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CYCLING DOF FACTOR 1 represses transcription through the TOPLESS co-repressor to control photoperiodic flowering in Arabidopsis

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Summary

CYCLING DOF FACTOR 1 (CDF1) and its homologs play an important role in the floral transition by repressing the expression of floral activator genes such as *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) in Arabidopsis. The day-length specific removal of CDF1-dependent repression is a critical mechanism in photoperiodic flowering. However, the mechanism by which CDF1 represses *CO* and *FT* transcription remained elusive. Here we demonstrate that Arabidopsis CDF proteins contain non-EAR motif-like conserved domains required for interaction with the TOPLESS (TPL) co-repressor protein. This TPL interaction confers a repressive function on CDF1, as mutations of the N-terminal TPL binding domain largely impair the ability of CDF1 protein to repress its targets. TPL proteins are present on specific regions of the *CO* and *FT* promoters where CDF1 binds during the morning. In addition, TPL binding increases when *CDF1* expression is elevated, suggesting that TPL is recruited to these promoters in a time-dependent fashion by CDFs. Moreover, reduction of TPL activity induced by expressing a dominant negative version of *TPL* (*tpl-1*) in phloem companion cells results in early flowering and a decreased sensitivity to photoperiod in a manner similar to a *cdf* loss-of-function mutant. Our results indicate that the mechanism of CDF1 repression is through the formation of a CDF-TPL transcriptional complex, which reduces the expression levels of *CO* and *FT* during the morning for seasonal flowering.

Keywords

Arabidopsis thaliana; photoperiodic flowering; transcriptional repression. *CONSTANS*; *FLOWERING LOCUS T*

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The authors declare that no conflict of interest exists.

Introduction

The transition from a juvenile to a reproductive stage of development is a critical regulatory process in plants. For annual flowering plants for which a single reproductive event defines the life cycle, timing of this developmental transition is especially important for the fitness of a given individual (Andrés and Coupland, 2012; Johansson and Staiger, 2015; Song *et al.*, 2015). The progression of the axial tilt of Earth's path throughout the year leads to a predictable yearly flux in temperature and in light duration (photoperiod). This change is especially dramatic at increasing distances from the equator (Thomas and Vince-Prue, 1996). For this reason, many plants measure the photoperiod change to predict the period in which to begin reprogramming from the vegetative to the reproductive phase of the life cycle.

In *Arabidopsis thaliana*, for which increasing day length (long days) is inductive towards flowering, the signal that initiates this process is the expression of the mobile protein FLOWERING LOCUS T (FT) (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Jaeger *et al.*, 2013). FT protein is produced in the phloem companion cells, and transported to the shoot apex (Corbesier *et al.*, 2007; Mathieu *et al.*, 2007; Notaguchi *et al.*, 2008; Liu *et al.*, 2012). There it interacts with downstream regulatory factors to commit the shoot apical meristem to a reproductive cell fate (Abe *et al.*, 2005; Wigge *et al.*, 2005; Taoka *et al.*, 2011; Jaeger *et al.*, 2013). Temporal and developmental regulation of *FT* transcription is critical for a proper timing of flowering.

For day-length information to be integrated into the expression of *FT*, several factors are required. The transcription factor CONSTANS (CO) is a primary activator of *FT* in long days (Suárez-López *et al.*, 2001). The activity of CO results in upregulation of *FT* toward the dusk of long days. This upregulation is due in part to the stabilization of CO protein by the blue-light photoreceptor FLAVIN BINDING, KELCH REPEAT, F-BOX 1 (FKF1) and GIGANTEA (GI) complex in the long-day afternoon (Song *et al.*, 2012; Song *et al.*, 2014). The stabilization of the CO protein is also accomplished through the action of TIMING OF CAB EXPRESSION 1 (TOC1), and PSEUDO RESPONSE REGULATOR 5 (PRR5), PRR7, and PRR9 proteins, which physically interact with CO and prevent its degradation (Hayama *et al.*, 2017).

The constraint of *FT* expression to only the afternoon of long days is accomplished through the action of CYCLING DOF FACTOR (CDF) transcription factors. CDF1, CDF2, CDF3, and CDF5 act redundantly to repress the expression of *CO* and *FT* (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). *CDF1*, *CDF2*, *CDF3* and *CDF5* are expressed during the mornings, and their expression is a direct output of the circadian clock (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). The core clock components, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), positively act in *CDF* expression during the late night and morning (Nakamichi *et al.*, 2007; Niwa *et al.*, 2007). *CDF* transcription is directly inhibited after zeitgieber time (ZT) 4 through the action of PRR5, PRR7, and PRR9 throughout the remainder of the day (Nakamichi *et al.*, 2007). During the afternoon of long days, FKF1-GI complex degrades CDF proteins through ubiquitin mediated degradation (Sawa *et al.*, 2007). This regulation of CDFs, both by the endogenous circadian clock, and

through blue light signaling, constrains the activity of these *CO* and *FT* repressors to the morning. This results in generating a day-length specific time-window before dusk in which *FT* can be specifically activated in long days.

CDF homologs in other flowering plant species appear to have largely conserved function. LATE BLOOMER 2 (LATE2) in *Pisum sativum* and StCDF1 in *Solanum tuberosum* function as repressors of flowering and tuberization, respectively, in response to photoperiod (Kloosterman *et al.*, 2013; Ridge *et al.*, 2016). CDF proteins belong to the larger family of plant specific DOF (DNA binding with one finger) transcription factors (Yanagisawa, 2002; Yanagisawa, 2004). CDFs compose a distinct subclade of the DOF protein family in Arabidopsis, of which 37 are present in the genome (Yanagisawa, 2002). The DOF DNA binding domain belongs to the C2C2-type zinc finger domain, but is unusual in that only a single zinc coordinating “finger” is present on each protein to assist in DNA binding (Yanagisawa, 2002). This single finger complicates the prediction of DOF *cis*-elements in target promoters, as the consensus binding sequence of “AAAG” is widely found in most promoters. At least at the *CO* promoter in Arabidopsis, the presence of a tandemerized DOF binding site upstream of the transcription start site is critical for CDF-dependent repression (Rosas *et al.*, 2014). Potentially, this multimerization could be a mechanism through which DOF target loci are regulated.

TOPLESS (TPL) proteins are common mediators of transcriptional repression in a variety of molecular pathways in Arabidopsis (Liu and Karmarkar, 2008). These include auxin, jasmonic acid, and ethylene hormone signaling, as well as several developmental pathways, including the circadian clock and flowering time regulation (Liu and Karmarkar, 2008; Wang *et al.*, 2013). The originally characterized dominant negative mutant *tpl-1* shows a pleiotropic phenotype that strikingly displays a lack of apical cell fate under higher temperatures (Long *et al.*, 2006). TPL and its homologs TOPLESS RELATED 1 (TPR1), TPR2, TPR3, and TPR4 are important co-repressors that assist transcription factors in mediating repression at their target loci (Causier *et al.*, 2012). This mechanism is perhaps most clear for TPL’s role in auxin signaling, where TPL and INDOLE-3-ACETIC ACID INDUCIBLE (IAA) transcription factors repress auxin inducible loci until the IAAs are degraded in response to auxin (Gray *et al.*, 2001). Although the direct mechanism of TPL repression is unclear, TPL interacts with several histone deacetylase (HDAC) enzymes, as well as the CDK8/HUA ENHANCER3 (HEN3) mediator complex module (Wang *et al.*, 2013; Ito *et al.*, 2016). A combination of these interactions likely facilitates the direct blocking and/or accessibility of target promoters to transcriptional activators (Ito *et al.*, 2016). The crystal structure of the N-terminal domain of TPL has been resolved; similar to how the analogous protein Groucho (Gro) functions in *Drosophila melanogaster*, TPL functions as a tetrameric complex (Ke *et al.*, 2015). The LisH (Lis homology domain) and CtLH (C-terminal to the LisH motif) domains of TPL are important for transcription factor binding. The sites on TPL that EAR (ethylene-responsive element binding factor-associated amphiphilic repression) motif containing factors bind to were identified, but not for other interaction motifs (Ke *et al.*, 2015). Several transcription factors that bind to TPL in a yeast two-hybrid assay are involved in the regulation of *FT* transcription, including TARGET OF EAT 1 (TOE1) and TOE2, SCHLAFMÜTZE (SMZ), SCHNARCHZAPFEN (SNZ) and TEMPRANILLO 1 (TEM1) and TEM2 (Schmid *et al.*, 2003; Causier *et al.*, 2012; Osnato *et*

al., 2012; Zhang *et al.*, 2015). TOE1 and TOE2 are involved in miRNA172 age-dependent regulation of flowering time (Jung *et al.*, 2007). TEM1, and TEM2 are involved in *FT* regulation during the afternoon of long days, are antagonistic to CO activity, and are downstream of GI and gibberellin hormones in the photoperiodic flowering pathway (Castillejo and Pelaz, 2008; Osnato *et al.*, 2012). Recently, it was also found that a B-box containing small protein MICROPROTEIN 1A (miP1a), and miP1b, interact with both TPL and CO during the mornings of long days to reduce CO activity (Graeff *et al.*, 2016).

Heretofore, the mechanism of CDF-dependent repression of the *CO* and *FT* genes was unknown. We enumerate here that CDF1 recruits TPL to reduce *CO* and *FT* transcription during the morning. We show that CDF1 and TPL together make a protein complex that likely forms mainly when CDFs are abundant. Additionally, our analysis of CDF1 and TPL interacting domains shows that the regions of CDF1 that interact with TPL are needed for full repressive activity. In long days, TPL associates with the *CO* and *FT* promoters mainly in the morning. Elevating the amount of CDF1 increases TPL binding even in the afternoon. Reduction of TPL activity specifically in phloem companion cells leads to early flowering. We demonstrate that TPL activity through CDFs during the mornings of both long days and short days is critical for constricting *CO* and *FT* expression to enable the correct interpretation of seasonal information by the plant.

Results

CDF proteins contain a conserved motif at their N-termini, which is important for binding to TPL

CDF1 and related CDF transcription factors all function as repressors of *CO* transcription and flowering time in Arabidopsis (Fornara *et al.*, 2009). To explore the possible mechanisms through which CDF transcription factors are able to confer repression on *CO* and *FT* transcription, we first looked for regions of high conservation at the amino acid level, as this would imply a retained function. We constructed an amino acid alignment of CDF proteins from Arabidopsis, functionally characterized CDFs from other plant species, as well as CDF-related DOF transcription factors from a variety of green plant lineages (Lijavetzky *et al.*, 2003; Yang *et al.*, 2010; Hernando-Amado *et al.*, 2012; Sugiyama *et al.*, 2012; Kloosterman *et al.*, 2013; Corrales *et al.*, 2014; Lucas-Reina *et al.*, 2015; Kang *et al.*, 2016; Ridge *et al.*, 2016). We found several stretches of amino acid conservation in CDF proteins (Figure S1). In addition to the DOF DNA-binding domain, we found a short conserved sequence (IKLFG) at the N-terminal ends of CDFs and nearly all CDF related DOF transcription factors (Figure S1). This motif exactly overlaps with a previously described (R/K)LFGV motif for binding to the co-repressor protein TPL (Causier *et al.*, 2012). The IKLFG residues are highly conserved among CDF-like DOF proteins found throughout land plants (Figure S1) (Cai *et al.*, 2013; Ma *et al.*, 2015; Kang *et al.*, 2016). While not the canonical EAR motif (the LxLxL amphiphilic domain which composes the majority of TPL interactors), CDFs possess the (R/K)LFGV motif, similar to TEM1 protein and present in 12.3 percent of the established TPL interactome (Causier *et al.*, 2012). Although no DOF domain transcription factors have been reported as being likely TPL interactors, we hypothesized that CDFs bring TPL to target loci to repress transcription.

We next investigated if these residues were critical for interaction with TPL. We first used a yeast two-hybrid (Y2H) approach to see if CDF1 interacted with TPL, and if corresponding mutations in the N-terminal motif of CDF1 impaired complex formation. We found that CDF1 interacted with TPL, but the incorporation of either a deletion of the N-terminal end residues (amino acid positions: 1–19, designated as CDF1-ΔN) including the IKLFG motif, or a replacement of the interior three residues into alanine (A) in the motif (IKLFG to IKAAA; designated as CDF1-mut), was sufficient to prevent the interaction (Figure 1a,b). In either case, the mutated CDF1 proteins could interact with the N-terminal fragment of the GIGANTEA (GI) protein, which binds to the CDF1 C-terminus (Kloosterman *et al.*, 2013). This indicates that these CDF1 variant proteins were expressed in this system, and were still able form complexes with GI. To verify the CDF1-TPL interaction results *in planta*, we used both Bimolecular Fluorescent Complementation (BiFC) and Co-immunoprecipitation (Co-IP) experiments. CDF1 bound to TPL in BiFC in epidermal cells of *Nicotiana benthamiana*, but the inclusion of these mutations in the CDF1 N-terminus abolished the interaction with TPL (Figure 1c,S2a). We confirmed that both CDF1-ΔN and CDF1-mut proteins were localized to the nucleus, similar to non-mutated CDF1 (Figure S2b). In addition, reciprocal Co-IP of TPL protein transiently co-expressed in tobacco leaves also showed a recovery of CDF1 protein bound to TPL (Figure 1d). These results together indicate that the N-terminal conserved motif of CDF1 functions as the binding site to TPL.

The TPL interaction motif at the N-termini of CDF family proteins is important for repression of the flowering time genes *CO* and *FT.*

To investigate whether the N-terminal motif in CDFs is required for the repressive function of the transcription factor, we generated transgenic plants that overexpress an epitope-tagged CDF1 protein (*35S:CDF1-3F6H*) or two mutated variants of tagged CDF1 proteins (*35S:CDF1-ΔN-3F6H* and *35S:CDF1-mut-3F6H*) (Figure 1a). We tested the flowering time response of transgenic lines with similar expression levels of *CDF1* transcripts (Figure 2a,d,e), and found that *35S:CDF1-3F6H* overexpression plants were late-flowering in long days compared to WT as previously shown (Imaizumi *et al.*, 2005). The flowering time of *35S:CDF1-ΔN-3F6H* and *35S:CDF1-mut-3F6H* lines in which similar or higher levels of *CDF1* transcripts was expressed (Figure 2b,c) were not significantly different from that of WT plants, although we noticed that the size of *35S:CDF1-ΔN-3F6H* and *35S:CDF1-mut-3F6H* rosettes seemed to be slightly smaller than wild-type plants (Figure 2d,e). This result suggests that these mutations at least attenuated the repressive function of the protein for floral induction. Flowering time among all of the lines under short-day photoperiods was similar, although *35S:CDF1-3F6H* lines and *35S:CDF1-mut-3F6H #30* were slightly delayed compared to WT plants (Figure S3).

To eliminate the possibility that the loss of floral repression in the *CDF1* variant overexpressors was caused by destabilization of the proteins, we analyzed the expression levels of these CDF1 variants. All CDF1 variant proteins in the transgenic lines with similar expression levels of *CDF1* transcripts were expressed at levels slightly higher than non-mutated CDF1 protein during the morning (Figure 2f). This result clearly indicates that the lack of flowering phenotype in *35S:CDF1-ΔN-3F6H* and *35S:CDF1-mut-3F6H* lines is not due to the lack of the expression of CDF1-ΔN-3F6H and CDF1-mut-3F6H proteins.

We also confirmed that these mutations did not affect the ability to bind to DNA. We used a yeast one-hybrid (Y1H) approach to ascertain if CDF1-ΔN and CDF1-mut could still bind to a 500 bp fragment of the *CO* promoter, which contains a cluster of DOF binding sites, where CDF1 binds (Imaizumi *et al.*, 2005). We found that the mutated CDF1 proteins fused with the GAL4 activation domain could activate the LacZ reporter similar to the normal CDF1 protein, indicating that these mutations did not interfere with their DNA binding activities (Figure 2g).

We then analyzed whether *CO* or *FT* expression was altered in *35S:CDF1-ΔN-3F6H* or *35S:CDF1-mut-3F6H* plants compared to Wild-type (WT) and *35S:CDF1-3F6H.* Under long-day photoperiods, *35S:CDF1-3F6H* have a reduction in *CO* mRNA in the afternoon and night, and *FT* mRNA levels are lower throughout the day (Figure 2h,k). In *35S:CDF1-ΔN-3F6H* and *p35S:CDF1-mut-3F6H* plants, levels of *CO* transcript were slightly lower but more similar to that in WT throughout the day, comparing against those in *35S:CDF1-3F6H* plants (Figure 2k,j). Overall *FT* expression patterns in *35S:CDF1-ΔN-3F6H* and *35S:CDF1-mut-3F6H* lines showed a similar trend throughout the day to that in WT, although a peak *FT* expression in those lines might be slightly lower (Figure 2l,m). These results suggest that repressive activity of CDF1 is largely ascribed to interaction with the TPL co-repressor through the N-terminal motif of CDF1. Taken together, the results imply that the conserved N-terminal TPL binding site among CDFs is important to confer on CDF1 its repression ability.

TPL associates with the *CO* and *FT* promoters during the mornings of long days and in a *CDF1* expression dependent manner

If CDF proteins are concerting repressive activity at the *CO* and *FT* promoters through recruiting TPL complex during the morning, when CDFs are abundant, we hypothesized that TPL protein should also exist at these loci at the same time as CDF1 (Sawa *et al.*, 2007; Song *et al.*, 2012). We analyzed whether TPL binds to the *CO* promoter especially in the morning. We performed ChIP-qPCR assays using *TPL:TPL-3HA* transgenic line in which *TPL-3HA* cDNA was expressed under its native promoter (Wang *et al.*, 2013). We harvested the samples at ZT4 and ZT16 time points, which correspond to an abundant CDF1 protein time point, and the peak level of *FT* expression (when CDF1 is low), respectively. Our results showed that TPL protein strongly associated with the *CO* promoter region at ZT4 (Figure 3a). The binding occurred specifically at amplicons within approximately the first 500 bp upstream of the transcription start site (amplicons 5 and 6) as well as several sites further upstream (amplicons 1 and 3) (Figure 3a). The −500 bp upstream region contains the cluster of DOF binding sites, where CDF1 binds (Imaizumi *et al.*, 2005), and has also been shown to be a highly conserved region among *CO* homologs in many Arabidopsis-related *Brassicaceae* species (Simon *et al.*, 2015). Therefore, it likely represents *bona fide* CDF1 binding sites on the *CO* promoter. This assertion is also supported by previous CDF1 ChIP experiments (Sawa *et al.*, 2007). Nevertheless, it is also possible that other factors may contribute to recruit TPL to these sites in the *CO* promoter. We found an insignificant amount of binding of TPL to the *CO* promoter (except at amplicon 6) during the ZT16 time point (Figure 3a). Similarly at the *FT* promoter, we found TPL binding to an amplicon upstream of the transcription start site at −800 bp, as well as binding to the 3′-UTR region

only in the samples harvested at ZT4 (Figure 3b). CDF1 has been found to bind to near the transcription start site (amplicons 12 and 13), and possibly around amplicon 8 (Song *et al.*, 2012). This indicates that other factors may also recruit TPL to the *FT* promoter. We found little binding of TPL to the *FT* promoter at the ZT16 time point (Figure 3b).

TPL is widely expressed throughout development (Wei *et al.*, 2015; Espinosa-Ruiz *et al.*, 2017); we wondered what mechanism led TPL protein to be differentially recruited to both *CO* and *FT* promoter regions between morning and night. A previous study demonstrated that TPL protein levels changed under 12-hour light/12-hour dark conditions (Wang *et al.*, 2013). Therefore, we analyzed the protein expression profiles of TPL in long days and short days using *TPL:TPL-3HA* plants. TPL was expressed at similar levels throughout the day in both day-length conditions, although we saw a slight increase in TPL levels around ZT10 in long days (Figure 3c). This result shows that the expression levels of TPL protein did not correlate with the degrees of association of TPL to specific regions of *CO* and *FT* loci between morning and night.

As CDF1 physically interacts with TPL, and because CDF1 binds to some overlapping DNA regions (such as *CO* amplicons 1, 5, and 6) during the morning, we hypothesized that the morning enriched binding of TPL to the *CO* and *FT* promoters was at least in part due to CDF1 binding to these promoter regions. To assess this possibility, we analyzed the binding of TPL to the *CO* promoter in plants with higher levels of CDF1 of which *CO* is repressed throughout the day. We used *TPL:TPL-3HA and TPL:TPL-3HA/35S:CDF1* plants with similar levels of TPL-3HA protein (Figure S4a,b). We chose the ZT12 time point, as CDF1 levels in WT should be low due to degradation through the FKF1-GI complex, but in the *35S:CDF1* line CDF1 levels are still sufficiently high enough to repress *CO* and *FT* (Imaizumi *et al.*, 2005). We saw increased enrichment of TPL in *35S:CDF1* background around amplicon 7, within the first 500 bp region upstream of the transcription start site (Figure 3d), where CDF1 was found to bind (Sawa *et al.*, 2007). This data suggests that the recruitment of TPL to specific regions of *CO* and *FT* promoter is likely conveyed by the binding of CDF protein that widely fluctuates in expression level throughout the day.

Loss of TPL function in phloem companion cells causes early flowering under long-day and short-day conditions

If CDF transcription factors repress target loci through TPL recruitment, we reasoned that the loss of TPL function should mimic the loss of CDFs. Dominant negative *tpl-1* mutants have been utilized in a variety of studies to observe the function of TPL and related TPRs (Long *et al.*, 2006). Although the molecular function of the *tpl-1* mutation is unknown, its presence seems to interfere with the function of normally expressed TPL and TPRs, resulting in a dominant negative phenotype (Long *et al.*, 2006). However, the pleiotropic nature of the *tpl-1* phenotype, and in particular the loss of meristematic function and lateral organ defects make detailed analysis of photoperiodic flowering time challenging. In order to more specifically characterize the role of TPL in flowering time regulation, we aimed to lessen the function of TPL and TPRs only in leaf phloem tissues where *CDF*s, *CO* and *FT* are expressed. We generated transgenic lines that expressed tpl-1 mutant protein from the control of a *SUCROSE-PROTON SYMPORTER 2 (SUC2)* phloem companion cell-specific

promoter (*SUC2:HA-tpl-1*). To our knowledge, this is this first study that utilizes a tissue-specific promoter driven *tpl-1* transgenic line to study the effect of reduction of TPL/TPR function in specific tissues. The *cdf1,2,3,5* mutant (hereafter referred to as the *cdfq* mutant) is very early flowering in both long days and short days, due to an upregulation of *CO* and *FT* during the morning (Fornara *et al.*, 2009). We anticipated that in *SUC2:HA-tpl-1* plants, CDFs would be unable to recruit functional TPL protein, and would thus be unable to repress their target loci. In other words, we predicted that a flowering time phenotype of *SUC2:HA-tpl-1* likely resembles that of *cdfq.*

We first analyzed the flowering time phenotype of these plants. Similar to *cdfq* mutants, *SUC2:HA-tpl-1* plants flower early in long days and especially short days (Figure 4). This result implies that having proper function of TPL/TPRs in phloem is important for photoperiodic flowering regulation.

Because TPL can interact with several other repressors of *FT* transcription, we wanted to check if the *cdfq* mutant phenotype was synergistic with *SUC2:HA-tpl-1.* We expected that in *cdfq* mutant backgrounds, if *CDF*s were genetically epistatic to *TPL*, then the flowering time phenotype of *SUC2:HA-tpl-1/cdfq* would not be additive. We generated *SUC2:HA-tpl-1/cdfq* lines with similar to or higher levels of *TPL* (*tpl-1*) transcripts than that in *SUC2:HA-tpl-1* (Figure S4c). We saw a slight hastening of flowering time under long-day conditions in several *SUC2:HA-tpl-1/cdfq* lines (which have higher amount of *TPL* (*tpl-1*) transcripts). Under short-day conditions, *SUC2:HA-tpl-1/cdfq* lines were the same or later flowering than *cdfq* (Figure 4). This suggests that any additional contribution from other TPL-interacting factors likely occurs under long-day conditions, however with flowering occurring at four to five total leaf number in long days, these plants may be encroaching on a developmental minimum of leaves before inflorescence initiation can occur. In short days, as several *SUC2:HA-tpl-1/cdfq* lines showed slightly later flowering than *cdfq*, TPL may work with other factors that also influence flowering time.

Conversely, we wanted to investigate if the expression of *tpl-1* in leaf phloem could mitigate the flowering effect caused by the overexpression of *CDF1.* If CDF1 represses its target loci mainly through TPL in leaf phloem, then introgression of the *SUC2:HA-tpl-1* transgene into a *35S:CDF1* genetic background should severely lessen the late flowering phenotype of the *35S:CDF1* plants. We found that in both long-day and short-day conditions the *SUC2:HA-tpl-1/35S:CDF1* plants flowered significantly earlier than the *35S:CDF1* plants (Figure 4). This indicates that CDF1 represses flowering mainly through the function of TPL/TPRs. The *SUC2:HA-tpl-1/35S:CDF1* plants, however, flowered later than their corresponding *SUC2:HA-tpl-1* plants in wild type background. This suggests that CDFs may also have some repressor activity unrelated to TPL function, or that increased CDF1 concentration can make use of TPL complexes that do not contain the tpl-1 mutant protein.

Taken together, these data suggest that the loss of TPL function impairs the native CDF proteins from being able to repress the photoperiodic flowering pathway.

Loss of TPL function in phloem interferes with CDF-dependent repression of *CO* and *FT*

We next investigated whether *SUC2:HA-tpl-1* plants had increased expression of CDF target loci, similar to the *cdfq* mutants. We measured the gene expression of *CO* and *FT* in both long days and short days in WT, *cdfq*, and *SUC2:HA-tpl-1* lines (Figure 5,S5). In long days, *SUC2:HA-tpl-1* plants showed increased levels of *CO* mRNA throughout the middle of the day (Figure 5a). Additionally, they showed increased expression of *FT* throughout the day, similar to *cdfq* mutants (Figure 5b). We also analyzed the expression levels of *FT* downstream genes, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *FRUITFUL* (*FUL*), *LEAFY* (*LFY*), and *APETALA 1* (*AP1*) (Andrés and Coupland, 2012; Song *et al.*, 2015) (Figure S5). Especially in the *SUC2:HA-tpl-1#4* line in which *TPL* (*tpl-1*) levels are relatively higher, the expression level of *SOC1*, *FUL*, *LFY*, and *AP1* were higher than those in WT plants (Figure S5). In short days, *CO* mRNA level was upregulated during the daytime in both *SUC2:HA-tpl-1* plants and *cdfq* mutants, and *FT* expression level was upregulated relative to WT (Figure 5c,d). These data support the notion that *SUC2:HA-tpl-1* affects flowering time and expression of *CO* and *FT* genes in a manner similar to *cdfq*.

Both *FT* and *CO* expression levels increase over developmental time (Kotake *et al.*, 2003). We wanted to ascertain whether the increase of *CO* and *FT* expression seen in *cdfq* mutants on a daily scale were present over developmental time, and whether *SUC2:HA-tpl-1*-dependent increases in *CO* and *FT* expression followed a similar trajectory across early development. We performed a gene expression analysis of WT, *cdfq*, and *SUC2:HA-tpl-1* plants from day 5 after germination until day 19. We harvested the tissues at the ZT13 time point, which is the daytime peak of *CO* expression, and close to the daily peak of *FT* expression. During the experiment, both *cdfq* mutants and *SUC2:HA-tpl-1* plants, but not WT, started flowering on day 15 (Figure 5f). We observed that *CO* expression increased in both *cdfq* and *SUC2:HA-tpl-1* plants from day 11 onward compared to WT, and *FT* levels increased gradually in both *cdfq* and *SUC2:HA-tpl-1* throughout the experimental period (Figure 5e,f). We saw a similar trend when looking at the expression of downstream targets of *FT*, *SOC1*, *LFY*, and *AP1* (Figure S6). These data suggest that CDF and TPL-dependent repression of flowering occurs throughout seedling development.

Both *SUC2:HA-tpl-1/gi* and *SUC2:HA-tpl-1/fkf1* plants flowered earlier than their parental mutants in long days and short days

In addition to CDF1 related genetic backgrounds, we wanted to further validate the potential CDF-dependent nature of the *SUC2:HA-tpl-1* phenotype. In *fkf1* and *gi* mutant backgrounds, increased levels of CDF1 protein accumulates in the middle of the day, lengthening the period of CDF dependent repression of *CO* and *FT*, which contributes in their later flowering phenotypes (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Fornara *et al.*, 2009). We introduced the *SUC2:HA-tpl-1* construct into *fkf1-2* and *gi-2* mutant backgrounds, and analyzed the flowering time phenotype. In long days, *fkf1-2* and *gi-2* mutants flower significantly later than WT plants, but mutants expressing the *SUC2:HA-tpl-1* transgene are comparable or earlier than WT (Figure 6a,b). In short days, we saw a similar phenotype to *SUC2:HA-tpl-1/35S:CDF1* transgenic plants, with a significant reduction in flowering time, but later than WT plants under long-day conditions as well as later than *SUC2:HA-tpl-1/*Col-0 plants of similar transgene expression level (Figure 6c).

Again, this suggests that some CDF activity independent of TPL may also be contributing to a delay in flowering time phenotype under long-day and short-day conditions.

TPL regulates *TSF* levels and overexpression of *tpl-1* in leaf phloem accelerates flowering time in *co* and *ft* mutants

If the introduction of the *SUC2:HA-tpl-1* transgene impairs the ability of CDFs to repress transcription, then we reasoned that the loss of the key activators which CDFs regulate would revert the early flowering phenotype seen in both *SUC2:HA-tpl-1* to a late flowering phenotype. To investigate this, we transformed *SUC2:HA-tpl-1* into the *ft-101* and *co-101* mutant genetic background, and analyzed the flowering time phenotype. We found that under long-day conditions, *SUC2:HA-tpl-1/ft-101 and SUC2:HA-tpl-1/co-101* plants flowered later than *SUC2:HA-tpl-1* plants, however, they flowered earlier than *ft-101* or *co-101* mutants (Figure 7a,b). In short days, we found this phenotype was attenuated and the *SUC2:HA-tpl-1/ft-101* and *SUC2:HA-tpl-1/co-101* plants flowered more similar to, but still earlier to that of *ft-101* and *co-101* plants (Figure 7a,c). TPL has been previously reported to act in repressing downstream components of the flowering pathway such as *LFY* at the shoot apex (Wu *et al.*, 2015). Due to the tissue specific activity of the *SUC2* promoter at these developmental stages however, it is unlikely that the *SUC2:HA-tpl-1* transgene is expressed at the shoot apex. We checked the gene expression of several downstream and parallel components of the flowering pathway in WT, *cdfq*, and *SUC2:HA-tpl-1* under long-day conditions to investigate the potential cause of early flowering in *ft-101* and *co-101* mutants (Figure S5). Upon checking the gene expression of the *FT* paralog *TWIN SISTER OF FT* (*TSF*), which is expressed in vascular tissues (Yamaguchi *et al.*, 2005), we found that *TSF* expression was highly upregulated in *SUC2:HA-tpl-1* plants throughout the day (Figure 7d). In *cdfq* mutants, we saw an upregulation of *TSF* transcripts during both the morning and afternoon, but not of the same magnitude as in *SUC2:HA-tpl-1* plants (Figure 7d). This suggested that CDFs may partially regulate TPL recruitment to the *TSF* gene, and another factor may also play an important role to regulate *TSF* through TPL in the afternoon of long days. To test whether CDF1-dependent recruitment of TPL to the *TSF* locus occurs, we performed a ChIP assay looking at the *TSF* promoter in WT, *TPL:TPL-3HA and TPL:TPL-3HA/35S:CDF1* plants at the ZT12 time point of long days. We found that TPL strongly associated with a region of the *TSF* promoter upstream of the transcription start site in *TPL:TPL-3HA/35S:CDF1* plants (Figure 7e), suggesting that TPL recruitment to the *TSF* promoter can directly or indirectly occur through CDF1. Taken together, these data suggest that the long-day specific early flowering phenotype of *SUC2:HA-tpl-1/ft-101 and SUC2:HA-tpl-1/co-101* plants may be in part due to the upregulation of *TSF*, and that CDF1 recruitment is partially responsible for TPL-dependent regulation of *TSF*.

Discussion

Here we show that the mechanism by which CDF1 represses transcription of photoperiodic flowering target genes is through interactions with a co-repressor protein TPL. CDF proteins contain a conserved motif responsible for interaction with TPL (Figure 1), and eliminating this motif attenuates the function of CDF1 as a transcriptional repressor (Figure 2). TPL protein binds to the *CO* and *FT* promoters during the morning, which occurs at the same

time as CDF1 binding to *CO* and *FT* (Figure 3). Increasing the expression of *CDF1* brings additional TPL to target loci (Figure 3), suggesting CDF dependent recruitment of TPL. Removing TPL dependent repression exclusively within leaf phloem causes early flowering and photoperiodic insensitivity, similar to *cdfq* loss of function (Figure 4,6,7). *SUC2:HA-tpl-1* plants are largely insensitive to changes in *CDF1* expression, highlighting that loss of co-repressor perturbs CDF function (Figure 4). These data together demonstrate that a CDF-TPL transcriptional complex regulates *CO* and *FT* during the morning to limit their expression to the afternoon (Figure 7f).

TPL-dependent repression mechanism and its similarity to auxin signaling

TPL-dependent repression is typified by potential interactions such as mediator blocking and chromatin remodeling, though the exact mechanisms of TPL activity remain unclear (Long *et al.*, 2006; Liu and Karmarkar, 2008; Wang *et al.*, 2013; Ito *et al.*, 2016). TPL interacts with several HDACs, and it has been postulated that TPL is able to recruit HDAC enzymes to target promoters to bring about transcriptional silencing through chromatin remodeling. In addition, TPL and LEUNIG (LUG), have both been shown to interact with HEN3/CDK8 (Gonzalez *et al.*, 2007; Ito *et al.*, 2016). This HEN3/CDK8 module of mediator has been implicated in a repressive role by preventing mediator holoenzyme formation (Tsai *et al.*, 2013). A recent study of *D. melanogaster* Gro, a general co-repressor that shares similar functional domains with TPL, points to discrete Gro binding rather than spreading of hypoacetylated histones and heterochromatin for long-range silencing (Chambers *et al.*, 2017). The same study found that *bona fide* Gro targets were enriched at loci having paused or stalled RNA polymerase II. This suggests that Gro plays a similar role to TPL-CDK8 in potentially preventing maturation of Mediator complex to initiate transcription. The TPL ChIP data presented here also implicates discrete binding rather than a spreading mechanism at *CO* and *FT* (Figure 3). How TPL-dependent histone deacetylation versus mediator interaction might function is unknown. Although recent work has progressed the understanding of histone modifications at the *FT* promoter, the temporal and cell specific nature of these changes still poses many questions (Turck *et al.*, 2007; Jiang *et al.*, 2008; Adrian *et al.*, 2010; Pazhouhandeh *et al.*, 2011; Cao *et al.*, 2014).

This CDF-TPL-dependent transcriptional regulatory module bears many similarities to the molecular architecture of the IAA-TPL auxin circuit; the specific transcriptional repressor brings in TPL to solidify the repressive status of target loci. Further, the removal of the transcription factor through ubiquitin dependent proteasomal degradation (in this case through FKF1 E3 ubiquitin ligase) relieves repression and enables activation in the afternoon of long days (Pierre-Jerome *et al.*, 2013). Due to the fact that several other *FT* regulators appear to bind to TPL, this positions TPL as a key mechanistic player in *CO* and *FT* regulation, and may play a more general role in mediating the accessibility of transcription factor binding sites over the relatively large space of the *FT* promoter (Causier *et al.*, 2012; Graeff *et al.*, 2016).

CDF and TPL function in the context of temporal and spatial regulation of transcription

Daily temporal regulation of photoperiodic regulators is critical for the timing and amplitude of *FT* expression, and thus the quantitative flowering time phenotype in Arabidopsis (Song

et al., 2015). Due to the transcriptional complexity of the *CO* and *FT* gene regulatory network, being able to predict the combinatorial effects of many regulators can be challenging, and knowing more about the interactions between multiple transcription factors has the potential to better characterize the system (Andrés and Coupland, 2012). CDF transcription factors are key regulators of the timing of *CO* and *FT* expression, and their position as direct outputs of the circadian clock makes them a primary means through which to shut down *CO* and *FT* expression during the morning. In general, morning dependent repression at *CO* and *FT* cannot be overcome by the presence of activators during this time period. Overexpression of various *CO* activators, such as FLOWERING BHLH (FBH) and class II TEOSINTE BRANCHED 1/CYCLOIDEA/PROLIFERATING CELL FACTOR 1 (TCP) transcription factors, is insufficient to strongly upregulate *CO* during the morning (Ito *et al.*, 2012; Kubota *et al.*, 2017; Liu *et al.*, 2017). Similarly at *FT*, overexpression of some activators, such as CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX 1 (CIB1), are unable to increase *FT* during the morning (Liu *et al.*, 2008). Potentially, CDF-TPL dependent repression can restrict the access of activators during the morning period. Either through physical blocking of mediator or the changing of chromatin structure at either the *CO* or *FT* locus (Wang *et al.*, 2013; Ito *et al.*, 2016), a CDF-TPL morning complex can prevent activation of transcription until the afternoon of long days, after FKF1-GI dependent degradation of CDFs.

Although proper temporal regulation of *CO* and *FT* transcription is crucial for inducing the photoperiodic flowering response, spatial regulation of their expression also plays an important role (Song *et al.*, 2013). To more precisely investigate the function of the TPL general repressor in the photoperiodic flowering pathway, reducing TPL/TPR function in leaf phloem companion cells was an effective method for understanding the tissue-specific roles of TPL/TPR. Generally speaking, as TPL is involved in many different pathways in various cell types and interacts with wide varieties of transcriptional regulators (Liu and Karmarkar, 2008), the combination of functional analysis of specific TPL interactors and temporal, spatial, and developmental modification of TPL function using ectopic expression of *tpl-1* will likely provide us with insight into the more specific roles of TPL in different regulatory networks.

Insights into evolution of the CDF-TPL module

Many of the components of the photoperiodic flowering pathway are highly conserved among flowering plants, suggesting a common module for regulation of target pathways in response to seasonal change (Song *et al.*, 2015). This is evident from both the conservation of factors in the photoperiodic flowering pathway, as well as its role in multiple different kinds of seasonal organ development. These include the regulation of bud burst in tree species such as *Populus triocharpa* as well as for tuberization in potato (Hsu *et al.*, 2011; Kloosterman *et al.*, 2013). CDF1 homologs in in *P. sativum* and *S. tuberosum* are repressors of photoperiodic flowering and tuberization, respectively (Kloosterman *et al.*, 2013; Ridge *et al.*, 2016). Recently, it has been shown that the FKF1 and GI components of the photoperiodic time sensing module are also present in the basal linage of the land plants, in the bryophyte *Marchantia polymorpha* (Kubota *et al.*, 2014). In addition, one DOF transcription factor in *Marchantia* is similar to CDFs and possesses the TPL binding site

(Figure S1), although its function is unknown. Moreover, the moss *Physcomitrella patens*, the fern *Selaginella moellendorffii*, as well as several gymnosperms possess CDF-related DOF transcription factors that contain the TPL binding sites (Figure S1) (Sugiyama *et al.*, 2012). These findings suggest that an ancient photoperiod mechanism including the DOF factors may have emerged and remained conserved with the evolution of land plants. There are also additional indications that CDF like proteins are part of this seasonal regulatory circuit. A recent study showed that a CDF-like protein in *Chlamydomonas reinhardtii* regulated growth in response to seasonal change (Lucas-Reina *et al.*, 2015). This algae DOF protein bears the most sequence homology to the DOF domain of the CDF clade, although this specific DOF protein lacks the TPL binding sequences described in this manuscript (Lucas-Reina *et al.*, 2015). The presence of this TPL binding domain is not universal amongst the DOF transcription factor family but is conserved within the CDF-like subclade of the DOFs through long periods of evolutionary time (Figure S1) (Cai *et al.*, 2013; Lucas-Reina *et al.*, 2015; Ma *et al.*, 2015; Kang *et al.*, 2016). The presence or absence of this domain likely determines other transcriptional interactions; indeed several DOF factors that were originally characterized function as activators, and these DOF factors lack the TPL binding motif (Cavalar *et al.*, 2003). Based on the similar function as repressors amongst the CDF clade in Arabidopsis as well as their orthologs in other plant species, the N-terminal TPL binding motif is likely a conserved mechanism through which DOF factors function as transcriptional repressors (Kloosterman *et al.*, 2013; Corrales *et al.*, 2014; Ridge *et al.*, 2016). While the conserved TPL binding domain is less common than the EAR-domain in TPL clients, its presence in transcription factors other than flowering regulators suggests that this domain is general rather than specific to flowering targets (Causier *et al.*,2012). Due to their conservation amongst many basal lineages of green algae and plants, it will be interesting to see if there is a functional CDF/TPL-FKF1/GI module that is part of a core conserved photoperiod circuit.

Experimental procedures

Plant materials

All genetic resources in this work are the Columbia-0 (Col-0) background, and Col-0 plants are used as wild type in all experiments. *TPL:TPL:3HA* (Wang *et al.*, 2013), *cdfq* (Fornara *et al.*, 2009), *35S:CDF1* (Imaizumi *et al.*, 2005), *fkf1-2* (Imaizumi *et al.*, 2003), *gi-2* (Fowler *et al.*, 1999), *co-101* and *ft-101* (Takada and Goto, 2003) were previously described. To generate *35S:CDF1-3F6H*, *35S:CDF1-ΔN-3F6H*, and *35S:CDF1-mut-3F6H* constructs, full length, truncated, and PCR mutagenized *CDF1* cDNAs were cloned using the following forward primers 5′-TCCCCATGGGACTGGAAACTAAAGATCCTGCGATAAAGC-3′ (for *CDF1-3F6H*), 5′- TCCCCATGGGAACGGTTTTAGAGGTTGCTGATGAAGA-3′ (for *CDF1-ΔN-3F6H*), 5′-TCCCCATGGGACTGGAAACTAAAGATCCTGCGATAAAGGCCGCTGCTATGAAAATTCCTTTCCCGAC-3′ (for *CDF1-mut-3F6H*, the mutated sequences that encode three alanines are underlined) and the same reverse primer 5′-GGAGGATCCCCATCTGCTCATGGAAATTGATTGATCTTG-3′. These fragments were then inserted using restriction enzyme sites (*Nco*I, *Bam*HI) into pENTR-3F6H (Ito *et al.*, 2012; Song *et al.*, 2014), which is modified pENTR D-TOPO vector (Invitrogen) containing

sequences encoding a 3xFLAG 6xHis (3F6H) peptides to generate the constructs that contains *CDF1* variant genes translationally fused to 3xFLAG 6xHis peptides. After confirming the sequences, these cDNAs were transferred into pH7WG2 (Karimi *et al.*, 2002) destination vector, which harbors a *35S* expression cassette, using the LR clonase II enzyme mix (Invitrogen), and subsequently transformed into plants using the conventional *Agrobacterium* mediated transformation method.

To generate *SUC2:HA-tpl-1*, we first cloned the wild-type version of *TPL* CDS into pENTR D-TOPO using cDNA as a template, forward primer 5′-CACCATGTCTTCTCTTAGTAGAGAGCTCGTTTTC-3′ and 5′-TCATCTCTGAGGCTGATCAGATGCAG as the reverse primer. We named this construct pENTR-TPL. The *tpl-1* allele contains two mutations in the 5′ part of *TPL* CDS (Long *et al.*, 2006). To generate a vector containing the *tpl-1* mutation cDNA, we planned to replace about a 500-bp fragment of 5′ part of *TPL* CDS, which can be cut by *Not*I and *Nco*I, with that of *tpl-1* CDS, which contains these mutations. We first amplified the 5′ fragment of the *tpl-1* gene using *tpl-1*(Ler) cDNA as a template, Forward primer 5′-CACCATGTATCCATATGATGTTCCAGATTATGCTATGTCTTCTCTTAGTAGAGAGCT CGTT-3′ (the underlined sequences encode HA-tag) and 5′-GTACCAACTAGAAGCAGGGTCTGTTTAAT-3′ as the reverse primer. This fragment also contains a *Not*I site plus an N-terminal single HA tag. This fragment was cloned into pCR-Blunt II-TOPO (Invitrogen). We named this vector pCR-HA-tpl-1. As one of two *tpl-1* mutations created one extra *Nco*I site, to replace the ca. 500 bp fragment of *TPL* with that of *tpl-1*, which contains two *Nco*I sites, instead of one, we decided to clone the 500 bp fragments in two steps. First, we digested pCR-HA-tpl-1 using *Not*I and *Nco*I (5′ half of ca. 500 bp fragment, size 303 bp), and ligated the fragment into the *Not*I-*Nco*I sites of pENTR-TPL vector. We named this vector pENTR-HA-tpl-1′. To place the remaining the *Nco*I-*Nco*I *tpl-1* fragment (size 252 bp) into the vector, the *Nco*I-*Nco*I *tpl-1* fragment was ligated into *Nco*I cut pENTR-HA-tpl-1′. After confirming the sequence and orientation of the *Nco*I-*Nco*I fragment, we named this vector pENTR HA-tpl-1. The finished pENTR HA-tpl-1 cassette was transferred into an pH7WG2 vector containing a *SUC2* promoter and 5′-UTR (Truernit and Sauer, 1995; Sawa and Kay, 2011) using LR clonase II enzyme mix (Invitrogen), and transformed into plants using the conventional *Agrobacterium* mediated transformation method. The transgenic plants used in this work were selected based on having similar expression levels of *CDF1* (variants), *tpl-1* genes, or TPL-3HA protein.

Flowering time analysis

For flowering time analysis, seeds were directly sown on the soil (Sunshine Mix #4; Sun Gro Horticulture) and stratified for 2–3 days at 4°C in darkness to synchronize the timing of germination. Plants were grown at 22°C under long days (16 h light/8 h dark) or short days (8 h light/16 h dark). Light was provided by full-spectrum white fluorescent light bulbs (F017/950/48′′ Octron; Osram Sylvania) with a fluence rate of 60–90 μmol photons m^{-2} sec^{-1} in long days and 75–115 μmol photons m^{-2} sec^{-1} in short days. Flowering time was measured by counting the number of rosette and cauline leaves on the main stem when they bolted and the inflorescence was between 3–5 cm. Plant lines were sown in rows in horticultural 32-cell flats, with 16 individual plants per line split into two flats.

Yeast 2-hybrid analysis

To generate full-length products (without epitope tags) of *CDF1*, *CDF1-ΔN*, and *CDF1-mut* for Y2H analysis, the respective forward primers 5′-CACCATGCTGGAAACTAAAGATCC TGCGATAAAGC -3′ (for *CDF1*), 5′-CACCATGACGGTTTTAGAGGTTGCTGATGAAGA-3′ (for *CDF1-ΔN*), and 5′ CACCATGCTGGAAACTAAAGATCCTGCGATAAAGGCCGCTGCTATGAAAATTCCT TTCCCGAC-3′ (for *CDF1-mut*) were used and the same reverse primer 5′-TCACATCTGCTCATGGAA ATTGATTGATC-3′, using an cDNA clone as template. These PCR products were then cloned into pENTR D-TOPO (Invitrogen) using the standard TOPO reaction. The pENTR-TPL clone described above was used for this analysis. Plasmid cassettes were then transferred to pACT2-GW and pAS-GW, two gateway compatible prey and bait vectors (Song *et al.*, 2014) using LR clonase II (Invitrogen). Yeast strains Y187 and AH109 were transformed with prey and bait vectors, respectively using the standard yeast PEG based plasmid transformation (Clontech). After colonies formed on –W or –L containing media, three independent colonies were grown together, then mated against their corresponding pairs for 3 days on YPDA media. After mating, yeast colonies were transferred onto –WL media. After checking for mating confirmation, yeast sectors were retransferred at the same time onto –WL and –WLH media. The *GI* clone contains the N-terminus of the GI protein used in this analysis was described previously (Sawa *et al.*, 2007).

Bimolecular fluorescence complementation assays

BiFC experiments were performed on 3-week-old *N. benthamiana* plants grown at 22°C under long days (16 h light/8 h dark) on soil (Sunshine Mix #3; Sun Gro Horticulture) according to (Martin *et al.* 2009). Light was provided by full-spectrum white fluorescent light bulbs (F017/950/48″ Octron; Osram Sylvania) with a fluence rate of 60–90 μmol photons m^{-2} sec^{-1}. pSITE vectors were used to generate BiFC constructs for CDF1, CDF1-ΔN, CDF1-mut, and TPL proteins (Martin *et al.*, 2009). In all cases the combinations are N-terminal fusions of either the nEYFP or cEYFP to the cDNA of CDF1 or TPL (Martin *et al.*, 2009). RFP fused Histone H2B was used as a nucleus marker (Goodin *et al.*, 2002). Injection of agrobacterium strains into tobacco leaves was performed as in (Goodin *et al.*, 2002), but the OD_{600} of the *Agrobacterium* culture used was adjusted to 0.1, and the ABI *Agrobacterium* strain was used. 3 days after transfection, plant leaves were imaged using a confocal microscope (TCS SP5; Leica Microsystems).

RNA isolation and gene expression analysis

For gene expression analyses, 2-week-old seedlings were grown on LS agar plates containing 3% sucrose in long days and short days. Plates were grown in a self-contained growth chamber (Percival), light was provided by full-spectrum white fluorescent light bulbs (F017/950/24″ Octron; Osram Sylvania) with a fluence rate of 60–90 μmol photons m^{-2} sec^{-1}. Tissue was harvested at every 3 hours during a 24-h period, and was used for RNA extraction using illustra RNAspin Mini kit (GE Healthcare). To synthesize cDNA, 2 μg of total RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio- Rad). The cDNA was diluted to 100 μl of water (1:9 ratio), and 4 μl of diluted cDNA was used for quantitative polymerase chain reaction (Q-PCR) using Bio-Rad real-time thermal cycler (MyiQ). Primers

and PCR conditions used for *IPP2*, *CDF1*, *CO*, *FT, SOC1, AP1, LFY*, and *TSF* amplification were described previously; *IPP2*, *CDF1*, *CO* and *FT* (Song *et al.*, 2012), *SOC1* and *AP1* (Han *et al.*, 2008), *LFY* (Kotake *et al.*, 2003), and *TSF* (Yan *et al.*, 2014). *TPL* and *FUL* Q-PCR primers are listed in supplementary table S1. The PCR conditions for detecting *TPL* transcripts were 45 total cycles using the protocol of 95°C for 10 sec, 55°C for 20 sec, and 72°C for 20 sec. For *FUL*, 45 cycles were run using the protocol of 95°C for 10 sec, 65.5°C for 15 sec, and 72°C for 15 sec. With the exception of the developmental time courses in Figure 5e,f and S6, *IPP2* expression was used as an internal control to normalize cDNA amount. For Figure 5e,f and S6, *IPP2* and *PP2A* were used as internal controls to minimize variation over the experimental period. All expression data were calculated from at least three independent biological experiments.

Immunoblot analysis

Total crude protein was extracted from frozen-ground seedlings in the extraction buffer [50 mM sodium phosphate (pH 7.0), 100 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate]. The supernatant was collected after centrifugation at 21,000 × ***g*** for 5 min. Then protein samples were separated by 8 or 12% SDS-PAGE gels (8% for TPL, and 12% for CDF1) and transferred to nitrocellulose membranes (for each sample, 5–10 μg of total protein was used). The 3×FLAG and 6xHis epitope-tagged CDF1, *CDF1-ΔN*, *CDF1-mut* and HA-tagged TPL, HA-tagged *tpl-1* fusion proteins were detected using HRP conjugated anti-FLAG (Sigma) and anti-HA (Roche) antibodies. Super Signal West Pico and Femto Chemiluminescent substrate kits (Thermo Fisher Scientific) were used to detect signals. All experiments were performed at least three times with independent biological replicates.

Chromatin immunoprecipitation

ChIP experiments were performed on 2-week-old seedlings, which were grown on the same conditions described in RNA isolation and gene expression analysis section. 600 mg of tissue was harvested and frozen in liquid nitrogen in small sachets and kept at −80°C until the ChIP procedure was started. ChIP experiments were performed as illustrated in (Yamaguchi *et al.*, 2014) with minor modifications. The initial extraction buffer used contains 0.4 M sucrose, 10 mM HEPES pH 8.0, 2 mM EDTA, 5 mM β-mercaptoethanol, EDTA-free protease inhibitor tablet (Pierce), 1% Formaldehyde. Plants were harvested, ground in liquid nitrogen into a fine powder, then the extraction buffer containing formaldehyde was added and incubated for 10 min at 4°C, then quenched with glycine to a total concentration of 200 mM glycine for 5 min, then filtered twice through a miracloth filter. For the qPCR analysis of DNA, the reaction sizes were scaled down to a 15 μl reaction size, and 1.5 μl of DNA was used for input into the reaction for both purified input and immunopurified samples. For the *CO* promoter, PCR reactions were run for 60 cycles using the protocol of 95°C for 10 sec, 57°C for 20 sec, and 72°C for 20 sec. For the *FT* promoter, PCR reactions were run for 65 cycles using the protocol of 95°C for 10 sec, 52°C for 20 sec, 72°C for 20 sec. For the *TSF* promoter, PCR reactions were run for 60 cycles using the protocol of 95°C for 10 sec, 57°C for 20 sec, 72°C for 20 sec. Primer sequences for each amplicon of *CO* and *FT* loci are previously listed (Ito *et al.* 2012, Song *et al.* 2012). Primer sequences for each amplicon of *TSF* locus are listed in supplementary table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance Statement

Temporal control of expression profiles of photoperiodic flowering genes is important for regulating timing of flowering. The mechanism of CDF-dependent repression on the *CO* and *FT* promoters is currently unknown. Here we demonstrate that CDFs recruit the co-repressor TPL to repress transcription during the morning. Understanding the time-dependent abundance of various transcriptional complexes that regulate *CO* and *FT* expression is key to understanding flowering time regulation.

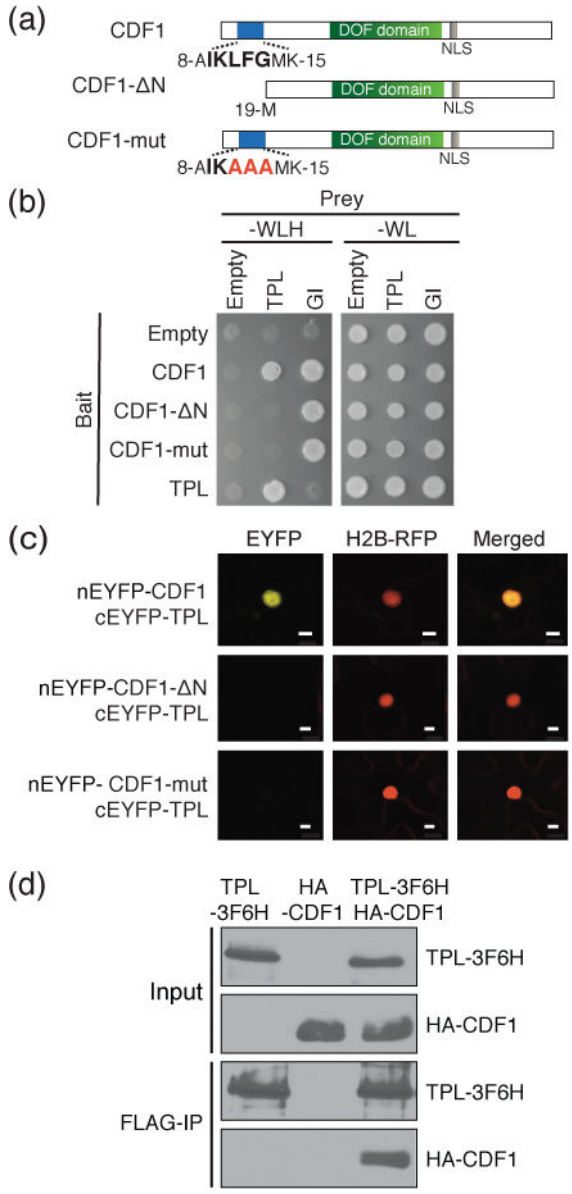

Figure 1. CDF1 and TPL form a protein complex through a conserved binding motif located at the N-terminus of CDF1

(a) Schematic representation of CDF1 full length protein and the N-terminal CDF variants used in this study. The N-terminal amino acid sequences (IKLFG) conserved in CDF family proteins (Figure S1), which overlap with the TPL binding motif (R/KLFGV), are shown in bold. The mutated amino acids in CDF1-mut protein are shown in red. The relative positions of DOF DNA binding domain (DOF domain) and nuclear localization signal (NLS) are indicated. (b) Y2H analysis of CDF1-TPL protein interaction. The –WLH plate shows the interaction of bait and prey proteins, while the –WL plate shows the presence of both bait and prey constructs. N-terminus of GI protein was used as a positive control for CDF1 variants. (c) BiFC interaction analysis in transiently transfected *N. benthamiana* leaves between full-length of CDF1 protein, CDF1-ΔN and CDF1-mut variants, and TPL protein. Histone H2B-RFP was used to determine the position of the nucleus in the same cell. Scale

bars show 10 μm. (d) TPL-CDF1 interaction using coimmunoprecipitation assay of transiently transfected *N. benthamiana* leaves.

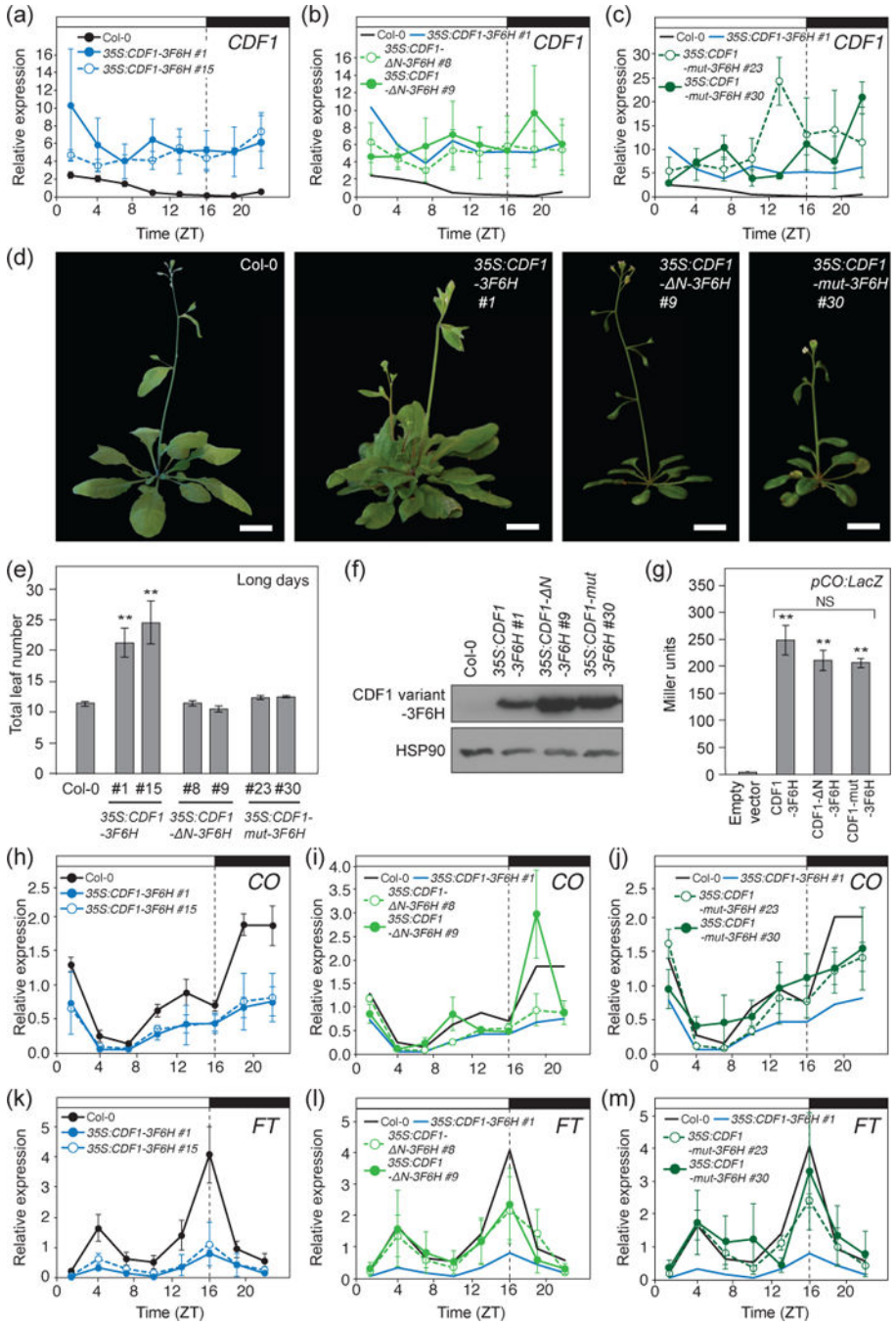

Figure 2. Removal of TPL interacting residues attenuates CDF1 repressor function

(a, b, and c) *CDF1* expression levels in Col-0, *35S:CDF1-3F6H* (a), *35S:CDF1-ΔN-3F6H* (b), and *35S:CDF1-mut-3F6H* plants (c) grown in long days for 14 days. Means +/− SEM were calculated from four independent experiments. The traces of diurnal *CDF1* expression changes in Col-0 and *35S:CDF1-3F6H* #1 shown in (a) are included in (b) and (c) for comparison. (d) Representative images of WT, *35S:CDF1-3F6H, 35S:CDF1-ΔN-3F6H*, and *35S:CDF1-mut-3F6H* plants at flowering in long days. Scale bars=2 cm. (e) Quantification of flowering time by total leaf number at bolting from (d) under long days. Means +/- SEM

were calculated from N=16 individuals. **P < 0.01 (one-tailed *t* test). (f) CDF1-3F6H, CDF1-ΔN-3F6H, and CDF1-mut-3F6H protein expression at ZT4 time point in 14-day-old long-day grown transgenic seedlings. HSP90 served as a loading control. (g) Y1H analysis of CDF1-3F6H, CDF1-ΔN-3F6H, CDF1-mut-3F6H proteins binding to a 500 bp region of the *CO* promoter (*pCO:LacZ*), which contains a previously characterized DOF binding *cis*-element repeats. LacZ activity is displayed in Miller units. Means +/− SEM were calculated from three independent experiments. **P < 0.01 (one-tailed *t* test), NS = non-significant. (h, i, and j) Gene expression analysis of *CO* in Col-0, *35S:CDF1-3F6H* (h), *35S:CDF1-ΔN-3F6H* (i), and *35S:CDF1-mut-3F6H* plants (j). Plants were grown in long days for 14 days. Experiments were repeated four times independently, and the means +/- SEM derived from four experiments are shown. The traces of diurnal *CO* expression changes in Col-0 and *35S:CDF1-3F6H* #1 shown in (h) are included in (i) and (j) for comparison. (k, l, and m) Gene expression analysis of *FT* in Col-0, *35S:CDF1-3F6H* (k), *35S:CDF1-ΔN-3F6H* (l), and *35S:CDF1-mut-3F6H* plants (m). Plants were grown in long days for 14 days. Experiments were repeated four times independently, and the means +/− SEM derived from four experiments are shown. The traces of diurnal *FT* expression changes in Col-0 and *35S:CDF1-3F6H* #1 shown in (h) are included in (i) and (j) for comparison.

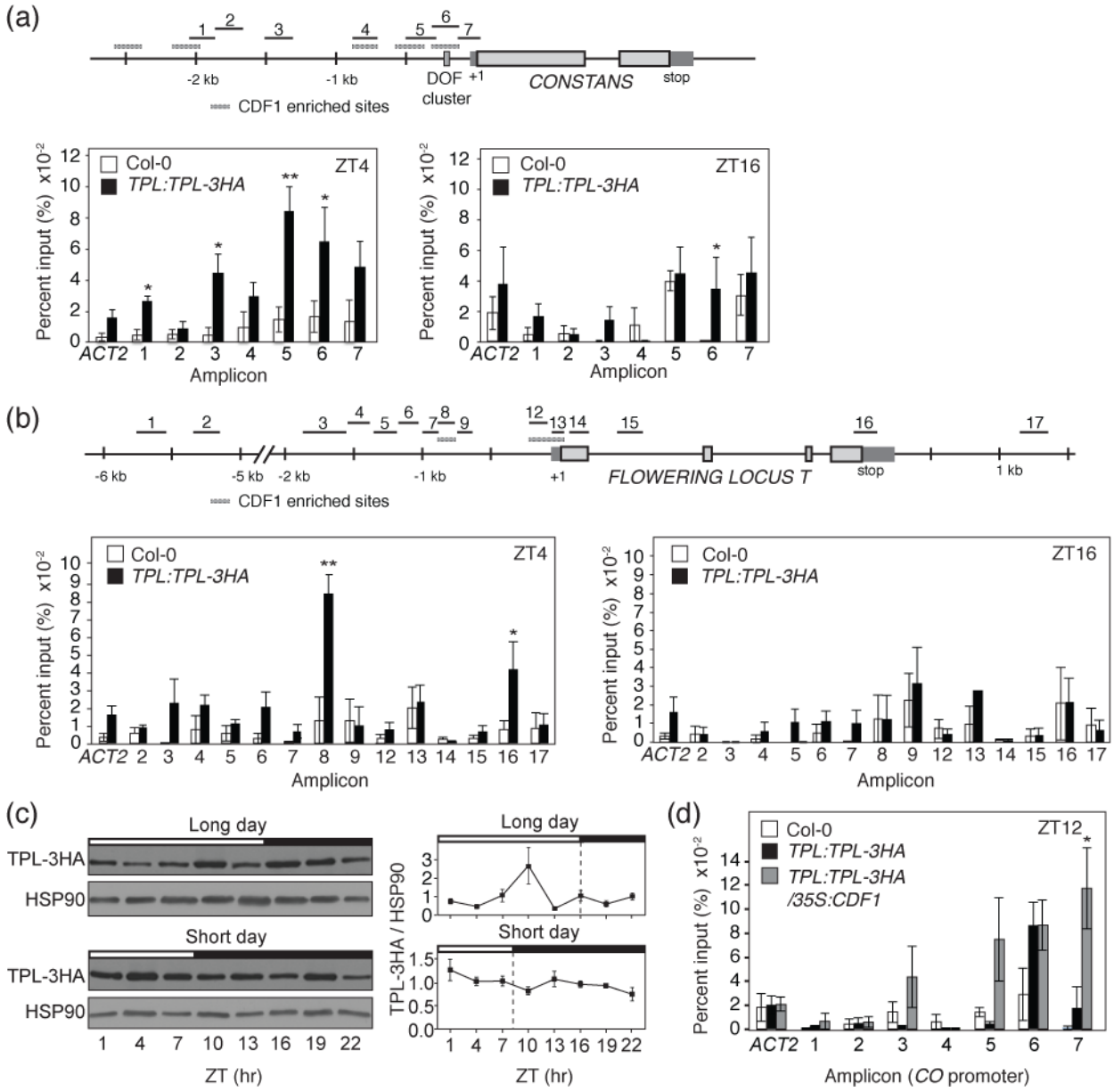

Figure 3. TPL associates with the *CO* and *FT* promoter regions during the morning in a CDF1-dependent manner *in vivo*

(a and b) ChIP assay-showing TPL binding to regions of the *CO* and *FT* genomic locus at ZT4 and ZT16 from 14-day-old *TPL:TLP-3HA* and Col-0 plants grown under long-day photoperiods. The schematic diagrams of positions of each ChIP amplicon scattered in *CO* and *FT* promoter regions are shown. The results are means and +/− SEM calculated from four independent experiments. Col-0 plants were used as negative controls. *P < 0.05,**P < 0.01 (one-tailed t test). Hatched grey boxes indicate regions of CDF1 enrichment from Sawa *et al.*, 2007 and Song *et al.*, 2012, respectively. (c) Daily protein expression profiles of TPL in long days and short days. 14-day-old *TPL:TLP-3HA* plants were used for experiments. Experiments were performed three times independently. Means +/− SEM were calculated from ratios of signal strength of TPL proteins and HSP90 loading control. (d) ChIP

experiment for TPL binding to the *CO* genomic locus at ZT12, in Col-0, *TPL:TPL:3HA*, and *35S:CDF1 TPL:TPL:3HA* plants. All calculations were performed as in (a) and (b). Plants were 14 days old and grown under long-day photoperiods.

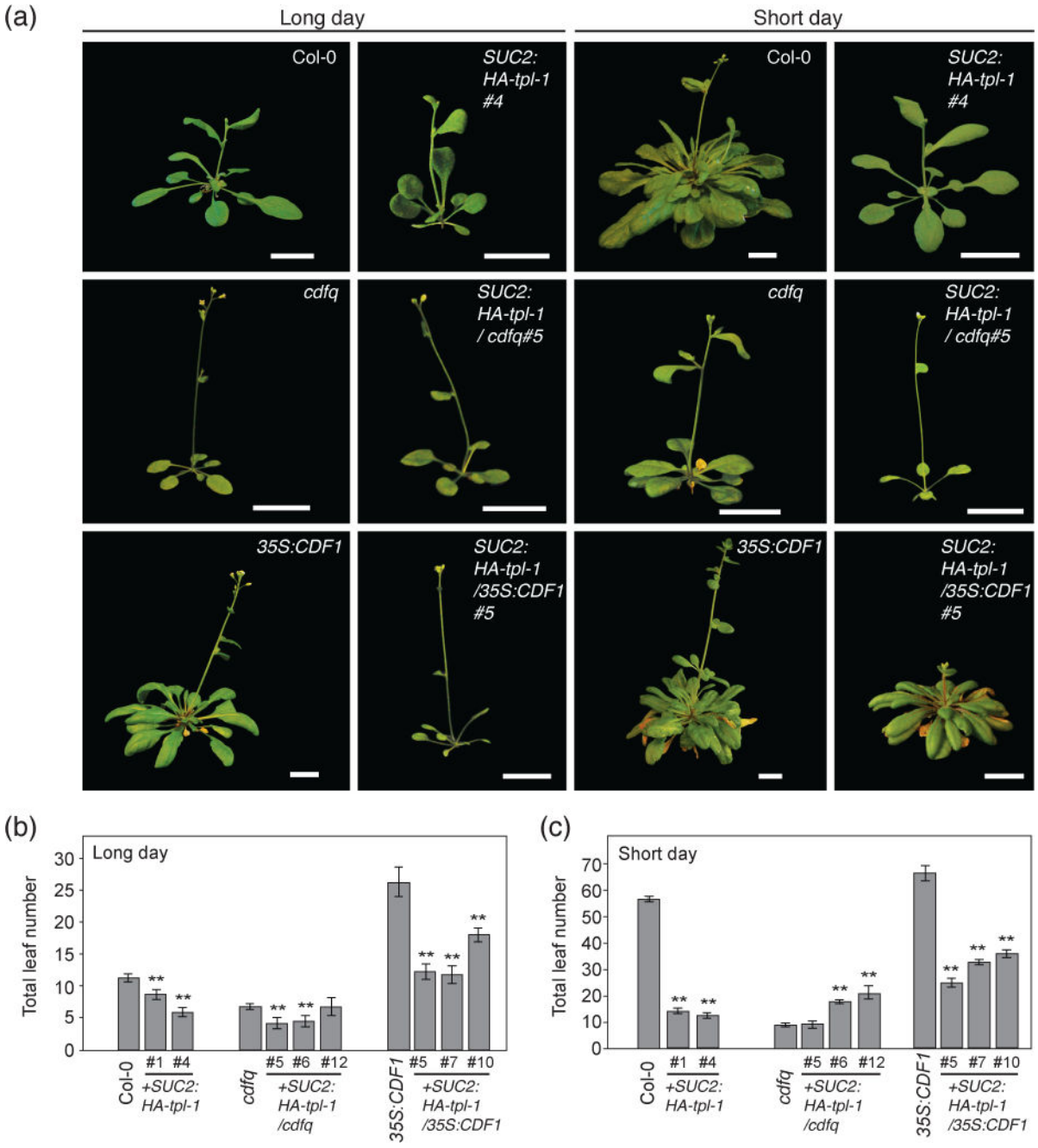

Figure 4. Expressing a dominant negative form of *TPL* in phloem companion cells phenocopies *cdfq* mutants, and lessens the late flowering phenotype of *CDF1* overexpressors

(a) Representative images of *SUC2:HA-tpl-1, SUC2:HA-tpl-1/cdfq, and SUC2:HA-tpl-1/35S:CDF1* plants under long-day and short-day photoperiods at flowering. Scale bars=2 cm. (b and c) Quantification of flowering time by total leaf number at bolting from (a) under long days (b) and short days (c). Means +/− SEM were calculated from N=16 individuals. **P < 0.01 (one-tailed *t* test).

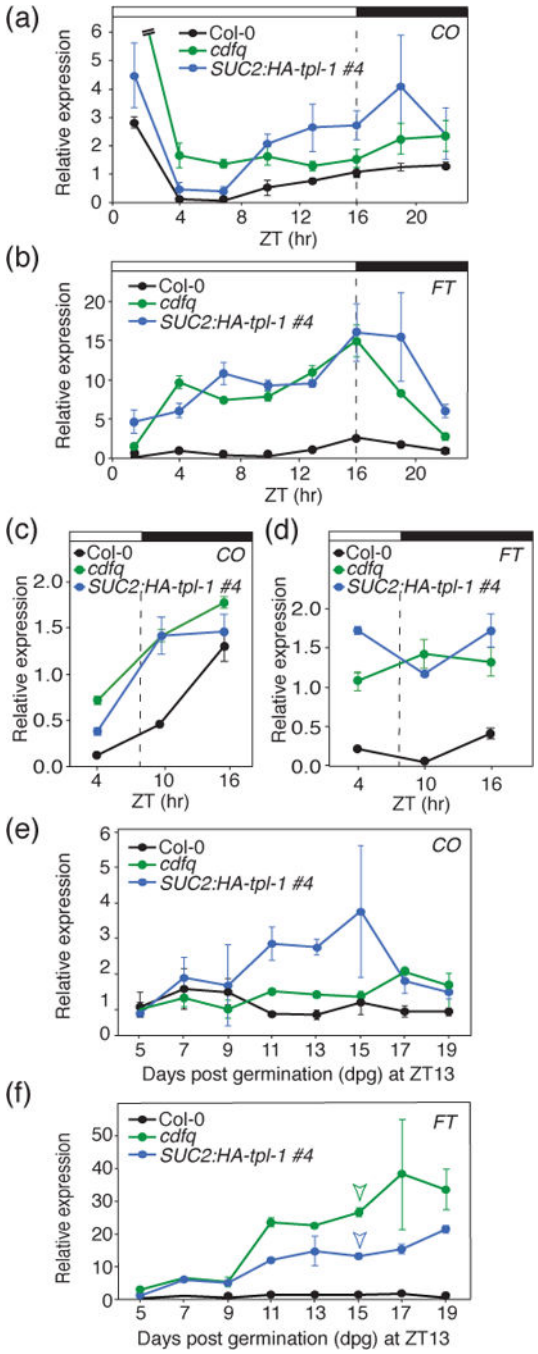

Figure 5. *SUC2:HA-tpl-1* transgenic plants have increased *FT* and *CO* expression in long days and short days, as well as over developmental time

(a) and (b) Diurnal gene expression analysis of *CO* and *FT* in Col-0, *cdfq*, and *SUC2:HA-tpl-1* plants. Experiments were performed on 14-day-old seedlings grown in long days, with 3-hour resolution. Means +/− SEM were calculated from four independent experiments. (c and d) Gene expression analysis of *CO* (c) and *FT* (d) in Col-0, *cdfq*, and *SUC2:HA-tpl-1* plants in short days. Experiments were performed on 14-day-old seedlings in short days, with 6-hour resolution. Means +/- SEM were calculated from four independent experiments. (e and f) Gene expression analysis of *CO* (e) and *FT* (f) in Col-0, *cdfq*, and *SUC2:HA-tpl-1*

plants over developmental time. Plants were harvested over time from 5-days to 19-days old at the ZT13 time point of long days. Means +/− SEM were calculated from four independent experiments. Arrowheads indicate the day at which either *cdfq* or *SUC2:HA-tpl-1* plants were first observed having a single plant begin flowering (the wild-type plants did not start flowering during the experiment).

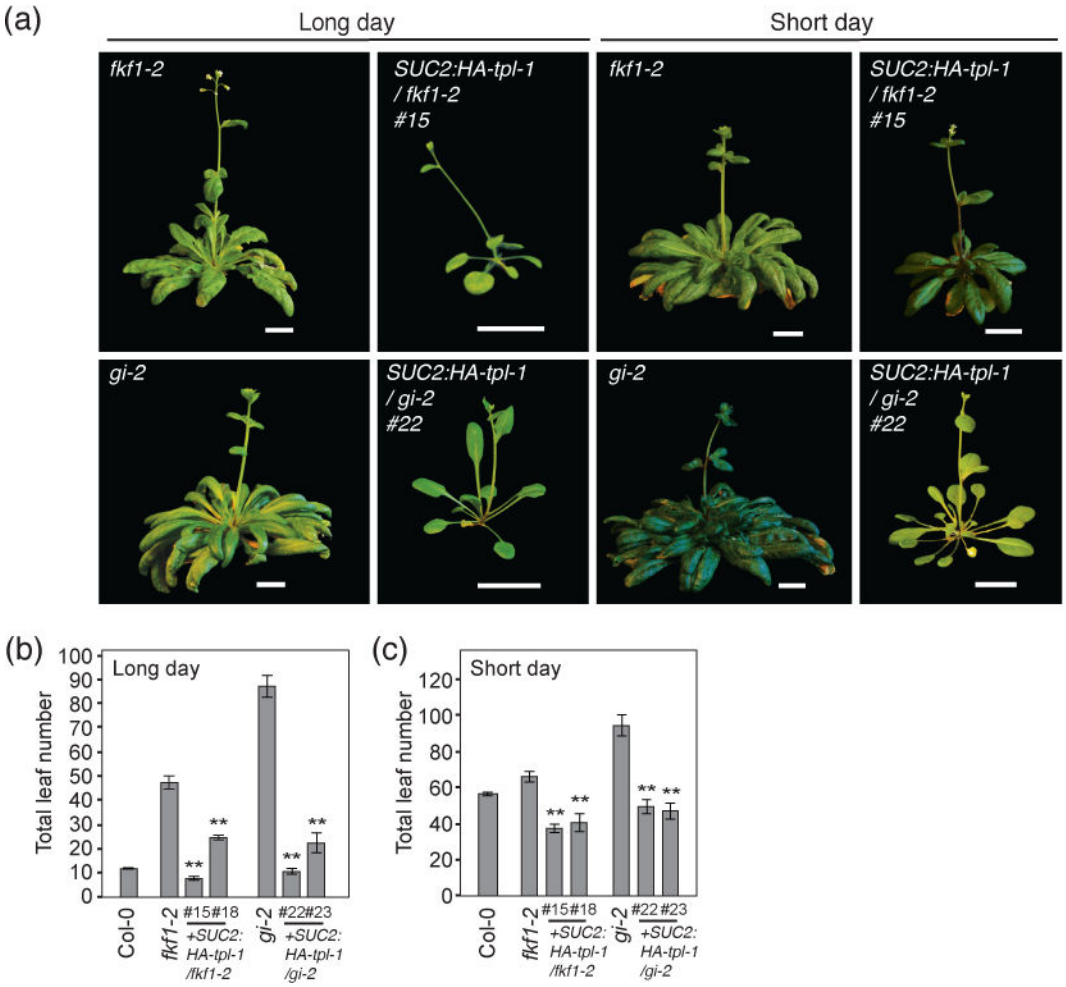

Figure 6. *SUC2:HA-tpl-1* plants in *fkf1* and *gi* backgrounds are early flowering in long days and short days

(a) Representative images of *SUC2:HA-tpl-1* plants (*SUC2:HA-tpl-1/fkf1-2*, and *SUC2:HA-tpl-1/gi-2*) and their respective genetic backgrounds (*fkf1-2*, and *gi-2*) under long-day and short-day photoperiods at flowering. Scale bars=2 cm. (b and c) Quantification of flowering time by total leaf number at bolting from (a) under long days (b) and short days (c). Means +/− SEM were calculated from N=16 individuals. **P < 0.01 (one-tailed t test).

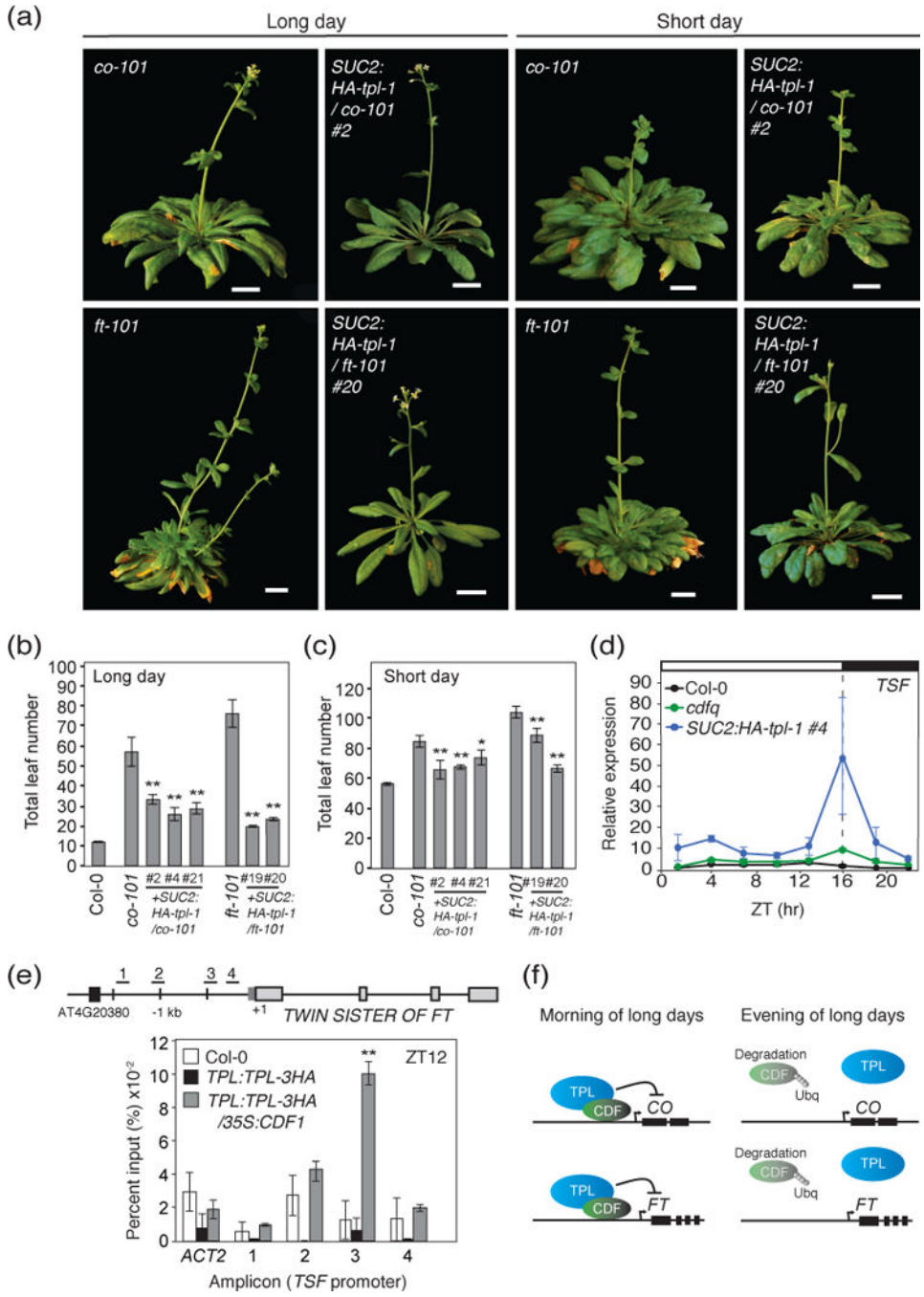

Figure 7. Introduction of *SUC2:HA-tpl-1* into *co* and *ft* mutants caused earlier flowering, and TPL directly regulates *TSF* expression in long days

(a) Representative images of *SUC2:HA-tpl-1/co-101* and *SUC2:HA-tpl-1/ft-101* plants and their parental genetic backgrounds (*co-101 and ft-101*) under long-day and short-day photoperiods at flowering. Scale bars=2 cm. (b and c) Quantification of flowering time by total leaf number at bolting from (a) under long days (b) and short days (c). Means +/− SEM were calculated from N=16 individuals. *P < 0.05,**P < 0.01 (one-tailed t test). (d) Diurnal gene expression analysis of *TSF* in Col-0, *cdfq*, and *SUC2:HA-tpl-1* plants in long days. Experiments were performed on 14-day-old seedlings grown in long days, with 3-hour

resolution. Means +/− SEM were calculated from four independent experiments. (e) ChIP experiment for TPL binding to the *TSF* genomic locus at ZT12, in Col-0, *TPL:TPL:3HA*, and *TPL:TPL:3HA/35S:CDF1*. Means and +/− SEM were calculated from four independent experiments. **P < 0.01 (one-tailed *t* test). Plants were 2 weeks old and grown under long-day photoperiods. (f) Model for CDF1-TPL dependent regulation of *CO* and *FT* in the morning and the evening.