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Arabidopsis DOF Transcription Factors Act Redundantly to Reduce *CONSTANS* Expression and Are Essential for a Photoperiodic Flowering Response

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SUMMARY

Flowering of *Arabidopsis* is induced by long summer days (LDs). The transcriptional regulator CONSTANS (CO) promotes flowering, and its transcription is increased under LDs. We systematically misexpressed transcription factors in companion cells and identified several DOF proteins that delay flowering by repressing *CO* transcription. Combining mutations in four of these, including CYCLING DOF FACTOR 2 (CDF2), caused photoperiod-insensitive early flowering by increasing *CO* mRNA levels. *CO* transcription is promoted to differing extents by GIGANTEA (GI) and the F-box protein FKF1. We show that GI stabilizes FKF1, thereby reducing CDF2 abundance and allowing transcription of *CO*. Despite the crucial function of *GI* in wild-type plants, introducing mutations in the four DOF-encoding genes into *gi* mutants restored the diurnal rhythm and light inducibility of *CO*. Thus, antagonism between GI and DOF transcription factors contributes to photoperiodic flowering by modulating an underlying diurnal rhythm in *CO* transcript levels.

INTRODUCTION

Plants occupy diverse environments, and most species that grow at higher latitudes synchronize their developmental program with seasonal changes in day length (or photoperiod). In *Arabidopsis*, flowering is induced during long days (LDs) characteristic of spring and early summer but is delayed during short winter days (SDs). Molecular genetic studies defined the photoperiodic flowering pathway, comprising at its core the *GIGANTEA* (*GI*), *CONSTANS* (*CO*), and *FLOWERING LOCUS T* (*FT*) genes (Kobayashi and Weigel, 2007; Turck et al., 2008), whose functions are highly conserved in distantly related species (Hayama et al., 2003). Mutations in any of these genes cause a strong delay in flowering under inductive LDs (Fowler et al., 1999; Kobayashi et al., 1999; Putterill et al., 1995), whereas their overexpression induces flowering independently of day length (Kardailsky et al., 1999; Mizoguchi et al., 2005; Onouchi et al., 2000).

GI encodes a protein predominantly present in the nucleus that acts early within the photoperiodic cascade to induce transcription of *CO* (Huq et al., 2000; Mizoguchi et al., 2005; Suarez-Lopez et al., 2001). Expression of *GI* mRNA is circadian clock regulated (Fowler et al., 1999; Park et al., 1999), and the GI protein accumulates with highest abundance 12 hr after dawn under LDs (David et al., 2006). Light regulates GI at the posttranscriptional level so that proteolysis occurs in the dark, but not in the light, possibly through direct interaction of GI with CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and EARLY FLOWERING 3 (ELF3) (David et al., 2006; Yu et al., 2008). In addition to being regulated by the circadian clock, *GI* in turn influences circadian rhythms in transcription of genes encoding clock components by regulating period and amplitude of their expression and indirectly modulating clock outputs (Fowler et al., 1999; Gould et al., 2006; Mizoguchi et al., 2005; Park et al., 1999). The dual effects of *gi* mutations on photoperiodic flowering and clock-regulated gene expression are separated by some mutant alleles (Gould et al., 2006; Martin-Tryon et al., 2007) and in plants overexpressing *GI* (Mizoguchi et al., 2005), suggesting that these effects are not dependent on one another.

CO acts downstream of the signaling cascade, which proceeds through the clock and GI (Mizoguchi et al., 2005; Suarez-Lopez et al., 2001). Under LDs, *CO* mRNA shows a biphasic pattern of expression in which transcript levels first rise after *GI* mRNA at the end of a LD, while plants are still exposed to light (Suarez-Lopez et al., 2001), and a second peak occurs during the night. Under SDs, only the peak during the night is observed (Corbesier et al., 2007; Imaizumi et al., 2003). The induction of *CO* mRNA in the light under LDs but not SDs is crucial for the promotion of flowering, because exposure to light is required for stabilization of CO protein (Jang et al., 2008; Valverde et al., 2004), activation of *FT* transcription in the leaves (An et al., 2004; Takada and Goto, 2003; Wigge et al., 2005; Yoo et al., 2005), and subsequent translocation of the FT protein to the shoot apical meristem (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007).

In addition to GI, the precise timing of *CO* expression requires the F-box protein FLAVIN BINDING, KELCH REPEAT, F-BOX PROTEIN 1 (FKF1) (Imaizumi et al., 2003; Nelson et al., 2000). However, in *gi* mutants *CO* mRNA is strongly reduced at all times under LDs and SDs (Suarez-Lopez et al., 2001), whereas in *fkf1* mutants, only the first peak of *CO* mRNA under LDs is abolished (Imaizumi et al., 2003). Both mutants show a late flowering phenotype under LDs. The molecular mechanism regulating *CO* transcription was recently proposed to rely on the formation of a protein complex including FKF1 and GI (Sawa et al., 2007). Interaction between FKF1 and GI occurred in vivo and preferentially at the end of a LD, showing reduced abundance in SDs (Sawa et al., 2007). Light is required to stabilize the interaction so that longer photoperiods cause enhanced accumulation of the GI-FKF1 complex and increased *CO* mRNA levels. Genetic evidence suggests that FKF1 activity depends on GI, because in a *gi-2* mutant, flowering is delayed even when *FKF1* is overexpressed from the *35S* promoter (Sawa et al., 2007). However, the molecular mechanism underlying this dependence is not known, and the roles of GI and FKF1 on *CO* expression may not be identical because mutations in these genes reduce *CO* mRNA levels to differing extents (Imaizumi et al., 2003; Suarez-Lopez et al., 2001).

FKF1 belongs to the F-box protein family and is predicted to interact directly with target proteins, leading to their ubiquitination and degradation via the proteasome. One such protein that interacts with FKF1 and regulates *CO* expression is the DOF transcription factor CYCLING DOF FACTOR 1 (CDF1) (Imaizumi et al., 2005). When overexpressed, CDF1 represses *CO* transcription, causing a strong delay of flowering under LDs. CDF1 protein accumulates at high levels at the beginning of the day, at a time when *CO* transcription is strongly reduced and the repression is likely to be direct, since CDF1 binds in vitro to a cluster of consensus DOF binding sites in the *CO* promoter. In vivo, CDF1 degradation depends on the activity of the GI-FKF1 protein complex so that in *fkf1* or *gi* mutants, CDF1 protein abundance is increased compared to wild-type plants at the end of the day. Increased levels of CDF1 are sufficient to repress *CO* and may partly explain the late-flowering phenotype of *fkf1* or *gi* mutants. A difficulty in ascribing a major role to CDF1 is that wild-type plants in which *CDF1* expression is reduced by RNA interference (*35S::CDF1-RNAi*) show only a slight acceleration of flowering and the diurnal pattern of *CO* expression is unaltered. Similarly, *fkf1* mutants carrying *35S::CDF1-RNAi* were hardly affected in flowering compared to *fkf1* mutants (Imaizumi et al., 2005). *CDF1* activity therefore cannot fully account for reduced *CO* expression in *fkf1*, suggesting that CDF1 might act redundantly with other unknown proteins.

We performed a systematic genetic screen to isolate genes affecting flowering from the leaves, and isolated additional members of the DOF family related to *CDF1*. We show that misexpression of these genes in phloem companion cells is sufficient to repress flowering under LDs and that release of this repression through combining loss-of-function alleles in four of these genes (*CDF1*, *2*, *3*, and *5*) causes photoperiod-insensitive early flowering. The abundance of endogenous CDF2 is regulated by GI and partially redundantly by FKF1, ZTL, and LKP2, explaining the different effects of *gi* and *fkf1* mutations on *CO* mRNA levels. Construction of a quintuple mutant carrying *gi* and mutations in the four *CDF* genes demonstrated that GI is required in wild-type plants to remove the CDFs, but not for the underlying rhythm in *CO* mRNA or for its response to photoperiod, which are restored in the quintuple mutant. Thus, we conclude that the layer of *CO* regulation represented by GI and the CDFs is not essential for light regulation of *CO* transcription under LDs, but rather that it modulates the amplitude and shape of an underlying rhythm.

RESULTS

Systematic Misexpression of *Arabidopsis* DOF Transcription Factors Identifies One Clade that Regulates Flowering Time

CO is expressed in phloem companion cells where it promotes *FT* expression only during LDs (An et al., 2004; Takada and Goto, 2003). In order to isolate additional proteins acting in the phloem to regulate flowering, a screen was performed in which a library of *Arabidopsis* transcription factors was systematically expressed in companion cells using the strong *SUCROSE TRANSPORTER 2* (*SUC2*) promoter (Imlau et al., 1999). The misexpressed library included 26 members of the DOF family (Yanagisawa, 2002), 5 of which caused a strong delay in flowering under LDs, but not SDs (Figure 1A; see Figure S1 available online). One of the remaining 21 (ADOF1; Figure S1) caused a much smaller delay in flowering, and the remaining 20 had no detectable effect on flowering. The 5 that strongly delayed flowering belong to the same phylogenetic clade, previously referred to as group II (Yanagisawa, 2002) or subfamily A (Moreno-Risueno et al., 2007) (Figure S1). In group II, CYCLING DOF FACTOR 2 (CDF2) and CYCLING DOF FACTOR 3 (CDF3) are the closest homologs of CDF1, and were previously shown to interact with FKF1 and LKP2 in yeast, but not to delay flowering when expressed from the *CaMV 35S* promoter (Imaizumi et al., 2005). COG1 is a negative regulator of phyA and phyB signaling that caused several altered photoresponses when overexpressed, but did not affect flowering time (Park et al., 2003). The systematic screen also identified two uncharacterized DOF genes, *At2g34140* (here named *CYCLING DOF FACTOR 4* or *CDF4)* and *At1g69570* (here named *CYCLING DOF FACTOR 5* or *CDF5*), that were not previously implicated in flowering-time control or photomorphogenesis.

The severe delay in flowering that we observed upon misexpression in the phloem was specific to LDs (Figure 1A) and suggests that these plants are impaired in the photoperiodic flowering pathway.

Spatial and Temporal Patterns of Expression of *CDF* Genes

To test whether group II *DOF* genes are expressed in the vascular tissue, transgenic plants that carried fusions of the promoter regions of *CDF2*, *CDF3*, and *CDF4* to the reporter gene *GUS* were analyzed. Seedlings grown under LDs showed expression of the GUS reporter in the vasculature of cotyledons and hypocotyls (Figures 1B–1D), true leaves, and roots (Figure S2). The same pattern was observed under short photoperiods in both cotyledons and adult leaves (Figure S2). We tested the diurnal expression profiles of the group II *DOF* genes under LDs and SDs using qRT-PCR (Figures 1E–1I). Three members, including

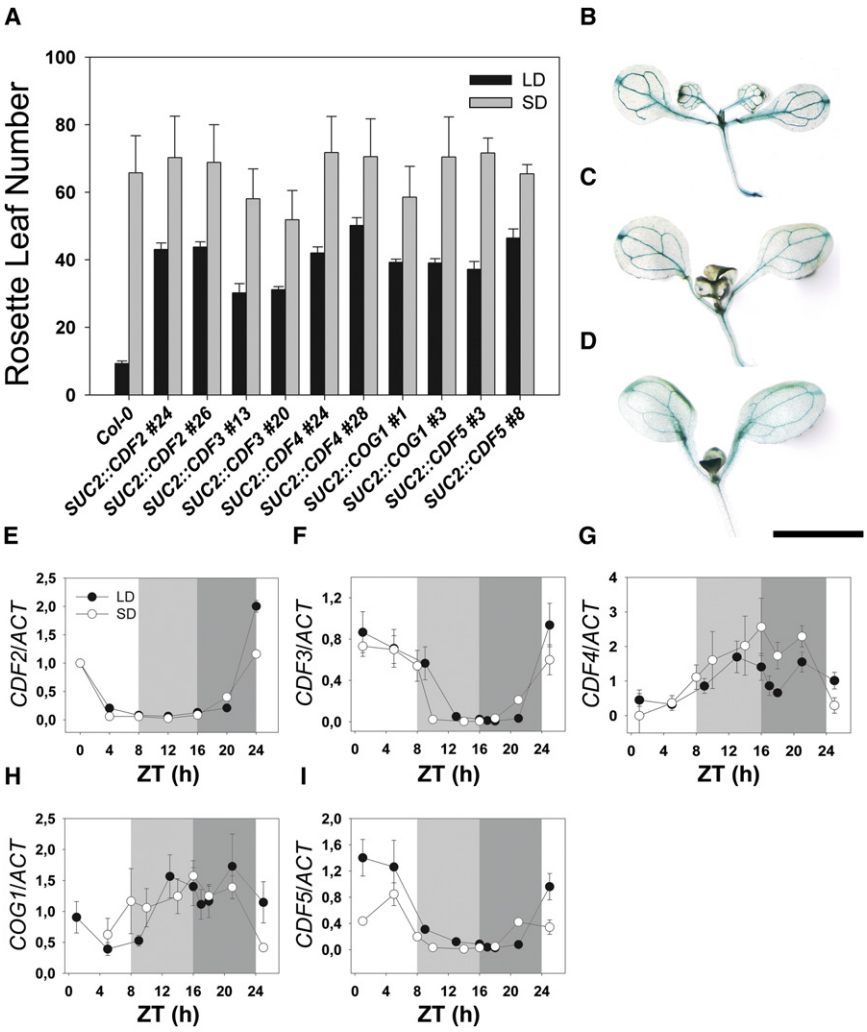

Figure 1. Misexpression of Group II DOF Transcription Factors Delays Flowering

Flowering time of plants overexpressing *CDF2*, *CDF3*, *CDF4*, *COG1*, and *CDF5* in phloem companion cells (A). The number of rosette leaves formed under long days (black bars) and short days (gray bars) is shown. Data for two independent transgenic lines for each construct are shown. Bars are mean ± standard deviation of at least 12 plants. Spatial expression of *CDF2* (B), *CDF3* (C), and *CDF4* (D) in 10-day-old seedlings grown in LDs. All plants express the *GUS* reporter in the vascular tissue of the cotyledons and leaves. Bar, 5mm. *CDF2* (E), *CDF3* (F), *CDF4* (G), *COG1* (H), and *CDF5* (I) mRNA levels were measured by qRT-PCR in 10-day-old seedlings, harvested throughout a long day (LDs; filled circles) or short day (SDs; open circles). Data are mean ± standard deviation of three independent amplifications (Experimental Procedures). All values are normalized to actin levels. Time (h) is expressed as hours from dawn (ZT, *zeitgeber*). The dark gray shadowing indicates the night period under LDs and SDs; the light gray represents night under SDs only.

CDF2, *CDF3,* and *CDF5*, showed a similar profile, with high mRNA levels at the beginning of the light period. Expression levels subsequently decreased, reaching a minimum between 16 and 20 hr after dawn before rising again at the end of the day (Figures 1E, 1F, and 1I). The patterns were similar under both LDs and SDs. Expression of *CDF4* and *COG1* showed different diurnal patterns with their transcript levels rising progressively from dawn and decreasing during the night (Figures 1G and 1H). Therefore, the mRNAs of several *CDF* genes show diurnal patterns in accumulation in the leaves, and their expression at elevated levels is sufficient to repress flowering.

Combinations of *cdf* Mutant Alleles Identify Layers of Redundancy in the Function of *CDF* Genes

Activation of *CO* transcription involves the cyclic degradation of CDF1, a repressor of *CO* (Imaizumi et al., 2005). However, reduction of *CDF1* expression by RNA interference caused only a slight acceleration of flowering under LDs and did not cause detectable increases in *CO* mRNA levels. We tested for genetic redundancy between *CDF1*, *CDF2*, *CDF3*, and *CDF5* because of the predicted similarities in their protein products and in their expression profiles as well as the capacity of all of these genes to strongly repress flowering when overexpressed in companion cells (Figure 1A). Mutant alleles were isolated (Experimental Procedures and Figure S3) and plants carrying combinations of mutations were constructed.

The *cdf2-1* and *cdf5-1* mutants showed early flowering when compared to wild-type plants, with a decrease in the rosette leaf number under both LDs and SDs (Figure 2A). In contrast, the single *cdf3-1* mutant grown in either LDs or SDs showed no obvious alteration in flowering time. The *cdf2-1 cdf5-1* double mutant showed an additive effect when compared to *cdf2-1* and *cdf5-1* under LDs or SDs. Crossing the *35S::CDF1-RNAi* transgene into the *cdf2-1 cdf5-1* double mutant produced a triple mutant flowering as early as the *cdf2-1 cdf5-1* double mutant under LDs and showing a further slight acceleration of flowering in short photoperiods (Figure 2A). Finally, in a quadruple mutant *cdf1-R cdf2-1 cdf3-1 cdf5-1*, flowering was strongly accelerated both in LDs and SDs, so that plants flowered at similar times under both photoperiods (Figure 2A). We conclude that *CDF1*, *CDF2*, *CDF3*, and *CDF5* redundantly repress the floral transition in wild-type plants and do so to a greater extent under SD conditions, thereby conferring a photoperiodic response.

CDF Proteins Act Redundantly to Reduce *CO* mRNA Abundance

Acceleration of flowering by lengthening photoperiods requires activation of *CO* transcription in the leaves at the end of the light period (Kobayashi and Weigel, 2007; Turck et al., 2008). To assess whether all of the CDFs can repress *CO* transcription, the abundance of *CO* mRNA was tested in plants overexpressing the CDFs from the *SUC2* promoter. *CO* transcript levels were

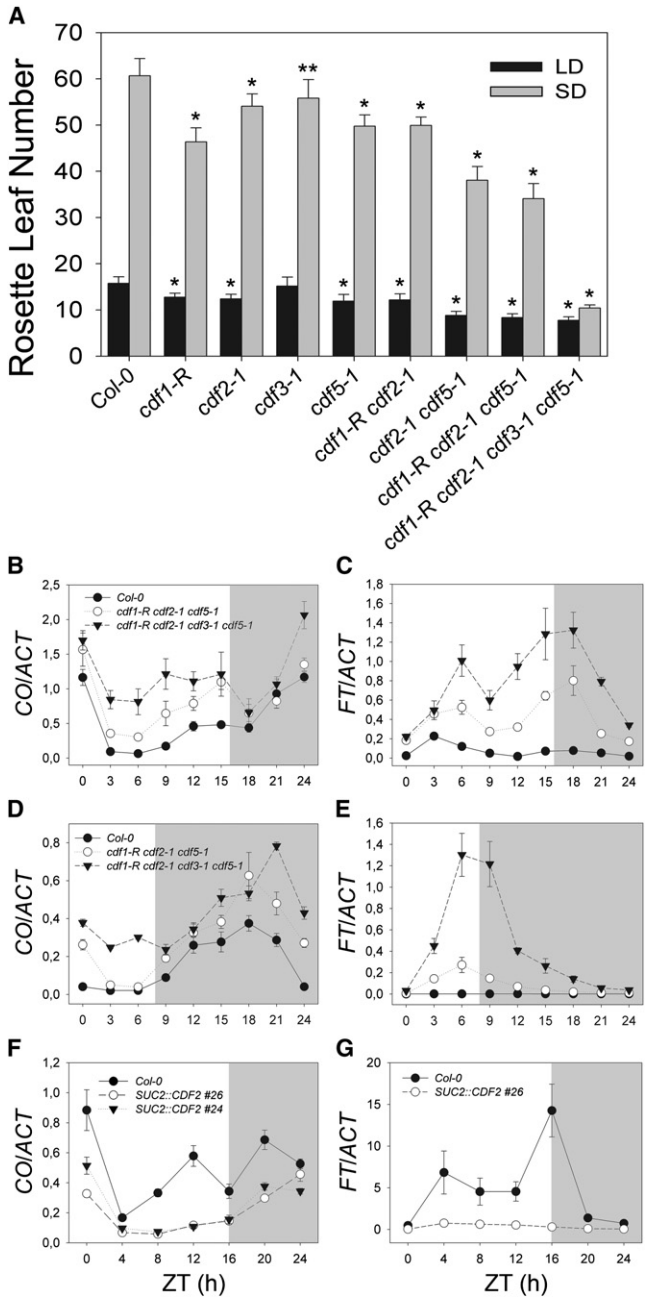

Figure 2. Characterization of *cdf* Mutants

Flowering time expressed as rosette leaf number at bolting of *35S::CDF1-RNAi* (*cdf1-R*), *cdf2-1*, *cdf3-1*, *cdf5-1* mutants and plants carrying combinations of the four mutant alleles under LDs (black bars) and SDs (gray bars) (A). Col-0 was used as a control. Data are mean ± standard deviation of at least 12 homozygous plants. P values for Student's t test were calculated for each line compared to wild-type Col-0: *p < 0,001, **p < 0,05. *CO* (B and D), and *FT* (C and E) mRNA levels were measured by qRT-PCR on 10-day-old Col-0, *cdf1-R cdf2-1 cdf5-1* and *cdf1-R cdf2-1 cdf3-1 cdf5-1* seedlings grown in LDs (B and C) and SDs (D and E). In *SUC2::CDF2* seedlings, *CO* (F) and *FT* (G) mRNA levels are reduced. Additional independent transgenic lines gave identical results (data not shown). Data are mean ± standard deviation of three independent amplifications (Experimental Procedures). White areas in the graphs indicate light periods; gray areas indicate dark periods. Time (h) is expressed as hours from dawn (ZT, *zeitgeber*).

decreased in these plants compared to Col-0, and rhythmic cycling of the mRNA was dampened. The data for *SUC2::CDF2* are shown in Figure 2F. Furthermore, *FT* transcription was abolished in *SUC2::CDF2* plants (Figure 2G). The late flowering of *SUC2::CDF2* plants was suppressed by introducing a *SUC2::CO* or *SUC2::FT* transgene (Figure S4), which increased the level of *CO* (Figure S4) or *FT* mRNA (data not shown), overcoming CDF2-mediated repression. These data suggest that CDF overexpressing plants are late flowering because *CO* mRNA levels, and consequently *FT* expression, are reduced.

To assess whether CDF1, CDF2, CDF3, and CDF5 regulate *CO* expression in wild-type plants, *CO* and *FT* mRNA levels were measured in seedlings carrying combinations of *cdf* mutations (Figures 2B–2E). *CO* transcript levels were increased at all time points compared to Col-0 in *cdf1-R cdf2-1 cdf5-1* and *cdf1-R cdf2-1 cdf3-1 cdf5-1*, both under LDs and SDs (Figures 2B and 2D). Nevertheless, *CO* mRNA levels still showed a diurnal rhythm even in the quadruple mutant. These data indicate that *CO* transcription is strongly reduced by the CDF transcription factors in wild-type plants, but that even when their activity is dramatically impaired in the quadruple mutant, *CO* mRNA levels still show a robust diurnal rhythm.

FT transcript levels respond rapidly to changing CO levels, particularly when plants are exposed to light and the CO protein is stabilized (Jang et al., 2008; Valverde et al., 2004). Under LDs, *FT* mRNA levels were increased compared to Col-0 in *cdf1-R cdf2-1 cdf5-1* and *cdf1-R cdf2-1 cdf3-1 cdf5-1* mutants (Figure 2C). Induction of *FT* mRNA levels occurred in *cdf1-R cdf2-1 cdf5-1* and *cdf1-R cdf2-1 cdf3-1 cdf5-1* plants earlier than in wild-type, presumably as a consequence of increased *CO* mRNA levels during the day leading to CO protein accumulation.

Finally, inactivation of the *CO* gene completely suppressed the early flowering of the *cdf1-R cdf2-1 cdf5-1* triple mutant, so that *co-10 cdf1-R cdf2-1 cdf5-1* plants flowered at the same time as *co-10* mutants (Figure S4). Taken together, these data indicate that *CDF1*, *CDF2, CDF3*, and *CDF5* act redundantly upstream of *CO* and that their effect on flowering time is dependent upon *CO*. In addition, these transcription factors modulate *CO* gene expression, but even in the quadruple mutant, a diurnal rhythm in *CO* mRNA levels occurs in LDs and SDs.

Posttranscriptional Regulation of CDF2 Protein Abundance by FKF1 and GI

Early flowering of *Arabidopsis* requires the activity of a protein complex containing GI and FKF1. This complex targets CDF1 for degradation, and since CDF2 also interacts with FKF1 in yeast and in vitro (Imaizumi et al., 2005), we tested whether it could also be a substrate for GI-FKF1-mediated degradation. An antiserum against CDF2 was raised that detects this protein in nuclear extracts (Figure S5 and Experimental Procedures). In wild-type plants grown in LDs, CDF2 accumulation shows a diurnal cycle with a peak of expression in the middle of the day (Figure 3A and Figure S5). In both *fkf1-2* and *gi-100* mutants, CDF2 accumulates to higher levels (Figures 3B and 3C and Figure S5). In contrast, plants in which *GI* is overexpressed never accumulate CDF2 to the levels found in wild-type plants, although small amounts of CDF2 protein are still detectable (Figure 3D and Figure S5).

Whether differences in CDF2 protein between genotypes could be explained by alterations in *CDF2* mRNA levels was then tested.

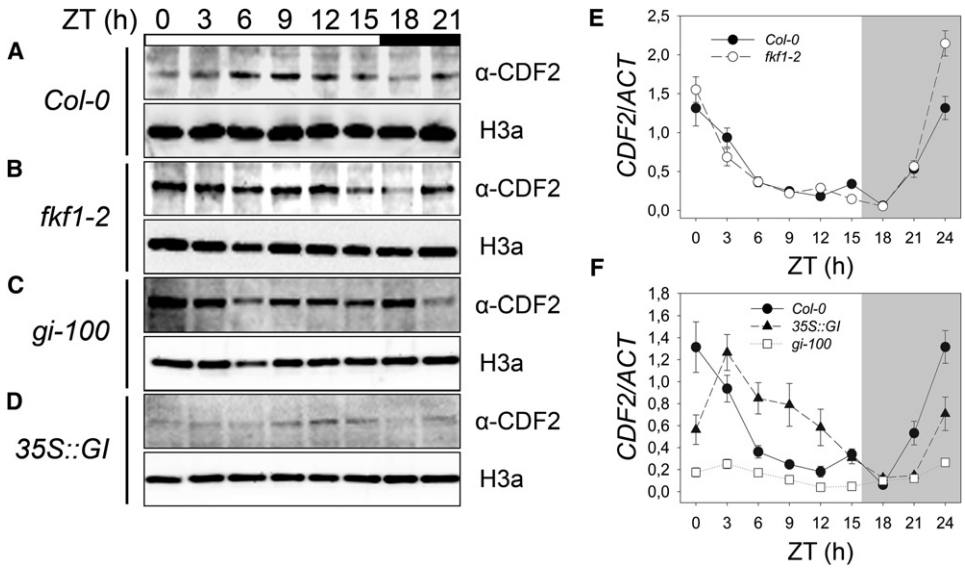

Figure 3. Diurnal Accumulation of CDF2 Protein Depends on GI and FKF1

(A–D) Western blots comparing the accumulation of CDF2 protein throughout a 24 hr time course in 10-day-old Col-0 (A), *fkf1-2* (B), *gi-100* (C), and *35S::GI* (D) plants. Proteins were extracted from a nuclei-enriched preparation and probed with an antibody against CDF2 (upper panels). H3a antibody was used as loading control (lower panels).

(E–F) The mRNA profile of *CDF2* was unchanged in *fkf1-2* (E), but altered in *gi-100* and *35S::GI* plants (F). Time (h) is expressed as hours from dawn (ZT, *zeitgeber*). White areas and bar indicate light periods; black areas and bar indicate dark periods. Data are mean ± standard deviation of three independent amplifications (Experimental Procedures).

The diurnal pattern of *CDF2* mRNA abundance is the same in *fkf1-2* and Col-0 (Figure 3E), indicating that CDF2 abundance is regulated by FKF1 posttranscriptionally. In *gi-100*, the amplitude and overall level of *CDF2* mRNA is reduced, in contrast to the higher protein abundance observed (Figure 3F). In *35S::GI*, the amplitude of *CDF2* mRNA is almost unchanged but the phase is shifted slightly later, whereas the abundance of the protein is greatly reduced. These data indicate that the major effect of *FKF1* and *GI* on CDF2 protein accumulation is at the post-transcriptional level.

GI Controls the Accumulation of FKF1 Protein and Can Promote Flowering Independently of FKF1

Plants constitutively expressing *GI* show strongly reduced CDF2 levels throughout the day (Figure 3D). To determine whether this effect was due to increased FKF1 abundance, the level of FKF1-TAP protein expressed from the *FKF1* promoter was tested during a 24 hr LD cycle in *gi-100*, *35S::GI*, and *fkf1* backgrounds (Figure 4 and Figure S6). FKF1-TAP protein shows dramatically reduced accumulation in *gi-100* (Figure 4B and Figure S6) compared to

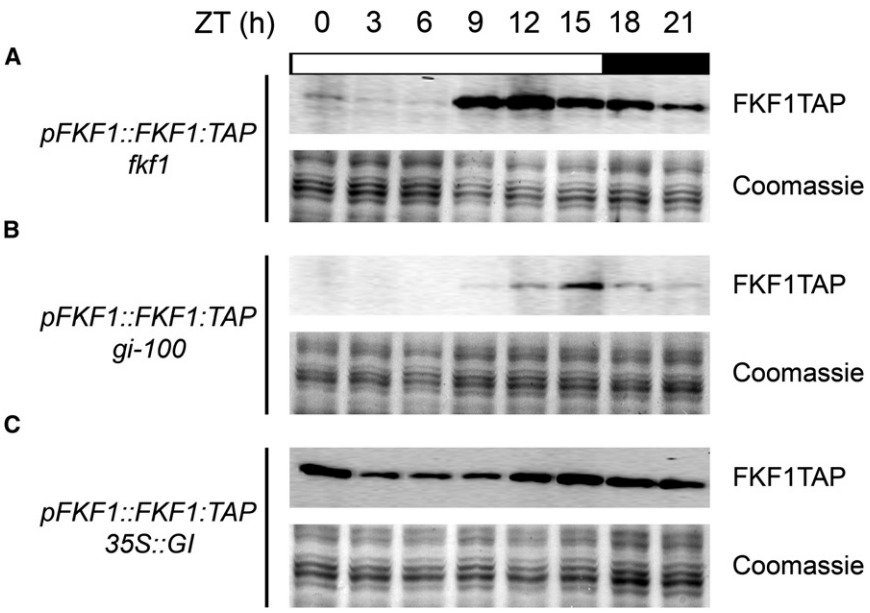

Figure 4. GI Regulates FKF1 Protein Abundance

Western blots comparing the levels of FKF1-TAP protein in 10-day-old *fkf1* (A), *gi-100* (B), and *35S::GI* plants (C) containing a *pFKF1::FKF1:TAP* transgene. A picture of the Coomassie-stained gel is shown as loading control (lower panels). White and black bars represent light and dark periods, respectively. Time (h) is expressed as hours from dawn (ZT, *zeitgeber*).

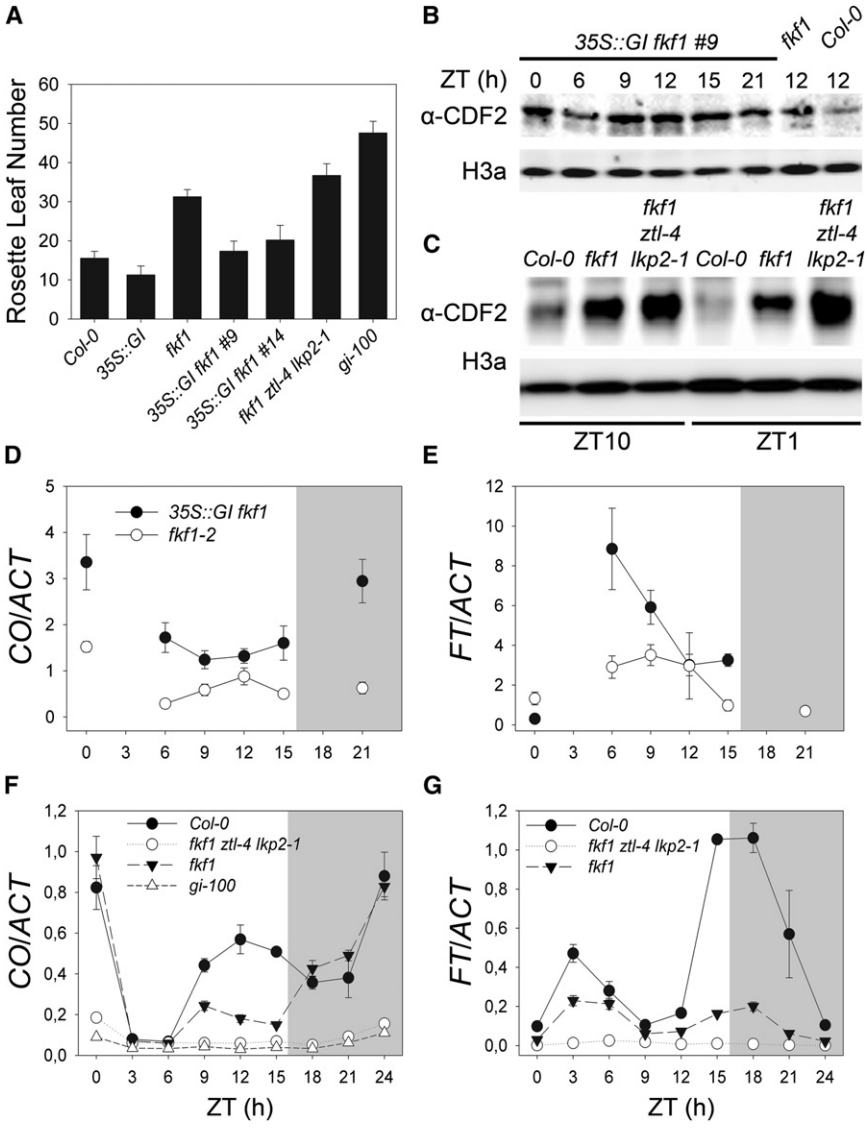

Figure 5. CDF2 Protein Levels Are Controlled by FKF1, ZTL, and LKP2

(A) Flowering time expressed as rosette leaf number of the indicated genotypes grown in LDs. Bars are mean ± standard deviation of at least 12 plants.

(B) *35S::GI fkf1 #9* plants were grown for 10 days in LDs and harvested at the indicated time points. Protein blots were probed with α-CDF2 (upper panel) or H3a (lower panel) as loading control. The *fkf1* and Col-0 samples harvested at ZT12 are shown for comparison.

(C) CDF2 protein accumulation at ZT10 and ZT1 in 10-day-old *fkf1 ztl-4 lkp2-1* triple mutant compared to *fkf1* and Col-0.

(D and E) Levels of *CO* (D) and *FT* (E) transcripts were determined by qRT-PCR on 10-day-old seedlings of *35S::GI fkf1 #9* and *fkf1-2* plants harvested at the indicated time of day.

(F and G) *CO* (F) and *FT* (G) mRNA expression levels were determined in 10-day-old *fkf1 ztl-4 lkp2-1* seedlings and compared to Col-0, *fkf1*, and *gi-100* single mutants. Time (h) is expressed as hours from dawn (ZT, *zeitgeber*). White areas in the graphs indicate light periods; gray areas indicate dark periods. Data are mean ± standard deviation of three independent amplifications (Experimental Procedures).

fkf1 (Figure 4A). In contrast, plants overexpressing *GI* accumulate FKF1-TAP protein throughout the 24 hr cycle, and increased abundance is evident during the light phase (Figure 4C and Figure S6). FKF1-TAP protein accumulation is not a consequence of altered mRNA levels (Figure S6), indicating that GI is required to stabilize FKF1 and for the correct timing of its accumulation. The increased abundance of FKF1-TAP in the light in *35S::GI* likely causes earlier formation of the GI-FKF1 complex, consistent with the strongly reduced accumulation of CDF2 protein in these plants (Figure 3D).

To test whether GI activity depends on FKF1, a *35S::GI* transgene was introduced into *fkf1* and CDF2 protein levels were determined under LDs (Figure 5B and Figure S7). CDF2 protein levels were increased at all time points analyzed, compared to Col-0 or *35S::GI* controls, and were similar to those of *fkf1* mutants. Therefore, when GI is overexpressed, most of GI-mediated degradation of CDF2 depends on FKF1.

In addition to regulating *CO*, GI was also shown to promote flowering by increasing *FT* mRNA levels independently of *CO* (Jung et al., 2007). To test whether acceleration of flowering in *35S::GI fkf1* plants is caused by increased *CO* transcription or activation of *FT* independently of CO, the level of *CO* mRNA was tested in these plants. *CO* mRNA levels were higher in *35S::GI fkf1* transgenic plants compared to *fkf1* (Figure 5D and Figure S7), and *FT* mRNA was also higher early in the day (Figure 5E and Figure S7).

These data indicate that despite the presence of higher levels of CDF1 and CDF2 floral repressors in plants that lack FKF1, *CO* transcription and flowering can be promoted by GI overexpression. Consistent with these data, *35S::GI fkf1* plants flower earlier than *fkf1* in LDs, albeit later than *GI* overexpressors (Figure 5A).

Redundant Role of FKF1, ZTL, and LKP2 in the Regulation of CDF2 Protein Accumulation and *CO* Transcription

Early flowering and increased *CO* mRNA expression in *35S::GI fkf1* plants must occur through a mechanism that does not rely on FKF1-mediated degradation of CDF1 and CDF2. We investigated the possibility that LKP2 or ZTL, which are close homologs of FKF1 (Jarillo et al., 2001; Somers et al., 2000), could act redundantly with FKF1 in the regulation of CDF2 and *CO* transcription. Indeed, in *fkf1 ztl-4 lkp2-1* triple mutants, CDF2 protein abundance was increased compared to *fkf1* at several times of day, suggesting that in *fkf1* mutants, CDF2 degradation can proceed via LKP2 and/or ZTL (Figure 5C and Figure S7).

To determine whether the limited effect of *fkf1* mutations on *CO* mRNA level was due to a redundant function of ZTL and

LKP2, *CO* transcript levels were measured through a 24 hr time course under LDs in *fkf1 ztl-4 lkp2-1* triple mutants (Figure 5F). *CO* and *FT* mRNA abundance were strongly suppressed in these plants, to levels similar to those observed in a *gi* mutant. Therefore, the residual *CO* mRNA rhythm observed in *fkf1* depends on *ZTL* and/or *LKP2* (Figures 5F and 5G). Consistent with these data, flowering of *fkf1 ztl-4 lkp2-1* is delayed compared to *fkf1* (Figure 5A). These data indicate that LKP2 and/or ZTL are redundant with FKF1 in the control of *CO* mRNA expression by affecting the stability of CDF2 and probably other CDFs.

GI Is Not Required to Produce the Light-Mediated Remodeling of *CO* mRNA Rhythm

The diurnal waveform of *CO* transcription is at least partly formed by the antagonistic activities of GI and CDF proteins. We constructed a quintuple mutant between *gi-100* and *cdf1-R cdf2-1 cdf3-1 cdf5-1* to assess whether GI activity fully depends on degradation of CDFs and to investigate *CO* transcription in plants in which both positive and negative regulators are strongly impaired. In *gi-100 cdf1-R cdf2-1 cdf3-1 cdf5-1* quintuple mutants, the biphasic rhythm in *CO* mRNA under LDs was similar to that observed in Col-0 plants (Figure 6B) and *FT* transcript was present at wild-type levels (Figure 6C). Similarly, under LDs, the flowering time of *gi-100 cdf1-R cdf2-1 cdf3-1 cdf5-1* resembles that of Col-0 plants (Figure 6A). These results indicate that *cdf1-R cdf2-1 cdf3-1 cdf5-1* are largely epistatic to *gi* in the regulation of *CO* expression and the control of flowering time. Also, introduction of *cdf1-R cdf2-1 cdf3-1 cdf5-1* mutations into the *gi-100* mutant restores the monophasic peak in *CO* mRNA under SDs at levels similar to those of *cdf1-R cdf2-1 cdf3-1 cdf5-1* quadruple mutants (Figure 6D). These results indicate that GI is not essential to activate *CO* transcription, but is required to remove the CDF repressors so that transcription of *CO* can proceed through a further layer of transcriptional regulation.

Activation of *CO* transcription at dusk was shown to be light inducible through the formation of the GI-FKF1 complex and CDF1 degradation (Sawa et al., 2007). Therefore, we tested whether the activation of *CO* transcription by light was abolished in *gi-100 cdf1-R cdf2-1 cdf3-1 cdf5-1* quintuple mutants, in which the GI-FKF1-CDF layer of regulation is strongly impaired. A population of SD-grown plants was shifted to LDs at ZT8 and harvested at the same time as the samples remaining in SDs. After the shift to LDs, *CO* mRNA transcription was remodeled within 4 hr from a monophasic to a biphasic waveform (Figure 6E), indicating that light-induction at dusk still occurs in *gi-100 cdf1-R cdf2-1 cdf3-1 cdf5-1*. Furthermore, *FT* expression is slightly induced early in the day in *gi-100 cdf1-R cdf2-1 cdf3-1 cdf5-1* grown in SDs (Figure 6F) and is strongly activated at dusk upon LD exposure (Figure 6G). Consistent with the gene expression data, a photoperiodic flowering response is restored in the quintuple mutant so that it flowers earlier under LDs than SDs (Figure 6A). Therefore, analysis of the quintuple mutant demonstrated that GI is not essential for photoperiodic regulation of *CO* transcription or a photoperiodic flowering response. Furthermore, as GI was previously shown to be essential for CDF1 mediated degradation by FKF1 (Sawa et al., 2007), our data indicate that the GI-F box layer of *CO* regulation can be removed without preventing photoperiodic activation of *CO* transcription.

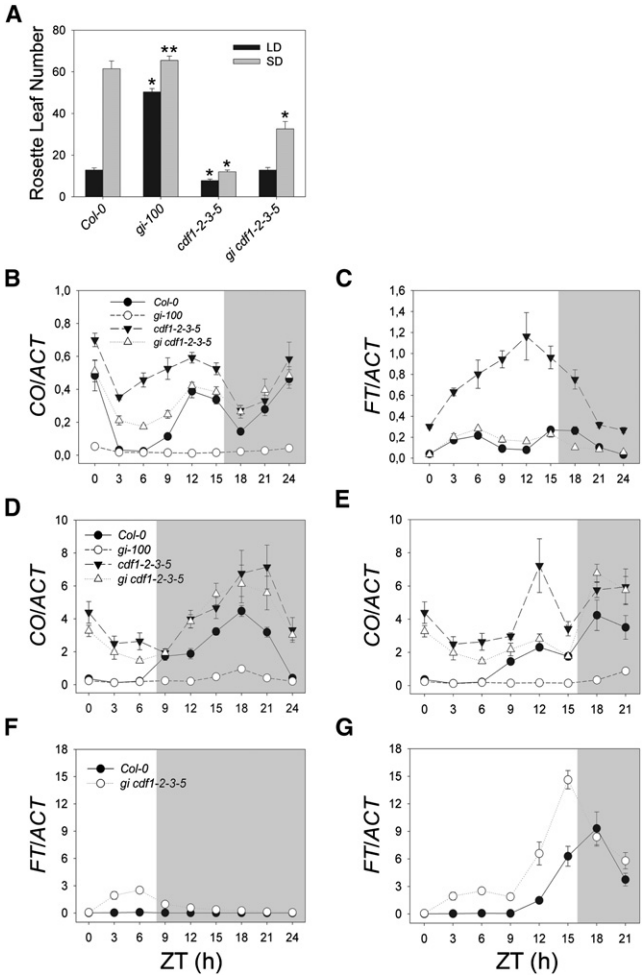

Figure 6. GI and CDFs Act Antagonistically to Modulate *CO* mRNA Amplitude

Flowering time expressed as rosette leaf number at bolting of Col-0, *gi-100*, *cdf1-R cdf2-1 cdf3-1 cdf5-1* (*cdf1-2-3-5*) and *gi-100 cdf1-R cdf2-1 cdf3-1 cdf5-1* (*gi cdf1-2-3-5*) (A). Bars are mean ± standard deviation of at least 12 plants. P values for Student's t test were calculated for each line compared to wild-type Col-0: *p < 0,001, **p < 0,05. *CO* (B, D, and E) and *FT* (C, F, and G) mRNA levels were determined by qRT-PCR on the indicated genotypes. Seedlings were grown in LDs (B and C) or SDs (D and F) and harvested at day 11. On the same day at ZT8, a group of seedlings from each genotype was shifted from SDs to LDs and the mRNA pattern of *CO* (E) and *FT* (G) was determined. Expression data are mean ± standard deviation of three independent amplifications (Experimental Procedures). Time (h) is expressed as hours from dawn (ZT, *zeitgeber*).

DISCUSSION

The regulation of *CO* transcription by photoperiod is one of the major determinants of a flowering response to day length in *Arabidopsis* (Kobayashi and Weigel, 2007; Turck et al., 2008). We have shown that there is extreme genetic redundancy in a class of DOF transcription factors that are negative regulators of *CO* transcription as well as in a family of F-box proteins that promote degradation of the DOFs. By constructing plants carrying complex combinations of up to five mutations, we clarified the roles of these proteins and of GI in *CO* transcriptional regulation.

The CDF DOF transcription factors are essential for a photoperiodic flowering response, while the F-box proteins have a more fundamental role in *CO* regulation than previously believed. This role is similar to that of GI, which probably acts on *CO* transcription only indirectly by stabilizing the F-box proteins. Although the interaction of GI and the F-box proteins contributes to light inducibility of *CO*, it is not essential for light induction, which still occurs in a quintuple mutant in which GI and CDF activity is strongly impaired. Our data, therefore, distinguish distinct layers of transcriptional regulation of *CO* by photoperiod that were not previously recognized.

CDF Transcription Factors Act Redundantly to Repress *CO* Transcription and to Modulate the Diurnal Rhythm in Its Expression

Exposure to longer photoperiods triggers flowering of *Arabidopsis* by inducing *CO* transcription at the end of the day and stabilizing CO protein (Jang et al., 2008; Liu et al., 2008; Yanovsky and Kay, 2002). Transcriptional induction ensures that under LDs of 16 hr of light, *CO* mRNA rises approximately 8 hr after dawn, reaching a peak at the end of the day and enabling CO to activate *FT* transcription (Imaizumi et al., 2003; Suarez-Lopez et al., 2001). We isolated several genes encoding CDF transcription factors that delayed flowering upon misexpression in the phloem. Impairment of four of these genes in a *cdf1-R cdf2-1 cdf3-1 cdf5-1* quadruple mutant caused early flowering that is independent of photoperiod, demonstrating that in wild-type plants, these genes are essential for a photoperiodic flowering response. The extreme phenotype of these plants compared to the single, double, and triple mutant combinations that were tested indicates that there is multilayered redundancy between these proteins in the regulation of flowering time (Figure 7). Nevertheless, the quadruple mutant may still retain some CDF activity. The *cdf2-1* mutant expresses a low level of *CDF2* mRNA and CDF2 protein, while the *cdf1-R* allele does not abolish *CDF1* mRNA. Also, there are other members of the clade for which no mutations are yet available and some of these, such as *CDF4*, are expressed in the vascular tissue. CDF4 is closely related to COG1, which influences hypocotyl elongation in response to phytochrome signaling (Park et al., 2003), and if CDF4 has a similar function, then these proteins would appear to influence hypocotyl elongation from the vascular tissue. In any case, further reduction of CDF activity in the quadruple mutant background is unlikely to cause much earlier flowering, because these plants are earlier flowering than Col-0 grown under LDs and almost as early flowering as plants strongly overexpressing *CO* from the *35S* or *SUC2* promoters.

In the quadruple mutant, *CO* mRNA levels were elevated throughout the day and during the night immediately before dawn. Therefore, in wild-type plants, the CDF transcription factors act under LDs and SDs to dampen *CO* transcription throughout the diurnal cycle. Previously, CDF1 was shown to be degraded late in the day by FKF1 and its mRNA peaks in abundance at dawn; therefore, although *cdf1-R* lines showed wild-type patterns of *CO* mRNA expression, CDF1 was proposed to repress *CO* transcription early in the day (Imaizumi et al., 2005). Our data indicate that the CDFs have a broader role suppressing *CO* throughout the day, so that, although the abundance of CDF1 and CDF2 is reduced by GI-FKF1 late in the day, there must still be sufficient CDF protein present at that time to reduce *CO* expression. Some of CDF1 or CDF2 might escape ubiquitination by GI-FKF1 or other members of the family may not be substrates for the ubiquitin ligase. Nevertheless, the high level of *CO* mRNA that occurs early in the day in the quadruple mutant during LDs and SDs is probably most important in causing the photoperiodic insensitive early flowering of these plants. Expression of *CO* mRNA early in the day would allow the protein to accumulate in the light under SDs when it is usually absent under these conditions (Jang et al., 2008; Valverde et al., 2004), and it would accumulate for longer than in wild-type plants under LDs. Therefore the CDFs play an essential role in conferring a photoperiodic flowering response on *Arabidopsis*, so that when their activity is strongly impaired, *CO* mRNA is present at higher levels at most times of the day, causing much earlier flowering.

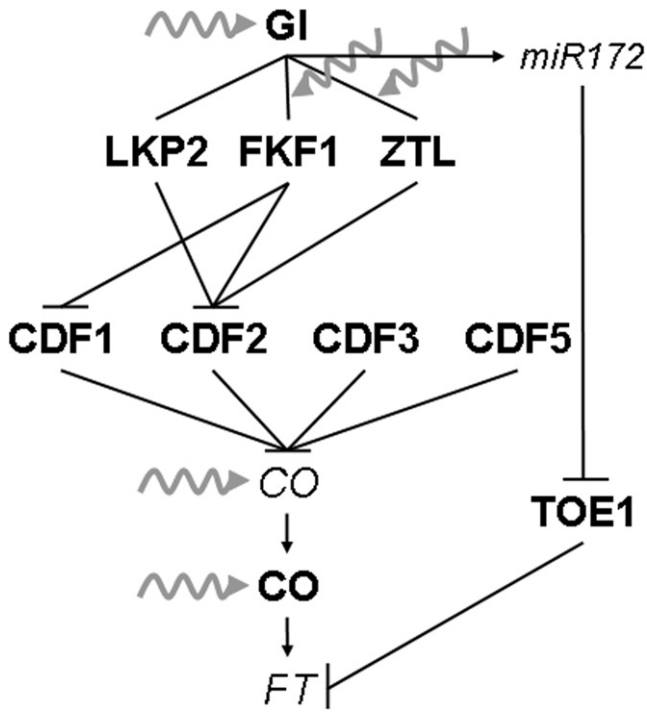

Figure 7. Model for the regulatory signaling cascade that proceeds from GI to *CO*

The diurnal phase of *GI* mRNA expression is regulated by the circadian clock and GI protein is stabilized by light so that under LDs it accumulates to higher levels than under SDs. At the end of the day under LDs, light promotes GI interaction with FKF1 and ZTL, increasing their stability. Whether the interaction of GI with LKP2 is light-dependent has not been determined. FKF1 targets CDF1 and CDF2 for ubiquitination and proteasome-mediated degradation. ZTL and/or LKP2 promote CDF2 degradation independently of FKF1. CDF1, CDF2, CDF3 and CDF5 coordinately repress *CO* transcription throughout the day. *CO* transcription is activated by light independently of the GI/F-box/CDF system. Downstream of this regulatory network, the CO protein induces *FT* transcription and flowering under LDs but not under SDs, because light promotes CO stability. GI also controls *FT* mRNA level through a direct pathway that does not depend on *CO*, whereby GI regulates the abundance of *miR172*, a microRNA targeting the mRNA of the *FT* repressor TOE1. Proteins are indicated in bold and genes in italics. Arrows represent transcriptional activation. Perpendicular lines indicate transcriptional repression. Lines represent protein-protein interactions. Wavy arrows indicate light input to the network.

Redundancy between FKF1 and Related Proteins in Regulating *CO* Transcription

FKF1 was shown to be required for the increase in *CO* transcript that occurs in the light at the end of a LD but not for the second peak that occurs in darkness (Imaizumi et al., 2003). The light-mediated interaction with GI at the end of the day is required to remove CDF1 contributing to transcriptional activation of *CO* (Sawa et al., 2007). This activity was assumed to be specific to FKF1 because although *fkf1* mutants were markedly late flowering, mutations in the related proteins ZTL or LKP2 had a minor or no effect on flowering, respectively (Imaizumi et al., 2005; Kim et al., 2005; Somers et al., 2000). However, we demonstrated that these proteins are partially redundant with respect to flowering, because the triple mutant *fkf1 ztl-4 lkp2-1* is later flowering than *fkf1*, shows dramatically lower *CO* mRNA levels at all time points, and has higher CDF2 levels. The effect on CDF2 levels is not due to an increase in *CDF2* mRNA. Therefore, the limited effect of *fkf1* mutations on *CO* expression is because ZTL and/or LKP2 are still present and carry out a related function in CDF2 degradation. This function presumably has no or only a small effect on flowering in single *ztl* or *lkp2* mutants because FKF1 is still present and carries out the predominant role in CDF degradation. Similarly, ZTL and LKP2 must have major functions in the ubiquitination of other target proteins that are not major targets of FKF1, because the overexpression of *ZTL* and *LKP2* causes late flowering, whereas the overexpression of *FKF1* causes early flowering (Kim et al., 2005; Nelson et al., 2000; Schultz et al., 2001). Previously ZTL was shown to promote the degradation of clock components TOC1 and PRR5 (Kiba et al., 2007; Kim et al., 2007; Mas et al., 2003), and as *prr5* mutants are late flowering (Nakamichi et al., 2005; Nakamichi et al., 2007), depleted PRR5 levels might explain the late flowering of *ZTL* overexpressors.

Our data do not indicate whether ZTL, LKP2 ,or both proteins regulate CDF levels redundantly with FKF1. However, only FKF1 and LKP2, but not ZTL, interacted with CDF1, CDF2, and CDF3 in yeast and in vitro (Imaizumi et al., 2005). Therefore, ZTL may also not recognize CDFs in vivo but may specifically interact with other substrates. Also, the cellular location of the F-box proteins may contribute to their specificity in vivo. The interaction between GI and ZTL occurs mainly in the cytosol (Kim et al., 2007), whereas the GI-FKF1 complex targets CDF1 in the nucleus at the *CO* locus (Sawa et al., 2007). GI interaction with LKP2 has not been tested in planta; however, LKP2 also localizes in nuclei when overexpressed (Fukamatsu et al., 2005; Yasuhara et al., 2004). Similarly, differences in substrate specificity could also cause FKF1, ZTL, and LKP2 to vary in their affinity for different members of the CDF family. Such differences in specificity could complicate interpretations based on the available data for CDF2 and CDF1 protein levels. For example, the early flowering of *35S::GI fkf1* plants was associated with higher *CO* mRNA levels but also with high abundance of CDF2 as observed in *fkf1* mutants, and this may be due to other CDFs being higher affinity substrates for ZTL or LKP2. Reduced abundance of these other CDFs in *35S::GI fkf1* plants may be responsible for the early flowering and increased *CO* expression.

The Major Function of GI in *CO* Regulation Is to Promote the Degradation of CDF Repressors

GI acts upstream of *CO*; in *gi* mutants, *CO* mRNA levels are strongly reduced throughout the diurnal cycle in LDs and SDs (Suarez-Lopez et al., 2001). GI was proposed to promote *CO* expression through a light-mediated interaction with FKF1 that promotes degradation of CDF1 (Sawa et al., 2007). We demonstrated that GI is also required for CDF2 degradation and that the light-mediated interaction between GI and FKF1 stabilizes FKF1 protein, thereby ensuring that it is present to ubiquitinate CDF2 as well as CDF1 and presumably other CDFs. The more severe effect of *gi* mutations on *CO* transcript levels compared to *fkf1* suggested that GI has additional functions in *CO* regulation. Our observation, discussed above, that the triple mutant *fkf1 ztl-4 lkp2-1* shows similar *CO* mRNA levels to *gi*, suggests that the additional function of GI is to act through one or both of the related F-box proteins to regulate *CO*. Consistent with this idea, GI was previously shown to interact with FKF1, ZTL, and LKP2 as well as to stabilize ZTL, suggesting that the major function of GI is to stabilize the three F-box proteins in this family (Kim et al., 2007). Furthermore, GI appears to be the limiting factor in the accumulation of the F-box proteins, because in *GI* overexpressing plants, both FKF1 and ZTL accumulate to higher levels throughout the day (this work and Kim et al., 2007). Also, the reduction of CDF2 levels caused by overexpression of GI is an indirect effect through the F-box proteins, because in *35S::GI fkf1* plants CDF2 levels are similar to those observed in *fkf1* mutants.

GI also regulates *FT* expression independently of *CO*. In *gi* mutants, levels of the *miR172* are reduced, and this increases the abundance of the mRNAs encoding AP2-like flowering transcription factors that repress flowering (Jung et al., 2007). These AP2-like proteins repress the transcription of *FT*. Our conclusions are mainly based on measuring *CO* mRNA levels and therefore are not influenced by this additional *CO*-independent function of *GI* in promoting flowering. It remains to be tested whether the effect of GI on *miR172* can also be explained by stabilization of the F-box proteins or whether this represents an entirely independent biochemical function of GI (Jung et al., 2007).

Impairing both GI and CDF Activity Reveals an Additional Layer of Transcriptional Regulation of *CO* by Photoperiod

In the *gi* mutant, *CO* mRNA levels are low at all time points, but in the quintuple *gi-100 cdf1-R cdf2-1 cdf3-1 cdf5-1* mutant, a diurnal rhythm in *CO* mRNA is restored as is light inducibility of *CO* at the end of the day. Therefore, GI is not required for cycling or light inducibility of *CO* mRNA, but it promotes the degradation of the CDF repressors, allowing the underlying rhythm to be expressed. The elevated levels of CDFs present in the *gi* mutant must suppress the underlying rhythm and light inducibility of *CO* mRNA. Similarly, *35S::GI* plants contain low levels of CDF and elevated *CO* mRNA, but the abundance of *CO* mRNA still shows a diurnal rhythm with a peak late in the day similar to wild-type plants (Mizoguchi et al., 2005). This rhythm is also likely to be caused by the additional layer of transcriptional regulation that we identified in the quintuple mutant.

The mechanism conferring this rhythm and light inducibility of *CO* mRNA is unknown. Although analysis of the quintuple mutant excludes a role for *GI* in creating this rhythm, we cannot rule out the possibility that CDFs contribute, because some CDF alleles

may retain expression, as described above. However, F-box mediated degradation of CDFs is unlikely to contribute because at least FKF1 and ZTL accumulate to very low levels in *gi* mutants, and the activity of overexpressed *FKF1* was shown to depend on *GI* (Kim et al., 2007; Sawa et al., 2007). Therefore, the rhythm and light inducibility of *CO* mRNA observed in the quintuple mutant is likely to depend on a mechanism independent of *GI* and the F-box proteins. This system is also likely to involve circadian clock regulation to drive the diurnal peak during the night observed under SDs and LDs and to confer the light inducible peak under LDs, which falls at 15h before the onset of darkness. These data suggest that an additional set of circadian-clock controlled transcriptional regulators of *CO* remain to be identified.

Four layers of diurnal regulation of *CO* ensure that it is activated specifically under LDs. At the transcriptional level, CDFs repress *CO* transcription, and these are themselves removed by GI and the F-box proteins discussed here. A further layer of transcriptional regulation activates *CO* transcription during the night and contributes light inducibility at the end of a LD. In addition, the photoreceptor phytochrome B promotes degradation of CO protein early in the day, whereas the ubiquitin ligase COP1 interacts with CO and promotes its degradation during the night. The complexity of this regulation may be a specific requirement of *CO* because of the need to ensure that its activation occurs only at certain times under particular photoperiods. However, perhaps more likely, *CO* regulation provides insight into how light signaling and circadian clock control combine to confer precise temporal and environmental regulation on a wide set of plant genes (Al-Sady et al., 2006; Michael et al., 2008; Nozue et al., 2007).

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

All plant material described was in the Col-0 accession. The *cdf2-1* (GK782H09) and *cdf3-1* (GK808G05) alleles are T-DNA insertion lines obtained from the GABI-Kat collection (Rosso et al., 2003). The *cdf5-1* allele corresponds to the insertion line SALK_076153 (Figure S3). All T-DNA insertion sites were confirmed by sequencing. The *35S::CDF1-RNAi #23* (*cdf1-R*) and the *pFKF1::FKF1:TAP fkf1* were kindly provided by Takato Imaizumi, and have been previously described (Imaizumi et al., 2005; Imaizumi et al., 2003). The *fkf1*, *fkf1-2*, *gi-100*, and *co-10* alleles have been previously described (Huq et al., 2000; Imaizumi et al., 2003; Laubinger et al., 2006; Nelson et al., 2000).

For flowering-time measurements, plants were grown on soil in controlled environment rooms under LDs (16 hr light/8 hr dark) or SDs (8 hr light/16 hr dark).

Plasmid Construction and Plant Transformation

All cDNAs from DOF transcription factors were from the REGIA collection in GATEWAY compatible vectors (http://www.jicgenomelab.co.uk/libraries/), except *COG1* and *CDF5*, which were amplified from Col-0 cDNA using primers described in Supplemental Data. All DOF cDNAs were cloned by recombination using LR clonase II (Invitrogen) into a *pSUC2::GATEWAY* destination vector under the control of a 2.1 kb fragment of the *SUCROSE TANSPORTER 2* (*SUC2*) promoter (Imlau et al., 1999). The *pCDF2*, *pCDF3*, and *pCDF4* promoters were amplified from Col-0 genomic DNA using the primers described in Supplemental Data. PCR products were recombined in pDONR201 using BP clonase II. The promoter fragments were subsequently recombined into *pMDC163* (Curtis and Grossniklaus, 2003) upstream of the *GUS* reporter gene. All binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) or GV3101 (pMP90RK) and transformed into *Arabidopsis* Col-0 by the floral-dip method (Clough and Bent, 1998).

Phylogenetic Tree Construction

The 52 amino acid sequence of the highly conserved DOF domain was used for the alignment as previously described (Pelucchi et al., 2002).

Quantification of mRNA Expression

RNA was isolated from whole seedlings using the Quiagen RNeasy extraction kit, and DNA contamination removed using Ambion's DNA-*free* kit. For cDNA synthesis, 3–5 μg total RNA was primed using oligo dT15 primer and reverse transcribed using the Invitrogen Superscript II kit (Invitrogen). cDNA was diluted to 200 μl with water, and 3 μl diluted cDNA was used for subsequent qRT-PCR reactions. Amplified products were detected using SyBR Green II in an IQ5 (Biorad) thermal cycler. Each data point shown in the figures is the average of three independent amplifications of the same RNA sample run in the same reaction plate. Each sample was also analyzed in three reaction plates (so a total of nine amplifications were run for each sample) and gave similar results. This process was repeated with at least two independent RNA samples (biological replicates) for each genotype and condition. Each biological replicate produced results similar to those shown. The primer pairs used for expression analyses are described in the Supplemental Data.

GUS Assay

Seedlings were grown under LDs in Murashige-Skoog medium or soil, placed in a Petri dish, and treated with heptane for 10 min. Subsequently, plants were incubated at 37°C in staining buffer containing 0.5 mg/mL X-Gluc, 0.1% Triton X-100, 50 mM Phosphate buffer (pH 7), and 0.5 mM Ferricyanate stock solution [422 mg of $K_4Fe(CN)_6.3H_2O$ and 329mg $K_3Fe(CN)_6.3H_2O$ in 10 ml water]. After staining, the samples were bleached and dehydrated with a series of 50%, 70%, and 100% ethanol for 1 hr each.

CDF2 Antibody Production

Peptide synthesis, purification, coupling, immunization, and affinity purification were performed by Eurogentec S.A Belgium. Two CDF2-specific peptides, C-DEEMGDSGLGREEGD corresponding to aa 49-62 (peptide 1) and CQEESLRNESNDVTT corresponding to aa 85-99 (peptide 2), were synthesized by solid-phase synthesis, purified by HPLC to 90% purity, and used as antigens to immunize two rabbits (SA5344 and SA5345). After five successive booster injections, the sera were collected and the antibody titer was estimated by ELISA. Peptide 2 produced a strong immune response in both animals. The antibody was affinity purified using a Peptide 2-Sepharose affinity matrix.

Protein Extraction and Detection

Ten-day-old *Arabidopsis* seedlings were grown on MS agar in temperature-controlled light cabinets and harvested at the indicated time points. Nuclear extracts were prepared as described previously (Valverde et al., 2004), but excluding the sonication step. Nuclear proteins (20 μg) were separated on 10% bis-Tris NuPAGE gels in MOPs/SDS buffer (Invitrogen) and transferred to nitrocellulose. Western analysis was performed as described in Jang et al. (2008), but at the primary antibody reaction step, the affinity-purified CDF2 antiserum was diluted in Tris-buffered saline containing 0.1% Tween 20 (TBST), followed by 1 hr incubation with horseradish-peroxidase-conjugated secondary antibody diluted in 5% milk-TBST. Immunoreactive proteins were visualized by a mixture of Femto and Dura chemiluminescence substrate system (Pierce). The membranes were subsequently reprobed with an antibody against histone H3a (Abcam) as loading control. For peptide competition experiments, diluted primary antibody containing the respective peptide at a final concentration of 20 μg per ml was incubated for 2 hr at room temperature with the blots, followed by subsequent steps of detection. Extraction and detection of the FKF1-TAP protein was carried out as previously described (Imaizumi et al., 2003). For TAP detection, Rabbit IgG was used as primary antibody, followed by HRP coupled to secondary antiserum. The signals were detected using the Pico substrate system (Pierce).

SUPPLEMENTAL DATA

Supplemental data include seven supplemental figures and primer sequences and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00256-1/.

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REFERENCES

Al-Sady, B., Ni, W., Kircher, S., Schafer, E., and Quail, P.H. (2006). Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. Mol. Cell *23*, 439–446.

An, H., Roussot, C., Suarez-Lopez, P., Corbesier, L., Vincent, C., Pineiro, M., Hepworth, S., Mouradov, A., Justin, S., Turnbull, C., and Coupland, G. (2004). CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of *Arabidopsis*. Development *131*, 3615–3626.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. *16*, 735–743.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., and Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science *316*, 1030–1033.

Curtis, M.D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol. *133*, 462–469.

David, K.M., Armbruster, U., Tama, N., and Putterill, J. (2006). *Arabidopsis* GIGANTEA protein is post-transcriptionally regulated by light and dark. FEBS Lett. *580*, 1193–1197.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G., and Putterill, J. (1999). GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. EMBO J. *18*, 4679–4688.

Fukamatsu, Y., Mitsui, S., Yasuhara, M., Tokioka, Y., Ihara, N., Fujita, S., and Kiyosue, T. (2005). Identification of LOV KELCH PROTEIN2 (LKP2)-interacting factors that can recruit LKP2 to nuclear bodies. Plant Cell Physiol. *46*, 1340–1349.

Gould, P.D., Locke, J.C., Larue, C., Southern, M.M., Davis, S.J., Hanano, S., Moyle, R., Milich, R., Putterill, J., Millar, A.J., and Hall, A. (2006). The molecular basis of temperature compensation in the *Arabidopsis* circadian clock. Plant Cell *18*, 1177–1187.

Hayama, R., Yokoi, S., Tamaki, S., Yano, M., and Shimamoto, K. (2003). Adaptation of photoperiodic control pathways produces short-day flowering in rice. Nature *422*, 719–722.

Huq, E., Tepperman, J.M., and Quail, P.H. (2000). GIGANTEA is a nuclear protein involved in phytochrome signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *97*, 9789–9794.

Imaizumi, T., Tran, H.G., Swartz, T.E., Briggs, W.R., and Kay, S.A. (2003). FKF1 is essential for photoperiodic-specific light signalling in *Arabidopsis*. Nature *426*, 302–306.

Imaizumi, T., Schultz, T.F., Harmon, F.G., Ho, L.A., and Kay, S.A. (2005). FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in *Arabidopsis*. Science *309*, 293–297.

Imlau, A., Truernit, E., and Sauer, N. (1999). Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell *11*, 309–322.

Jaeger, K.E., and Wigge, P.A. (2007). FT protein acts as a long-range signal in *Arabidopsis*. Curr. Biol. *17*, 1050–1054.

Jang, S., Marchal, V., Panigrahi, K.C., Wenkel, S., Soppe, W., Deng, X.W., Valverde, F., and Coupland, G. (2008). Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. EMBO J. *27*, 1277–1288.

Jarillo, J.A., Capel, J., Tang, R.H., Yang, H.Q., Alonso, J.M., Ecker, J.R., and Cashmore, A.R. (2001). An Arabidopsis circadian clock component interacts with both CRY1 and phyB. Nature *410*, 487–490.

Jung, J.H., Seo, Y.H., Seo, P.J., Reyes, J.L., Yun, J., Chua, N.H., and Park, C.M. (2007). The GIGANTEA-regulated microRNA172 mediates photoperiodic flowering independent of CONSTANS in *Arabidopsis*. Plant Cell *19*, 2736–2748.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J., and Weigel, D. (1999). Activation tagging of the floral inducer FT. Science *286*, 1962–1965.

Kiba, T., Henriques, R., Sakakibara, H., and Chua, N.H. (2007). Targeted degradation of PSEUDO-RESPONSE REGULATOR5 by an SCFZTL complex regulates clock function and photomorphogenesis in *Arabidopsis thaliana*. Plant Cell *19*, 2516–2530.

Kim, W.Y., Hicks, K.A., and Somers, D.E. (2005). Independent roles for EARLY FLOWERING 3 and ZEITLUPE in the control of circadian timing, hypocotyl length, and flowering time. Plant Physiol. *139*, 1557–1569.

Kim, W.Y., Fujiwara, S., Suh, S.S., Kim, J., Kim, Y., Han, L., David, K., Putterill, J., Nam, H.G., and Somers, D.E. (2007). ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. Nature *449*, 356–360.

Kobayashi, Y., and Weigel, D. (2007). Move on up, it's time for change–mobile signals controlling photoperiod-dependent flowering. Genes Dev. *21*, 2371–2384.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. Science *286*, 1960–1962.

Laubinger, S., Marchal, V., Le Gourrierec, J., Wenkel, S., Adrian, J., Jang, S., Kulajta, C., Braun, H., Coupland, G., and Hoecker, U. (2006). *Arabidopsis* SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability. Development *133*, 3213–3222.

Liu, L.J., Zhang, Y.C., Li, Q.H., Sang, Y., Mao, J., Lian, H.L., Wang, L., and Yang, H.Q. (2008). COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in *Arabidopsis*. Plant Cell *20*, 292–306.

Martin-Tryon, E.L., Kreps, J.A., and Harmer, S.L. (2007). GIGANTEA acts in blue light signaling and has biochemically separable roles in circadian clock and flowering time regulation. Plant Physiol. *143*, 473–486.

Mas, P., Kim, W.Y., Somers, D.E., and Kay, S.A. (2003). Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. Nature *426*, 567–570.

Mathieu, J., Warthmann, N., Kuttner, F., and Schmid, M. (2007). Export of FT protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. Curr. Biol. *17*, 1055–1060.

Michael, T.P., Breton, G., Hazen, S.P., Priest, H., Mockler, T.C., Kay, S.A., and Chory, J. (2008). A morning-specific phytohormone gene expression program underlying rhythmic plant growth. PLoS Biol. *6*, e225.

Mizoguchi, T., Wright, L., Fujiwara, S., Cremer, F., Lee, K., Onouchi, H., Mouradov, A., Fowler, S., Kamada, H., Putterill, J., and Coupland, G. (2005). Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in *Arabidopsis*. Plant Cell *17*, 2255–2270.

Moreno-Risueno, M.A., Martinez, M., Vicente-Carbajosa, J., and Carbonero, P. (2007). The family of DOF transcription factors: from green unicellular algae to vascular plants. Mol. Genet. Genomics *277*, 379–390.

Nakamichi, N., Kita, M., Ito, S., Sato, E., Yamashino, T., and Mizuno, T. (2005). The Arabidopsis pseudo-response regulators, PRR5 and PRR7, coordinately play essential roles for circadian clock function. Plant Cell Physiol. *46*, 609–619.

Nakamichi, N., Kita, M., Niinuma, K., Ito, S., Yamashino, T., Mizoguchi, T., and Mizuno, T. (2007). Arabidopsis clock-associated pseudo-response regulators PRR9, PRR7 and PRR5 coordinately and positively regulate flowering time

through the canonical CONSTANS-dependent photoperiodic pathway. Plant Cell Physiol. *48*, 822–832.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A., and Bartel, B. (2000). FKF1, a clock-controlled gene that regulates the transition to flowering in *Arabidopsis*. Cell *101*, 331–340.

Nozue, K., Covington, M.F., Duek, P.D., Lorrain, S., Fankhauser, C., Harmer, S.L., and Maloof, J.N. (2007). Rhythmic growth explained by coincidence between internal and external cues. Nature *448*, 358–361.

Onouchi, H., Igeno, M.I., Perilleux, C., Graves, K., and Coupland, G. (2000). Mutagenesis of plants overexpressing CONSTANS demonstrates novel interactions among *Arabidopsis* flowering-time genes. Plant Cell *12*, 885–900.

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim, H.K., Soh, M.S., Kim, H.J., Kay, S.A., and Nam, H.G. (1999). Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis* GIGANTEA gene. Science *285*, 1579–1582.

Park, D.H., Lim, P.O., Kim, J.S., Cho, D.S., Hong, S.H., and Nam, H.G. (2003). The Arabidopsis COG1 gene encodes a Dof domain transcription factor and negatively regulates phytochrome signaling. Plant J. *34*, 161–171.

Pelucchi, N., Fornara, F., Favalli, C., Masiero, S., Lago, C., Pè, M.E., Colombo, L., and Kater, M.M. (2002). Comparative analysis of rice MADS-box genes expressed during flower development. Sex. Plant Reprod. *15*, 113–122.

Putterill, J., Robson, F., Lee, K., Simon, R., and Coupland, G. (1995). The CONSTANS gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell *80*, 847–857.

Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K., and Weisshaar, B. (2003). An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol. Biol. *53*, 247–259.

Sawa, M., Nusinow, D.A., Kay, S.A., and Imaizumi, T. (2007). FKF1 and GIGANTEA complex formation is required for day-length measurement in *Arabidopsis*. Science *318*, 261–265.

Schultz, T.F., Kiyosue, T., Yanovsky, M., Wada, M., and Kay, S.A. (2001). A role for LKP2 in the circadian clock of *Arabidopsis*. Plant Cell *13*, 2659–2670.

Somers, D.E., Schultz, T.F., Milnamow, M., and Kay, S.A. (2000). ZEITLUPE encodes a novel clock-associated PAS protein from *Arabidopsis*. Cell *101*, 319–329.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and Coupland, G. (2001). CONSTANS mediates between the circadian clock and the control of flowering in *Arabidopsis*. Nature *410*, 1116–1120.

Takada, S., and Goto, K. (2003). Terminal flower2, an Arabidopsis homolog of heterochromatin protein1, counteracts the activation of flowering locus T by constans in the vascular tissues of leaves to regulate flowering time. Plant Cell *15*, 2856–2865.

Turck, F., Fornara, F., and Coupland, G. (2008). Regulation and Identity of Florigen: FLOWERING LOCUS T Moves Center Stage. Annu. Rev. Plant Biol. *59*, 573–594.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and Coupland, G. (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science *303*, 1003–1006.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U., and Weigel, D. (2005). Integration of spatial and temporal information during floral induction in *Arabidopsis*. Science *309*, 1056–1059.

Yanagisawa, S. (2002). The Dof family of plant transcription factors. Trends Plant Sci. *7*, 555–560.

Yanovsky, M.J., and Kay, S.A. (2002). Molecular basis of seasonal time measurement in *Arabidopsis*. Nature *419*, 308–312.

Yasuhara, M., Mitsui, S., Hirano, H., Takanabe, R., Tokioka, Y., Ihara, N., Komatsu, A., Seki, M., Shinozaki, K., and Kiyosue, T. (2004). Identification of ASK and clock-associated proteins as molecular partners of LKP2 (LOV kelch protein 2) in *Arabidopsis*. J. Exp. Bot. *55*, 2015–2027.

Yoo, S.K., Chung, K.S., Kim, J., Lee, J.H., Hong, S.M., Yoo, S.J., Yoo, S.Y., Lee, J.S., and Ahn, J.H. (2005). CONSTANS activates SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 through FLOWERING LOCUS T to promote flowering in *Arabidopsis*. Plant Physiol. *139*, 770–778.

Yu, J.W., Rubio, V., Lee, N.Y., Bai, S., Lee, S.Y., Kim, S.S., Liu, L., Zhang, Y., Irigoyen, M.L., Sullivan, J.A., et al. (2008). COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability. Mol. Cell *32*, 617–630.