,3

1State Key Laboratory of Genetic Engineering, Collaborative Innovation Center of Genetics and Development, Institute of Plant Biology, Center for Evolutionary Biology, School of Life Sciences, Fudan University, Shanghai 200438, China; 2Ministry of Education Key Laboratory of Biodiversity and Ecological Engineering, Institute of Biodiversity Sciences, Fudan University, Shanghai 200438, China; 3Institutes of Biomedical Sciences, Fudan University, Shanghai 200438, China

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

Supplemental material is available for this article.

Received August 22, 2014; revised version accepted April 7, 2015.

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 (Baurle 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). Premature flowering reduces the period of vegetative development and decreases reproductive fitness and 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
manner in the long-day afternoon ([maizumi 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 ([maizumi et al. 2007; Fornara et al. 2009]. Consequently, the expression of CO increases in the early morning and again in the afternoon ([Sauz-Lopez 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, SCHLAFMUTZEN (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 (EREBP), 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. SI A] 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. SI]. We found that most of the truncated CO proteins could interact with TOE1 except the truncated COs that lacked the activation domain [Supplemental Fig. SI A], 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. SI B]. 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. SI C; 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. SI D]. 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. SI E]. 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 TOEs 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.
TOEs 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 TOEs 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].
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 to 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.

TOEs 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 TOEs 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 TOEs and CO under short days, TOEs 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 TOEs 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].
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).

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.

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 fkh1-3 transgenic plants under long days. However, the TOE1-10MYC protein did not show an obvious increase in fkh1-3 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 fkh1-3 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
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 (Izaiumi 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.
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.

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 miR172a 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 TOEs 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

#### 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
The vector (Gateway) using LR Clonase II enzyme mix (Invitrogen) cDNA was amplified using primers BLZ47 and BLZ48. All FKF1 cDNAs were transferred into pGADT7 (prey); verified by primers BLZ3 to BLZ8, cloned into pGBKT7, and the LOV domain of ZTL, LKP2, and FKF1 were amplified individually, 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 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 BLZ3 and BLZ4 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-CFP, 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% TritonX-100, 10% glycerol, 50 mM MG132, protease inhibitor cocktail]. The protein suspensions

**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 BLZ3 and BLZ4. For the 35S::CO-ECFP transgenic plants, the full-length of CO cDNA was amplified using primers BLZ5 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 pGBW20 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 Arabidopsis 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.

**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). 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. 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.
were centrifuged at 20,000g for 10 min, the resultant supernatant was incubated with prewashed anti-Myc agarose beads (Agma) 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% PAGE gel, transferred to nitrocellulose membrane (Millipore), and detected with corresponding antibodies.

**Gene expression analysis**

To detect TOEs 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 [Ttransgen]. 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 20,000g 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 antibodies. 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 mercaptoethanol, 1 M hexylene glycol]. To cross-link proteins and DNA, 2% of the mixture 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 mercaptoethanol, 1 M hexylene glycol, 10 mM MgCl2, 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 pre-cleared 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 NaHCO3 + 1 mL of 10% SDS + ddH2O 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 glyogen 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 pET28a-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-HCl at pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 50 µg/µL poly(dI-dC)–poly(dC-dI), 100 µg/µL BSA] and ddH2O 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 µJ/cm² for 20 sec [Feng et al. 2012].

**Acknowledgments**

We thank Professor Jianxiang Liu for providing BiFC plasmids, and Professor Jinbiao Ma for providing the pSUMO plasmid. We also thank Professor Xuemei Chen for her kind support of this work. This work was supported by grants from the National Natural Sciences Foundation of China (31130006 and 91131007) and
Zhang et al.

the Ministry of Science and Technology (2011CB944600) and funds from the State Key Laboratory of Genetic Engineering.

References


SUETSUGU N, Wada M. 2013. Evolution of three LOV blue light receptor families in green plants and photosynthetic


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

Bailong Zhang, Liang Wang, Liping Zeng, et al.

Genes Dev. 2015 29: 975-987
Access the most recent version at doi:10.1101/gad.251520.114

Supplemental Material
http://genesdev.cshlp.org/content/suppl/2015/04/30/29.9.975.DC1

References
This article cites 45 articles, 19 of which can be accessed free at:
http://genesdev.cshlp.org/content/29/9/975.full.html#ref-list-1

Open Access
Freely available online through the Genes & Development Open Access option.

Creative Commons License
This article, published in Genes & Development, is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.

To subscribe to Genes & Development go to:
http://genesdev.cshlp.org/subscriptions

© 2015 Zhang et al.; Published by Cold Spring Harbor Laboratory Press