Seasonal Circuitry: transcriptional and post-translational regulation of photoperiodic flowering in *Arabidopsis thaliana*

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ABSTRACT

Seasonal Circuitry: transcriptional and post-translational regulation of photoperiodic flowering in *Arabidopsis thaliana*

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For plants, the transition towards reproductive development is timed so that it coincides with the most optimal external conditions. For many plant species, day length is an important cue in mediating this process. At the molecular level, the perception of day length for flowering is accomplished through the a variety of mechanisms, but the regulatory control of the genes encoding the CONSTANS (CO) transcription factor and the FLOWERING LOCUS T (FT) florigenic protein are most critical in Arabidopsis. Temporal control of the *CO* and *FT* daily expression pattern is mediated by the CYCLING DOF FACTOR (CDF) family of transcriptional repressors, and the degradation of CDFs by the FLAVIN BINDING KELCH REPEAT F-BOX 1 (FKF1) photoreceptor in the evening of long days. This dissertation is an examination of the mechanisms by which CDFs perform their repressive function on their target genes *CO* and *FT*, and the further details of FKF1 function mediated through stability of the photoactive form of the light sensing LOV (Light Oxygen Voltage) domain.

In this dissertation, first I review the framework of photoperiodic flowering through a presentation of the history of flowering time research and its context in circadian biology. Then, I move on to provide a review of the major factors and processes that have been found to play a role in the regulation of *CO* and *FT* in Arabidopsis. Later on, I describe our experimental findings about how the CDF transcription factors utilize the general co-repressor protein TOPLESS (TPL) to exert repression on *CO* and *FT*. I then discuss the implications of our findings on *CO* and *FT* regulation in Arabidopsis as well as other plant species. Next, I detail our characterization of a novel CDF transcription factor in Arabidopsis, CDF6, and its role in the repression of photoperiodic flowering. Finally, I present our preliminary findings that show the importance of photocycle rate kinetics of the FKF1 LOV domain on photoperiodic flowering regulation, demonstrating that the conversion of FKF1 into a shorter photocycle variant perturbs its function by reducing the light-memory into dusk and early night of long days.

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CHAPTER I.

INTRODUCTION: SEASONAL CIRCUITRY IN PLANTS

Segments of this chapter appeared previously and were revised and modified from:
Photoperiodic Flowering Regulation in *Arabidopsis thaliana.* Greg Golembeski, Hannah A. Kinmonth-Schultz, Young Hun Song and Takato Imaizumi. *Adv Bot Res*. 2014; 72:1-28. Article PMCID: PMC4326075

Visually, seasonal changes in plant development and physiology are some of the most striking in nature. Climactic variables on earth, under which plant growth and development is constrained, are in continual flux day by day, and throughout the year. Plants have evolved an array of responses through which to constrain, promote, or adapt their development in synchrony with the external environment (Thomas and Vince-Prue, 1996). To a resident of the US Pacific Northwest, perhaps the most familiar examples of this would be the onset of dormancy and subsequent spring flush in deciduous trees, and the emergence and flowering of the ubiquitous daffodils. From a biological perspective, a key question related to these casual observations is how such a diverse array of responses can be regulated. Phrased another way: how are the seasons perceived by the plant, and then connected and relayed to the various outputs required to induce changes in physiology and development?

Flowering time has been a key part of the study of seasonal responses both for its relevance in the agricultural context as well as due to the radical changes required to transition into reproductive development (Zeevaart, 2006). Timing of floral initiation is a key agricultural characteristic with large impacts on yield, and has shaped the domestication of a variety of crop species as they have moved throughout the world and most importantly to different latitudes (Song *et al.*, 2015). Without the partial or total suppression of the photoperiodic response from species endemic to the tropics, their growth, flowering and seed set in the far northern or southern hemisphere would be compromised (Doebley *et al.*, 2006). This is especially clear in species such as sorghum, potato, and rice, where their responses and

domestication have been characterized on a molecular genetic level (Ishikawa *et al.*, 2005; Xue *et al.*, 2008; Murphy *et al.*, 2011; Kloosterman *et al.*, 2013).

The topic of how plants are able to recognize what constitutes optimal conditions for flowering has been an active area of research for almost a century. Wightman Garner and Henry Allard were the first to empirically describe that the duration of light in a 24-hour period is a key cue for the induction of flowering in many plant species. Originally interested in explaining why soybeans planted sequentially over the summer decreased in days to flower as they were planted later in the season, they sought to find the causal variable behind the phenomenon. Over the course of two years from 1918 to 1920, they experimentally manipulated exposure of plants to light and dark cycles by moving plants from a common outdoor plot into darkened sheds. Through the careful control of light and dark duration to simulate different seasonal light conditions, they were able to determine critical durations of light or darkness that are required for induction of flowering in over 12 plant species and many different cultivars. This general principle of an exhibited response triggered by a change in day length, they coined “photoperiodism” (Garner and Allard, 1920). This revolutionary idea changed the thinking about seasonal responses by suggesting that the mechanism for sensing seasonal changes could be tied specifically to the sensing of duration of light in a given day, not intrinsically growth or metabolism. In addition, they found that plants could be grouped into three different groups by their flowering response. Some plants flower as day length increases in late spring (long-day plants), some flower as day length wanes as autumn begins (short-day plants), and some plants flower at certain times regardless of the photoperiods (day-neutral plants) (Garner and Allard, 1920). Similar studies from the same era further deepened the breadth of descriptions of plant photoperiodism, including in basal land plants such as *Marchantia polymorpha* (Wann, 1925).

The determination of day-length as a critical regulator of flowering time left several questions with regard to the physiology of the flowering response. Where was day-length sensed in the plant and how is the signal for floral induction carried throughout the organism? Elegant grafting experiments performed first by the Russian physiologist Mikhail Chailakhyan determined that a mobile signal from leaf scions exposed to inductive photoperiods could induce flowering in non-induced graft stock (Chailakhyan, 1937; Chailakhyan, 1968). Experimental evidence suggested that the transmissible signal could be universal or

nearly universal among flowering plants, for instance grafts in which leaves from induced short-day *Kalanchoë blossfeldiana* (stock) and long-day *Sedum spectabile* plants (scion) were able were able to induce flowering when grafted to plants of the opposite response type (Wellensiek, 1967; Zeevaart, 2006). Grafts between different species were also often found to lead to flowering induction (Zeevaart, 1976; Zeevaart, 2006). These observations led Denis Carr and Lloyd Evans to propose a model for two-step floral induction (Carr, 1967; Evans, 1971). The first stimulus would be involved in the sensing of photoperiod and the incorporation of other endogenous and environmental factors, which would induce the secondary stimulus that was potentially universal and transmitted from the leaf. Advances in the characterization of plant hormones further identified the Gibberellins (GA) as general promoters of the flowering response, but the incompleteness of GA as an explanatory variable in flowering led to the conclusion that the promotion of flowering was accomplished through the activity of GA, and a hitherto unknown molecule "anthesin" that was graft transmissible (Evans, 1971; Chaïlakhyan, 1975).

The search for the chemical basis of anthesin (or "florigen") remained elusive and gradually fell out of favor until contributions from *Arabidopsis* that facilitated the discovery of FT protein as a key candidate. The discovery FT as a mobile signal in *Arabidopsis* along with recognition that its function is conserved in a range of distantly related plant species (Corbesier *et al.*, 2007), has cemented the role of FT as a universal florigen (Kojima *et al.*, 2002; Abe *et al.*, 2005; Wigge *et al.*, 2005; Kobayashi and Weigel, 2007; Tamaki *et al.*, 2007). Increasingly, as our understanding of the photoperiodic sensing mechanism has expanded, we have found that similar regulatory networks govern flowering plant species other than *Arabidopsis*, and that the mechanism of photoperiodic flowering induction is highly conserved (Song *et al.*, 2010). A key interest of mine, explored throughout this thesis, has been how the *FT* gene is regulated to be off under non-inductive photoperiods and on under inductive ones.

CONNECTING PHOTOPERIODIC FLOWERING TO THE CIRCADIAN CLOCK

One of the key questions that emerged with the discovery of photoperiodic flowering responses was the mechanism for how photoperiod was sensed. Since the early 18th century with the experiments of the astronomer Jean-Jacques d'Ortous De Mairan, plants have been known to have oscillatory leaf

movements that occur in 24-hour cycles even in the absence of light, as if a light stimulus was present (De Mairan, 1729). Carl Linnaeus was able to demonstrate similar, but crude principles, through his novel "flowering clock". An impressive display; the clock was arranged as a common garden of different species, in which floral buds from each species opened and closed sequentially, throughout the day (Linnaeus, 1751). These rhythms, which show a period of around 24 hours (hence circadian), show an inherited entrainment to the rotation of the earth that persists even after many generations of exposure to alternative day lengths in the laboratory (Bünning, 1960). This internal "clock" has selective value in the regulation of daily output changes to the internal biochemical processes of the cell and the organism, which we can now appreciate given the advances in molecular biology in the last decades (Baudry and Kay, 2008). The connection between the internal clock and photoperiodic responses, however, was not immediately clear. First proposed by Erwin Bünning in 1936, and refined later by Colin Pittendrigh the "external coincidence" model, as it came to be known, proposed that photoperiodic phenomena could be explained by the interaction of light stimuli and the clock (Bünning, 1936; Pittendrigh and Minis, 1964). The clock would set the pace of the 24-hour rhythm, and define a period of photosensitivity to which exposure to light would be inductive for a photoperiodic response (Pittendrigh, 1972). In non-inductive photoperiods, the presence of darkness during the sensitive period of the response would result in no elicited reaction. In contrast, the encroachment of light into the photosensitive part of the circadian cycle, brought upon by longer inductive photoperiods, would cause a physiological response (Figure 1-1).

For more than thirty years, it remained controversial that the endogenous circadian clock regulated the photoperiodic flowering response. Key experiments that unequivocally linked flowering to the clock were performed by Murray Coulter and Karl Hamner on the short-day plant *Glycine max* in 1964, by giving light pulses at different time points after transfer of plants into continuous darkness. One of the prevailing counter-hypotheses of the time posited that night duration was the primary cue for the photoperiodic response, and that this was mediated by the turnover kinetics of the photoreceptor phytochrome. According to this hypothesis, for short-day plants, in which photoperiods below a certain threshold are inductive, directing light pulses at different times of night should affect the photoperiodic flowering response equally as long as a certain night length was prevented. It was found, instead, that light pulses during the night (referred to as night breaks) affected the flowering response in a rhythmic

fashion (Carpenter and Hamner, 1964; Coulter and Hamner, 1964). Additional experiments performed by Halaban in 1968 in the short day plant *Coleus frederici* showed that the phases in which flowering was inhibited by night break pulses of plants always correlated with leaf movement position rather than the duration of night (Halaban, 1968b; Halaban, 1968a). This was true for plants placed under several different photoperiods. These early findings helped to cement the clock as a crucial component in determining photoperiodic flowering responses.

CONSERVED MACHINERY IN PERCEPTION AND COORDINATION OF SEASONAL DEVELOPMENT

Extensive studies have now been performed in other plant species, using some of the tools and framework originally characterized in Arabidopsis. As anticipated from earlier physiological studies, the developmental switch to flowering is modulated by the production of homologues of FT protein, which is highly conserved in function across the flowering plant lineages (Lifschitz *et al.*, 2006; Rodríguez-Falcón *et al.*, 2006; Yan *et al.*, 2006; Tamaki *et al.*, 2007). While some flowering plants appear to use a very similar FT module utilizing the transcription factor CONSTANS (CO) as an activator in response to photoperiod (a mechanism I will detail in the subsequent chapter), many grasses for instance have coopted PSUEDO RESPONSE REGULATOR (PRR) –like transcription factors (Ppd-1 like TFs) as direct activators or repressors of *FT* homologues (Hd3a-like proteins) (Murakami *et al.*, 2003; Murphy *et al.*, 2011; Andrés and Coupland, 2012; Song *et al.*, 2015).

In many cases *CO* and *FT* appear to have acquired new function by being co-opted into separate organogenesis events in the plant life cycle. *CO-FT* is utilized for the control of growth cessation and bud burst in *Populus triocharpa*, and has been shown to be involved in latitude specific adaptation through differential expression of *CO* (Böhlenius *et al.*, 2006). In *Solanum tuberosum* (potato), *CO* and *FT* are both involved in the timing of tuberization (Kloosterman *et al.*, 2013). In *Fragraria* species (strawberry) *CO* and *FT* regulation is further complicated by the additional photoperiodic regulation of the repressive *FT* homologue *TERMINAL FLOWER 1* (*TFL1*) in both seasonal regulation of flowering and stolon development (Koskela *et al.*, 2012).

While *CO* and *FT* seem commonly involved in seasonal responses in flowering plants, they appear to be a largely derived character among land plants (Serrano *et al.*, 2009; Lucas-Reina *et al.*, 2015). *FT* likely evolved in function within gymnosperms, and although *CO*-like genes can be found in *Chlamydomonas reinhardtii* and have a photoperiodic expression pattern, it does not appear to regulate the timing of reproduction in a similar manner and likely has different function (Gyllenstrand *et al.*, 2007; Serrano *et al.*, 2009; Lucas-Reina *et al.*, 2015; Liu *et al.*, 2016). Recent studies in *Marchantia polymorpha* and *Physcomitrella patens,* however, have shown that the photoperiodic flowering factors *GIGANTEA (GI) FLAVIN-BINDING KELCH REPEAT F-BOX1 (FKF1)*, and *CYCLING DOF FACTORS (CDFs)* (a mechanism I will detail in the subsequent chapter) are involved in the control of the vegetative to reproductive phase of development (Sugiyama *et al.*, 2012; Kubota *et al.*, 2014). This suggests that many of these factors could have conserved function as part of a "core photoperiod module" shared across all land plants. While not a major experimental goal of this thesis, I will discuss some of the implications of my findings in the context of this module throughout.

BASIC MODELS AND AN OUTLINE

What I have detailed here is a simple history of plant photoperiodic flowering research and broad arcs of the flowering time response, and I will go into more detail about the mechanism of photoperiodic flowering that has emerged as a consequence of molecular studies in Arabidopsis in the next chapter (Golembeski and Imaizumi, 2015). An item that I would like to stress, however, is that a major difficulty in the study of the flowering time response has been the complexity (i.e. number of regulators) present in the regulation of particularly the *FT* gene. Like many pathways where a hub in the network serves as a focal point for a broad number of inputs, the interconnectedness can make genetic analysis a challenge. We have tried to clarify this issue by focusing on the key regulators CDFs and FKF1, which primarily regulate *CO* and *FT* through controlling the timing of their transcription, in addition to FKF1's ability to assist in CO protein stabilization.

In chapter III, I will discuss findings that show a novel mechanism where CDFs require the co-repressor protein TOPLESS (TPL) to concert their repressive activity (Goralogia *et al.*, 2017). In chapter

IV I will discuss the discovery of a hitherto uncharacterized DOF in Arabidopsis, CDF6, which has function similar to other Arabidopsis CDFs. In chapter V I will discuss a preliminary study looking into the kinetics of FKF1's light sensing domain, and implications of its function in signaling during the determination of flowering initiation. In chapter VI I will discuss some of the overall conclusions found in chapters III, IV, and V.

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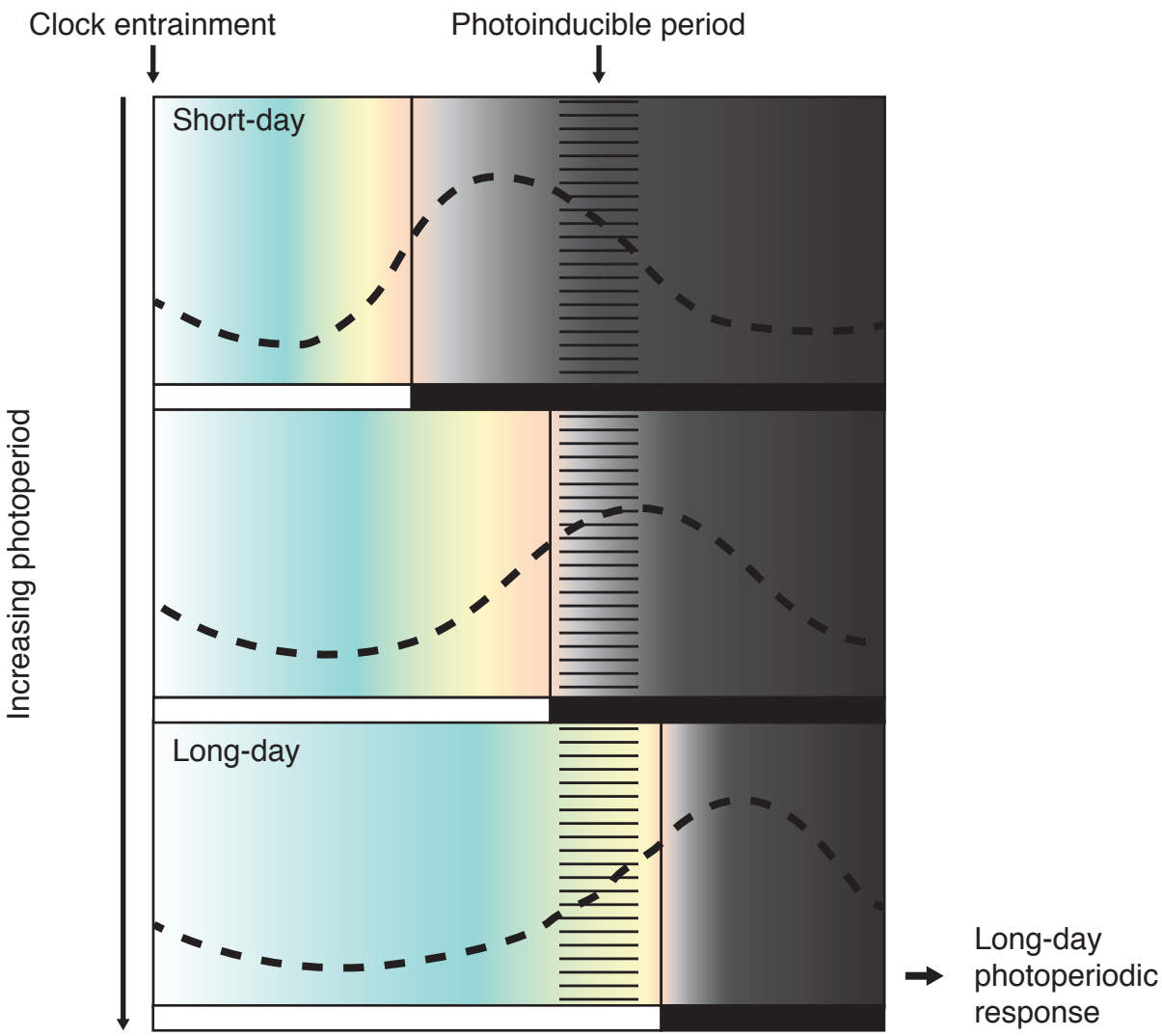

Figure 1-1. The external coincidence model for photoperiodic phenomena. The following example represents a photoperiodic response that occurs in the afternoon of long days, as in photoperiodic flowering in Arabidopsis. The circadian clock generates a rhythm that determines a specif-ic period of the day in which a light signal can induce the response. This period is similar regardless of day length. In short day conditions the photoinducible period does not coincide with a light signal, so no response occurs. As days lengthen with the coming of spring and summer, light begins to encroach on the photoinducible period, eliciting the photoperiodic response. Light serves a dual purpose; to reset the clock at dawn and dusk and to be present or absent during the photoinducible phase, to promote or halt the response.

CHAPTER II.

PHOTOPERIODIC FLOWERING IN *ARABIDOPSIS*

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SUMMARY

Flowering in response to seasonal change is brought about through sensing of fluctuation in day-length or photoperiod. Flowering induction occurs through the production of the florigenic protein FLOWERING LOCUS T (FT) and its movement from the phloem companion cells in the leaf vasculature into the shoot apex, where meristematic reprogramming occurs. *FT* activation in response to photoperiod conditions is accomplished largely through the activity of the transcription factor CONSTANS (CO). Regulation of *CO* expression and protein stability, as well as the timing of other components via the circadian clock, is a critical mechanism through which plants are able to respond to photoperiod to initiate the floral transition. Modulation of *FT* expression in response to external and internal stimuli via components of the flowering network is critical to mediate a fluid flowering response to a variety of environmental parameters. In addition, the regulated movement of FT protein between the phloem and the shoot apex, and interactions, which determine floral meristem cell fate, constitute novel mechanisms through which photoperiodic information is translated into flowering time.

INTRODUCTION

In plants, entirely new structures which house the germline of an individual must be formed post-embryonically (Evans, 1971). Once the formation of reproductive organs is specified, the process is largely irreversible (Evans, 1971). This necessitates that the phase transition between the vegetative and

reproductive periods of plant development is regulated so that optimal endogenous and exogenous factors are synchronized for maximal reproductive fitness (Kinmonth-Schultz et al., 2013). Changes in season preclude many environmental conditions that plants must prepare or react to. Anticipation of seasonal change is perceived through changes in day length (the photoperiod), that are the causal agent of seasonal climate (Thomas and Vince-Prue, 1996). Season, then, and its associated changes in day-length (photoperiod) is thus an excellent integrated variable through which many other environmental conditions can be interpreted to determine the timing of the floral transition (Kinmonth-Schultz et al., 2013). For this reason, photoperiodic change is a common cue which many plant species utilize to coordinate their flowering time (Song et al., 2010).

A great deal is now known about the molecular mechanisms through which flowering in response to photoperiod in *Arabidopsis* is accomplished. At the basic level, photoperiod regulates the expression and activity of CONSTANS (CO: At5g15840), a key transcriptional activator of the gene that encodes the "florigen" protein FLOWERING LOCUS T (FT: At1g65480), which is crucial in specifying the conversion of vegetative cell fate at the shoot apex into a reproductive identity (Putterill et al., 1995; An et al., 2004; Abe et al., 2005; Mathieu et al., 2007; Tiwari et al., 2010; Andrés and Coupland, 2012) (Figure 2-1). For over a decade, uncovering how *CO* and *FT* are regulated by photoperiod has been a key goal in the understanding of how photoperiodic information determines flowering output, and considerable advancement has been made in understanding the photoperiodic regulatory network in *Arabidopsis* (Song et al., 2013). In this review, we will discuss our current understanding of photoperiodic input sensing, ambient temperature and light quality effects on photoperiodic flowering, the chromatin landscape at flowering component loci, florigen movement from the leaf to the shoot apex, and the influence of carbohydrate status on flowering in response to photoperiod.

OVERVIEW OF THE FLOWERING TIME PATHWAYS

The regulation of the timing of the floral transition is an intricate one. Multiple pathways are able to regulate the expression of "floral integrator genes" (Moon et al., 2005). These integrators are network hubs that link the pathways that measure environmental and developmental competence to the

downstream targets that cause the patterning of reproductive structures and floral organs (Song et al., 2013). Because of their unique placement within these networks, they are able to filter multiple inputs into a single output. Commonly described floral integrator genes in *Arabidopsis* are *FT, SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1:* At2g45660)*, AGAMOUS LIKE 24* (*AGL24:* At4g24540)*, FRUITFULL* (*FUL:* At5g60910) *SQUAMOSA PROMOTER BINDING LIKE* (*SPLs*)*, LEAFY* (*LFY*: At5g61850), *and APETALA 1* (*AP1*: At1g69120); incremental expression of these integrators strongly increases competency to flower (Simon et al., 1996; Ruiz-Garcia et al., 1997; Samach et al., 2000; Yanovsky and Kay, 2002; Michaels et al., 2003b; Teper-Bamnolker and Samach, 2005). Of particular interest is *FT*, as *FT* is the main component that links the environmental sensory machinery that is present within the leaf vasculature, to the shoot apex where reproductive structures eventually form (Turck et al., 2008). Since the 1930s, it has been known that a mobile signal present within the leaves of many plant species is able to induce flowering (Chailakhyan, 1937; Chailakhyan, 1968; Chailakhian, 1970; Evans, 1971). Experimental evidence determined that FT protein is the long sought "florigenic" signal that moves through the phloem stream and initiates flowering at the shoot apex. The *FT* gene is the primary target of several of these pathways, including photoperiod, vernalization, hormone signaling, temperature and plant age (Song et al., 2013). Vernalization is a crucial sensory mechanism to prevent flowering in the fall and increase flowering competence in spring after prolonged cold, and will not be discussed in detail here [see (Kim and Sung, 2014) for review]. Among the summer annual accessions of *Arabidopsis* from which many of the common laboratory strains are derived, photoperiod is the most important factor in dictating the timing of the floral transition (Song et al., 2010). It should be noted, however, that for the vast majority of winter annual *Arabidopsis* accessions, variation in flowering time predominantly is explained by variation in vernalization pathway components (Johanson et al., 2000; Michaels et al., 2003a; Lempe et al., 2005; Strange et al., 2011). While the concept of graded floral integrator expression and the input of multiple pathways in this response is useful, the discrete nature of many of these pathways has been called into question in recent years, and significant cross-talk between pathways is now known (Amasino, 2010). Throughout this review we will discuss the implications of the links between pathways on the architecture of the flowering time network.

PHOTOPERIODIC SENSORY MECHANISM

In *Arabidopsis*, photoperiodic information is specified through the interaction of the circadian clock and light, and both of these factors converge to regulate the expression and the activity of the CO transcription factor. The CO protein contains the B-box and CCT (CO, COL, and TOC1) domains, and is the core activator of *FT* in response to photoperiod (Strayer et al., 2000; Robson et al., 2001; Suárez-López et al., 2001; Khanna et al., 2009) (Figure 2-1). *CO* mRNA has a unique daily expression pattern that has a global minima in the morning, and has a maxima at night (Suárez-López et al., 2001). The control of the daily oscillation is a direct output of the circadian clock (Suárez-López et al., 2001). Additionally, under long day conditions (16h light/8h dark), there is a local maximum point of gene expression at ZT 16. It is this maxima that is responsible for a corresponding increase in *FT* activation, as CO protein activity is nullified in the dark (Laubinger et al., 2006). It is the coincidence of light with the local maximum in gene expression of *CO* in the late afternoon that is critical for the sensing of optimal long day conditions in *Arabidopsis* (Imaizumi and Kay, 2006). For this reason, the system of *CO* activity can be seen as a poised response in which the circadian clock determines the window in which a time dependent light signal can activate flowering. This system follows closely to the external coincidence model for photoperiodic phenomena, and remains a paragon example of a clock regulated developmental output (Pittendrigh, 1972; Kinmonth-Schultz et al., 2013).

CO TRANSCRIPTIONAL REGULATION

As mentioned, *CO* transcriptional regulation is highly dependent upon the circadian clock in order to set the pace for its oscillation. Repression of *CO* expression during the morning, which results in the daily global minimum, is mediated by the CYCLING DOF FACTOR (CDF) family of DOF domain transcription factors (Imaizumi et al., 2005). *CDF1* (At5g62430)*, CDF2* (At5g39660)*, CDF3* (At3g47500)*, CDF5* (At1g69570) expression is tightly regulated by the circadian clock, and show concurrent peaks of expression during the late night and morning (Fornara et al., 2009). *CDF* expression is a direct output of the circadian clock, and is activated through the action of the core clock component CIRCADIAN CLOCK

ASSOCIATED 1 (CCA1: At2g46830) and LATE ELONGATED HYPOCOTYL (LHY: At1g01060) MYB domain transcription factors (Schaffer et al., 1998; Wang and Tobin, 1998). *CDF* expression is repressed in the afternoon and evening through the action of PSUEDO RESPONSE REGULATORs (PRRs) PRR5 (At5g24470), PRR7 (At5g02810), and PRR9 (At2g46790) (Nakamichi et al., 2007; Ito et al., 2008). The functions of individual CDF proteins, CDF1, CDF2, CDF3 and CDF5, is largely additive and redundant in repression of the *CO* genomic locus (Fornara et al., 2009). DOF binding sites near the transcriptional start site in the *CO* promoter are composed of a short repetitive element, the copy number of which varies by ecotype (Rosas et al., 2014). The greater the number of CDF binding sites, the greater the sensitivity to photoperiodic inputs, as the peak and trough difference of *CO* expression is highest with additional copies of CDF binding sites (Rosas et al., 2014). CDF repression of *CO* is released in the afternoon through the action of a blue light induced complex composed of the GIGANTEA (GI: At1g22770) protein and the LOV domain blue-light photoreceptor E3 ubiquitin ligase FLAVIN BINDING KELCH REPEAT F-BOX 1 (FKF1: At1g68050) (Sawa et al., 2007). Both *GI* and *FKF1* mRNA expression is regulated by the circadian clock (Fowler et al., 1999; Mizoguchi and Coupland, 2000; Imaizumi et al., 2003; Imaizumi et al., 2005; Song et al., 2012a). This light induced complex targets CDF proteins for ubiquitin mediated proteasomal degradation through the F-box domain of FKF1, which removes the repressor from the *CO* promoter (Imaizumi et al., 2005). In addition to direct evidence, the combination of clock output and the activator potential of FKF1 through CDF degradation are sufficient to mathematically replicate the *CO* expression pattern (Salazar et al., 2009; Song et al., 2012a). In the future, additional modeling of *CO* transcription may be necessary for determining individual component contributions to specific transcriptional outputs.

FKF1 homologs, ZEITLUPE (ZTL: At5g57360) and LOV KELCH PROTEIN 2 (LKP2: At2g18915), degrade CDF2 protein, and also interact with other CDFs in yeast, although a comprehensive survey as to their role in CDF protein destabilization remains to be performed (Fornara et al., 2009). CDF2 protein, in addition to negative regulation through ZTL/FKF1/LKP2 family proteins, is also post-translationally modified with the SUMO ubiquitin-like peptide that is deposited by the SUMO-targeted ubiquitin ligase 4 (STUBL4: At1g66650) protein (Elrouby et al., 2013). It is currently unknown how the sumoylation of CDF2

or other CDFs might affect *CO* transcription in a time dependent fashion or whether CDFs are the target of other post translational modifications which might affect their activity.

Upon CDF protein removal, the FLOWERING BHLH (FBH1: At1g35460, FBH2: At4g09180, FBH3: At1g51140, and FBH4: At2g42280) group of bHLH transcription factors promotes the activation of *CO* transcription. FBH family members are able to bind to E-box elements within 5' region of the *CO* promoter (Ito et al., 2012b). The combination of CDF removal and FBH activation allows for the local maxima of *CO* expression in the afternoon of long days important for *FT* activation and the promotion of flowering under inductive conditions (Ito et al., 2012a). To date FBH's are the only known direct activators of *CO* transcription, it remains to be examined whether other factors are critical for *CO* transcriptional activation or what factors are responsible for time dependent activation of *CO* expression during the night of long and short days (Ito et al., 2012b).

CO POSTTRANSLATIONAL REGULATION

LIGHT QUALITY EFFECTS ON CO PROTEIN

In addition to the requisite accumulation of an abundance of *CO* transcripts and CO protein to the afternoon of long days, the relative activity and stability of CO protein is critical for its function in the afternoon induction of *FT*. CO protein stability changes under a variety of external light conditions, and external light quality inputs are important factors through which CO protein activity is regulated (Valverde et al., 2004) (Figure 2-2). Generally, CO is stabilized under blue and far-red light, and destabilized under red light and darkness (Valverde et al., 2004). Under blue light conditions, FKF1, in addition to its described role in CDF degradation, also stabilizes CO protein (Song et al., 2012a). Exactly how FKF1 stabilizes CO protein at the molecular level remains unknown.

In darkness, the CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1: At2g32950) and SUPPRESSOR OF PHYA-105s (SPA1: At2g46340, SPA2: At4g11110, SPA3: At3g15354, and SPA4: At1g53090) form a protein complex which actively degrades CO through ubiquitin mediated proteasomal degradation (Jang et al., 2008) (Figure 2-2). Inhibition of CO protein by COP1/SPAs prevents CO protein

produced at night from activating *FT* expression, further constraining the window of *FT* expression to the late afternoon (Laubinger et al., 2006; Jang et al., 2008). In order to limit the inhibitory effect of COP1/SPAs complex on CO protein during the day time, blue light activated and phosphorylated CRYPTOCHROME 2 (CRY2: At1g04400) photoreceptors bind to SPA1 protein and abolish their antagonistic activity on CO; thus, blue light can stabilize CO through two independent mechanisms, late afternoon stabilization by FKF1 and prevention of COP1/SPAs interference by CRY2 (Pokhilko et al., 2011; Zuo et al., 2011). The phytochrome photoreceptor PHYA (At1g09570) stabilizes CO protein under far red light enriched conditions (Valverde et al., 2004) (Figure 2-2). PHYB (At2g18790) has an antagonistic role to PHYA in CO protein stabilization, as CO protein is stabilized in *phyB* mutants (Valverde et al., 2004) (Figure 2-2). PHYTOCHROME DEPENDENT LATE FLOWERING (PHL: At1g72390), an unknown domain protein, is also involved in stabilization of CO protein, but the stabilizing effect it mediates is absent in *phyB* mutants (Endo et al., 2013). Molecular evidence suggests that PHL may be involved in sequestration of CO away from the inhibitory effects of PHYB dependent destabilization (Endo et al., 2013) (Figure 2-3). Taken as a whole, light dependent regulation of CO protein stability is critical for its function, but there is little evidence yet as to how light quality effects that are found in nature might affect the flowering time response; i.e. whether spectral qualities of long days or short days found under natural conditions have real, quantitative effects on flowering time. This is an important aspect of the photoperiodic response that remains to be analyzed in greater detail, in particular to determine the sum of mutual effects of photoreceptors on flowering time regulation.

TEMPERATURE AFFECTS ON CO PROTEIN STABILITY

In addition to light dependent regulation of CO protein, ambient temperature can also affect its stability. A RING-finger E3 ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1: At2g39810) negatively regulates CO protein accumulation during the morning, through binding of CO and targeting it for degradation by the proteasome (Lazaro et al., 2012). CO degradation by HOS1 is both time and temperature dependent. HOS1 dependent degradation of CO occurs primarily during the morning (Lazaro et al., 2012). CO degradation by HOS1 is increased under low temperature

conditions (4 degrees) (Lee et al., 2012). Due to HOS1 genetic relationship with PHYB signaling, it is possible that PHYB connects both low temperature and red light signaling in order to negatively regulate CO protein stability (Lee et al., 2012). At increased temperature, *co* mutants flower appreciably earlier compared to normal growth conditions, which suggests that *FT* activation under higher temperatures is likely not caused by increased stability of CO protein, but through other mechanisms (Kumar et al., 2012). Thus control of CO protein stability is broadly regulated by a combination of photoperiod, light signaling and ambient temperature (Figure 2-2).

FT TRANSCRIPTION

FT is critical in signaling for the transition into flowering, as a floral integrator and the primary signal through which the sensory machinery in the leaf vasculature is linked with the shoot apex (Andrés and Coupland, 2012). Many factors have been found to regulate its expression; it is through the action of these many factors that an incremental response to optimal environmental conditions can push or delay the floral transition (Schwartz et al., 2009).

ACTIVATION OF *FT* TRANSCRIPTION IN RESPONSE TO PHOTOPERIOD

Primary activation of *FT* in the late afternoon of long day conditions is accomplished through the action of CO protein (Kobayashi et al., 1999; Onouchi et al., 2000; Yoo et al., 2005). This activation occurs through two mechanisms of CO, the first being direct DNA binding to CO-responsive elements (CORE) in the *FT* promoter (Tiwari et al., 2010); and the second being the recruitment of additional proteins that compose a CO activator complex to assist in transcriptional activation. CO is able to recruit and form a protein complex with ASYMETTRIC LEAVES 1 (AS1: At2g37630) (through its B-box domain), as well as members of the transcriptional co-activator family NUCLEAR FACTOR Y (NF-YA1: At5g12840, NF-YB1: At2g38880, NF-YC1: At3g48590) (through its CCT domain) to CCAAT elements within the *FT* promoter (Ben-Naim et al., 2006; Kumimoto et al., 2008; Song et al., 2012b). Incremental increases in *CO* expression directly correlate with a corresponding increase in *FT* expression and an acceleration of

flowering time (Putterill et al., 1995). *CO* is a member of the plant specific B-box transcription factor family and is the primary activator of flowering among the homologues. *CO* is closely related to the CONSTANS LIKE 1 (COL1: At5g15850) COL1-5 subclade and recent domain replacement experiments have determined that the B-box domain differences between the proteins is essential for CO function (Hassidim et al., 2009; Khanna et al., 2009; Kim et al., 2013b). Replacement of the CO B-box domain into the COL1 and COL2 (At3g02380) proteins caused earlier flowering than wild type COL1 or COL2 protein resulting from increased *FT* transcription, which suggests that it is the B-box domain that is critical for CO dependent flowering function (Kim et al., 2013a). Several *COL* genes have been implicated in other environmental responses such as cold acclimation and light signaling so it could also be that COL proteins have regulatory roles that have not been activated under standard laboratory conditions (Hannah et al., 2005; Datta et al., 2006). Another B-box domain transcription factor B-BOX DOMAIN PROTEIN 19 (BBX19: At4g38960), which is regulated by the circadian clock, is able to interact with CO during the morning when its own expression is high, and to nullify CO activation of *FT* through interactions with the B-box domain of CO. BBX19 thus is able to further constrain the window of CO activity through depletion of any morning expressed CO protein activity (Wang et al., 2014). Under short day conditions the activity of CO protein is largely attenuated, likely through the action of BBX19 and the diminished presence of FKF1 during the light period. The instability of CO protein in the absence of light activated FKF1 and the presence of active COP1/SPAs abolishes CO protein activity and prevents *FT* transcription.

CLOCK DEPENDENT REPRESSION OF *FT* TRANSCRIPTION

In addition to clock regulation of *CO* transcription and protein stability, several clock-regulated factors contribute to repression of *FT* transcription. CDF transcription factors, in addition to their binding to the *CO* promoter during the morning, are able to bind to the *FT* promoter at the 5' proximal region around the transcription start site (Song et al., 2012a). Presumably, the same regulatory mechanism for CDF removal by light activated GI-FKF1 complex is able to free the *FT* promoter of repressive activity so that CO activation can be completed under inductive long day conditions. Two members of the APETALA 2 / ETHYLENE RESPONSE FACTOR family of transcription factors TEMPRANILLO 1 (TEM1: At1g25560)

and TEM2 (At1g68840) are able to repress *FT* transcription (Castillejo and Pelaz, 2008); TEM1 binds directly to the proximal 5' region of the *FT* promoter to repress its transcription. *TEM1* transcript abundance peaks around dusk in long day conditions concurrently with *CO* expression, and the circadian clock regulates the oscillation of its expression, as its oscillation perpetuates upon transfer in continuous light (Castillejo and Pelaz, 2008). Due to its daily timing, TEM1 and TEM2 may represent a brake on the activity of *CO* activation, where the pendulum shift between both can balance the *FT* transcriptional response. In addition to their regulation of *FT* transcription, TEMs are able to regulate flowering time through the gibberellin hormone signaling pathway through the repression of *GA-3 OXIDASE1* (*GA3OX1*: At1g15550) and *GA3OX2* (At1g80340), both of which encode biosynthetic enzymes that convert inactive GA hormone in to biologically active GA_4 (Osnato et al., 2012). GI also interacts with TEMs in tobacco, and this interaction may change TEM activity (Sawa and Kay, 2011). Thus TEM proteins serve as a point of integration between the day length dependent and independent regulation to modify the flowering output.

In addition to clock-regulated outputs, many basic questions about the direct regulation of circadian clock components also remain undetermined due to the enmeshed nature of the circadian network. *CCA1* and *LHY* overexpression, for instance, results in a late flowering phenotype, yet it is unknown whether the effect of the overexpression is due to corresponding increase in *CDF* expression, reduced *GI* expression, a combination of both, and/or the regulation of other clock components (such as *PRRs*) or other outputs (Wang and Tobin, 1998; Park et al., 1999; Nakamichi et al., 2007). In short, genetic analyses of phenotypic effects on circadian clock outputs could be the result of many parallel interactions, and may require modeling approaches in order to disentangle the effects of multiple inputs.

LIGHT DEPENDENT REGULATION OF *FT* TRANSCRIPTION

Photoperiod and clock dependent inputs are critical for mediating the time dependent regulation of *FT* transcription, and maintaining a window for *FT* activation so that long days can successfully initiate the floral transition. Similar to CO protein regulation, input of light quality through the action of photoreceptor proteins is important for modulation of *FT* transcription (Figure 2-3). Members of the

CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP HELIX (CIB) family of bHLH transcription factors activate *FT* expression (Liu et al., 2008). CIB1 (At4g34530), CIB2 (At5g48560) and CIB5 (At1g26260) form functional complexes with CRY2 *in vivo* and at least CIB1 directly binds to the *FT* promoter via E-box elements in the 5' proximal region to activate *FT* transcription (Liu et al., 2008; Liu et al., 2013). In addition to activation by CRY2, CIB protein abundance is positively affected by ZTL and LKP2, as CIB1 protein is unstable in *ztl* mutants (Liu et al., 2013). This suggests that an integration of positive blue light activating signals occurs to affect flowering time response (Figure 2-3). Thus, blue light inputs on *FT* are doubly activating through the degradation of CDF proteins by FKF1 and subsequent activation by CIB proteins (Imaizumi et al., 2005; Liu et al., 2013) (Figure 2-3). At present it is unclear if CIB transcription or its activity are regulated by the circadian clock; indirect regulation through ZTL and LKP2 covers most of the daytime of long and short days, so the time dependent nature of CIB activity in relation to other regulatory mechanisms still needs to be determined. Presumably due to CDF repressive activity, the activation of *FT* through CIBs occurs mainly in the afternoon of long days, as CIB1 overexpressing plants have a greatly enhanced peak of *FT* expression in the late afternoon, but similar levels of *FT* expression to wild type in the morning (Liu et al., 2008).

Contrary to red light's role in CO protein stabilization, VASCULAR PLANT ONE ZINC FINGER 1 (VOZ1: At1g28520) and VOZ2 (At2g42400), two NAC (NAM, ATAF1/2 and CUC2) domain transcription factors, activate *FT* expression under long day conditions (Yasui et al., 2012). VOZ proteins interact with PHYB, and like PHYB, their movement between the cytoplasm and the nucleus is tightly regulated. As *voz1,2* double mutants have elevated expression of the MADS-BOX *FT* repressor FLOWERING LOCUS C (FLC: At5g10140), it remains to be determined whether activation of *FT* by VOZ proteins is direct, indirect, or both (Yasui et al., 2012) (Figure 2-3). VOZ1 also regulates the expression of the nuclear pore protein MODIFYER OF SNC1 (MOS3/SAR3: At1g80680) a protein known to be involved in flowering time regulation and which is required for the movement of mRNA out of the nucleus (Celesnik et al., 2013). This suggests that control of transcript export of flowering components may be important in the regulation of flowering time or a mechanism through which photoreceptors can post-transcriptionally regulate the flowering response.

TEMPERATURE DEPENDENT REGULATION OF *FT* EXPRESSION

Regulation of flowering time through vernalization in winter annual *Arabidopsis* coordinates strict control of flowering, directly shutting down *FT* expression until long periods of cold have been attained. In contrast to the responses to drastic change in winter to spring temperature, ambient temperature change during spring can affect the flowering time response. A regulation of *FT* expression in response to shorter-term temperature change has become apparent. Several members of the MADS-box binding transcription factor family are critical in mediating this response: FLC and its homologues FLOWERING LOCUS M/ MADS AFFECTING FLOWERING 1 (FLM/MAF1: At1g77080), MAF2-5, and another MADS-box protein SHORT VEGETATIVE PHASE (SVP: At2g22540) are floral repressors which bind to CArG motifs in the *FT* and *SOC1* promoters and repress transcription (Michaels and Amasino, 1999; Scortecci et al., 2003; Helliwell et al., 2006). As MADS box DNA binding TFs form homo or heterodimeric complexes, posttranslational regulation through the formation of different complexes has emerged as an important thermosensory mechanism (Song et al., 2013). Interestingly, *FLM* transcripts are alternatively spliced in response to temperature. Two splice variants are produced FLM-ß and FLM-∂ under low and high temperatures, respectively (Figure 2-3). Under low ambient temperature conditions (16), the FLM- ß splice variant and SVP form a functional complex that can inhibit *FT* and *SOC1* expression (Lee et al., 2013; Pose et al., 2013). Under high ambient temperatures above 22 degrees, FLM-∂ and SVP complexes have impaired DNA binding potential and are not able to repress *FT* and *SOC1* transcription, leading to an increase in expression and an acceleration of flowering under higher temperatures (Lee et al., 2013; Pose et al., 2013). In terms of the daily timing of this regulation, though a comprehensive survey has not been performed, an increase in *FT* transcription at only ZT 16 in *flm maf3* double mutants suggests that their primary effect on *FT* expression occurs in the afternoon (Gu et al., 2013). Regulation by these complexes is likely also developmentally specific, as complex formation between SVP and FLC occurs earlier in development (7 day old vs 11 day old) (Li et al., 2008). This reinforces the idea that these MADS-box proteins are functioning as a modulator to try to stop the floral transition from happening too early in development, but to allow for flowering if thermal conditions are optimal.

How does low ambient temperature induction of MADS domain transcription factors occur? At least at *FLC*, the autonomous pathway protein FVE (At2g19520) (a retinoblastoma related protein) is able to physically associate with the histone deacetylase complex component HISTONE DEACETYLASE 6 (HDA6: At5g63110) and confer repressive activity upon the *FLC* chromatin (Ausin et al., 2004; Kim et al., 2004; Jeon and Kim, 2011; Pazhouhandeh et al., 2011). Thus, FVE mutants are late flowering due to the increase in *FLC* transcription. In response to lower temperatures HOS1 is able to form a protein complex with FVE and HDA6 and antagonize their repressive effect at the *FLC* genomic locus, causing an increase in *FLC* transcription and later flowering (Gu et al., 2011). Correspondingly, *hos1* mutants show a decrease in *FLC* transcription, which increases *FT*. HOS1 thus represents a dual factor in inhibiting flowering under lower temperatures both through increasing *FLC* transcription and through CO protein degradation. HOS1 also has a role in the regulation of cold responses, so its position in the network may be closest to cold input independent of the vernalization dependent sensing of cold temperatures (Figure 2-3).

In addition to its regulation of *FT* and *SOC1*, SVP binds to the promoters of several upstream components in multiple flowering pathways. Among the photoperiod pathway, SVP binds to the *GI* and *PRR7* promoters (Gregis et al., 2013) SVP also binds to several pathway components in the autonomous pathway and in members of the polycomb repressive complex 2 (PRC2), which suggests that SVP confers regulatory adjustments in response to temperature at multiple intersecting points in the flowering time pathways (Gregis et al., 2013). SVP regulation in these instances may be context dependent; SVP negatively regulates the ability of *GI* to accelerate flowering in response to drought through an ABA-mediated response (Riboni et al., 2013). Examples such as this suggest that SVP or other regulators may preferentially regulate flowering at different points in the pathway under different environmental conditions, which implies that untested environmental parameters that shape flowering time may change the flux of the flowering network at different points (through both upstream and downstream effects). Further work needs to be done to further clarify SVP's role in this regard.

Under high ambient temperature conditions, another important regulator of the acceleration of flowering is the bHLH transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4: At2g43010) (Kumar et al., 2012) (Figure 2-3). Under short day conditions, ambient temperature increase

to 27 degrees causes early flowering. In *pif4* mutants, flowering under 22 and 27 degree conditions occurs at the same time, as the plants are unable to respond to higher temperatures (Kumar et al., 2012). PIF4 directly binds to the *FT* promoter to activate transcription, but its binding site accessibility is strongly inhibited in the presence of the nucleosomes containing the H2A.Z histone variant (Kumar et al., 2012) (Figure 2-4). As with SVP dependent regulation, components of the chromatin regulatory machinery may play an active role in the activity of PIF4 to regulate *FT* under higher temperatures. H2A.Z deposition is also critical in mediating *FLC* repression, so the addition of H2A.Z histone variants is capable of regulating *FT* in through direct (*FT* through PIF4) and indirect means (*FT* though FLC) (Kumar et al., 2012). As *PIF4* expression is also regulated by the GA hormone pathway, PIF4 may serve as an important integrator between temperature dependent PHYB mediated responses as well as the developmental competency of the plants to flower (Nomoto et al., 2012b; Nomoto et al., 2012a).

Ambient temperature regulation through the circadian clock is another input point that may affect the floral transition. In particular *early flowering 3 (elf3*: At2g25930*)* mutants are insensitive to the delay of flowering times that takes place at 16 degrees (Strasser et al., 2009). ELF3, along with EARLY FLOWERING 4 (ELF4: At2g40080) and LUX ARRYTHMO (LUX: At3g46640), forms the “evening complex” (EC), a protein complex that regulates the expression of *PIF4* and *PIF5* to constrain the hypocotyl elongation response to the early evening (Nusinow et al., 2011). This circadian gating mechanism additionally serves to regulate *PIF4* expression in response to changes in ambient temperature (Figure 2-3) (Mizuno et al., 2014). The EC is normally able to repress *PIF4* expression in lower ambient temperatures, but transcription of *PIF4* increases as EC activity is attenuated under higher temperature conditions (Mizuno et al., 2014). ELF3 also acts as a repressor of *PRR9* expression, and thus could act in a temperature dependent manner by increasing CDF expression to repress flowering under low ambient temperature conditions (Dixon et al., 2011). Additionally, modulation of the core clock by temperature may also affect the flowering time response. CASEIN KINASE II BETA CHAIN 3 (CKB3: At3g60250) is involved in temperature dependent modulation of the circadian clock period, and preferentially phosphorylates CCA1 and LHY (Sugano et al., 1998; Daniel et al., 2004). Overexpression of a regulatory b-subunit of *CKB3* shortened the period length of clock and induced earlier flowering in both long days and short days (Sugano et al., 1999). This temperature dependent circadian effect on

flowering could be through multiple points of clock output, and more testing will be needed to tease apart clock dependent effects on ambient temperature signaling.

ENDOGENOUS FACTORS AFFECTING *FT* EXPRESSION

In addition to exogenous, environmental inputs in the modulation of flowering time through *FT* transcription, several endogenous cues are also important in determination of the floral transition, to sense the developmental competency and resources of the plant to be able to fully commit to flowering. This explains why flowering occurring directly subsequent to photomorphogenesis is a rare phenomenon.

THE AGE DEPENDENT FLOWERING PATHWAY

A separate pathway that is primarily regulated through the actions of miRNAs brings upon sensing of juvenility and its influence on *FT* and other floral integrator expression (Figure 2-3). Concomitant with GI's position within many parts of the flowering pathway, GI positively regulates the expression of a miRNA *miR172* under long day conditions, while SVP negatively regulates its expression (Jung et al., 2007; Cho et al., 2012). *miR172* reduces the transcript abundance of the AP2/ERF family regulators *SCHAFLMÜTZE* (*SMZ*: At3g54990), *SCHNARCHZAPFEN* (*SNZ*: At2g39250), *TARGET OF EAT 1* (*TOE1*: At2g28550), and *TOE2*: At5g60120, which are upregulated in juvenility to prevent the floral transition from moving onward too quickly (Aukerman and Sakai, 2003; Schmid et al., 2003; Mathieu et al., 2009). These AP2 family transcription factors bind to the 3' region of the *FT* gene in order to repress transcription (Mathieu et al., 2009) (Figure 2-4). *miR156* has an opposite role in the flowering time response. *miR156* targets members of the S*QUAMOSA PROMOTER BINDING LIKE* (*SPL*) family of transcription factors *SPL3* (At2g33810) and *SPL9* (At2g42200), which positively regulate the expression of other floral integrator genes such as *SOC1*, *AGL24, FUL*, *AP1* and *LFY* at the shoot apex (Wang et al., 2009) (Jung et al., 2011; Yu et al., 2012; Wang, 2014). *miR156* transcripts decrease in abundance as developmental time progresses, opposite that of *miR172* (Wu et al., 2009). How the development-dependent expression of *miR156* is regulated is currently unknown. The combined action of these two

miRNA dependent pathways allows for the increase of expression of *FT* in leaves, and increase in other floral integrators at the shoot apex as the plants go through development, while acting as a stopgap to prevent flowering in the absence of resources required to form floral organs and an inflorescence. Interestingly, the action of this pathway appears critical in several species for the maintenance of longer juvenile periods in perennials, and adds an additional layer through which modulation of flowering can occur within optimal seasonal conditions (Bergonzi and Albani, 2011).

FT CHROMATIN STRUCTURE

Due to the large array of factors that serve as inputs into *FT* expression, an intriguing question has been how all of these diverse factors interact and recruit or repel basal transcriptional machinery within the space of the *FT* promoter region. Particularly, how the composition and abundance of separate complexes, regulated by combinatorial changes in environment, dynamically change on a spatial level in response to a given set of stimuli. While the pace of the field is quickly speeding up, we are currently limited in scope with the ability to fully answer these questions. Still we have gained several clues into the structural constraints on *FT* transcription. Firstly, we know that components of the Polycomb repressive complex 2 (PRC2), whose role in floral regulation has already been well characterized in the vernalization response, are involved in *FT* transcriptional regulation (Turck et al., 2007; Farrona et al., 2011). Mutations in several components, including the SET domain protein CURLY LEAF (CLF: At2g23380), EMBRYONIC FLOWER 2 (EMF2: At5g51230), LIKE HETEROCHROMATIN PROTEIN 1/ TERMINAL FLOWER 2 (LPH1/TFL2: At5g17690) and FERTILIZATION INDEPENDENT ENDOSPERM 1 (FIE1: At3g20740) result in a pronounced decrease in the accumulation of the repressive histone mark H3K27me3 throughout the *FT* promoter region, and several of these components physically associate with *FT* chromatin in ChIP assays (Takada and Goto, 2003; Saleh et al., 2008; Adrian et al., 2010; Farrona et al., 2011; Shafiq et al., 2014). Corresponding to their role as repressors of *FT* transcription through interactions with chromatin, many of the mutants of this complex are early flowering. Loss of function mutations in *clf* in particular are coupled with an enormous increase in *FT* transcription (Shafiq et al., 2014). This evidence, coupled with the fact that the required minimal promoter fragment to replicate wild

type *pFT:GUS* expression is a large 5.7kb fragment, suggests that stable repression and or maintenance of a section of condensed chromatin around the *FT* locus is important for proper expression. Currently, it has been hypothesized that H3K27me3 deposition occurs throughout much of the *FT* promoter, with accessible regions to transcription factors to enable proper *FT* induction (Andrés and Coupland, 2012). There also may be a developmental component however, as *pFT:GUS* expression in *lhp1* mutant plants greatly expanded the range of *FT* expression in vascular tissues (Adrian et al., 2010). This evidence suggests that the domain of *FT* expression in the phloem companion cells to the distal part of the leaf may be controlled through LHP1 (Adrian et al., 2010; Farrona et al., 2011). The functional relevance of the domain of *FT* expression compared to the total amount of *FT*, however, has yet to be looked at in great detail.

In addition to the repressive machinery which affects *FT* chromatin status, components of the mediator complex which acts in transcription initiation is important for control of flowering time (Figure 2-4). PHYTOCHROME AND FLOWERING TIME 1 (PFT1: At1g25540) the MED25 subunit of the mediator complex, is critical for transcription of several photoperiodic flowering components and acts downstream of phytochrome photoreceptors (Elfving et al., 2011; Inigo et al., 2012b). Genetic evidence suggests that PFT1 serves an activating role both in *CO* transcription as well as *FT* transcription, through independent mechanisms (Inigo et al., 2012b). At least at *FT*, PFT1 is degraded by the proteasome through the action of two interacting proteins MED25-BINDING RING-H2 PROTEIN1 (MBR1: At2g15530) and MRB2 (At4g34040) (Inigo et al., 2012a). Unexpectedly, degradation of PFT1 is important for activation of *FT*, as inhibition of PFT1 degradation prevents *FT* activation. MED18 (At2g22370) also is involved as an activator of *FT* expression, although the possibility of a regulatory mechanism similar to MED25 has not been investigated (Zheng et al., 2013). The tailoring of specific effects of *FT* expression modulated by the PolII holoenzyme suggests that the basal transcriptional machinery and/or the structure of the activator complexes required for *FT* transcription are important in determining the flowering output. At this point whether or not repressive machinery at the chromatin level inhibits the conformation of the larger *FT* activation complex is still a largely open question, but it will be interesting to try to piece together how the activation machinery might conform around the *FT* genomic locus.

Presently lacking, in addition, is whether or not time dependent (within a day) or photoperiod dependent (long day vs. short day) changes in *FT* locus structure occur. At least in long days, recent work has further elucidated the role of CO activation within the chromatin structure of the *FT* promoter (Figure 2-4). CO, together with its transcriptional cofactors NF-YA, NF-YB and NF-YC, associates with the *FT* promoter regions that contains 2 CCAAT sequences, one at -5.3kb position and a second at the -2kb position upstream of the *FT* transcriptional start site (Ben-Naim et al., 2006; Adrian et al., 2010). Using chromatin conformational capture experiments to investigate whether loops in chromatin structure form in order to bring CO activator complexes directly to the transcriptional start sites, a double loop in the *FT* chromatin structure forms (Cao et al., 2014). This conformation is analogous to the chromatin organization that can typically be found between transcriptional start sites and distal enhancer elements. Because CO protein is unlikely to be found on the *FT* promoter under short day conditions, this conformation is likely to only take place under inductive long day conditions (Cao et al., 2014). Overexpression of CO also removes LHP1 binding to the 5' proximal region, so CO recruitment may serve as an additional mechanism with which to allow other factors to exert influence over *FT* transcription (Adrian et al., 2010). Still also unresolved is how other proteins, either activators or repressors, interact within this structural framework either at the transcriptional start site or in the 3' region (SMZ, SNZ, TOE1 and TOE2), and particularly how these change under different environmental parameters.

PHOTOSYNTHETIC STATUS

In addition to developmental age, carbohydrate status has long been implicated in the flowering time response (Evans, 1971). Though this area has remained largely unexplored, recent evidence has implicated photosynthetic output as an effect on flowering time (Wahl et al., 2013). Since changes in light intensity or the application of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], (a chemical inhibitor of photosynthesis) effects both *FT* transcription as well as the pace of the circadian clock (Corbesier et al., 1998; Corbesier et al., 2002; King et al., 2008; Haydon et al., 2013). Mutations in the gene that encodes the biosynthetic enzyme TREHALOSE-6-PHOSPHATE SYNTHASE 1 (TPS1: At1g78580), which

produces Trehalose 6-phosphate (T6P) a photosynthetic byproduct, have greatly impaired *FT* expression (Wahl et al., 2013). Although the mechanism for *FT* activation by T6P remains unknown, it appears that its activator activity may act in a photoperiod dependent manner to sense optimal carbohydrate status in long days prior to flowering (Wahl et al., 2013) Presumably, the signal for T6P must be transduced or translocated from mesophyll cells into the phloem companion cells, where *FT* transcription occurs, but how this occurs is also unknown. In addition to *FT* transcriptional changes, transcription of *SPL* family genes is reduced in the *tps1* mutant background, partially due to an increase in *miR156* levels early in development (Wahl et al., 2013). This new information implies that photosynthetic status may act at several levels of the flowering response to provide endogenous input as to whether or not to commit to flowering. Though it has not been determined in detail, it would be interesting to see if new leaves acting as carbon sink in relation to mature leaves are able to contribute to *FT* production equally compared to each other, as this has implications for growth effects on the competency to flower.

FT MOVEMENT

FT protein, once synthesized in the phloem companion cells of leaves, acts as a mobile signal that translocates to the shoot apex where downstream interactions are able to specify the transformation of the shoot apical meristem into an inflorescence meristem (Figure 2-5). As such, FT represent the long sought florigenic signal that links the leaf sensory mechanism with the floral conversion, and is a conserved mechanism for floral initiation throughout the angiosperm lineage (Andrés and Coupland, 2012). FT movement has been difficult to characterize, and indeed considerable effort has been taken to prove that it is indeed FT protein and not *FT* mRNA that constitutes the mobile signal after conflicting early reports (Corbesier et al., 2007; Jaeger and Wigge, 2007; Lin et al., 2007). Because of technical issues surrounding the size exclusion limit of tagged FT protein leaving through the plasmodesmata or through active transport into the phloem stream and the difficulty in obtaining enough phloem sap for molecular analysis, many of the pioneering experiments performed so far have occurred in *Cucurbita moschata* (Lin et al., 2007). Based on these experiments, FT likely moves into the phloem stream from the phloem companion cells by simple diffusion. Its uptake, however, into the shoot apical meristem

appears to require an active transport mechanism. Mutations in several *FT* mutants which attenuate the ability of its transport into the shoot apex move into the phloem stream by diffusion freely, which suggests that other factors are required to channel FT protein towards the shoot apex (Liu et al., 2012; Yoo et al., 2013a; Yoo et al., 2013b). Several candidates for involvement in the active transport of FT into the shoot apex were identified, but the order and operations at the molecular level that is required for FT movement remain unresolved (Liu et al., 2012; Yoo et al., 2013a). Structural analysis of mutagenized protein identified two residues in close proximity near the N-terminal part of the FT protein which appear critical for mediating FT protein import into the shoot apex, as mutations at these sites expressed under the vascular specific *SUCROSE SYMPORTER2* (*SUC2*: At1g22710) promoter have opposite effects of those expressed under TFL1 promoter fragments, suggesting that the mutated protein cannot be moved out of the vasculature and into the shoot apex (Ho and Weigel, 2014).

FT is an phosphatidylethanolamine (PE)-binding protein (PEBP) similar to those sound in mammals, and binds to phospholipids as has been anticipated due to its similarity to animal orthologous of the protein (Nakamura et al., 2014). Many phospholipids present within cells at the shoot apex show a diurnal oscillation of phospholipid abundance, several of which correlate with the peak of daily expression of FT. Phospholipid binding to residues on parts of the FT protein surface may also be important in the transport of FT into the nucleus of shoot apical cells once it arrives there, possibly through vesicular trafficking (Nakamura et al., 2014). In total, this now suggests that both active transport into the shoot apex as well as subcellular trafficking into the nucleus is an important part of FT protein regulation in the initiation of flowering.

INTERACTIONS AT THE SHOOT APEX

A balance of factors ensures that during juvenile development in *Arabidopsis*, the shoot apical meristem is committed only towards vegetative production. This vegetative cell fate is specified through the action of the FT related protein TERMINAL FLOWER 1 (TFL1: At5g03840), which acts antagonistically to FT protein in shifting the balance towards or away from reproductive development (Ruiz-Garcia et al., 1997; Abe et al., 2005; Taoka et al., 2011; Jaeger et al., 2013). When FT protein

arrives at the shoot apex, it forms of protein complex with the bZIP transcription factor FD (At4g35900), which is facilitated through docking with the arms of the 14-3-3 (At4g09000) adapter protein (Abe et al., 2005; Taoka et al., 2011). This FT-FD-14-3-3 activator complex then interacts with the promoters of *LFY* and *AP1* whose expression locks the shoot apical meristem into an inflorescence cell fate (Jaeger et al., 2013) (Figure 2-5).

TFL1 and FT proteins are highly conserved throughout flowering plant evolution and still retain very closely related protein structure. In contrast to *FT*, *TFL1* is only expressed at the shoot apex (Bradley et al., 1997). Because of their similar structure, the mechanism through which TFL1 acts as an inhibitor of FT mediated floral conversion is as a competitor for FD-14-3-3 docking, which is rendered non-functional upon complex formation with TFL1 (Ho and Weigel, 2014). Structural analysis through randomized mutagenesis of FT protein found several regions of critical importance for FT function, as well as the conversion of FT protein into one with TFL1-like function in the regulation of flowering time (Hanzawa et al., 2005). It appears that two regions in particular are crucial, one is a potential ligand-binding pocket which is now thought to mediate protein-protein interactions with a members of the TEOSINTE BRANCHED 1/CYCLOIDEA/ PCF transcription factor family. In particular the residues that control the surface charge of the area around the outside of the ligand-binding pocket appear important for function (Ho and Weigel, 2014). Mutations at these sites specifically affected the binding to a subset of TCP transcription factors, as FD and 14-3-3 *in vivo* binding is unaffected by these mutations (Ho and Weigel, 2014). In addition, mutations in an area outside of the ligand-binding pocket on FT, in an external loop of an adjacent alpha helix, are also sufficient to convert FT into a TFL1-like protein. This effect is likely due to interference of these new residues that are normally required for additional protein-protein interactions (Ho and Weigel, 2014).

Another important series of interactions that occurs at the shoot apex is the balance between FT, TFL1, and LFY and AP1 transcription factors. FT and FD together activate the expression of AP1 and LFY, and also LFY and AP1 positively regulate the expression of each other (Liljegren et al., 1999) (Figure 2-5). LFY additionally activates *FT* expression in the shoot apex. TFl1 and FD act antagonistically to all of these interactions by repressing the expression of LFY and AP1 (Figure 2-5). Modeling of this interaction at the shoot apex has determined that accumulation of FT via transport from the leaves is what

enables this highly buffered positive feed back loop to push the network into a net floral committed one through reinforcing expression of AP1 and LFY (Jaeger et al., 2013). Because the inflorescence of *Arabidopsis* is indeterminate, the inflorescence meristem must maintain some vegetative cell-like properties and for this purpose, *TFL1* expression in the center mass of the inflorescence meristem is still required (Andrés and Coupland, 2012). On the flanks of the new meristem, the efforts of AP1-LFY feed forward loop is able to initiate the formation of floral primordia from which sexual organs will form, thus patterning the new floral tissues of the inflorescence (Figure 2-5).

In addition to the FT-TFL-AP1-LFY module, SPL3, SPL4 (At1g53160), and SPL5 (At3g15270) act as activators of flowering at the shoot apex. *SPL*s expression is induced by photoperiod, and they are negatively regulated through the actions of *miR156* (Wang et al., 2009; Wu et al., 2009). In addition to age dependent regulation by *miR156*, the net effect of which is increasing *SPL* expression with plant age, *SPL*s are negatively regulated by the DELLA proteins REPRESSOR OF GA1-3 1 (RGA: At2g01570), GA INSENSITIVE (GAI), RGA-LIKE 1 (RGL1: At1g66350), and RGL2 (At3g03450) (Yu et al., 2012). This convergence point serves to integrated age and GA pathways to regulate the floral transition. In addition to regulation of Inflorescence meristem (IM) specification, SPLs and GA also play a role in the IM to floral meristem (FM) transition, as mutations in either pathway show aberrations in the development of the IM during bolting (Yamaguchi et al., 2014). Feedback between these pathways, both the floral specification genes and their downstream targets integrate all of the factors including photoperiod, age, GA, light quality, and temperature to bring about the formation of the inflorescence.

MAINTENANCE OF INFLORESCENCE ORGAN IDENTITY?

Even after the inflorescence has become specified, additional inputs from flowering pathway components are required to prevent the reversion of the inflorescence back into a vegetative structure. Mutations in members of the PRC2 complex *CLF, EMF2,* and *SWINGER* (*SWN*: At4g02020) result in an increase in *FLC* expression in the inflorescence (Muller-Xing et al., 2014). This increase in *FLC* expression occurs concomitantly with a rise in *SVP* expression, the complex of which facilitates repression of *FT* in newly formed tissues in the inflorescence. The loss of *FT* in these tissues is enough to

prevent the continued production of floral organ identity and reverts the inflorescence back into a vegetative state (Muller-Xing et al., 2014).

Many of the aforementioned regulators of *FT* expression have effects on inflorescence architecture, branching, and phyllotaxy, and relatively few of them have been characterized in detail. For example TCP transcription factors often have branching phenotypes in *Arabidopsis*. BRANCHED 1 (BRC1: At3g18550) for instance is involved in the suppression of axillary meristems at the shoot apex, though interactions with FT that suppress the ability of FT to promote axillary meristem formation (Niwa et al., 2013). Because FT and TCP transcription factors interact at the shoot apex in inflorescence meristem specification, their interaction may have important consequences for phyllotaxy of the new inflorescence (Figure 2-5b) (Ho and Weigel, 2014). CO was characterized as being a major mapped QTL for branching phenotype in *Arabidopsis* (Ungerer et al., 2002; Ho and Weigel, 2014). Likely many of the factors involved in specifying flowering time in the flower transition may behave in similar manners to regulate the production of reproductive organs even after the floral transition has already been specified.

PERSPECTIVE

The regulatory network that controls the floral transition in *Arabidopsis* has expanded greatly over the past decade, and indeed the connectedness between what were very discrete pathways has made the genetic analysis of different components difficult to interpret. While this has led to a wealth of very sophisticated analyses, we are still limited phenotypically in determining the role of individual factors in flowering time regulation (Andrés and Coupland, 2012; Kinmonth-Schultz et al., 2013). In addition to the complexity of the network, the number of environmental and endogenous inputs that can modify the flowering output should be a constant reminder that consistency in environmental conditions must be a continual goal, as the interpretation of relatively subtle phenotypes can be confounded by numerous environmental factors that relate to ambient temperature fluctuations, light quality, biotic and abiotic stresses, and others. In addition to the role of environmental effects on the floral transition, developmental considerations also appear to be at play in many areas. Once the floral transition is initiated, many recent studies point the idea that inputs such as photoperiod are still important in mediating inflorescence

architecture, maintenance of the inflorescence cell identity, and the propagation of the floral signal (Smith et al., 2010; Jaeger et al., 2013; Muller-Xing et al., 2014). This suggests that the same components also could be interacting with new partners in different tissues or under different environmental parameters.

While we are beginning to understand how different flowering regulatory factors change their activity or override other parts of the pathway under specific environmental conditions, the upstream control of these well characterized flowering components by environmental signals is relatively unknown. For instance, if temperature dependent splicing is an important factor in determining the abundance of active proteins or protein complexes, how is this splicing guided in response to temperature? What factors are responsible for their function? Likewise for factors in which temperature affects protein activity, such as the HOS1-FVE module, are the temperature based effects intrinsic properties of the proteins themselves or are other unknown cold-regulatory machinery required for their function?

Many factors have been identified which can modulate the transcription of floral integrator genes, but how these factors interact within the structure of each genomic locus has not yet been determined in great detail. In particular, we know that many factors are required to facilitate the proper *FT* expression pattern both in terms of tissues specificity as well as in a temporal fashion. How do these factors work for and against each other at the spatial level? Because of the large size of the *FT* promoter, and the implication of chromatin remodeling and other machinery is critical for *FT* expression, studying the effects of modified T-DNA *FT* promoter fragments may lose some of the context important information that may be conferred upon the original genomic locus. The advent and streamlining of genomic editing techniques such as CRYSPR/CAS9 may make studying changes in transcription factor binding sites or important structural pieces of the DNA more feasible. In addition to studying these changes, additional insight into natural variation in the promoter sequences of *FT* or other floral integrator genes may shed light on differences in flowering time output that do not directly correlate with SNPs in coding regions of flowering time regulatory factors (Schwartz et al., 2009).

A next major step in the study of the photoperiod pathway and the flowering pathways in general will be determination of the signaling outputs as a system, the robustness of the network and the changes that happen in connections and strength of interactions under changes in environment and photoperiod. Modeling of parts of the pathway has already been completed for *CO* expression and for the FT-TFL-

AP1-LFY regulatory pathway at the shoot apex. While these types of studies lose analytical power when many factors are included, more comprehensive analyses which seek to understand network dynamics under temperature or light quality fluctuations may shed additional light on to the phenotypes and genetic analyses which have already been performed. This knowledge will be critical to the pursuit of modification of flowering time in crop species, and in particular in trying to design ways in which to allow plants to compensate to changes in temperature and precipitation in agronomically important ways. While this goal is currently a very distant one, manipulation of flowering time is likely one that will greatly contribute to crop yields through tailoring of cultivars to specific climates or to changes in climate we anticipate will occur.

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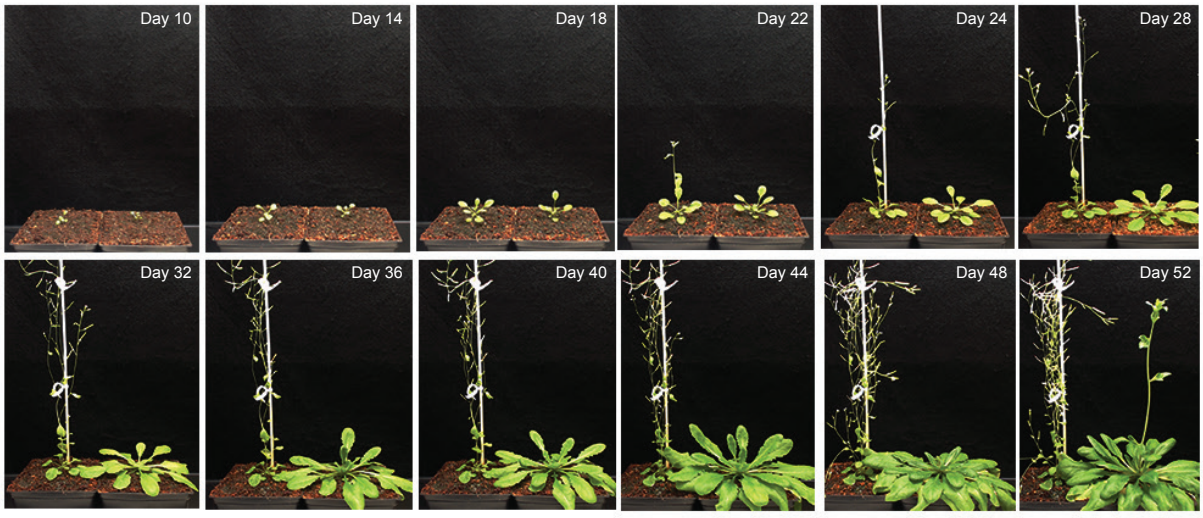

Figure 2-1. Flowering in response to photoperiod is mediated by *CONSTANS*. Time lapse imaging of WT (Col-0) plant (left in frame) and *co-101* mutant plant (right in frame) grown under long-day condi-tions (16hr L/8hr D) from 10 days old seedlings until flowering of *co-101* mutants. Flowering in response to the inductive photoperiod is severely delayed in *co-101* mutants.

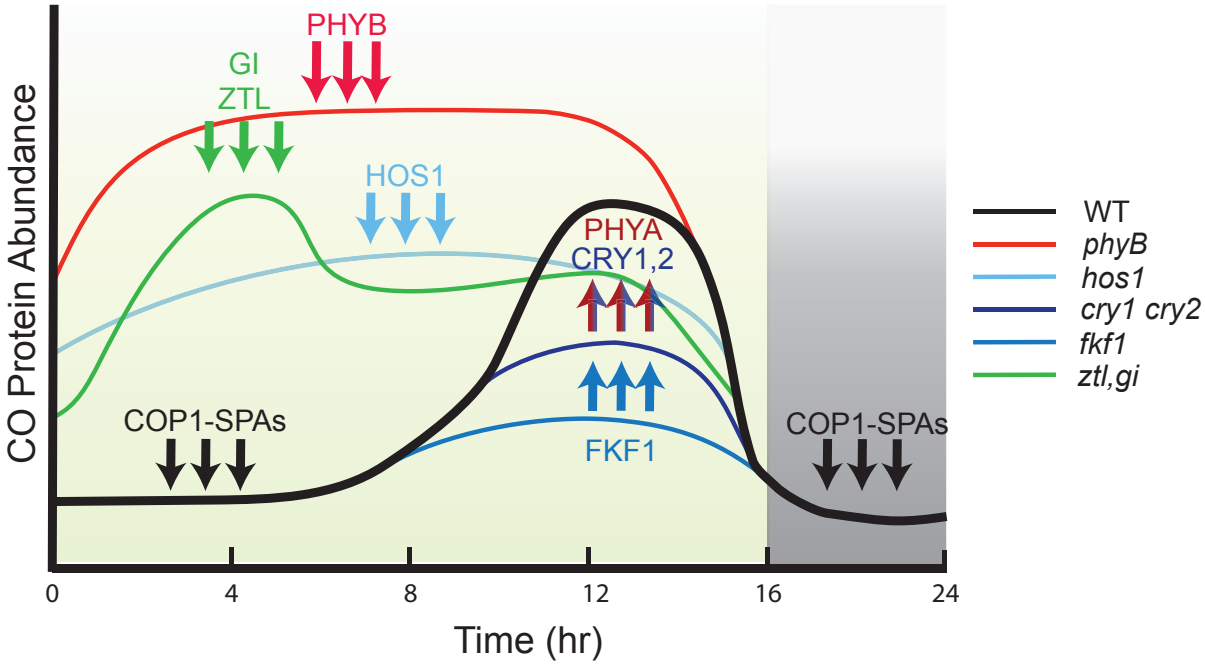

Figure 2-2. Post-translational control of CO determines the strength and temporal domain of CO activity to activate flowering. CO protein is highly unstable. Action of COP1-SPA complexes during both the day and the night degrades CO protein through ubiquitin mediated proteasomal degradation. During the daytime, CRY1 and CRY2 alleviate COP1-SPA activity on CO through protein-protein interactions. CO protein is also stabilized by PHYA through an unknown mechanism. PHYA and CRY1/CRY2 activity in the late afternoon increases CO protein abundance. Correspondingly, CO protein abundance is reduced in the afternoon in *cry1 cry2* and *phyA* mutants. HOS1 protein activity destabilizes CO protein, but only during the morning of long days. Mutations in hos1 shift the peak of CO abundance earlier in the day. FKF1 also stabi-lizes CO protein through an unknown mechanism. Concomitant with its daily expression in the afternoon of long days, FKF1 protects CO protein from degradation. PHYB acts antagonistically with CO, and desta-bilizes CO protein through an unknown mechanism. PHYB destabilization effects both the amplitude and temporal domain of CO abundance, as CO protein is highly abundant throughout the day in *phyB* mutants. Thus, CRY1, CRY2, PHYA, and PHYB contribute to CO amplitude, while HOS1 and FKF1 contribute to the temporal domain of CO protein abundance. *Note: Absolute CO abundance in phyB vs. *hos1* mutants are extrapolated from different genetic backgrounds and therefore may be different from the above representation.

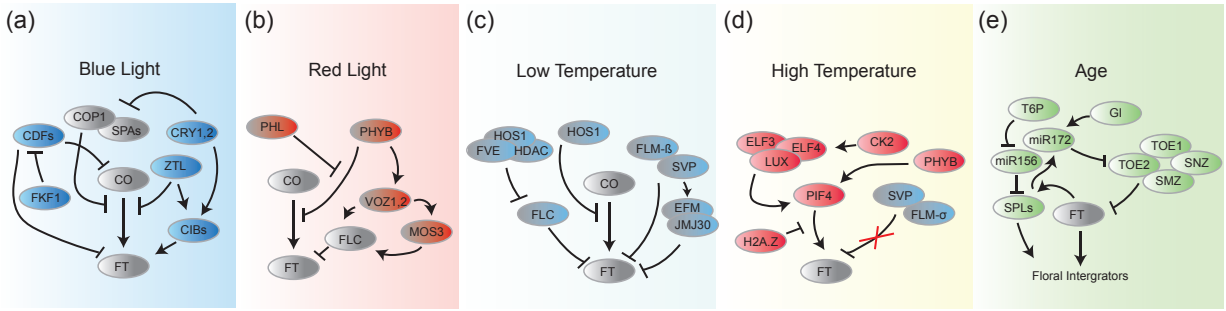

Figure 2-3. Regulation of *CO* and *FT* in response to environmental and endogenous cues. (a) Under blue-light enriched conditions, *CO* and *FT* transcription is increased due to increased degradation of CDFs by FKF1. CO protein is stabilized through inhibition of COP1/SPAs activity by CRY1 and CRY2. CRY1 and CRY2 also are able to increase *FT* expression directly through activation of CIB transcription factors. CO protein is also stabilized through FKF1 activity in a blue light dependent manner. (b) Under red light condi-tions, PHYB is able to destabilize CO protein. PHYB activity on CO is hampered through the action of PHL. PHYB is also able to indirectly repress *FT* expression through an increase in *FLC* transcription, either through direct activation of *FLC* by VOZ1 and VOZ2, or through and indirect effect whereby VOZ1/2 increase *FLC* transcripts through regulation of the nuclear pore component MOS3. (c) Under low ambient tempera-ture conditions, HOS1 is able to facilitate degradation of CO protein during the morning. HOS1 is also able to effect *FT* transcription through protein complex formation with FVE and HDAC, which represses *FLC* tran-scription. This reduction in FLC indirectly increases *FT* expression. SVP is able to form functional heterodimers with a splice variant of the transcription factor FLM (FLM- ß) which represses *FT* expression. FLM- ß is preferentially spliced under low temperature conditions. (d) Under high temperature conditions, PIF4 protein is able to upregulate *FT* expression, but its binding site is inhibited by the deposition of the histone variant H2A.Z to the *FT* 5' proximal region. PIF4 is also activated in response to high temperature by PHYB. Under high temperatures, SVP-FLM repression of *FT* is impaired by the preferential splicing of FLM-∂, which forms a non-functional complex with SVP. ELF3, ELF4, and LUX are circadian clock components which regulate hypocotyl elongation through PIF4 activity in the evening, and may be involved in PIF4 regulation of flowering in response to high temperature. CK2 is involved in adjusting the pace of the circadian clock in response to temperature and may be involved in temperature dependent changes in clock output that indirectly effect *FT* expression through PIF4. (e) Relative abundance of *miR172* and *miR156* changes with age, as *miR172* decreases with age and *miR156* increases. *miR172* activity reduces transcription of AP2-related repressors of *FT* expression, *TOE1,2*, *SMZ* and *SNZ*, and is positively regulated by GI activity. Conversely, *miR156* targets *SPLs* transcripts, which reduces the activity of SPLs to activate other floral integrator genes at the shoot apex. These pathways enable the prevention of flowering before developmental competency, and improve sensitivity to environmental conditions as the plants further develop.

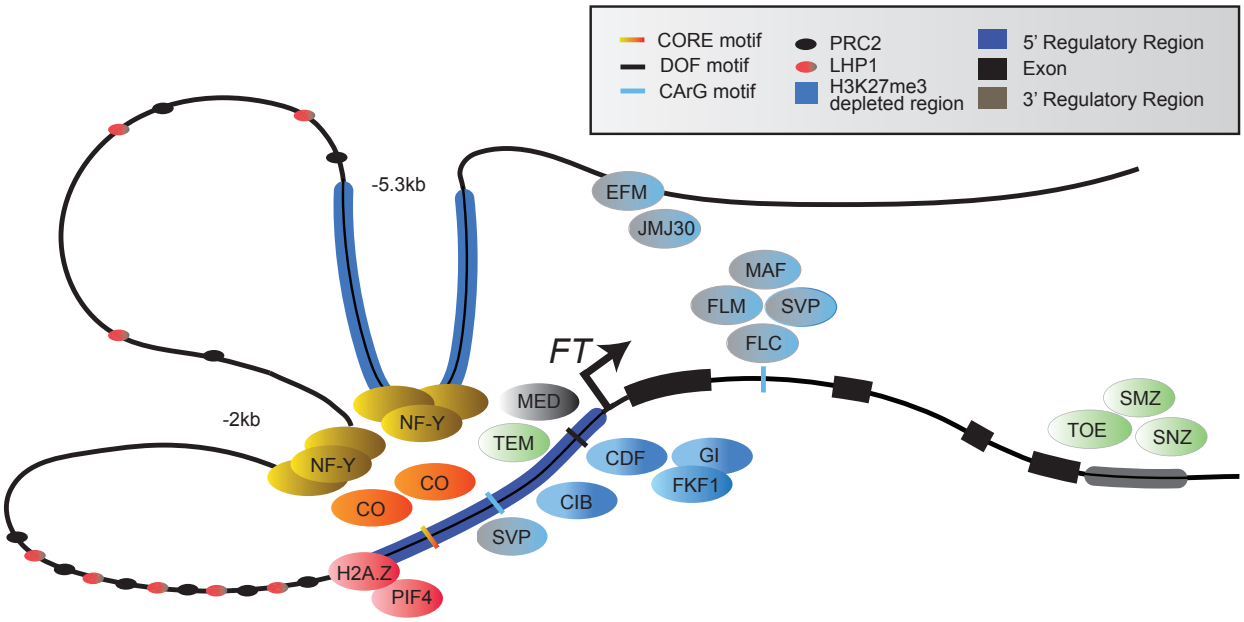

Figure 2-4. Regulation of *FT* expression in a spatial context. The proximal 5' region of the *FT* promoter is the binding site for many transcription factors which can repress or activate *FT* in response to external parameters.CDFs and TEMs are able to repress *FT* expression and are regulated by the circadian clock. CIB activators are able to induce *FT* under blue light enriched conditions. SVP is able to repress *FT* expres-sion under low temperature, and PIF4 increases it under high temperature. This regulatory region however, is normally not accessible to transcription factors through the activity of LHP1, which is enriched in this region, and PRC2, which is able to trimethylate lysine 27 residues on histones in this region. Only the -5.3kb CCAAT box motif is free from the actions of LHP1-PRC2. Once NF-Y factors are able to bind to the upstream CCAAT box sites and recruit CO to the *FT* locus, CO activity is able to remove the LHP1 presence in the 5' proximal region, which enables the activity of other *FT* regulators. EFM and JMJ30 form a complex that regulates *FT* through demethylation of histones in the FT locus. MADS domain factors FLC, SVP, FLM, and MAFs, are able to bind to the first *FT* intron to repression transcription, both in response to low ambient tem-perature as well as prior to vernalization. AP2 repressors TOE1,2, SMZ and SNZ are able to bind to the 3' regulatory region near the *FT* 3' UTR to regulate *FT* expression.

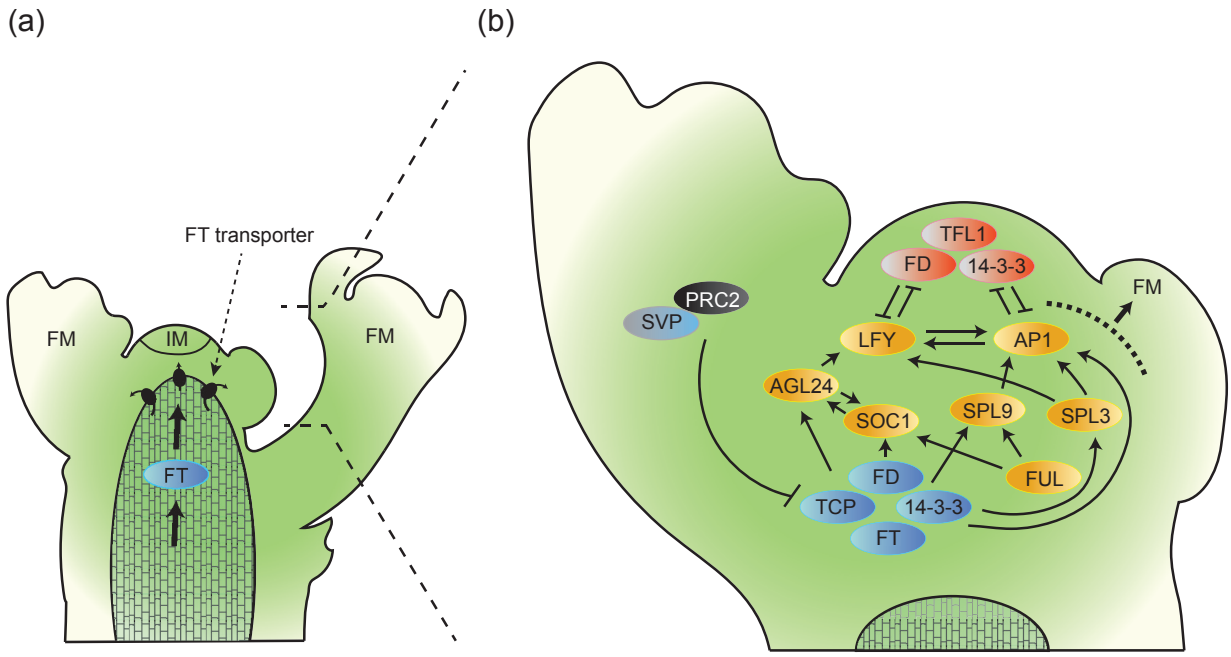

Figure 2-5. Specification of an inflorescence meristem and floral meristems at the shoot apex. (a) FT protein, after being synthesized in the leaf phloem companion cells, enters into the phloem stream through plasmodesmata and move to the shoot apex. Upon arrival at the shoot apex, an active transport mechanism uptakes FT protein into the cells surrounding the shoot apical meristem. (b) FT protein, along with TCP transcription factors, binds to the 14-3-3 adapter protein and to the transcription factor FD. FT competes for interaction with 14-3-3 and FD with TFL, which prevents FD from transcriptional activity. Active FT-14-3-3-FD complex activates *LFY* and *AP1*, which feed forward to increase each other's expression. The action of this feed forward loop initiates commitment to inflorescence cell fate. As the meristem continues in development,TFL activity is required to maintain the indeterminacy of the inflorescence, and a gradient is set whereby FT and other floral integrators are able to specify the placement of floral meristems (FM) on the flanks of the inflorescence meristem (IM). FT-14-3-3-FD complex activates expression of the floral integrator genes *SOC1*, *FUL*, and *SPL3*, *SPL4*, and *SPL5*. These, in turn, induce *AGL24* and *SPL9*, resulting in downstream activation of *AP1* and *LFY*. After floral meristem specification, FT is still required to maintain floral meristem identify; repression of *FT* is carried out by SVP and the PRC2 complex. If sufficient repression of *FT* is maintained, floral meristem identity can be converted back into a vegetative cell fate.

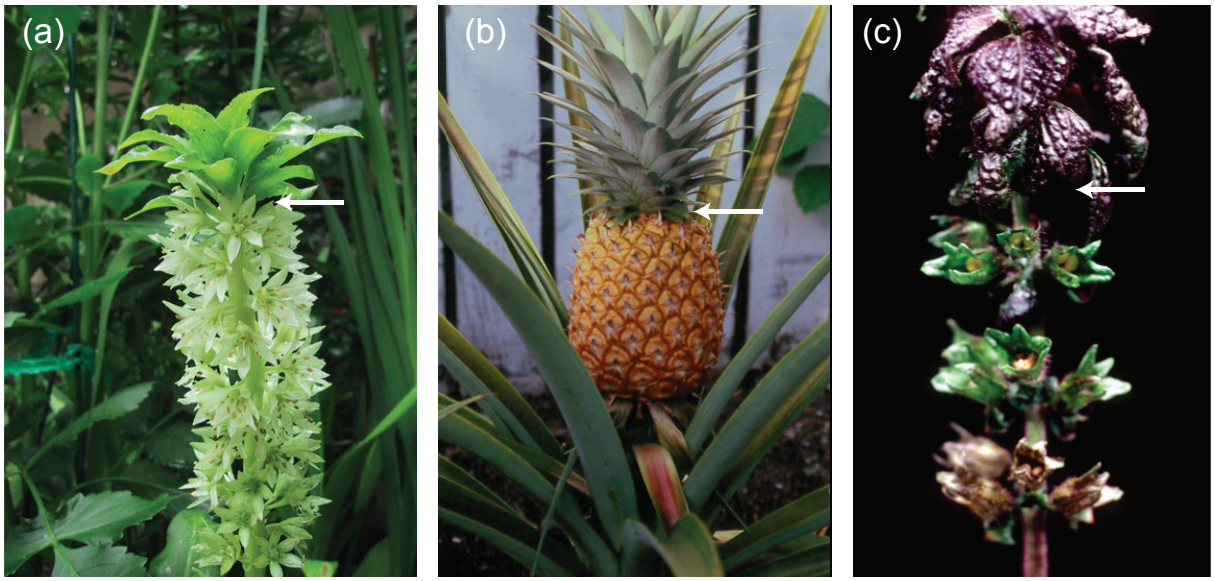

Figure 2-6. Reversion of the inflorescence meristem to a vegetative meristem in flowering plants. Once inflorescence meristematic identity is specified, plants are usually committed irreversibly to the flowering response. (a) *Eucomis autumnalis* (b) *Ananas comosus* and (c) *Perilla fructescens* illustrate an inflorescence meristem with the capability to revert into a vegetative meristem after floral specification is complete. White arrows indicate the point at which the inflorescence reverts to vegetative identity.

CHAPTER III.

CYCLING DOF FACTOR 1 REPRESSES TRANSCRIPTION THROUGH THE TOPLESS CO-REPRESSOR TO CONTROL PHOTOPERIODIC FLOWERING IN ARABIDOPSIS

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Goralogia, G.S., Liu, T.-K., Zhao, L., Panipinto, P.M., Groover, E.D., Bains, Y.S. and Imaizumi, T. (2017) CYCLING DOF FACTOR 1 represses transcription through the TOPLESS co-repressor to control photoperiodic flowering in Arabidopsis. *Plant J.*, 92, 244-262.

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.

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.

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 zeitgeber 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, SCHLAFM**Ü**TZE (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 3-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 3-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 3-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 3-1a,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 3-1c,3-S2a). We confirmed that both CDF1-ΔN and CDF1-mut proteins were localized to the nucleus, similar to non-mutated CDF1 (Figure 3-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 3-1d). 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 3-1a). We tested the flowering time response of transgenic lines with similar expression levels of *CDF1* transcripts (Figure 3-2a,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 3-2b,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 3-2d,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 3-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 3-2f). 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 3-2g).

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 3-2h,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 3-2k,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 3-2l,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 3-3a). 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 3-3a). 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 3-3a). 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 3-3b). 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 3-3b).

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 3-3c). 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 3-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 3-3d), 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 3-4). 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 3-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 3-4). 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 3-4). 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 3-5,S5). In long days, *SUC2:HA-tpl-1* plants showed increased levels of *CO* mRNA throughout the middle of the day (Figure 3-5a). Additionally, they showed increased expression of *FT* throughout the day, similar to *cdfq* mutants (Figure 3-5b). 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 3-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 3-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 3-5c,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 3-5f). 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 3-5e,f). We saw a similar trend when looking at the expression of downstream targets of *FT*, *SOC1*, *LFY*, and *AP1* (Figure 3-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 3-6a,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 3-6c). 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 3-7a,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 3-7a,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 3-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 3-7d). 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 3-7d). 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 3-7e), 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 3-1), and eliminating this motif attenuates the function of CDF1 as a transcriptional repressor (Figure 3-2). 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 3-3). Increasing the expression of *CDF1* brings additional TPL to target loci (Figure 3-3), 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 3-4,6,7).

SUC2:HA-tpl-1 plants are largely insensitive to changes in *CDF1* expression, highlighting that loss of co-repressor perturbs CDF function (Figure 3-4). 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 3-7f).

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 3-3). 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 3-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 3-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 3-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'-TCCCCATGGGACTG GAAACTAAAGATCCTGCGATAAAGGCCGCTGCTATGAAAATTCCTTTCCCGAC-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'-CACCATGTATCCATATGATGTTCCAGATTATGCTATGTCTTCTCTTAGTAGAGAGCTCGTT-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-∆N*, and *CDF1-mut* for Y2H analysis, the respective forward primers 5'- CACCATGCTGGAAACTAAAGATCC TGCGATAAAGC -3' (for *CDF1*), 5'- CACCATGACGGTTTTAGAGGTTGCTGATGAAGA-3' (for *CDF1-∆N*), and 5' CACCATGCTGGAAACTAAAGATCCTGCGATAAAGGCCGCTGCTATGAAAATTCCTTTCCCGAC-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 3-5e,f and S6, *IPP2* expression was used as an internal control to normalize cDNA amount. For Figure 3-5e,f and 3-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.

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SHORT LEGENDS FOR SUPPORTING INFORMATION

Figure 3-S1. Amino acid sequence alignment of CDF related proteins.

Figure 3-S2. BiFC negative controls and subcellular localization of TPL, CDF1, CDF1-ΔN, and CDF1-mut.

Figure 3-S3. Flowering phenotype of Col-0, *35S:CDF1-3F6H*, *35S:CDF1-∆N-3F6H*, and *35S:CDF1-mut-3F6H* transgenic lines in short days.

Figure 3-S4. Protein expression level of TPL in *TPL:TPL-3HA/35S:CDF1* transgenic line, *CDF1* expression in *TPL:TPL-3HA/35S:CDF1* transgenic line, and *TPL* expression in *SUC2:HA-tpl-1* transgenic lines.

Figure 3-S5. Gene expression profiles of *TPL*, *LFY*, *AP1*, *FUL*, and *SOC1* in WT, *cdfq* mutants, and *SUC2:HA-tpl-1 transgenic lines*.

Figure 3-S6. Gene expression levels of *TPL*, *SOC1*, *LFY*, and *AP1* over developmental time in WT, *cdfq* mutants, and *SUC2:HA-tpl-1* transgenic lines.

Table S1. ChIP and Q-PCR primers used in this study.

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Supplementary table 1. ChIP and Q-PCR primers used in this study.

TSF ChIP 1F	CAAAACCCGCGAAAGCTAGA
TSF ChIP 1R	CGCGTTTATGACCGAGAAAA
TSF ChIP 2F	ATCGTACATGCTGCCAAACA
TSF ChIP 2R	TGTCCATTTCCCCAATTTGT
TSF ChIP 3F	TGTGAACAATTATGGCGTCAA
TSF ChIP 3R	CACGCATTTTGCGTCACAT
TSF ChIP 4F	TGGCTAGCAAGAAACAAGTGG
TSF ChIP 4R	CATTACCAGGGTCTTTTCGTG
TPL Q-PCR F	GAGTTTTATGGATGCAACAGTTTG
TPL Q-PCR R	TAGTGGATGTACGTTTGAATTGCT
FUL Q-PCR F	TTGCAAGATCACAACAATTCGCTTCTC
FUL Q-PCR R	GTAACATCCAAGCCGGAAGC

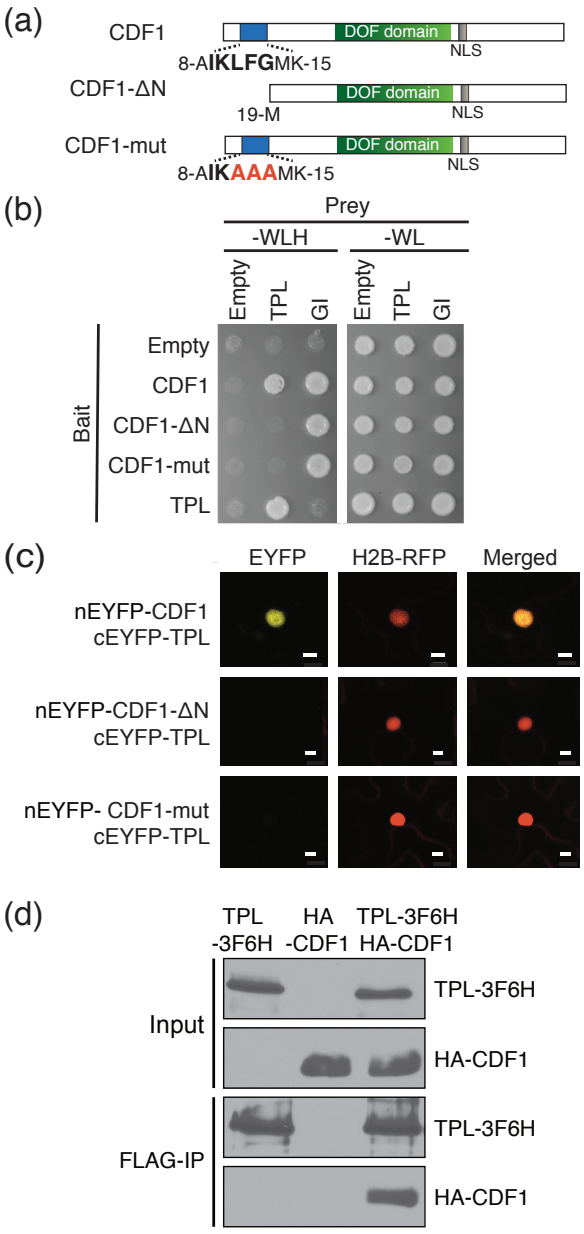


Figure 3-1. CDF1 and TPL form a protein complex through a conserved binding motif located at the N-terminus of CDF1. (a) Schematic representation of CDF1 full length protein and the N-terminal CDF variants used in this study. The N-terminal amino acid sequences (IKLFG) conserved in CDF family proteins (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 bind-ing 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 coimmuno-precipitation assay of transiently transfected *N. benthamiana* leaves.

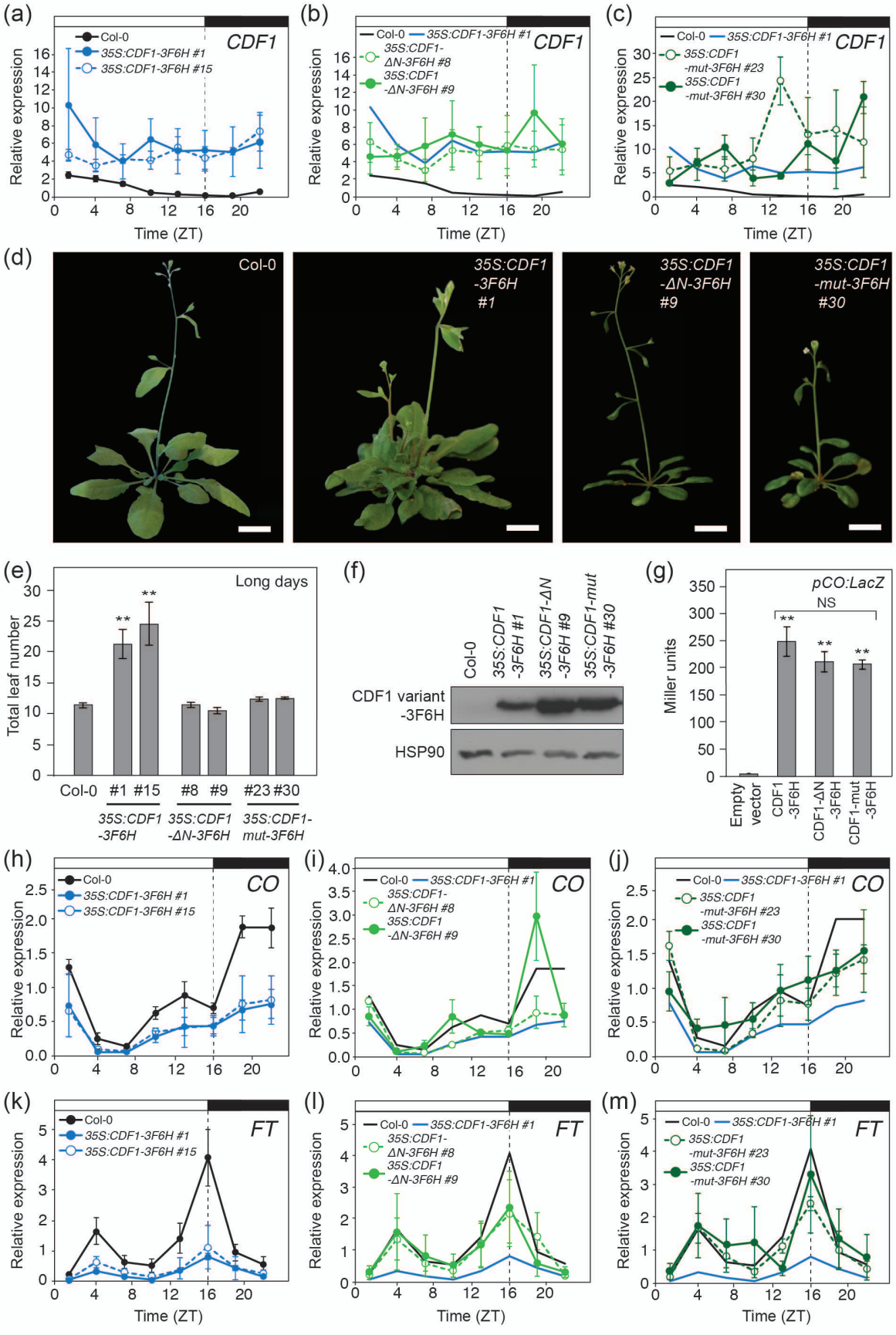

(a)
CDF1
Col-0
35S:CDF1-3F6H #1
35S:CDF1-3F6H #15
Relative expression
Time (ZT)
(b)
CDF1
Col-0
35S:CDF1-3F6H #1
35S:CDF1-ΔN-3F6H #8
35S:CDF1-ΔN-3F6H #9
Relative expression
Time (ZT)
(c)
CDF1
Col-0
35S:CDF1-3F6H #1
35S:CDF1-mut-3F6H #23
35S:CDF1-mut-3F6H #30
Relative expression
Time (ZT)
(d)
Col-0
35S:CDF1-3F6H #1
35S:CDF1-ΔN-3F6H #9
35S:CDF1-mut-3F6H #30
(e)
Long days
Total leaf number
Col-0
#1
#15
#8
#9
#23
#30
35S:CDF1-3F6H
35S:CDF1-ΔN-3F6H
35S:CDF1-mut-3F6H
(f)
Col-0
35S:CDF1-3F6H #1
35S:CDF1-ΔN-3F6H #9
35S:CDF1-mut-3F6H #30
CDF1 variant-3F6H
HSP90
(g)
pCO:LacZ
NS
Miller units
Empty vector
CDF1-3F6H
CDF1-ΔN-3F6H
CDF1-mut-3F6H
(h)
CO
Col-0
35S:CDF1-3F6H #1
35S:CDF1-3F6H #15
Relative expression
(i)
CO
Col-0
35S:CDF1-3F6H #1
35S:CDF1-ΔN-3F6H #8
35S:CDF1-ΔN-3F6H #9
Relative expression
(j)
CO
Col-0
35S:CDF1-3F6H #1
35S:CDF1-mut-3F6H #23
35S:CDF1-mut-3F6H #30
Relative expression
(k)
FT
Col-0
35S:CDF1-3F6H #1
35S:CDF1-3F6H #15
Relative expression
Time (ZT)
(l)
FT
Col-0
35S:CDF1-3F6H #1
35S:CDF1-ΔN-3F6H #8
35S:CDF1-ΔN-3F6H #9
Relative expression
Time (ZT)
(m)
FT
Col-0
35S:CDF1-3F6H #1
35S:CDF1-mut-3F6H #23
35S:CDF1-mut-3F6H #30
Relative expression
Time (ZT)

Figure 3-2. 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.

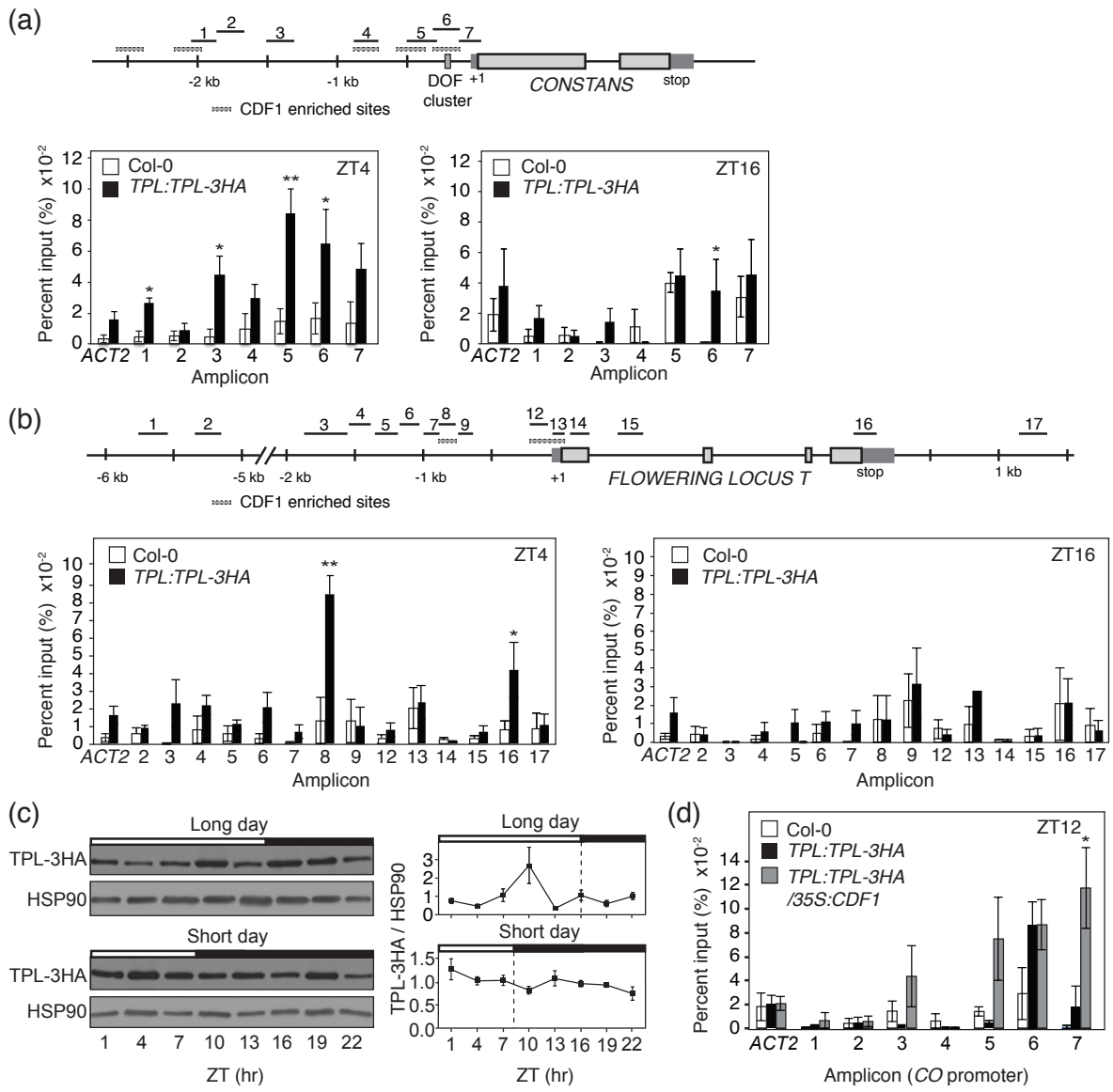


Figure 3-3. TPL associates with the *CO* and *FT* promoter regions during the morning in a CDF1-dependent manner *in vivo*. (a and b) ChIP assay-showing TPL binding to regions of the *CO* and *FT* genomic locus at ZT4 and ZT16 from 14-day-old *TPL:TLP-3HA* and Col-0 plants grown under long-day photoperiods. The schematic diagrams of positions of each ChIP ampli-con 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.

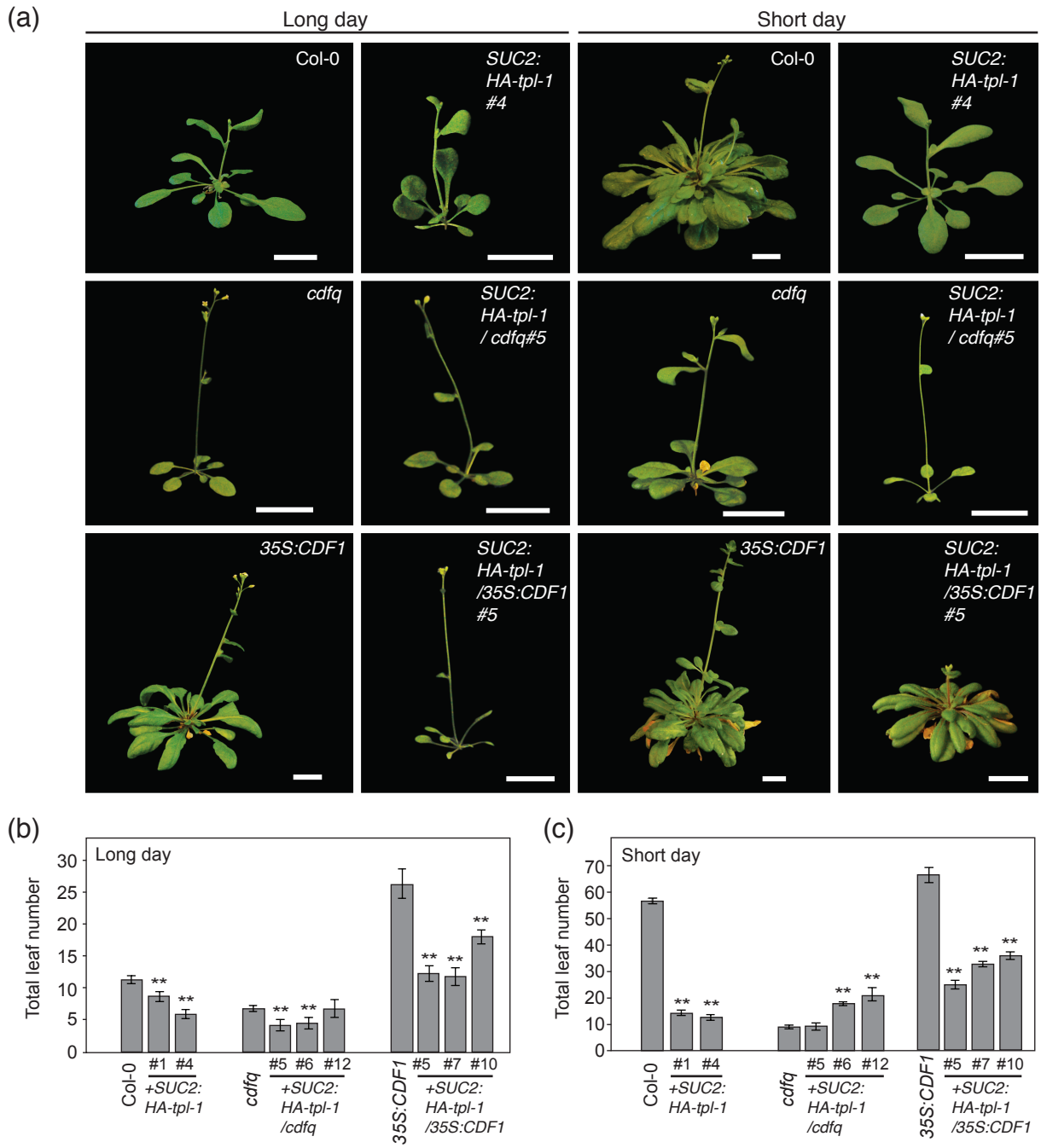


Figure 3-4. Expressing a dominant negative form of TPL in phloem companion cells phenocopies *cdfq* mutants, and lessens the late flowering phenotype of *CDF1* overexpressiors. *(a)* Representative images of images of *SUC2:HA-tpl-1, SUC2:HA-tpl-1/cdfq, andSUC2:HA-tpl-1/35S:CDF1* plants under long-day 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).

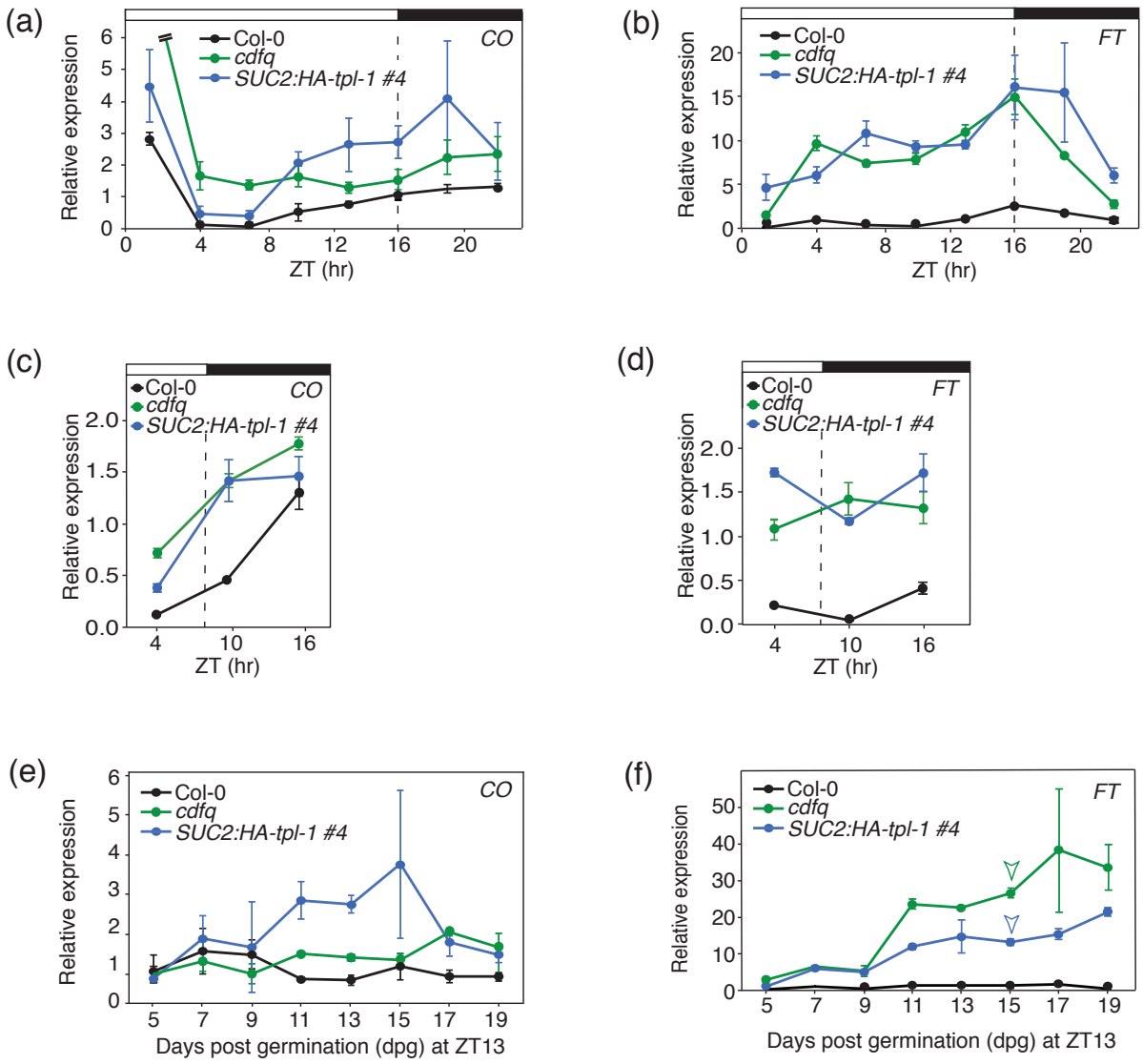


Figure 3-5. *SUC2:HA-tpl-1* transgenic plants have increased *FT* and *CO* expression in long days and short days, as well as over developmental time. (a) and (b) Diurnal gene expres-sion analysis of *CO* and *FT* in Col-0, *cdfq*, and *SUC2:HA-tpl-1* plants. Experiments were performed on 14-day-old 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 flow-ering (the wild-type plants did not start flowering during the experiment).

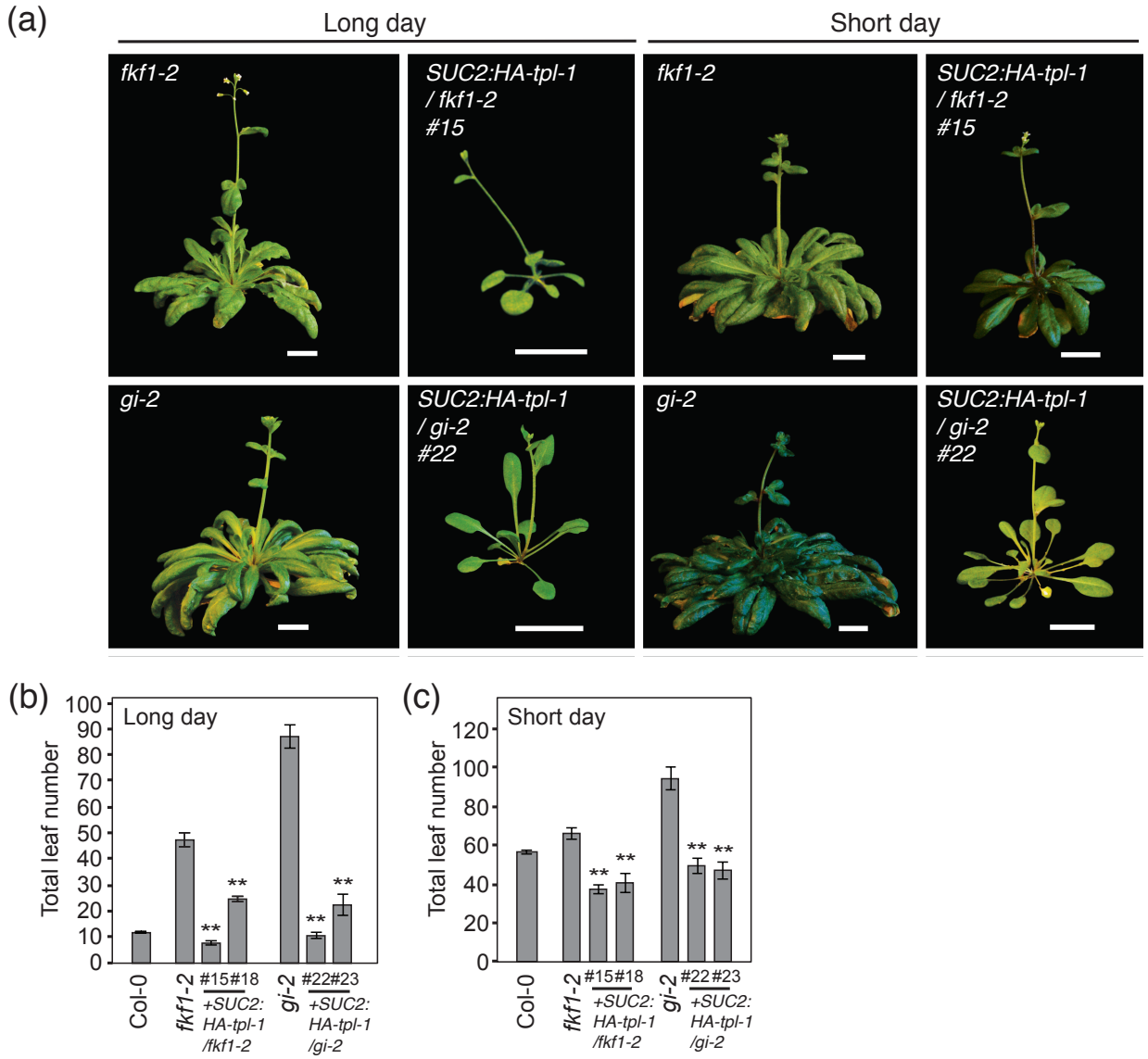


Figure 3-6. *SUC2:HA-tpl-1* plants in *fkf1* and *gi* backgrounds are early flowering in long days and short days. (a) Representative images of *SUC2:HA-tpl-1* plants (*SUC2:HA-tpl-1/fkf1-2*, and *SUC2:HA-tpl-1/gi-2*) and their respective genetic back-grounds (*fkf1-2*, and *gi-2*) under long-day and 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 calculat-ed from N=16 individuals. **P < 0.01 (one-tailed t test).

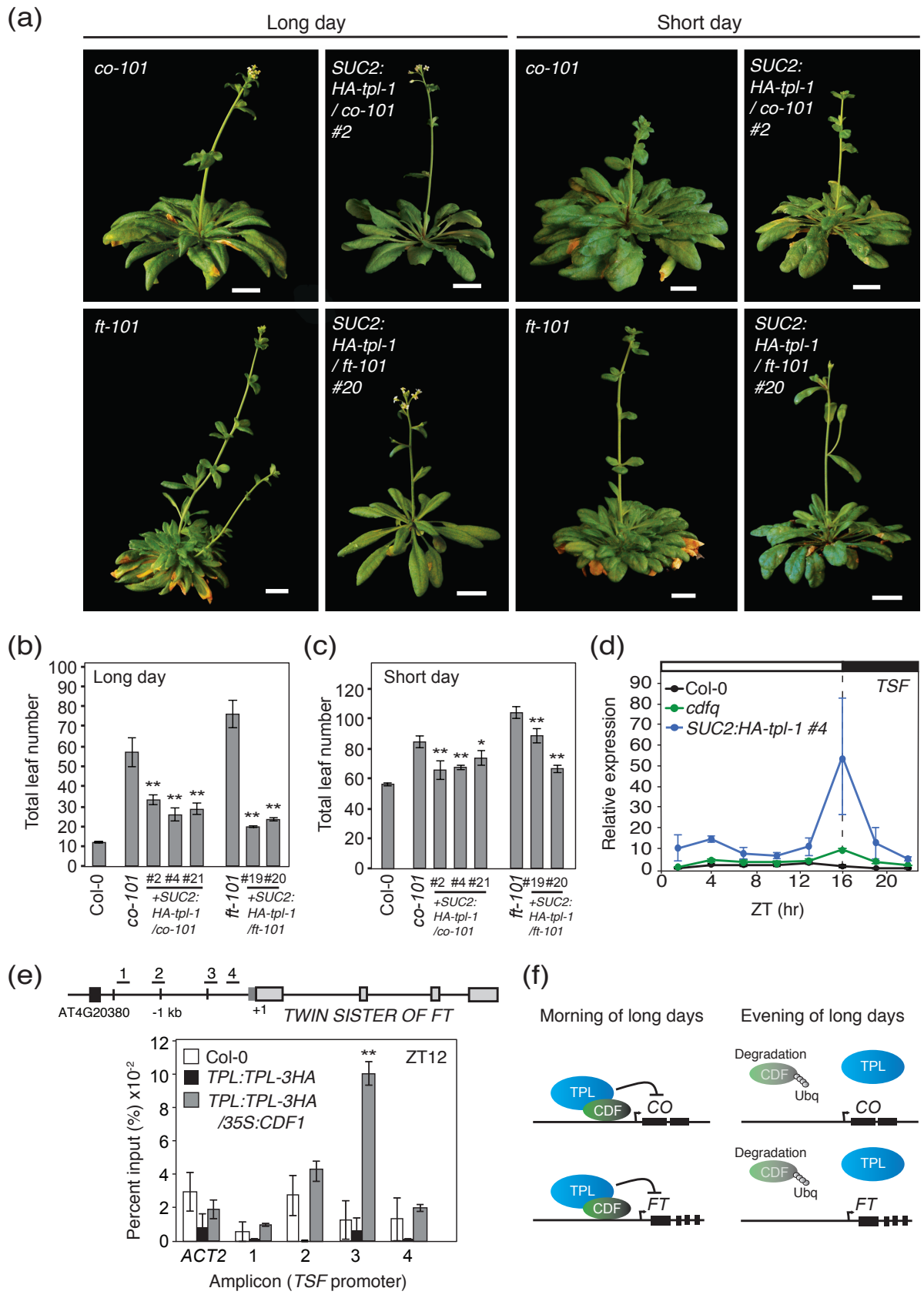

(a)
Long day
Short day
co-101
SUC2: HA-tpl-1 / co-101 #2
ft-101
SUC2: HA-tpl-1 / ft-101 #20
(b)
Long day
Total leaf number
Col-0
co-101
#2 #4 #21 +SUC2: HA-tpl-1 /co-101
ft-101
#19#20 +SUC2: HA-tpl-1 /ft-101
(c)
Short day
Total leaf number
(d)
Col-0
cdfq
SUC2:HA-tpl-1 #4
TSF
Relative expression
ZT (hr)
(e)
AT4G20380
-1 kb
+1
TWIN SISTER OF FT
Col-0
TPL:TPL-3HA
TPL:TPL-3HA /35S:CDF1
ZT12
Percent input (%) x10-2
ACT2
Amplicon (TSF promoter)
(f)
Morning of long days
Evening of long days
TPL
CDF
CO
FT
Degradation
Ubq

Figure 3-7. 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 back-grounds (*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 seed-lings grown in long days, with 3-hour resolution. Means +/- SEM were calculated from four inde-pendent 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.

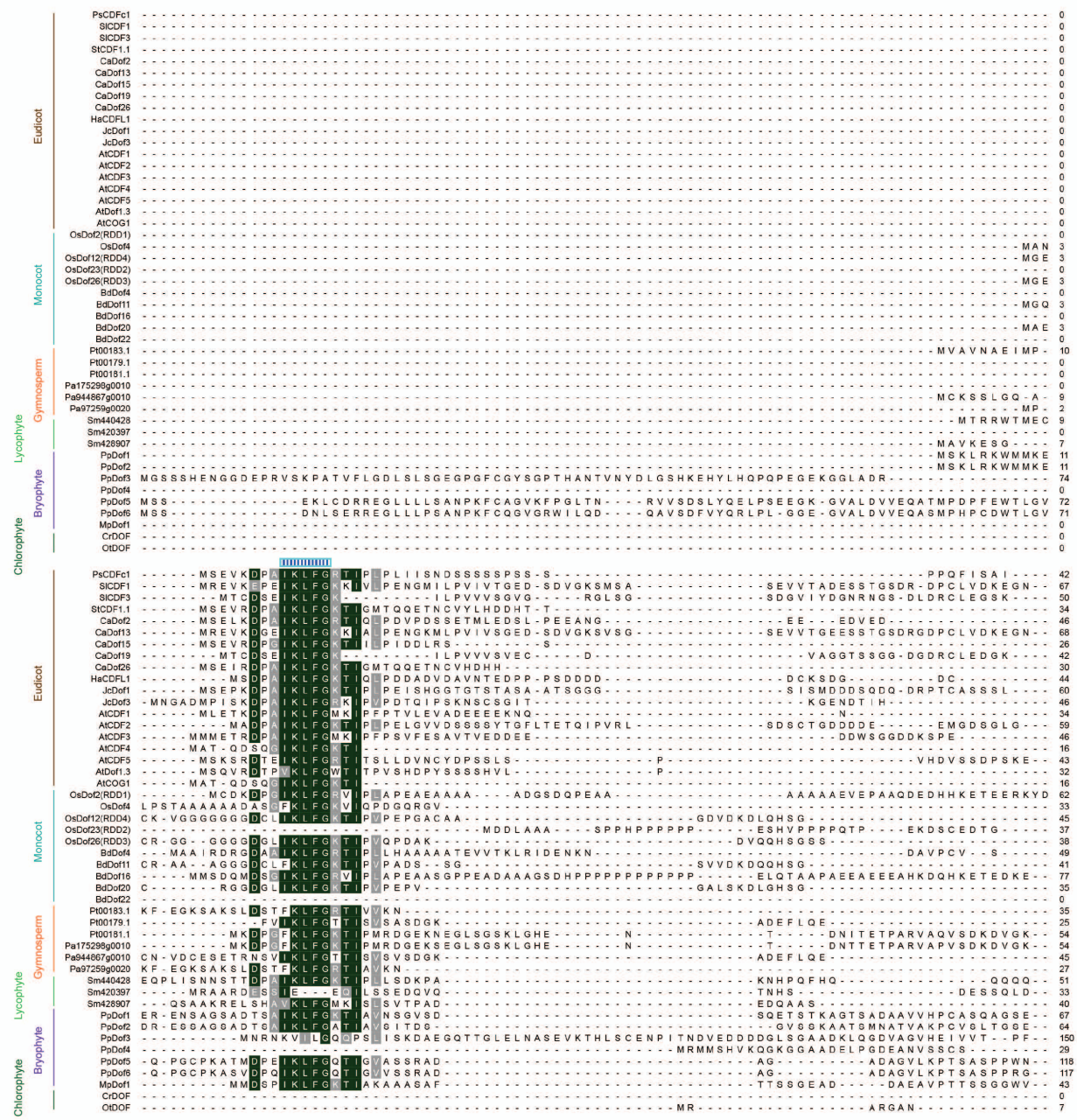


Figure 3-S1.1 Amino acid sequence alignment of CDF related proteins.

Figure 3-S1.2 Amino acid sequence alignment of CDF related proteins.

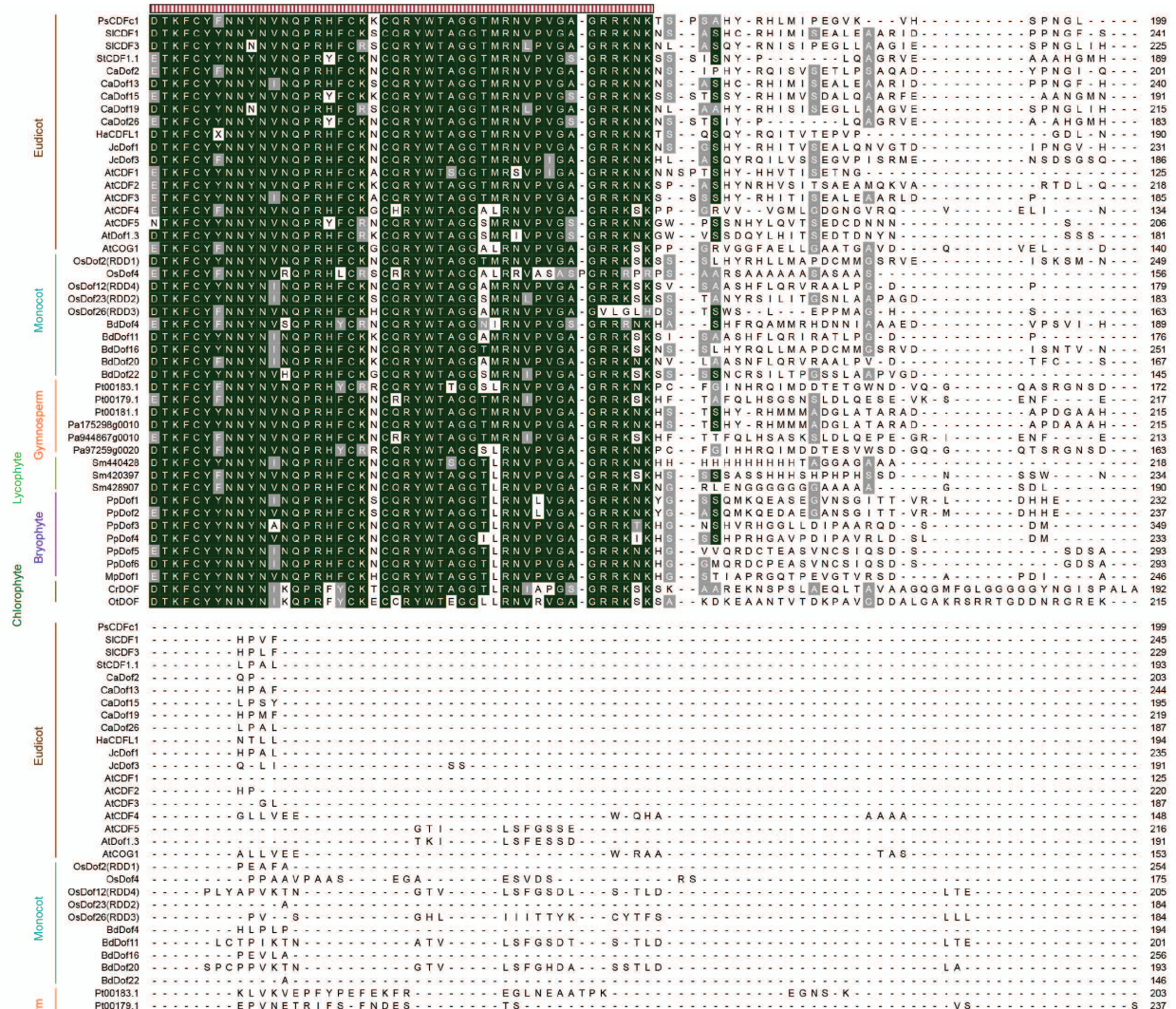


Figure 3-S1.3 Amino acid sequence alignment of CDF related proteins.

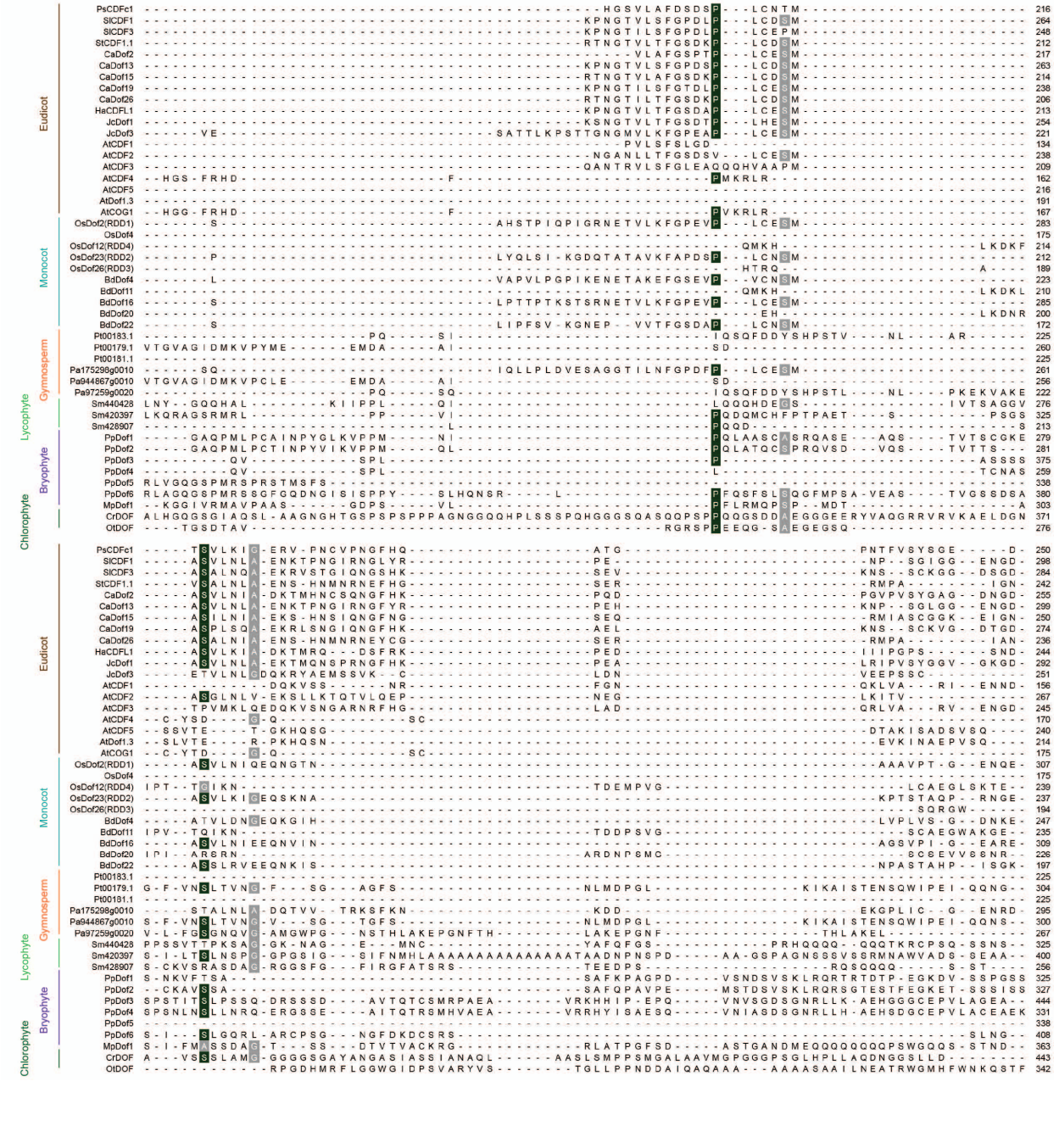


Figure 3-S1.4 Amino acid sequence alignment of CDF related proteins.

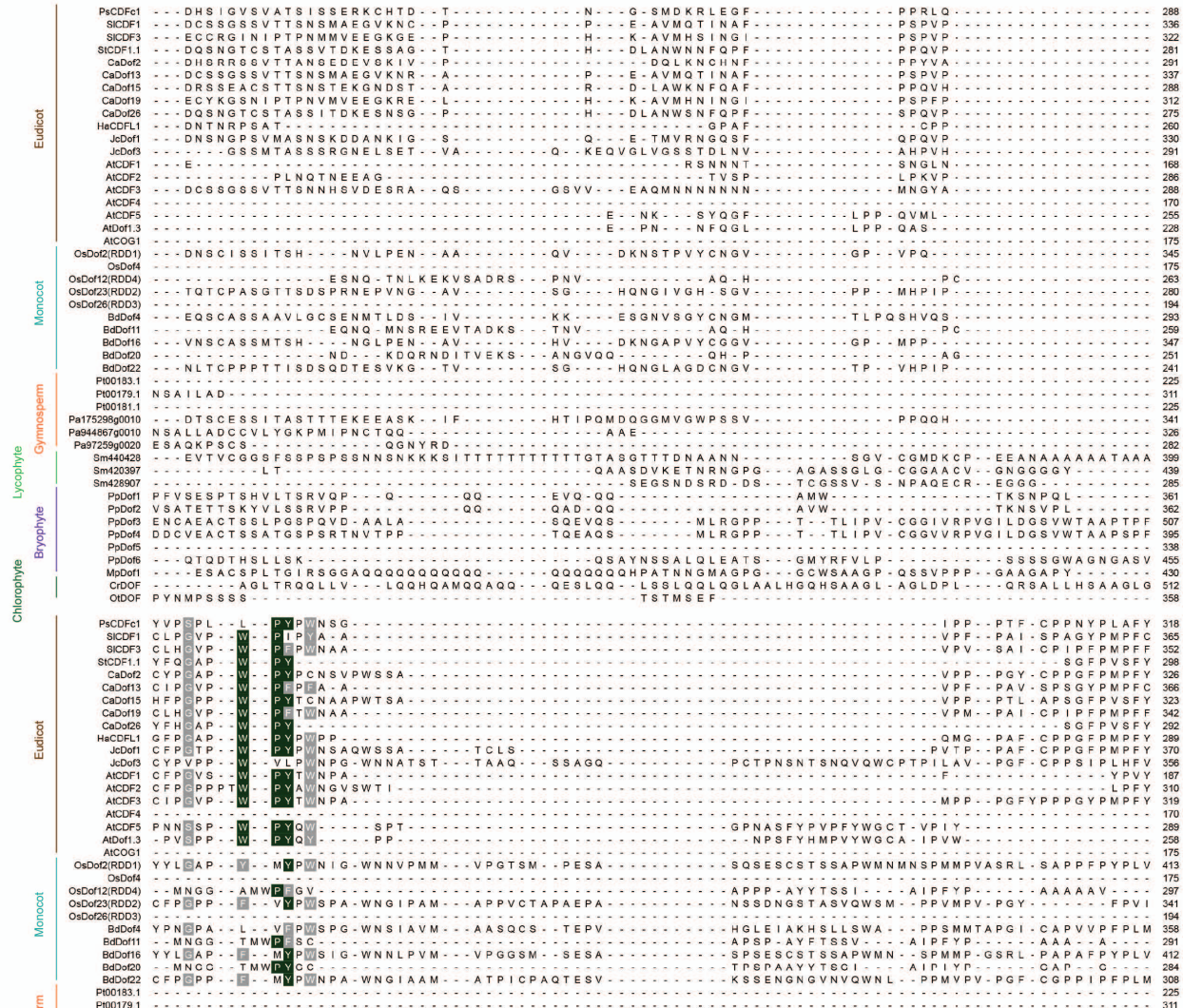
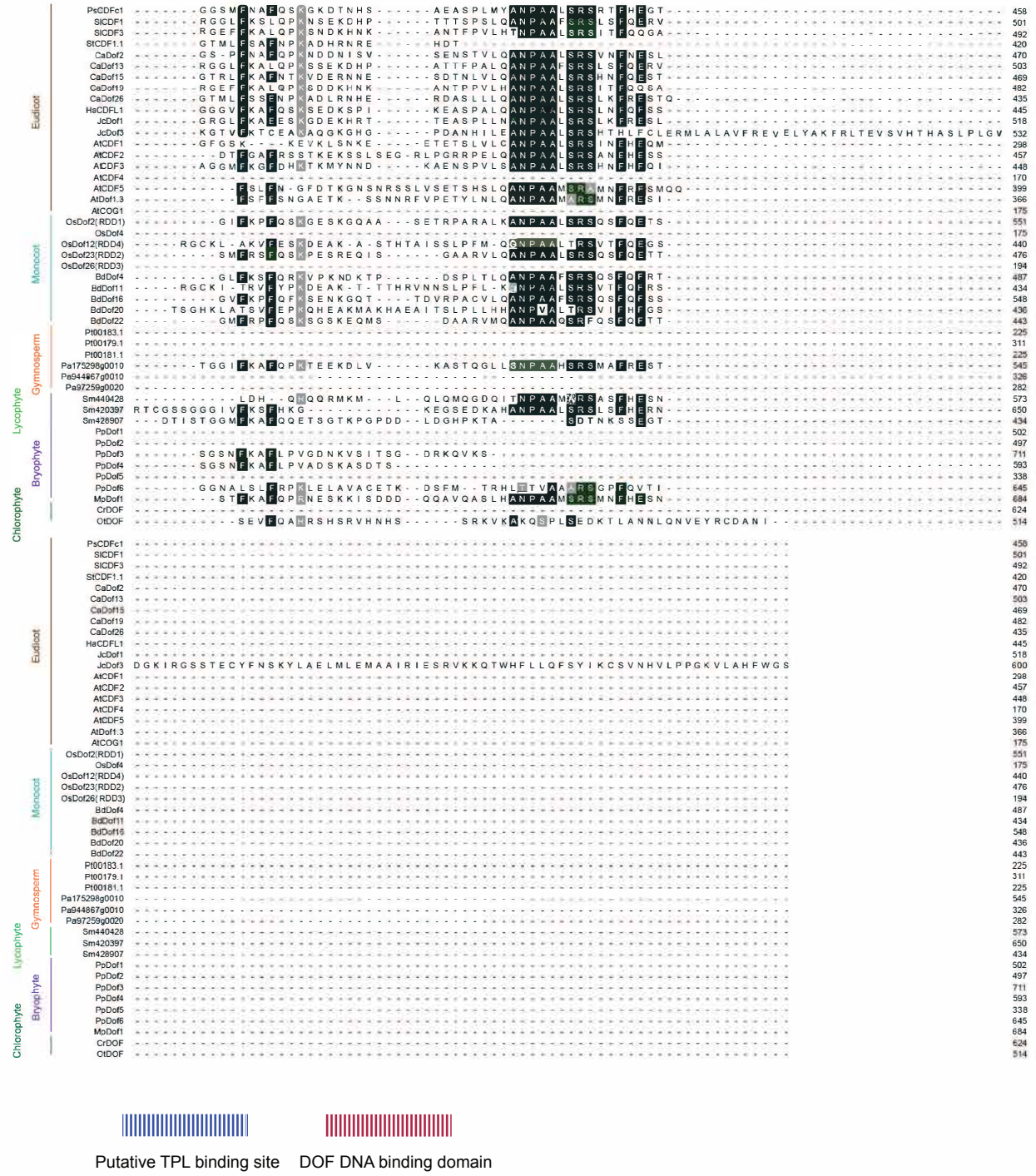


Figure 3-S1.5 Amino acid sequence alignment of CDF related proteins.

Figure 3-S1.6 Amino acid sequence alignment of CDF related proteins.



Putative TPL binding site DOF DNA binding domain

Figure 3-S1.7 Amino acid sequence alignment of CDF related proteins.

Figure 3-S1. Amino acid sequence alignment of CDF related proteins. CDF group DOF domain proteins were obtained from published studies, and BLAST analyses using the Phytosome 12 browser (https://phytozome.jgi.-doe.gov/pz/portal.html), or the plant transcription factor database (http://planttfdb.cbi.pku.edu.cn). *Arabidopsis* CDF1,2,3, and 5 proteins were used as a query when necessary. Specific proteins were chosen due to phylogenet-ic position within the CDF clade or by previously reported functional characterization. Protein sequences subse-quently were assembled and aligned using the CLUSTAL omega program. The species included are as follows: Ps= *Pisum sativum*, Sl= *Solanum lycopersicum*, St= *Solanum tuberosum*, Ca= *Capsicum annuum*, Ha= *Helianthus annuus*, Jc= *Jatropha curcas*, At= *Arabidopsis thaliana*, Os= *Oryza sativa*, Bd= *Brachypodium distachyon*, Pt= *Pinus taeda*, Pa= *Picea abies*, Sm= *Selaginella moellendorffii*, Pp= *Physcomitrella patens*, Mp= *Marchantia poly-morpha*, Cr= *Chlamydomonas reinhardtii*, Ot= *Ostreococcus tauri*. Dark Green color indicates amino acid posi-tions with more than 50% identical residues.Gray color indicates 50% of amino acid positions are similar. Red dashed bar indicates the consensus sequences of the DOF DNA binding domain. The blue dashed bar indicates the putative TPL interaction motif. Relative amino acid position number is indicated on the right side of the figure.

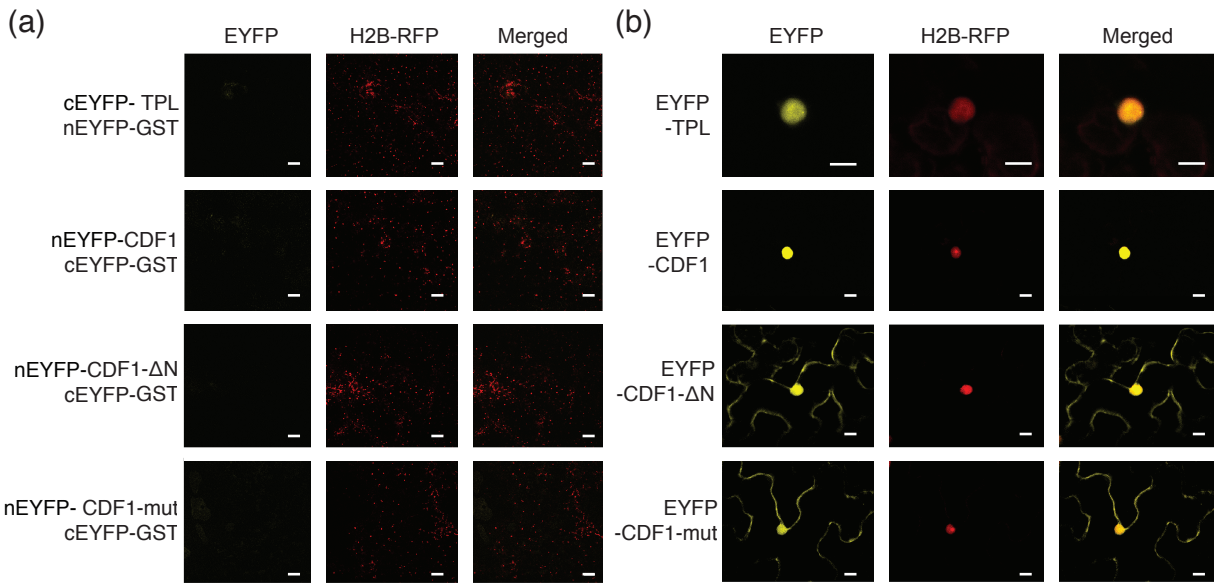


Figure 3-S2. BiFC negative controls and subcellular localization of TPL, CDF1, CDF1-ΔN, and CDF1-mut. (a) BiFC interaction analysis in transiently infected N. benthamiana leaves between full-length of CDF1 protein, CDF1-ΔN, CDF1-mut variants, TPL, and GST protein (a negative control). Scale bars show 50 μm. (b) Representative images of full-length YFP fusions of CDF1 protein, CDF1-ΔN, CDF1-mut variants, and TPL protein in transiently infected N. benthamiana leave epidermal cells. Scale bars show 10 μm. For both (a) and (b) Histone H2B-RFP was used to determine the position of the nucleus in the same cell.

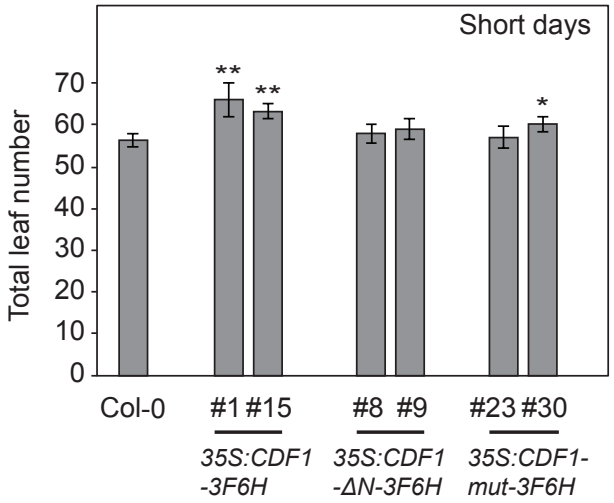


Figure 3-S3.Flowering phenotype of Col-0, *35S:CDF1-3F6H*, *35S:CDF1-∆N-3F6H*, and *35S:CDF1-mut-3F6H* transgenic lines in short days. Quantification of flowering time by total leaf number at bolting from figure 2d under long days. Means +/- SEM were calculated from N=16 individuals. *P < 0.05, **P < 0.01 (one-tailed t test).

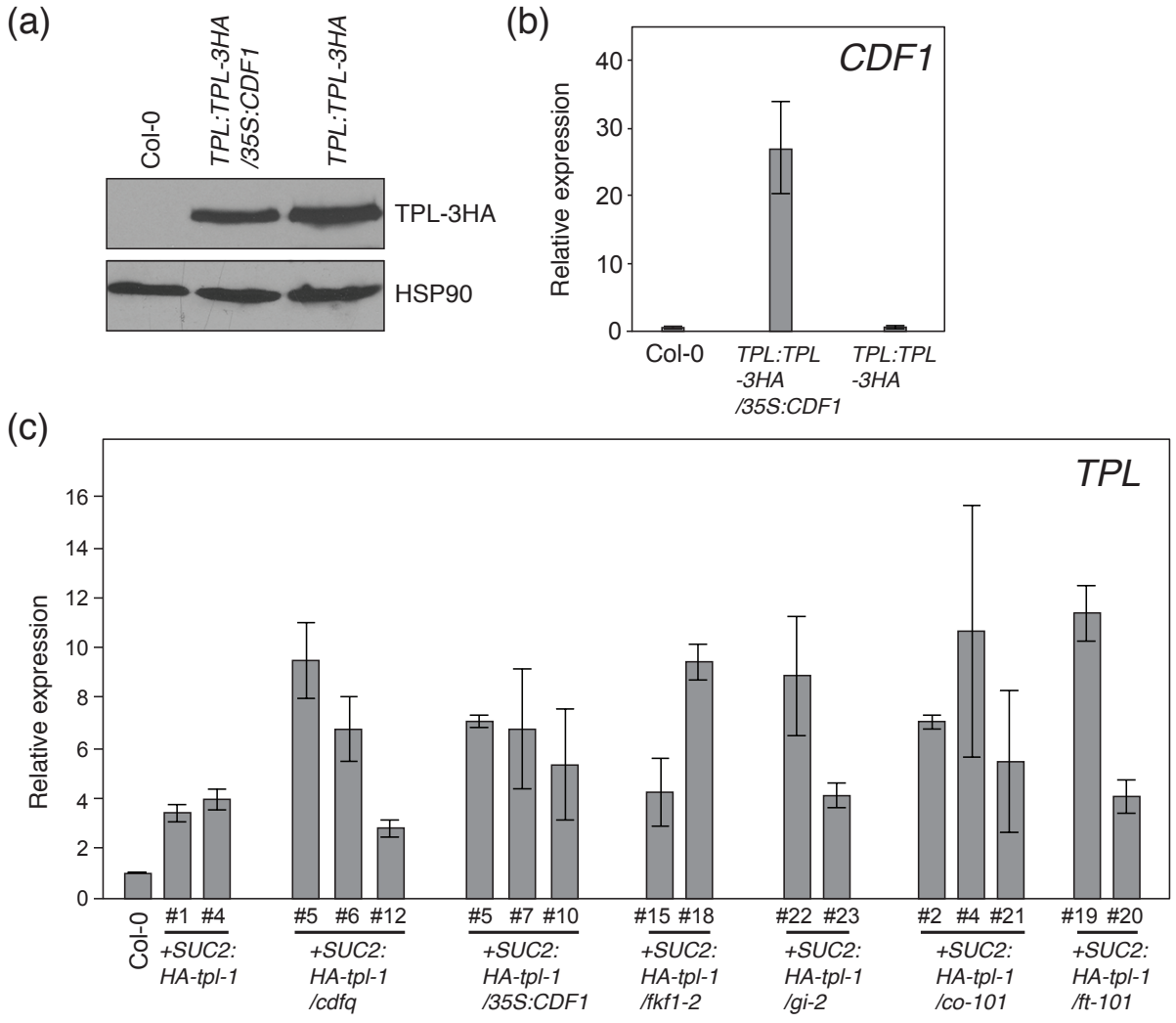


Figure 3-S4. Protein expression level of TPL in *TPL:TPL-3HA* and *TPL:TPL-3HA/35S:CDF1* transgenic line, *CDF1* gene expression in *TPL:TPL-3HA/35S:CDF1* transgenic line, and *TPL* gene expression in *SUC2:HA-tpl-1* transgenic lines. (a) Protein expression levels of TPL-3HA in Col-0, *TPL:TPL-3HA*, and *TPL:TPL-3HA/35S:CDF1*. The long-day grown 14-day-old seedlings were harvested at the ZT12 time point. HSP90 was used as a loading control. (b) Gene expression analysis of *CDF1* in Col-0, *TPL:TPL-3HA*, and *TPL:TPL-3HA/35S:CDF1* plants. The long-day grown 14-day-old seedlings were harvested at the ZT4 time point. (c) Gene expression analysis of *TPL* in *SUC2:HA-tpl-1* transgenic lines. The long-day grown 14-day-old seedlings were harvested at the ZT12 time point. For (b) and (c), means +/- SEM were calculated from three independent experiments.

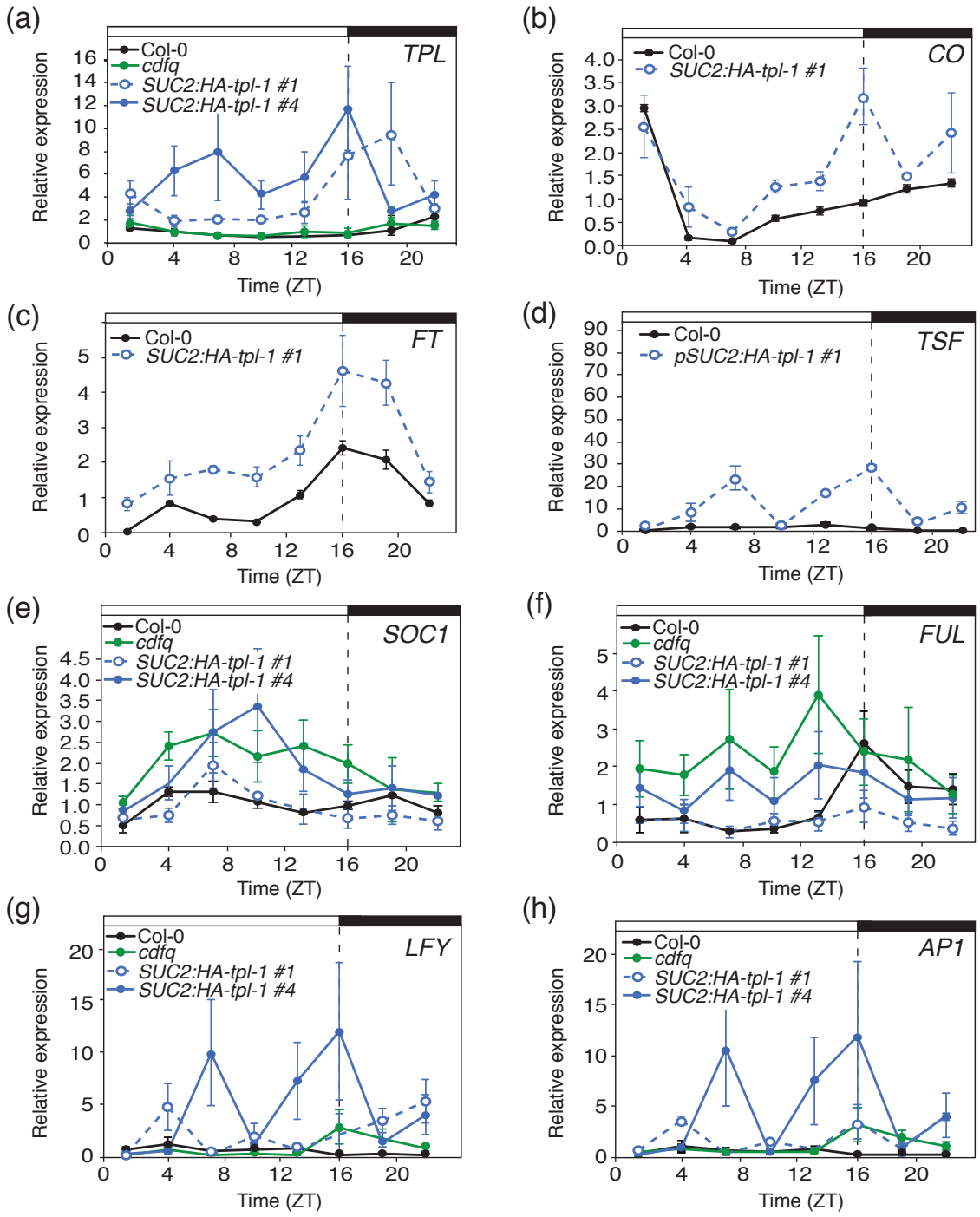


Figure 3-S5. Gene expression profiles of *TPL*, *LFY*, *AP1*, *FUL*, and *SOC1* in WT, *cdfq* mutants, and *SUC2:HA-tpl-1* transgenic lines. (a,e,f,g,h) Diurnal gene expression analysis of *TPL*, *SOC1*, *FUL*, and *AP1* in Col-0, *cdfq*, and *SUC2:HA-tpl-1* plants. Experiments were performed on 2-week-old seedlings grown in long days, with 3-hour resolution. Means +/- SEM were calculated from four independent experiments. (b,c,d) Diurnal gene expression analysis of *CO*, *FT*, and *TSF* in Col-0 and the *SUC2:HA:tpl-1* #1 transgenic line.

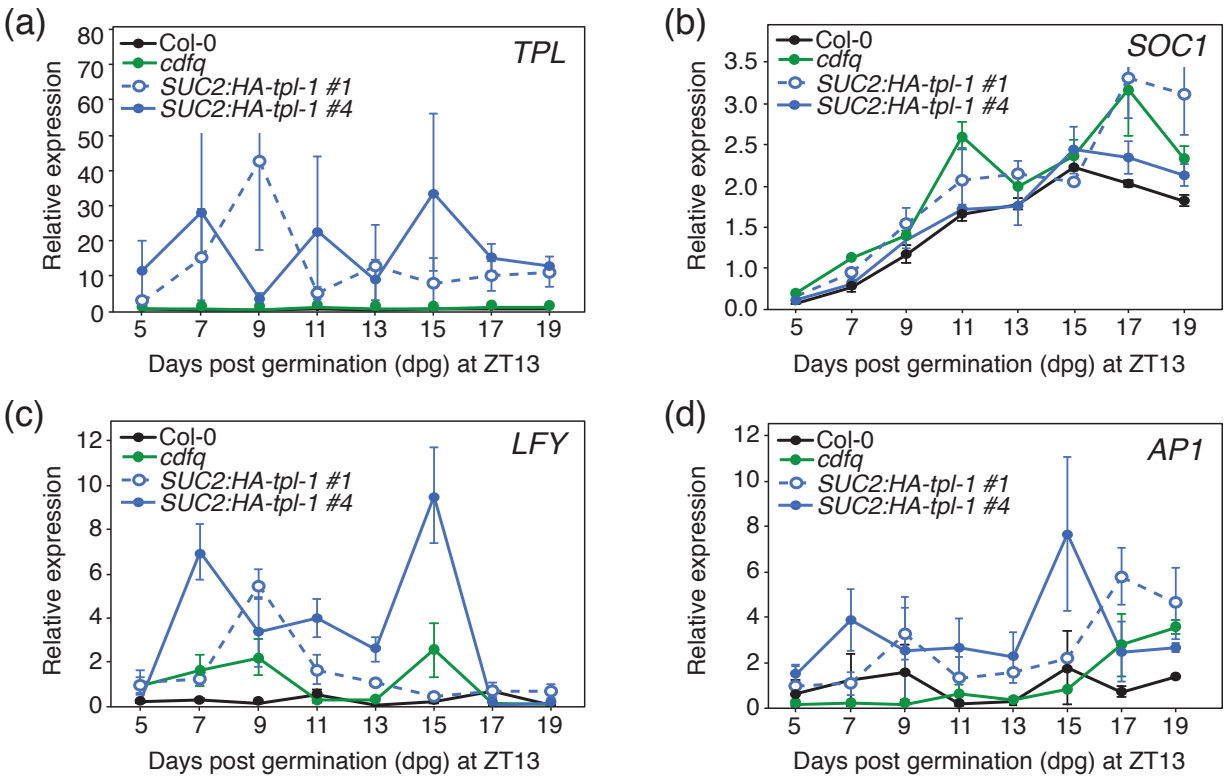


Figure 3-S6. Gene expression levels of *TPL*, *SOC1*, *LFY*, and *AP1* over developmental time in WT, *cdfq* mutants, and *SUC2:HA-tpl-1* transgenic lines. (a,b,c,d) Gene expression analysis of *TPL* (a), *SOC1* (b), *LFY* (c), and *AP1* (d) in Col-0, *cdfq*, and *SUC2:HA-tpl-1* plants over developmental time (days post germination). 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.

CHAPTER IV.

CYCLING DOF FACTOR 6 (CDF6) IS A CIRCADIAN CLOCK REGULATED REPRESSOR OF FLOWERING

Segments of this chapter previously appeared in preprint on BioRxIV:

Time-resolved interaction proteomics of the putative scaffold protein GIGANTEA in *Arabidopsis thaliana*. Johanna Krahmer, Greg Goralogia, Akane Kubota, Karen Halliday, Michael J. MacCoss, Thierry LeBihan, Takato Imaizumi, Andrew Millar.
Article DOI: 10.1101/162271

INTRODUCTION

Regulation of the timing of flowering is a critical process in plants (Thomas and Vince-Prue, 1996). In *Arabidopsis thaliana*, particularly amongst summer annual accessions, longer photoperiods quickly accelerate the initiation of flowering (Song *et al.*, 2015). Exposing Arabidopsis plants to longer photoperiods increases the expression of the florigen-encoding gene *FLOWERING LOCUS T* (*FT*), where the subsequent movement of FT protein to the shoot apical meristem leads to downstream interactions which facilitate a developmental switch from a vegetative cell fate to a reproductive one at the shoot apex (Figure 2-5) (Andrés and Coupland, 2012).

Temporal regulation of the *FT* gene is of critical importance in the coordination of this response, and is a combinatorial process determined by interaction downstream of the endogenous circadian clock and light perception through photoreceptor signaling (Figures 2-3, 2-4) (Song *et al.*, 2015). This process largely follows the external coincidence model for photoperiodic phenomena, first described by Colin Pittendrigh, although elements of the pathway also resemble an internal coincidence mechanism (Figure 1-1) (Pittendrigh, 1972; Imaizumi and Kay, 2006). A key factor in the activation of the *FT* gene is the transcription factor CONSTANS (CO), a B-box and CCT-domain protein which can bind to both proximal

and distal *cis*-elements of the *FT* promoter and is largely required to activate transcription of *FT* (Figure 2-4) (Putterill *et al.*, 1995; Suárez-López *et al.*, 2001; Adrian *et al.*, 2010). Under long-day conditions, CO is able to activate *FT* expression during the dusk period (Suárez-López *et al.*, 2001). After the dark period begins, CO protein is quickly degraded through a COP1-SPA dependent mechanism (Figure 2-2) (Laubinger *et al.*, 2006).

The timely initiation of *CO* and *FT* transcription is dependent upon a relief of repression mechanism facilitated by the CYCLING DOF FACTOR (CDF) family of transcription factors, which directly bind to both the *CO* and *FT* promoters (Figure 3-7f) (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Song *et al.*, 2012). *CDF* genes are regulated as a direct output of the endogenous circadian clock, and are highly expressed during the late night and early morning (Imaizumi *et al.*, 2005). Activation of *CDF*s is accomplished through the activity of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) during this time period (Nakamichi *et al.*, 2007). Repression of *CDF*s later in the day is performed by the PSEUDO RESPONSE REGULATOR (PRR) proteins PRR5, PRR7 and PRR9 (Nakamichi *et al.*, 2007). This expression of *CDF*s during the morning allows for transcriptional repression of both the *CO* and *FT* loci (Figure 3-7f). An absence of CDF removal during the daytime prevents the initiation of flowering during shorter photoperiods.

In order to remove CDFs from the promoters of floral activation genes *CO* and *FT*, the blue light receptor E3 ubiquitin ligase protein FLAVIN BINDING KELCH REPEAT F-BOX 1 (FKF1) and its protein complex partner GIGANTEA (GI), target CDFs for degradation once they become highly expressed during the afternoon of long-days (Figure 2-3) (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Fornara *et al.*, 2009). Both *GI* and *FKF1* expression is also regulated by the circadian clock (Fowler *et al.*, 1999; Imaizumi *et al.*, 2003; Mizoguchi *et al.*, 2005). This removes the CDF repressors from the *CO* and *FT* promoters, increasing *CO* expression after ZT13, and subsequently resulting in a peak of *FT* expression during dusk (ZT16).

The DOF (DNA binding with One Finger) family of transcription factors is a plant specific group of transcription factors that serves a variety of regulatory functions in plants ranging from vascular development, seed germination, stress responses, and flowering time (Yanagisawa, 2002; Yanagisawa, 2004; Noguero *et al.*, 2013). The DOF domain is of the zinc finger type, in the C_2C_2 configuration. Strangely, unlike other members of the zinc-finger TF superfamily, DOF domain proteins only contain a single zinc

ion-coordinating site (Yanagisawa, 2004). The mechanism of binding, whether single direct or in multimeric, is currently unknown. In Arabidopsis, there are 37 DOF transcription factors, divided into four distinct subclades (Yanagisawa, 2002). The CDF-like members cluster together in subclade (3b) or (A), and contain a number of unique shared domains (Yanagisawa, 2002; Lucas-Reina *et al.*, 2015). These include a TOPLESS (TPL) coordinating motif at their N-terminus (Figure 3-1a), as well as C-terminal sequences required for GI and FKF1 binding (Kloosterman *et al.*, 2013; Lucas-Reina *et al.*, 2015; Goralogia *et al.*, 2017). All Arabidopsis CDFs contain an NLS sequence adjacent to the DOF domain (Figure 3-1a) (Noguero *et al.*, 2013; Lucas-Reina *et al.*, 2015). CDFs together additively regulate the photoperiodic flowering response, and loss of function of multiple *CDF* genes shows a progressively earlier flowering phenotype in both short days and long days (Fornara *et al.*, 2009). At least at the *CO* gene, multiple copies of the DOF cis-element in tandem improve the ability of CDFs to repress transcription (Rosas *et al.*, 2014). The copy number of these tandemerized DOF repeats varies amongst Arabidopsis accessions (Rosas *et al.*, 2014).

In this study, we performed a GI protein interaction anaylsis in different cellular compartments (nuclear vs. cytosolic) and at different times of day. Through the course of this analysis (time series data not shown), we identified AtDof1.3 (At1g26790) as a potential interactor of GI. I will enumerate here how this hitherto uncharacterized DOF gene encodes a CDF-like protein, is expressed during the morning similar to other CDF TFs in Arabidopsis, is regulated by the circadian clock, interacts with FKF1-group proteins as well as GI, and negatively regulates flowering time through repression of *CO* and *FT*.

RESULTS

CYCLING DOF FACTOR 6 (*CDF6/ AtDof1.3*) ENCODES A CDF-LIKE PROTEIN

Our concurrent study of GI protein-protein interactions using an IP-mass spectrometry approach isolated a DOF-type transcription factor AtDof1.3 as a putative interactor. AtDof1.3 was found within the time-series dataset (data not shown), but not the nuclear vs. cytosolic dataset (Table 4-1). So, far *CDF1,2,3,4*, and *CDF5* have been isolated and shown to additively regulate the photoperiodic flowering

response (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). We wondered whether *AtDof1.3* was a member of the *CDF* subclade and had escaped previous analysis, or whether it belonged to another subclade of Arabidopsis *DOF*s. We performed a phylogenetic analysis on protein sequences of a subset of Arabidopsis DOF transcription factors, and found that AtDof1.3 is located within the CDF subclade, and is most closely related to CDF5 (Figure 4-1). Due its close relationship to other Arabidopsis *CDFs*, we decided to name AtDof1.3 CYCLING DOF FACTOR 6 (CDF6). We then performed a protein alignment to observe whether CDF6 contained or lacked the similar domains and motifs present on other Arabidopsis CDFs. We found that CDF6 also contains a TPL binding motif, and has high sequence conservation in the C-terminal sequences, similar to CDF1,2,3, and CDF5 (Figure 4-2).

CDF6 IS EXPRESSED DURING THE MORNING AND IS REGULATED BY THE CIRCADIAN CLOCK

A key feature of CDF transcription factors is their expression during the morning to inhibit photoperiodic flowering genes until they can be removed during the afternoon of long days (Imaizumi *et al.*, 2005). We sought to determine if *CDF6* was similar to other *CDF*s in this regard. We performed a gene expression analysis of *CDF6* under long day conditions, and we found that *CDF6* expression was high during the late night and morning, similar to other *CDF*s. We next wanted to determine if the circadian clock regulates *CDF6* expression. To investigate this, we performed a gene expression analysis on Wild type (Col-0) plants, where the plants were grown and entrained to normal long-day conditions for a total of 7 days, and then subsequently transferred to continuous light for 3 days. On the third day, seedlings were harvested at three-hour intervals. We found that under the continuous light transfer conditions, *CDF6* expression continues to oscillate, suggesting that *CDF6* expression is under the control of the circadian clock.

CDF6 INTERACTS WITH LOV DOMAIN FKF1-GROUP PROTEINS AND GI. OVEREXPRESSION OF *CDF6* RESULTS IN DECREASED *CO* AND *FT*, AND DELAYED FLOWERING

Normally under long-day conditions, as FKF1 and GI complex accumulates during the afternoon, CDFs are degraded to allow the expression of their targets, *CO* and *FT* (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Song *et al.*, 2012). Based on our protein sequence alignments, we anticipated that CDF6 is similarly regulated by FKF1 and GI through protein interaction and subsequent degradation. To test if the proteins indeed interacted, we performed a Yeast 2-hybrid (Y2H) analysis. We found that CDF6 likewise interacts with FKF1, LKP2, ZTL, and GI, as has been reported for other CDFs (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009).

Next, we wanted to determine whether or not CDF6 acts as a floral repressor similar to other CDFs. To test this, we constructed transgenic Arabidopsis *SUC2:HA-CDF6* plants; these overexpression lines drove the HA epitope tagged CDF6 protein from a *SUCROSE PROTON SYMPORTER 2* (*SUC2*) promoter, which expresses only within the phloem companion cells (Truernit and Sauer, 1995). First, we tested whether *SUC2:HA-CDF6* plants had a delayed flowering time phenotype compared with wild type plants. Indeed, we found that *CDF6* tissue specific overexpressors had significantly delayed flowering compared to wild type. Additionally, we expected that this delay in the flowering time was due to CDF6 repression of *FT* and *CO* expression, so we performed a gene expression analysis of wild type and *SUC2:HA-CDF6* transgenic lines under long day conditions. As expected, we found that *CO* and *FT* expression is highly diminished in the *SUC2:HA-CDF6* transgenic plants throughout the day. Taken together, these data suggest that CDF6 acts as a floral repressor through decreasing *CO* and *FT* expression.

DISCUSSION

Here we show that the Arabidopsis DOF transcription factor CDF6 is a circadian regulated repressor of flowering, similar to other CDF-group transcription factors in Arabidopsis. Like its counterparts, CDF6 interacts with FKF1, GI, ZTL and LKP2 and has similar C-terminal sequences to other CDF proteins. Likely, CDF6 is degraded through similar mechanisms as other CDFs (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009; Kloosterman *et al.*, 2013).

CDF transcription factors have been characterized genetically as having an additive role in the repression of *CO* and *FT*, as the phenotype progressively becomes more drastically early flowering as

additional *CDF* genes are nullified (Fornara *et al.*, 2009). Construction of a *cdf1,2,3,5,6* quintuple mutant might further detail the full contribution of CDFs towards floral repression, as this will remove all of the morning expressed CDFs. Hopefully this will further inform us as to the minimal developmental competency to flower while the photoperiod pathway is in a permissive state.

Major questions still remain about how CDF group proteins might act as a transcriptional complex on their target promoters. Although only CDF1 has been shown to directly bind to the *CO* and *FT* promoters, most of the CDFs likely occupy the same sites (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Song *et al.*, 2012). Our understanding of the DOF domain itself is also limited; although the canonical DOF binding sequence is known, some studies have suggested that flanking sequences are important in binding kinetics (Yanagisawa and Sheen, 1998; Yanagisawa and Schmidt, 1999; Yanagisawa, 2002; Yanagisawa, 2004). More detailed studies regarding the preference of DOF proteins or the diversity of DOF cis-elements throughout the larger family will have to be performed to answer this question. For example, there is a discrepancy even between *CO* and *FT*, as *CO* contains at least 1 multimerized DOF site, whereas *FT* has no similar site within its promoter (Rosas *et al.*, 2014; Simon *et al.*, 2015; Goralogia *et al.*, 2017). This begs the question of how CDFs operate together at *CO* and *FT*. Are CDF6 and other CDFs able to form heterodimers or homodimers? Can single proteins indeed bind DNA through only the single zinc-finger? Do different CDFs have distinguishing binding times throughout the day or are they completely additive? In this case *CDF6* appears to have an overlapping daily expression pattern with other *CDF*s, and thus likely has a very similar role in its association with the *CO* and *FT* promoters.

Our approach in this study, utilizing IP-mass spectrometry as a discovery method for GI interacting proteins, was able to reveal another CDF group protein that had not been characterized. Due to the functional redundancy of *CDF*s, this approach has the benefit of being unbiased based on gene ontology or phylogenetic relationship. Additionally, this approach uncovered CDF6 due to the time-series data we performed. Because our nuclear vs. cytosolic experiments were performed at ZT13, when GI protein levels are highest, we were unable to isolate any CDF proteins in the nuclear fraction, likely because CDFs had been degraded by ZT13. This further validates a combinatorial approach to IP-mass spec approaches in circadian regulated pathways. Relative gene expression levels of *CDF6* are quite low, so other methods dependent upon transcript levels may not have identified it. Being able to capture the temporal dynamics of

interaction has the key benefit of being able to categorize compartmentalized functions, especially in a protein with such diverse functions as GI (Cao *et al.*, 2005; Jung *et al.*, 2007; Kim *et al.*, 2007; Riboni *et al.*, 2013).

EXPERIMENTAL PROCEDURES

GENERATION OF PLANT MATERIALS

To generate *SUC2:HA-CDF6* plants, the *CDF6* CDS was PCR-amplified using cDNA derived from long-day grown plants as a template, and cloned into pENTR D-TOPO (Invitrogen), to form pENTR HA-CDF6. 2.3 kbp of the SUC2 5' upstream promoter region was amplified and cloned into the pENTR 5'-TOPO vector (Invitrogen), to form pENTR 5' SUC2. Using a sequential LR clonase II reaction (Invitrogen), we integrated the pENTR 5' SUC2, pENTR HA-CDF6 into the R4pGWB501 vector (Nakagawa et al. 2008), to form *pSUC2:HA-CDF6*. After confirming the sequence, this vector was transformed into Col-0 wild-type plants using by *Agrobacterium*-mediated transformation. Transgenic plants were selected based on the expression level of *CDF6* gene expression.

PLANT GROWTH CONDITIONS

For flowering time experiments, seeds were sown and stratified at 4 °C for 3 days on soil (Sunshine Mix #4; Sun Gro Horticulture), containing Osmocote Classic time-release fertilizer (Scotts, Marysville) and Systemic Granules: Insect Control (Bionide, Oriskany). Growth was at 22 °C under long-day conditions (16h light, full-spectrum white fluorescent light bulbs (F017/950/24 Octron; Osram Sylvania, 70-80 μmol·m−2·s−1). Flowering time was measured as the mean number of rosette leaves, for at least 16 plants per genotype, ± the standard error of the mean (SEM). For qPCR analysis, 10-day old seedlings were grown on 1× Linsmaier and Skoog (LS) media (Caisson), supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, under long-day conditions at 22°C in growth chambers (CU-36L5; Percival Scientific; lighting conditions as for flowering time) and harvested at 3-h intervals from 1 h after dawn.

PROTEIN EXTRACTION AND ENRICHMENT BY TAP FOR MASS SPECTROMETRIC ANALYSIS, AND BIOINFORMATICS ANALYSIS

Frozen plant tissue was ground to a fine powder in a liquid nitrogen and dry ice-cooled mortar and processed essentially as described (Song et al., 2014). For protein extraction, one tissue volume of SII buffer (100mM sodium phosphate pH7.4, 150mM KCl, 5mM EDTA, 5mM EGTA, 0.1% TritonX-100) or RIPA buffer (50mM Tris pH 7.5, 150mM KCl, 1% NP- 40, 0.5% Dexoycholate) with 1 Complete protease inhibitor Cocktail EDTA free mini tablet (Roche) per 10ml, PhosStop phosphatase inhibitor mixture (Roche), 50μM MG-132 and 1mM PMSF was added to the tissue and tubes were vortexed vigorously. Crude extracts were sonicated with a sonicating probe at 10μM amplitude for 10s three times, cleared at 3220xg twice and filtered through 0.45μm syringe filters. Protein was quantified with a standard Bradford assay. The remaining TAP procedures and mass spectrometry were as described (Song *et al.*, 2014).

For the qualitative study, database searches were performed using Comet (Eng et al. 2013), searching against the Uniprot Arabidopsis protein sequence database, and using Percolator (Matrix Science, Boston, USA) with a q-value cutoff of 0.01. Cysteine residue masses were considered statically modified by iodoacetamide, and methionine dynamically modified by a single oxidation. Precursor mass tolerance was 10 ppm, and product ion tolerance 0.5 Da. The principle of parsimony was used for protein inference, and at least two unique peptides were required for each identified protein.

QUANTITATIVE PCR (QPCR) ANALYSIS

Fresh seedlings were ground into powder using an automated mill with liquid nitrogen, and total RNA was isolated by using an illustra RNAspin Mini kit (GE Healthcare) according to the manufacturer's instructions. Two μg of total RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. cDNA was diluted to 5 times with water, and 2 μl was used as a template for quantitative PCR (qPCR) analysis using primers shown in Table S1. Isopentenyl pyrophosphate/dimethylallyl pyrophosphate isomerase (*IPP2*) was used as an internal control for

normalization. The average value from WT was set to 1.0 to calculate the relative expression of other lines. To amplify *CO* and *CDF6*, three-step PCR cycling program was used: 1 min at 95 °C, followed by 40–50 cycles of 10 s at 95 °C, melting temperatures for 15 or 20 s, and 72 °C extension for 15 s. To amplify *GI*, *FT*, and *IPP2*, a two-step PCR cycling program was used: 1 min at 95 °C, followed by 40–50 cycles of 10 s at 95 °C and 20 s at 60 °C. Data show the average of three biological replicates with SEM; each measurement had two technical replicates.

YEAST-2-HYBRID ASSAY

Full-length *CDF6* coding sequence was PCR-amplified using cDNA as template with primers shown in Table S1, cloned into pENTR D-TOPO (Invitrogen) and sequence-verified. The plasmid cassette was transferred to pAS-GW, a gateway compatible bait vector (Nakayama et al. 2002) using LR clonase II (Invitrogen). The GI-N, GI-C, FKF1, LKP2, and ZTL clones not peer-reviewed) used in this analysis were described previously; GI-N and GI-C (Sawa *et al.*, 2007), and FKF1, LKP2, and ZTL (Imaizumi et al. 2005). Yeast strains Y187 and AH109 were transformed with prey and bait vectors, respectively using the standard yeast transformation protocol (Clontech). After colonies formed on –W or –L containing media, three independent colonies were grown together, and 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 experiments were repeated several times with the same results

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		Number of peptides identified in qualitative TAP experiment		GI-TAP timeseries enrichment (max. GI / WT)	
Accession	**Name**	**GI-TAP /RIPA**	**GI-TAP /SII**	**t-test q value**	**Fold-enrichment**
AT1G22770	**GIGANTEA**	**309**	**299**	**0.00043**	**76**
AT5G57360	**ZTL**	**47**	**61**	**0.00025**	**32**
AT5G06600	UBP12	15	20	0.00029	19
AT5G02500	**HSP70-1**	**12**	**16**	**0.092***	**2.5**
AT3G13920	EIF4A1	5	10	n.d.	n.d
AT1G68050	**FKF1**	**6**	**9**	**0.00052**	**12**
AT2G18915	**LKP2**	**6**	**9**	**0.00097**	**13**
AT1G75950	**ASK1**	**7**	**8**	**0.0013**	**8.6**
AT1G47128	RD21A	8	7	0.00025	19
AT3G08530	CHC2	1	5	0.40*	2.4
AT5G17920	MS1	3	4	0. 0.095*	1.8
AT3G12780	PGK1	2	3	0.0046	4.63
AT5G43060	RD21B	4	2	n.d.	17247
AT5G60390	EFTuEF1-A	4	2	n.d.	n.d.
AT2G44060	AT2G44060	3	2	n.d.	n.d
AT5G42190	**ASK2**	**2**	**2**	**0**	**n.d**
AT1G56070	LOS1	1	2	0.0037	2.9
AT3G17390	SAM4	2	1	0.016	1.9
AT1G16030	**HSP70-5**	**1**	**1**	**0.092***	**2.5**
AT4G03550	CALS12	1	1	n.d.	n.d
AT1G70490	ARF2-B	1	1	n.d.	n.d
AT1G80870	AT1G80870	1	1	n.d.	n.d.
AT2G29420	GSTU7	1	1	n.d.	n.d.
AT3G58350	RTM3	1	1	n.d.	n.d.
AT5G23540	AT5G23540	1	1	0.150*	1.9

Table 4-1. Candidate interacting proteins identified in the nuclear vs. cytoplasmic qualitative study. Control (WT tissue) and GI-TAP samples were extracted in RIPA or SII buffer. 25 Arabidopsis proteins were identified by at least one peptide in each GI-TAP sample and none in the controls, excluding likely contaminant proteins that are abundant (ribosome, cytoskeleton) or localized to other compartments than GI (chloroplast, mitochondria). Bold: known direct or indirect interactors and homologues. *: below threshold in time series study. n.d., not detected.

Gene	Forward primer (5'->3')	Reverse primer (5'->3')	Melting temp.	time (sec)	Aim
CDF6	CGAGTCTTCGGACTCTTTGG	GACGGGCATGTGGTAGAAAC			qPCR
CO	CTACAACGACAATGGTTCCATTAAC	CAGGGTCAGGTTGTTGC	56	20	qPCR
FT	CTGGAACAACCTTTGGCAAT	TACACTGTTTGCCTGCCAAG	55	20	qPCR
GI	GGGTAAATATGCTGCTGGAGA	CAGTATGACACCAGCTCCATT			qPCR
IPP2	GTATGAGTTGCTTCTCCAGCAAAG	GAGGATGGCTGCAACAAGTGT			qPCR
HACDF6	CACCATGTACCCATACGATGTTCCAGATTACGCTATGTCTCAAGTTAGAGAT	TCATTATATGCTCTCTCTGAAGTTCATAG			Cons.

Primers used in the course of this study.

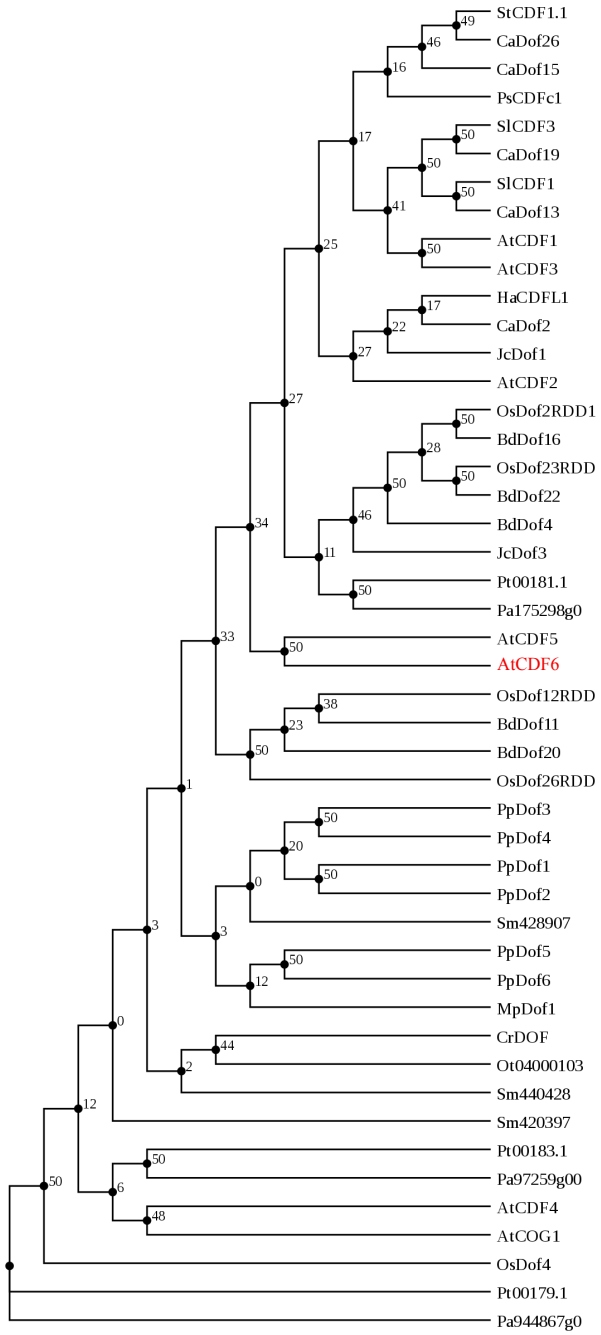

Figure 4-1. CDF6 is most closely related to Arabidopsis CDF5 among CDF group proteins. Phylogenetic analysis was performed using MUSCLE alignment, then subsequently analyzed using a neighbor-join-ing algorithm. Bootstrapping was performed 50 iterations, and bootstrapping support for each clade is listed with the support/50. The species included are as follows: Ps= *Pisum sativum*, Sl= *Solanum lycopersicum*, St= *Solanum tuberosum*, Ca= *Capsicum annuum*, Ha= *Helianthus annuus*, Jc= *Jatropha curcas*, At= *Arabidopsis thaliana*, Os= *Oryza sativa*, Bd= *Brachypodium distachyon*, Pt= *Pinus taeda*, Pa= *Picea abies*, Sm= *Selaginella moellendorffii*, Pp= *Physcomitrella patens*, Mp= *Marchantia polymorpha*, Cr= *Chlamydomonas reinhardtii*, Ot= *Ostreococcus tauri*.

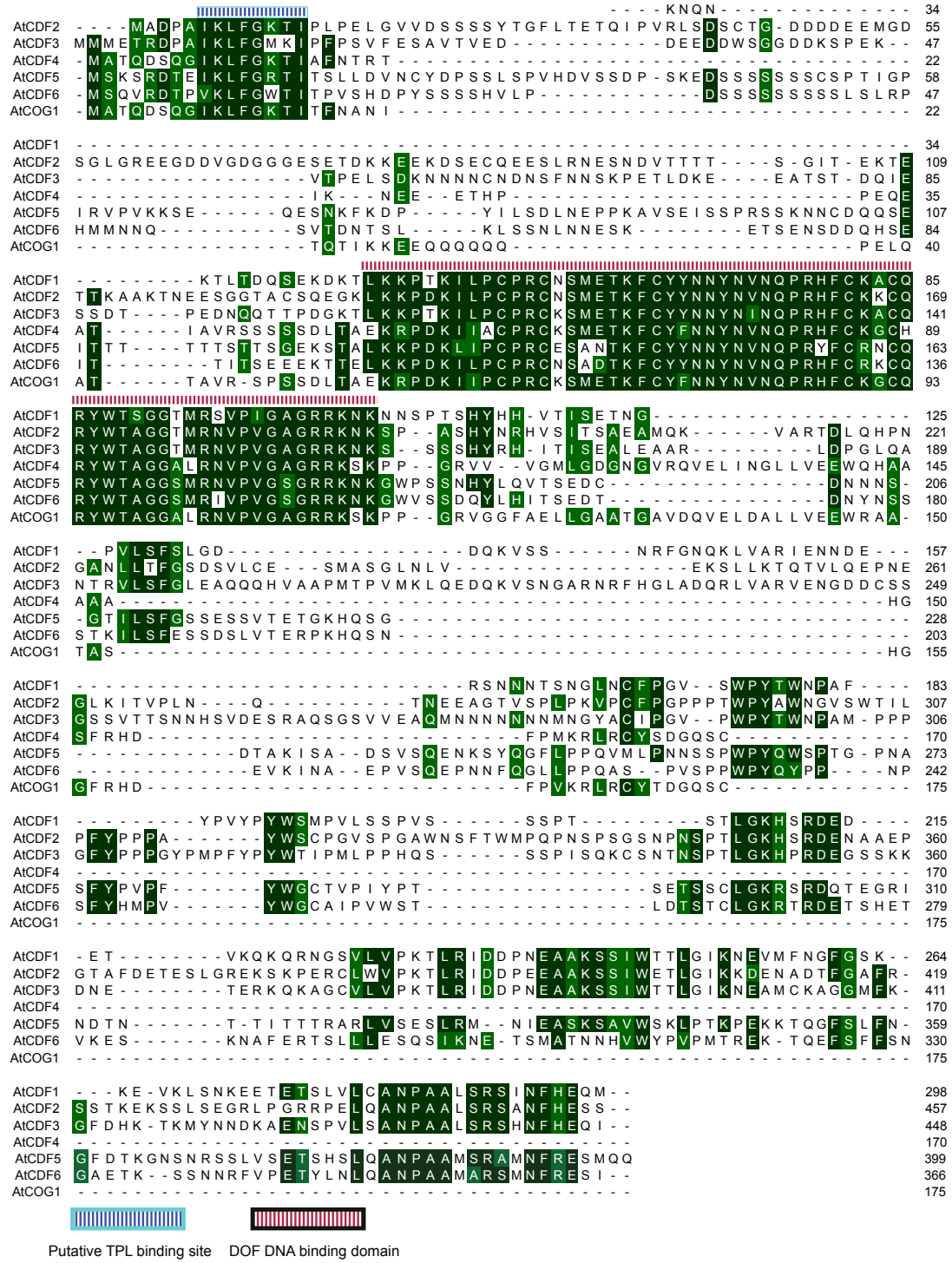

Figure 4-2. CDF6 contains similar protein motifs to other Arabidopsis CDFs. Dark Protein sequences subsequently were assembled and aligned using the CLUSTAL omega program. Dark green color indicates amino acid positions with more than 50% identical residues. Light green indicates 50% of amino acid positions are similar. Red dashed bar indicates the consensus sequences of the DOF DNA binding domain. The blue dashed bar indicates the putative TPL interaction motif. Relative amino acid position number is indicat-ed on the right side of the figure

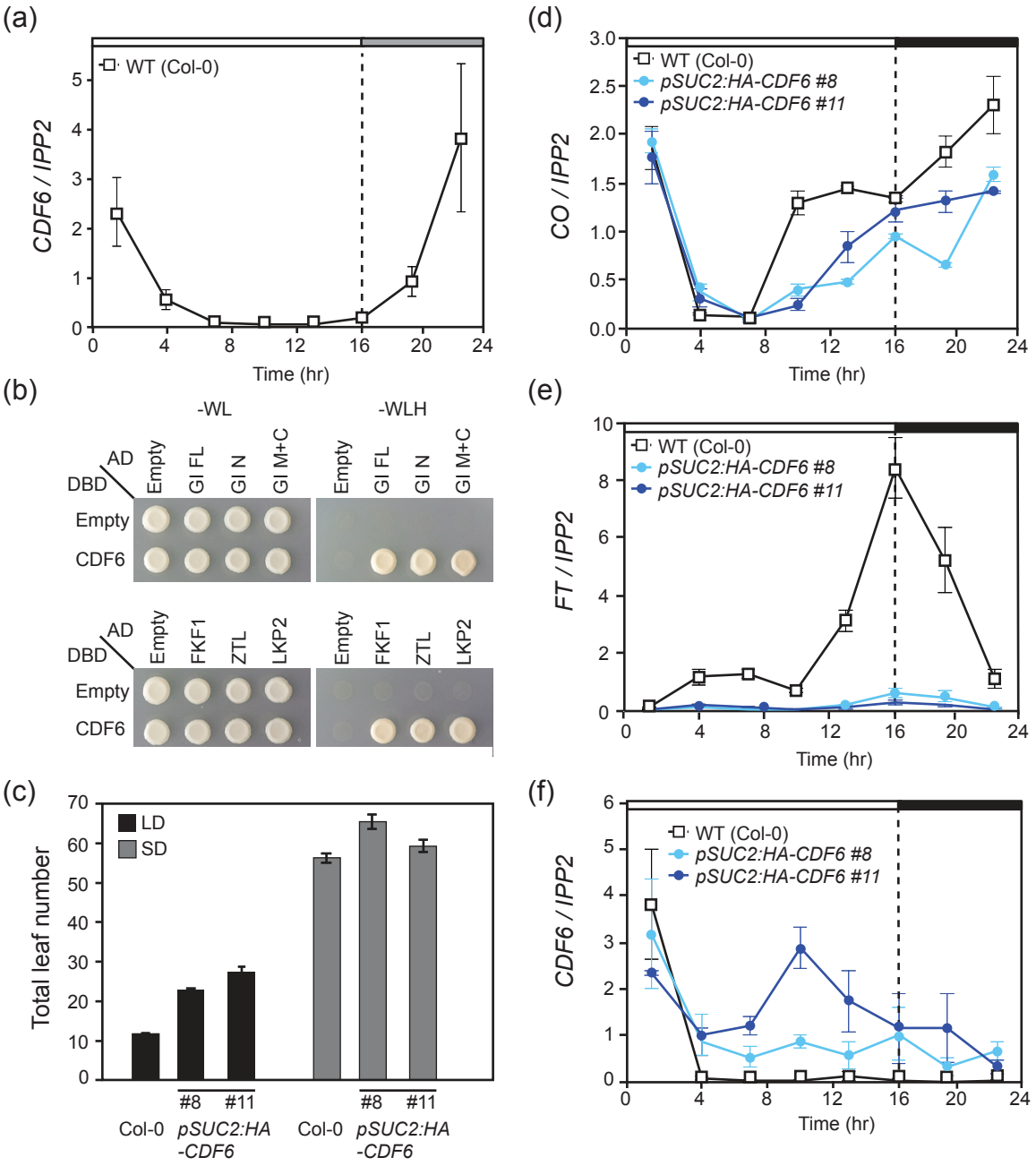

Figure 4-3. *CDF6* is regulated by the circadian clock, interacts with FKF1 group proteins, and acts as a transcriptional repressor of *CO* and *FT*. (a) Circadian expression profile of *CDF6* mRNA, in WT plants 3 days after transfer to constant light. (b) Yeast two-hybrid assays validate interaction of CDF6 with full-length GI, N- and C-terminal domains of GI, as well as ZTL, FKF1 and LKP2. (c) Constitutive *CDF6* expression delays flowering of transgenic *pSUC2-HA:CDF6* lines more under long days, compared to WT control, than under short days. Each transgenic line differed significantly from WT, t-test $p<0.0001$, except #8 in SD, not signif-icant. (d-f) RNA expression profiles of *CO*, *FT* and *CDF6* were tested by qPCR in WT and *pSUC2-HA:CDF6*, confirming that CDF6 suppresses evening *CO* and *FT* expression.

CHAPTER V.

PHOTOCYCLE KINETICS OF FLAVIN BINDING KELCH REPEAT F-BOX1 (FKF1) REGULATES PHOTOPERIODIC FLOWERING IN ARABIDOPSIS.

A PRELIMINARY STUDY

INTRODUCTION

A key characteristic of photoperiodic responses that are examples of external coincidence is the ability to perceive light during a critical period set by the circadian clock (Figure 1-1) (Pittendrigh, 1972). In Arabidopsis the light receptor protein and E3-ubiquitin ligase FLAVIN BINDING KELCH REPEAT F-BOX1 (FKF1) fulfills this role through multiple functions (Figures 2-2, 2-3) (Ito *et al.*, 2012). FKF1 expression is controlled by the circadian clock, and is expressed during the afternoon, peaking at about 12 hours after dawn (Nelson *et al.*, 2000; Imaizumi *et al.*, 2003). When FKF1 is expressed, it is able to form a protein complex with the GIGANTEA (GI) protein (Sawa *et al.*, 2007). *GI* is also regulated by, and is a component of the circadian clock, and is expressed concomitantly (Park *et al.*, 1999; Mizoguchi *et al.*, 2005). GI-FKF1 complex is able to bind to the CYCLING DOF FACTOR (CDF) family of transcriptional repressors, and target them for degradation through polyubiquitination (Figure 2-4) (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). FKF1 has an additional role in stabilization of a key transcriptional activator of photoperiodic flowering, CONSTANS (CO) (Figure 2-2) (Song *et al.*, 2012). Normally, CO protein is highly unstable in the dark through degradation by CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) and SUPPRESSOR OF PHYA-105s (SPAs) (Figure 2-2) (Laubinger *et al.*, 2006; Liu *et al.*, 2008). Through interactions in the light-sensing domain of photoactive FKF1, CO protein is stabilized (Song *et al.*, 2012; Song *et al.*, 2014). CO protein expressed during the afternoon of long days is thus able to activate the expression of the *FLOWERING LOCUS T (FT)* gene, which encodes a mobile protein capable of mediating the

transition to flowering through interactions at the shoot apical meristem (Figures 2-4, 2-5) (Andrés and Coupland, 2012). In these ways, FKF1 acts as a key mediator of photoperiodic flowering through external coincidence, by acting as the photo-sensory and clock regulated factor.

Plants have evolved a wide array of photoreceptor proteins to enable the perception of different intensities and wavelengths of light, and the function of these photoreceptors is critical in a diverse set of physiological and developmental responses (Chen *et al.*, 2004). These range from photomorphogenesis, to phototropism, and shade avoidance, just to mention a few (Chen *et al.*, 2004). These photoreceptor proteins include the phytochromes (red and far-red), cryptochromes (blue), phototropins (blue), and ultraviolet-B receptors (UV) (Christie and Briggs, 2001; Chen *et al.*, 2004; Thomas, 2006). FKF1 is a blue light photoreceptor that utilizes the LOV (Light, Oxygen, or Voltage) sensory domain similar to phototropins (Imaizumi *et al.*, 2003; Nakasako *et al.*, 2005; Ito *et al.*, 2012). Unlike phototropins, which primarily function through phosphorylation from their kinase domains, FKF1 functions through interactions between its F-box and Kelch repeat domains, and other target proteins (Briggs and Christie, 2002; Imaizumi *et al.*, 2005; Ito *et al.*, 2012). FKF1 also has two homologs in Arabidopsis that include the circadian clock factor ZEITLUPE (ZTL), and LOV KELCH PROTEIN 2 (LKP2) (Somers *et al.*, 2000; Schultz *et al.*, 2001). As mentioned, FKF1, ZTL, and LKP2 function primarily as light activated E3-ubiquitin ligases, and are thus able to bind other components of the SCF (SKP1/ Cullin / F-box) ubiquitin ligase complex through their F-box domain, to concert proteasomal degradation upon their targets (Briggs, 2007; Ito *et al.*, 2012).

To date, several analyses have been performed on the mechanism of LOV domain light perception, and the crystal structures of several LOV domains have been resolved (Pudasaini *et al.*, 2017). LOV domain proteins mainly utilize the flavin mononucleotide (FMN) molecule as a chromophore for light sensing (Salomon *et al.*, 2000; Kasahara *et al.*, 2010). The FMN molecule sits within a pocket of the LOV domain, and upon light irradiation, a covalent interaction is formed between the FMN molecule and an adjacent cysteine (Salomon *et al.*, 2000; Zoltowski *et al.*, 2009; Kasahara *et al.*, 2010). This covalent interaction is referred to as a “C4 adduct” and facilitates changes the overall structure of the LOV domain so as to be in the photoactivated state

(Ito *et al.*, 2012). Absorption of light in the dark state occurs at a maximum of 450nm (D_{450}) and upon excitation, the photoactive LOV domain shifts in its absorption spectrum towards a maximum at 390nm (S_{390}) (Salomon *et al.*, 2000; Zikihara *et al.*, 2006). In phototropins, the formation of the C4 adduct happens rapidly within 1-2 μs, after which dark reversion back to the ground state occurs within around 5 s to 3 min (Ito *et al.*, 2012). In FKF1, adduct formation is marginally slower at about 6 μs but the decay rate of dark reversion from photoactive to ground state is extremely slow at >65 hr (Ito *et al.*, 2012; Pudasaini *et al.*, 2017). This rate of conversion between the photoactive state back to the LOV ground state is referred to as the photocycle rate. Due to the turnover rate of FKF1 protein throughout the day, this largely means that FKF1 remains in the photoactive state for the entire period in which it remains a functional protein (Ito *et al.*, 2012). The Arabidopsis ZTL photocycle rate is significantly faster than FKF1, with a time value of about 1.4 hr (Pudasaini *et al.*, 2017).

Since the photoactive state of FKF1, ZTL, and LKP2 are very long compared especially against the phototropins (and other photoreceptors), we wondered how important having an extremely long dark recovery rate is for the function of the protein. For ZTL, the conversion rate between the two states has an important function, as ZTL has roles in both its dark state as well as its photoactive state (Más *et al.*, 2003; Pudasaini *et al.*, 2017). When ZTL is in its photoactive state, it can bind strongly to GI and HEAT SHOCK PROTEIN 90 (HSP90) (Pudasaini *et al.*, 2017). When ZTL reverts back to the ground state, it is no longer able to bind strongly to GI and HSP90, and instead targets the core clock proteins TIMING OF CAB EXPRESSION 1 (TOC1) and PSUEDO RESPONSE REGULATOR 5 (PRR5) for degradation by ubiquitin mediated proteasomal degradation (Más *et al.*, 2003; Han *et al.*, 2004; Kim *et al.*, 2007; Fujiwara *et al.*, 2008; Baudry *et al.*, 2010). This allows for the nighttime degradation of these afternoon phased clock regulators. As far as is known, FKF1 does not have a dark state function.

A recent study interrogated the result of changing the photocycle rate of ZTL on its function in circadian clock regulation (Pudasaini *et al.*, 2017). Based on the crystal structure and analysis of amino acids that are distinct between ZTL and FKF1, the major residues that account for the differences in photocycle rate between FKF1 and ZTL have been identified (Pudasaini *et*

al., 2017). Functional analysis of ZTL protein that had been converted from a wild type 1 hr photocycle, to progressively more FKF1-like photocycle showed several abnormalities that affected the function of the protein. One important amino acid, the G80R (R89 on FKF1) variant is a purely kinetic rate mutation. Because these ZTL variants have a slower transition back to ground state, there is less degradation of PRR5 and TOC1 during the evening (Pudasaini *et al.*, 2017). Another variant position V48I (I57 in FKF1) effects both the photocycle rate and has an allosteric defect relative to wild type ZTL (Pudasaini *et al.*, 2017). V48I variants bias another residue (Q154) involved in C4-adduct stabilization towards a more inward configuration, which mimics the formation in which ZTL is in the dark state. This results in a ZTL protein that is constitutively active in degrading PRR5 and TOC1 targets. Variants that include the V48I mutation show an arrhythmic clock phenotype dude to low levels of PRR5 and TOC1 (Pudasaini *et al.*, 2017).

FKF1's role among the FKF1/ ZTL/ LKP2 group is largely unique in its regulation of photoperiodic flowering, and the interaction of photocycle rate kinetics on the function of FKF1 is unknown. Here, we show that changing the photocycle rate of the very slow FKF1 LOV domain into a faster photo-cycling LOV through the incorporation of ZTL-like mutations negatively affects FKF1 flowering time function and results in late flowering in inductive photoperiods. We additionally show that the late flowering phenotype of faster photocycle FKF1 is due to decreased expression of *CO* and *FT* during the afternoon and evening, implying that CDF degradation is incomplete. We show that increasing the photocycle rate of FKF1 inhibits strong protein complex formation with typical interactors such as GI, ASK2, and CDF1.

RESULTS

INCREASING THE PHOTOCYCLE RATE OF FKF1 RESULTS IN LATE FLOWERING, SIMILAR TO *fkf1* MUTANT.

Because FKF1 is unique in both its functions in regulating photoperiodic flowering in Arabidopsis, and it has a very slow photocycle rate compared against similar LOV domain proteins, we wanted to test the functional significance of faster photocycle FKF1 variants on the photoperiodic flowering response. We constructed several transgenic Arabidopsis lines that expressed epitope-tagged FKF1 protein and FKF1 photocycle variants driven by a constitutive 35S promoter, and transformed them into the *fkf1* mutant background. These include *35S:HA-FKF1/ fkf1*, *35S:HA-FKF1(I57V)/ fkf1*, *35S:HA-FKF1(R89G)/ fkf1*, and *35S:HA-FKF1(I57V R89G) / fkf1.* We then selected individual transformants by mRNA and protein expression level. We found that while the expression of *FKF1* correlated with FKF1 protein for HA-FKF1, HA-FKF1(I57V) and FKF1(R89G), much higher levels of *HA-FKF1(I57V R89G)* expression were required to obtain similar protein levels to the other constructs (data not shown). While we were able to obtain similar amounts of protein accumulation of HA-FKF1(I57V R89G), this does suggest that the protein stability of this construct is weaker than other FKF1 variants. Photocycle rates for each construct are listed in (Figure 5-1c). We then analyzed the flowering time phenotype of the different transgenic plants under long day photoperiods. We found that *35S:HA-FKF1* / *fkf1* plants complemented the flowering time phenotype of the *fkf1* mutants, and had a similar flowering time phenotype compared with wild type (Figure 5-1e). The *35S:HA-FKF1(I57V R89G) / fkf1* double variant had a late flowering time phenotype, similar to *fkf1* mutant (Figure 5-1e). *35S:HA-FKF1(I57V)/ fkf1* and *35S:HA-FKF1(R89G)/ fkf1* plants showed an intermediate phenotype, with a delay of flowering over the wild type and *35S:HA-FKF1/ fkf1* line (Figure 5-1e). Taken together, this suggests that the fast photocycle of *35S:HA-FKF1(I57V R89G) / fkf1* is highly disruptive of FKF1 function in flowering time regulation and that slower photocycle kinetic rate is necessary for FKF1 function.

FASTER PHOTOCYCLE VARIANTS OF FKF1 DISRUPT AFTERNOON AND EVENING TRANSCRIPTION OF *CO* AND *FT*

Due to the flowering time phenotype of the faster FKF1 variant transgenics, two likely scenarios that would explain the loss of FKF1 function would be incomplete degradation of CDF transcription factors during the middle of the day, and the second would be that FKF1-dependent CO protein stabilization was disrupted, resulting in lower activation of *FT* towards the dusk of long days. In order to begin to investigate whether this could be case, we performed a gene expression analysis on 14 day old seedlings grown under long-day conditions and analyzed the expression of the *CO*, *FT* and *FKF1* genes. We found that at *CO,* which normally increases in expression at around ZT12, *35S:HA-FKF1(I57V)/ fkf1* lines had very low expression compared to wild type throughout the middle of the day, but levels increased during the nighttime (Figure 5-2f). Similarly, *35S:HA-FKF1(R89G)/ fkf1* transgenics had low levels of *CO* during the afternoon (Figure 5-2g). *35S:HA-FKF1(I57V R89G) / fkf1* plants had very low levels of *CO* during the afternoon, similar to *fkf1* mutants, but levels gradually increased during the night time (Figure 5-2h). *CO* expression levels in all of the faster photocycle variant lines follow a very similar trace pattern to *CDF1* overexpression transgenic plants (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009; Goralogia *et al.*, 2017). At *FT*, *35S:HA-FKF1(I57V)/ fkf1, 35S:HA-FKF1(R89G)/ fkf1, and 35S:HA-FKF1(I57V R89G) / fkf1* all had low levels of *FT* during the evening relative to the wild type and *35S:HA-FKF1/ fkf1* lines (Figure 5-2j-l). A small induction of *FT* occurred during the morning of all of the FKF1 variant lines, similar to the wild type (Figure 5-2j-l). In general, for both *FT* and *CO*, the *35S:HA-FKF1/ fkf1* line had very similar expression trace to wild type, but the line was not able to replicate the nighttime expression level of *CO* (Figure 5-2e). As we expected, *CO* and *FT* expression patterns were indicative of a loss of normal FKF1 function, which suggests inefficient degradation of CDF1 and a loss of CO protein stability during the afternoon and evening of long days.

PROTEIN COMPLEX FORMATION BETWEEN FKF1, GI, AND ASK2 IS ATTENUATED IN FASTER PHOTOCYCLE VARIANTS

Based on the gene expression phenotype of faster photocycle FKF1 variants, we anticipated that there might be an effect on FKF1's ability to interact with normal complex partners. The mechanism of FKF1-dependent degradation requires the photoactive form to degrade CDFs. This is distinct from ZTL, where the dark form LOV conformation facilitates the ability to degrade PRR5 and TOC1. Since faster FKF1 photocycle variants are more likely to have reverted to ground state relative to the wild type protein, we anticipated that some of the protein interactions would be attenuated, and this might explain the late flowering phenotype. To investigate this, we constructed Arabidopsis transgenic lines which express *35S:HA-FKF1* and *35S:HA-FKF1(I57V R89G) / fkf1,* and were transformed into the *pGI:GI-TAP/ gi-2* genetic background. We performed a co-immunoprecipitation assay during the dusk period before and after ZT16 of long days (Figure 5-3a). When GI protein was immunoprecipitated and FKF1 was subsequently detected, we found that FKF1 protein was strongly bound to GI during the daylight period and into the beginning of the night for the wild type FKF1 (Figure 5-3a). When we compared this against the *35S:HA-FKF1(I57V R89G) / fkf1* plants, we found only a very small amount of FKF1 protein bound to GI, and only during the light period (Figure 5-3a). This suggests that GI binding is highly attenuated by the increase in FKF1 photocycle rate.

Since FKF1 is an E3 ubiquitin ligase and requires interactions with other components of the SCF complex in order to degrade CDF repressors, we wondered whether the incorporation of faster photocycle variants would affect binding of FKF1 to components of the SCF. We performed a transient co-immunoprecipitation experiment in *N. benthamiana* leaves to check for interaction between ARABIDOPSIS SKP1 LIKE 2 (ASK2) and variants of FKF1. We found that the I57V FKF1 variant did not significantly negatively affect FKF1 interaction compared with wild type FKF1, but the R89G variant did attenuate the binding of FKF1 to ASK2. The I57V R89G double resembled the R89G single variant, suggesting that the R89G contributed the major effect of protein complex inhibition for ASK binding. Although additional experiments need to be performed, it is possible that, similar to ZTL, the V48/I57 residue could have different allosteric effects based on the protein target in question. This could explain why I57V incorporation

negatively affected GI binding to FKF1 but did not substantially affect ASK2 binding (Pudasaini *et al.*, 2017).

DISCUSSION AND FUTURE DIRECTIONS

LONGER STABILITY OF PHOTOACTIVE FKF1 PROTEIN IS IMPORTANT FOR FLOWERING FUNCTION

Here we have endeavored to find out whether or not the very slow rate at which FKF1 shifts back to ground state after light excitation is an important characteristic for FKF1 in its regulation of flowering in response to photoperiod. We created faster photocycling FKF1 variants with key residues swapped to more ZTL-like LOV domain kinetics, and assessed whether the protein remained functional. Plants expressing faster photocycle FKF1 variants were not able to rescue the late flowering phenotype of *fkf1* mutants, and this was attributable to a large decrease in *CO* and *FT* transcription during the afternoon and evening. We investigated whether the perturbation of slow photocycle rate could affect the formation of protein complexes required for the normal regulation of flowering time, and we found that both GI and ASK2 binding was attenuated in faster photocycle FKF1 variants. This suggests that the intrinsic photocycle rate of FKF1 of greater than 65 hr does have a function, and is useful for FKF1 to properly regulate the flowering time response.

Presumably, the mechanism by which faster photocycle FKF1 proteins are less efficient at FKF1-like roles is that the quicker decay back to the ground state creates a pool of FKF1 protein towards dusk that is not in the photoactive conformation. Because FKF1 does not have a dark-linked function like ZTL, the association of proteins required for the induction of flowering at the end of long days does not occur at the same level. This causes a decrease in transcription probably due to an increased amount of CDFs bound to both *CO* and *FT* loci. In addition to the different functionality of ZTL, the data that we have procured in changing the FKF1 photocycle rate suggests that the divergence of photocycle time between ZTL and FKF1 is an important one

for the function of each protein. Because ZTL has this switch between light dependent binding to GI and HSP90, and dark-mediated degradation of the clock proteins PRR5 and TOC1, a shorter photocycle makes sense; If ZTL had a slower photocycle, similar to FKF1, then too much photoactive ZTL would accumulate at night, which would prevent the timely degradation of PRR5 and TOC1.

One question in particular that this line of inquiry brings up is: how slow of a photocycle is too slow? In comparison to other flowering plant species, Arabidopsis FKF1 likely has a significantly slower photocycle rate. The presence of the I57 residue is rare among any other plants, and many species appear to have the V48 (ZTL-like) residue at that position (Pudasaini *et al.*, 2017). One potentially interesting characteristic is that potentially, changing the rate of FKF1 photocycle could make the plant more or less sensitive to longer or shorter photoperiods. For a long-day plant such as Arabidopsis, having a faster rate of FKF1 photocycle would make the plant less likely to flower during shorter intermediate photoperiods. Potentially this could be useful for facilitating the rational tweaking of the flowering time response. Although we do not have data at the moment as to the percentage of photoactivated FKF1with the normal slow photocycle *in vivo*, potentially having an even slower photocycle still could accelerate the transition to flowering by having more photoactive FKF1 earlier in the day.

DOMAIN SWAPPING OF THE ZTL LOV DOMAIN INTO FKF1

The major question posed by this study is whether or not the photocycle rate itself, alone, is important in the function of these photoreceptor proteins in regulating the transition to flowering. While the FKF1 and ZTL LOV domains are quite similar, they also have some unique features. Potentially the photocycle rate changing mutations that we have incorporated also require some context specific information from other residues within or outside the LOV domain (for instance in the F-box). In order to deal with some of these issues, an additional series of experiments that would be helpful would be to construct transgenic Arabidopsis plants that express FKF1 proteins with a ZTL LOV domain. Comparing the whole ZTL LOV domain swap in an otherwise FKF1

context next to the fast photocycle FKF1 will help in determining the effects of residues outside of the key rate determining ones. Additionally, if our hypothesis is correct regarding the need for a slow photocycle FKF1 for flowering function, progressively slowing down the photocycle rate of a FKF1-ZTL LOV chimeric protein back to FKF1 normal slow photocycle should rescue the *fkf1* mutants and restore the flowering time response. This would firmly cement the idea that the *rate* of the photocycle is the primary delimiter of function between the FKF1 and ZTL proteins.

EXPERIMENTAL PROCEEDURES

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. *fkf1* (Imaizumi *et al.*, 2003) and *gi-2* (Fowler *et al.*, 1999) were previously characterized. To generate each of the FKF1 photocycle variants, a megaprimer approach was utilized to perform the site directed mutagenesis. 5'CACCATGTACCCATATGATGTTCCAGATTATGCTATGGCGAGAGAACATGCGATC 3' was utilized as the forward primer and to incorporate the HA epitope tag. 5' CAGAGCATCGGAAACAACGAACGAAGGCGGAGT3' was used as a reverse primer to generate the I57V mutated megaprimer. 5'TAGGAATCGACAGTTACCACCAAGAACTTCATC3' was used as a reverse primer to generate the R89G mutated megprimer. After the first round of PCR and purification, the previous forward primer and 5' TTACAGATCCGAGTCTTGCC 3' as a reverse primer were used to generate the full length modified HA-FKF1 CDS. After purification, the products were incorporated into pENTR D-TOPO (Invitrogen) using the standard TOPO reaction protocol. 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. Individual transgenic lines were

chosen for experimental analysis based on protein levels detected by western blot at ZT13 (data not shown).

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). 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. 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.

RNA ISOLATION AND GENE EXPRESSION ANALYSIS

For gene expression analyses, 2-week-old seedlings were grown on LS agar plates in long 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*, *FKF1, CO* and *FT* (Song *et al.*, 2012), *IPP2* expression was used as an internal control to normalize cDNA amount.

CO-IMMUNOPRECIPITATION AND IMMUNOBLOT ANALYSIS

Co-immunoprecipiation experimental protocols were described previously (Song *et al.*, 2012; Song *et al.*, 2014). For immunoblots, 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% SDS-PAGE gels and transferred to nitrocellulose membranes (for each sample, 5–10 μg of total protein was used). 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.

ACKNOWLEDGEMENTS

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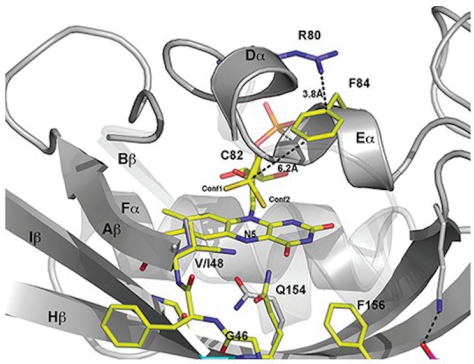
(a)



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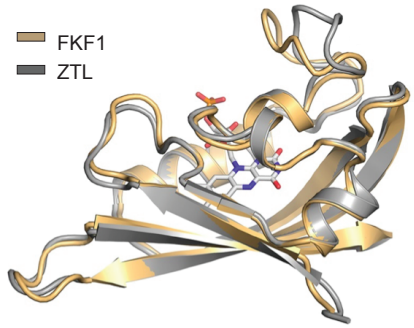
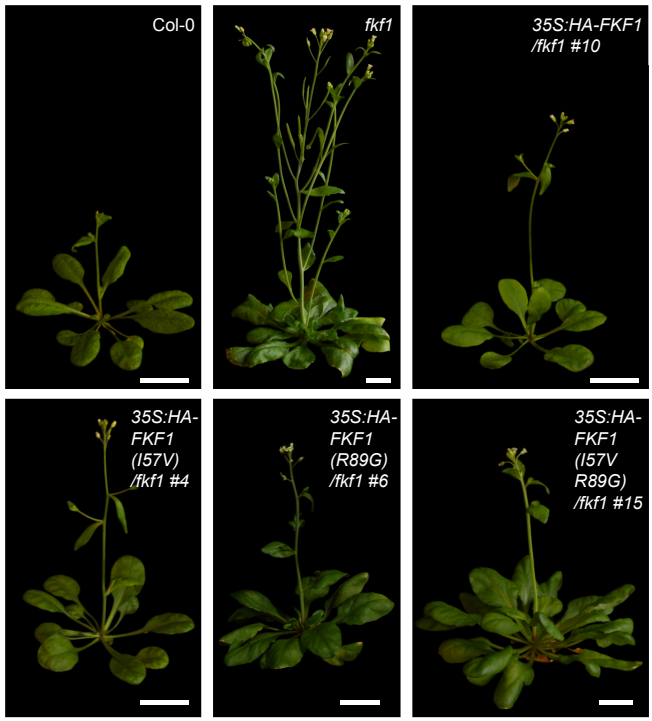


(c)

Construct	time constant (hr)
FKF1	>65
ZTL	1.4
FKF1(I57V)	15
FKF1(R89G)	31
FKF1(I57V/R89G)	4

(d)



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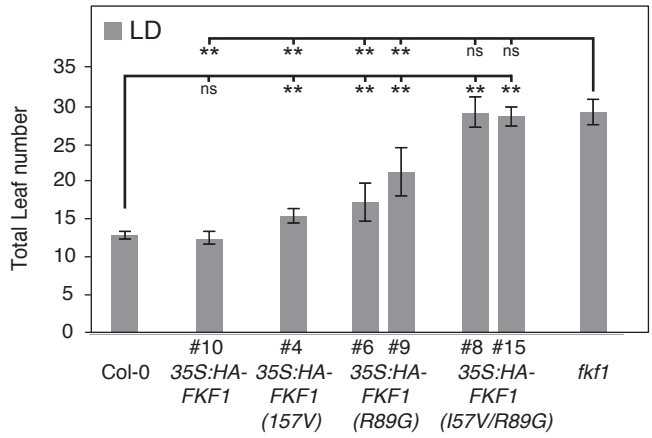

Figure 5-1. Complementation of *fkf1* mutants with fast photocycle FKF1 variants does not rescue the flowering time phenotype. (a) Structure of ZTL LOV domain FMN chromophore binding active site. (b) Overlay of predicted structure of FKF1 LOV over the ZTL LOV structure. (c) Photocycle times of proteins and variants used in this study. (d) Representative images of Col-0, *fkf1*, *35S:HA-FKF1/fkf1*, *35S:HA-FKF1(I57V)/fkf1*, *35S:HA-FKF1(R89G)/fkf1*,and *35S:HA-FKF1(I57V R89G)/fkf1* lines at flowering under long-day photoperiods. Scale bars=2cm. (e) Quantification of flowering time by total leaf number at bolting under long day photoperiods. Means +/- SEM were calculated from N=16 individuals *P < 0.05, **P < 0.01 (two-tailed t test) ns=non significant.

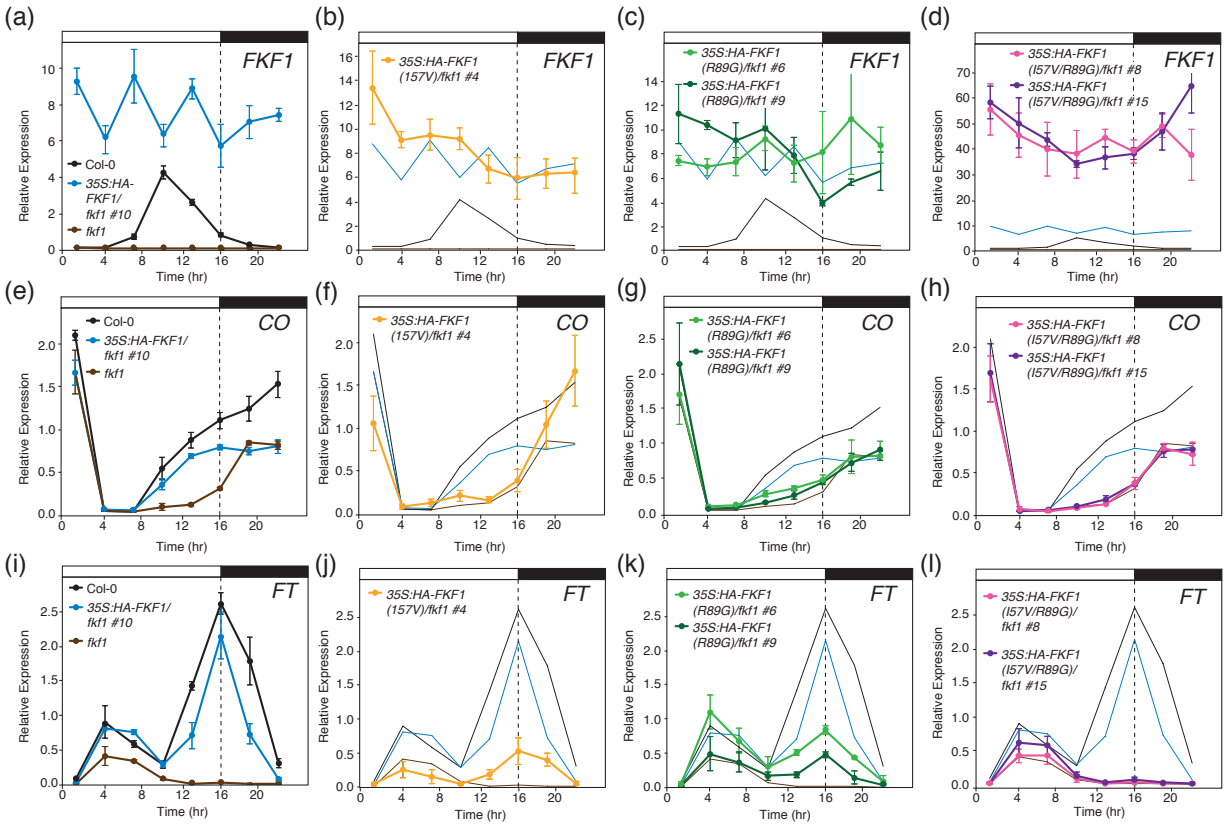

Figure 5-2. Faster photocycle variants of FKF1 have reduced *CO* and *FT* expression throughout the afternoon. Diurnal gene expression analysis of *FKF1* (a-d), *CO* (e-h), and *FT* (i-l) in Col-0, *fkf1*, *35S:HA-FKF1/fkf1, 35S:HA-FKF1(157V)/fkf1, 35S:HA-FKF1(R89G)/fkf1,* and *35S:HA-FKF1(I57V/R89G)/fkf1* plants. Tracings of Col-0, *fkf1*, and *35S:HA-FKF1/fkf1* appear in the same respective colors as in (a,e,i) in each panel as a reference. Experiments were performed on 14-day-old seedlings grown in long days, with 3-hour resolution. Means +/- SEM were calculated from four independent experiments.

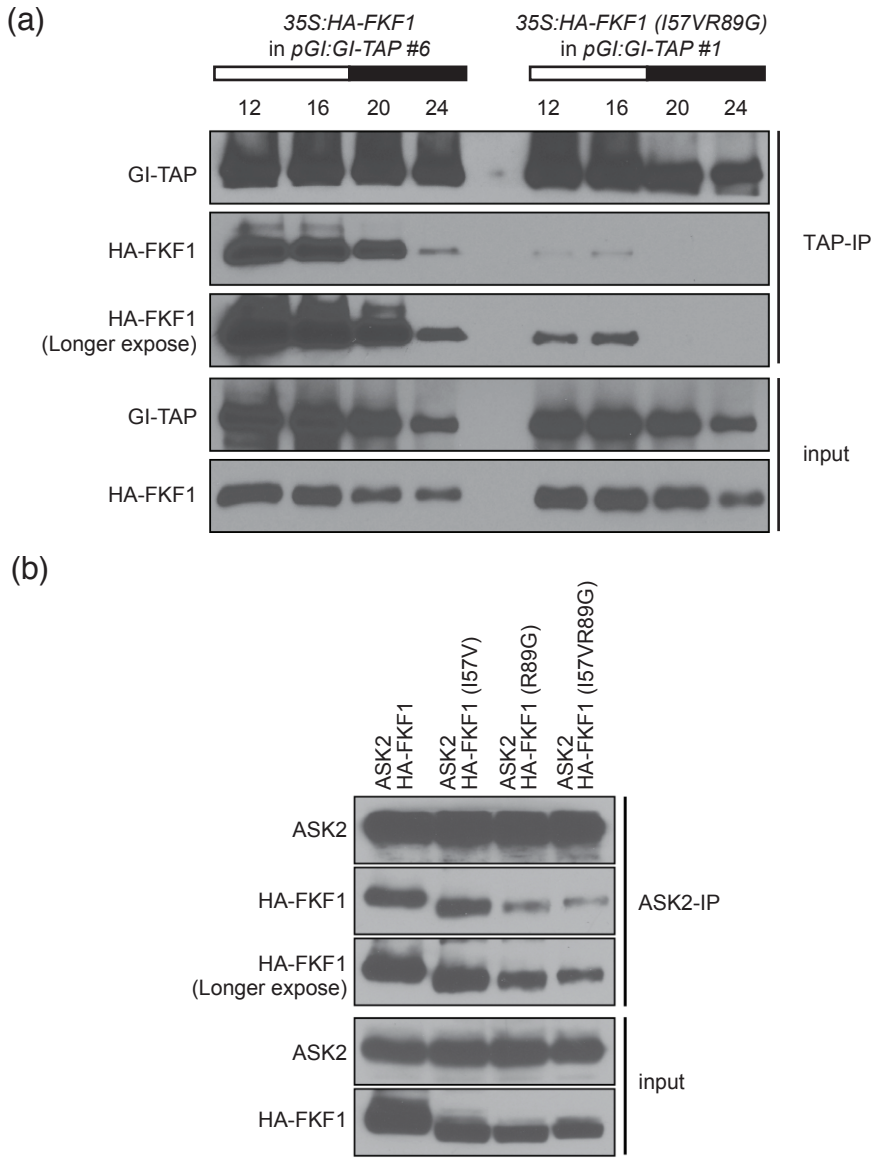

Figure 5-3. Fast photocycle FKF1 variants have attenuated protein-protein inter-actions with GI and ASK2. (a) GI-FKF1 interaction using coimmunoprecipitation assay of Arabidopsis 14 day old seedlings grown under long-day conditions. *35S:HA-FKF1* in *pGI:GI-TAP* and *35S:HA-FKF1 (I57VR89G)* in *pGI:GI-TAP* were used to compare slow and fast photocycle rates of FKF1, respectively. Harvesting time points were between ZT12 and ZT24, at 4 hour intervals. (b) ASK2-FKF1 interac-tion using coimmuno-precipitation assay of transiently transfected *N. benthamiana* leaves.

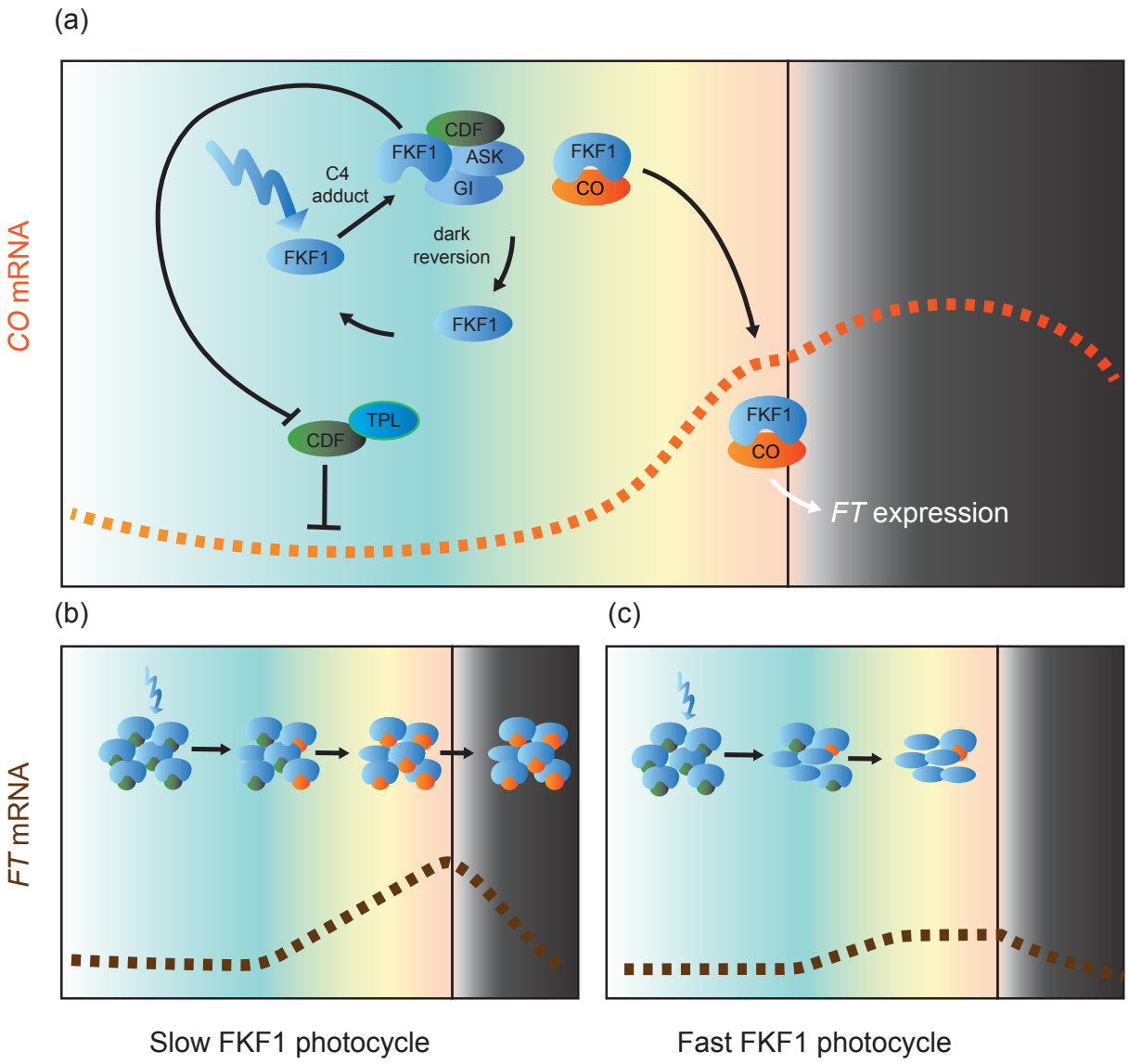

Figure 5-4. Hypothetical model of the affect of photocycle kinetics of FKF1 on the regulation of flowering time in response to photoperiod. (a) Light activated FKF1 protein forms a C4 adduct with the FMN chromophore, causing the FKF1 LOV domain to shift into the photoactive state. Once activated, FKF1 can strongly bind to CDF1, GI, and components of the SCF complex to target CDFs for degradation. Photoactive FKF1 assists in CO protein stabalization towards the dusk period of long-days, allowing for an increase in CO protein, which in turn goes on to increase *FT* expression and promote the transition to flow-ering. (b) Normal slow photocycle FKF1 protein remains stably in the photoactive state, degrading CDFs and stabalizing CO during the entire light period and into the early night. (c) Fast photocycle FKF1 variants more quickly undergo dark reversion to the ground state. During the later hours of daylight, ground state FKF1 species out number photoactive FKF1 proteins and CDFs remain to repress *CO* and *FT* transcription. Due to increased CDF-dependent repression, less CO protein is translated, and those that are have fewer photo-active FKF1 proteins that can prevent them from being actively degraded. This combined effect results in lower levels of *FT* transcription, and delayed flowering.

CHAPTER VI.

CONCLUSIONS: TIMING THE RITE OF SPRING

In late May of 1913, a group of patrons waited patiently to view the next ballet work of an up-and-coming composer, Igor Stravinsky, at the Théâtre des Champs-Élysées in Paris. Perhaps many (excluding perhaps the Bohemian contingent) were expecting to see an elegant work portraying the interpretation of a folk Russian ode to seasonal rebirth: a mathematical and layered concert between the many instruments in the orchestra pit, displaying the beauty and unity of spring through the lens of music and choreography. What the audience faced however, was a cacophony of sounds, dissonance, and dancers huddled, crouching to the sacrifice of a young girl to the gods of spring. If the urban legends are to be believed, a riot ensued after the performance. I find this to be a useful metaphor for the course of this project, and also to my view of this orchestral work. What attracted me to photoperiodism, the circadian clock, and flowering time was the elegance of an intricate pathway: cycles of repeating patterns that have the beautiful complexity *and* outward simplicity of a physical motion diorama, as well as major questions about how such a system operates. The photoperiodic flowering time response, while it contains elements of this wonder, is also wonderfully complicated. A dizzying array of factors are required for its function, which can at times cause ones' one hypotheses to be lost in a sea of possibilities. Interrogating the *seemingly* chaotic nature of this pathway has been both frustrating but also very rewarding.

REGULATION OF FLOWERING TIME: HOW MANY IS TOO MANY?

As discussed in chapters I and II, the number of factors required for the proper expression and stability of CO and FT genes and proteins is large (Golembeski and Imaizumi, 2015). A major question that emerged in my mind was: how is photoperiod often such a strict determinant of the flowering time response (as in the classical experiments) when dozens of transcription factors compete for space on the *FT* promoter? The integration of other inputs is critical for the modulation of the transition to flowering in response to

environmental cues, but in many cases the overall contribution between these pathways, together, was still very much an open question. When I began working on this project, a major goal was that hopefully, the more detailed mechanistic characterization of some key repressors would reveal something about the transcriptional landscape around the *CO* and *FT* loci. Perhaps through these factors, gatekeeping of modulatory inputs from environmental and other signals could be temporally regulated by controlling access to elements in the *CO* or *FT* promoters. Factors like FVE (a potential CDF1 interactor) and LHP1 seemed like promising candidates for study due to their interactions with larger chromatin modifying complexes as well as the interesting nature of LHP1's role in controlling the tissue specific domain of *FT* expression (Turck *et al.*, 2007; Pazhouhandeh *et al.*, 2011). After some discouraging original surveys, a simple protein alignment of the CDF group of repressors helped to open the door into a larger window of *CO* and *FT* transcriptional regulation. The presence of a potential TPL-dependent mechanism tied to CDF transcription factor recruitment fit in nicely within the context of this overall model we sought to test.

Prior to our study detailed in chapter III, the CDF transcription factors were thought to directly regulate the *CO* and *FT* genes together, to shut down their expression during the morning. Our analysis further indicated that the presence of TPL during the morning at several CDF cis-elements is a key process by which CDFs can concert transcriptional repression upon their target loci. Although there are many potential CDF binding sites found throughout both the *CO* and *FT* promoter regions, TPL binding during the morning appears to be at discrete locations near previously characterized CDF-enriched binding sites. This mechanism of TPL binding mirrors more recent studies of Groucho/TUP1/ TPL, which indicate that discrete binding and interactions with the mediator complex are more likely than the spreading of TPL binding and HDAC associated condensation of chromatin throughout the promoter regions that were previously suggested in the literature (at least at *CO* and *FT*) (Long *et al.*, 2006; Chambers *et al.*, 2017). This makes sense in the context of our original model of temporal gatekeeping: clock regulated CDF transcription factors could bring TPL to the *CO* and *FT* promoters, and TPL dependent interactions with basal transcriptional components could deny entry of environmental or developmental modulators of *FT* transcription during the morning period, to prevent early flowering during sub-optimal photoperiods.

This mechanism of CDF and TPL dependent repression of the seasonal reproductive transition appears to be highly conserved. CDF-like DOF proteins within the basal lineages of land plants have the same TPL-coordinating residues found across angiosperms. In *Physcomitrella patens,* CDF-like proteins are involved in the growth-phase transition, and in *Marchantia polymorpha*, a GI-FKF1 module conrtols the seasonal initiation of reproductive development (Sugiyama *et al.*, 2012; Kubota *et al.*, 2014). Although more functional characterization of GI, FKF1, and CDFs in basal land plants will need to be looked at, these components likely represent a core photoperiodic output module that evolved with land plants. As alluded to in chapter I, the compoinents of this module were likely expanded to include factors like CO and FT, and have since been co-opted for a variety of output seasonal responses in angiosperms (Song *et al.*, 2015).

Although the CDF-TPL characterization is a part of a larger story regarding the network-level interactions that determine the relative contribution of input pathways towards *FT* expression, the work in this thesis does not substantially answer those higher-level questions. Still, an emerging story regarding this point is that the many transcription factors that regulate *FT* expression must do so in the context of certain daily changes in *FT* chromatin. During this morning period, TPL-dependent repression likely blocks access from any morning expressed activators that could potentially bind to the 5' proximal region in the *FT* promoter. In the afternoon of long days, CO binding to distal sites in the *FT* promoter causes a change in the chromatin conformation to include a double loop (Cao *et al.*, 2014). This active conformation of the *FT* gene likely improves the accessibility of the 5' proximal region of the *FT* promoter to factors that can modulate expression in response to additional environmental or developmental cues (Cao *et al.*, 2014). Ultimately, this means that CO and CDFs/TPL act like temporal gatekeepers in this case. CO provides a context in which the many activators and repressors can act upon *FT* in the afternoon of long days, and CDFs/TPL can prevent access when the photoperiod is too short through acting during the morning (Takada and Goto, 2003; Adrian *et al.*, 2010). In the context of my previous analogy, this means that in some ways, what appears as a chaotic mess of dozens of interactors competing for binding sites in the *FT* promoter is more like a facilitated dance. During the right times of day, these additional factors can potentially have their place to modify the flowering time and exert the best possible outcomes for plant reproduction based on internal and external conditions.

One other major question about *FT* transcription has to do with the cell specific expression patterns. For the most part, the analyses I have performed investigating the role of CDFs and TPL in the regulation of *FT* transcription have been dealing with *CDF* and *CO* expressing phloem companion cells, which are widely spread throughout the major and minor veins of leaves. Only a fraction of these cells, however, ultimately express *FT* towards the distal part of the leaves (Takada and Goto, 2003). We know some of the factors that control the boundary of *FT* expression, including LHP1 (Which plays a role in depositing H3K27me3 marks throughout the distal parts of the *FT* promoter), and other inputs that may increase the *FT* expression domain within a leaf as individual leaves age (Takada and Goto, 2003; Adrian *et al.*, 2010). Presumably the determination of *FT* expression domain also requires interactions with CDF-TPL or with CO to shut down or activate *FT* expression in certain cells, but these interactions are largely missing from our overall models. The same is true for FT protein as it exits the leaf phloem and moves towards the shoot apex and is off loaded. We know several of the factors which may assist in FT movement in the phloem, but the transporter through which FT is off-loaded at the shoot apex has not been characterized (Liu *et al.*, 2012; Yoo *et al.*, 2013). Many of the interactions that happen at the shoot apex once FT is off-loaded are also relatively unknown (Andrés and Coupland, 2012; Golembeski and Imaizumi, 2015). Many of the factors at the shoot apex may be additionally regulated by photoperiod through GA accumulation or through some other mechanism, but very few studies so far have directly compared what is occurring in the leaf vasculature with what is going on simultaneously at the shoot apex.

PHOTOCYCLE RATE KINETICS OF FKF1

While a major focus of my work on this project has been on the transcriptional regulation of photoperiodic flowering, protein level regulation of the various components is a critical part of the overall picture, and few molecules are more central to this in Arabidopsis than FKF1 (Imaizumi *et al.*, 2003; Imaizumi *et al.*, 2005; Song *et al.*, 2012). There have already been a large number of studies that have clarified the roles of FKF1 and its related proteins ZTL and LKP2, but there are still large questions about how they function (Kim *et al.*, 2007; Baudry *et al.*, 2010; Song *et al.*, 2014; Pudasaini *et al.*, 2017). As I enumerated in chapter V, our approach and that of our collaborators has been to try and understand what

makes FKF1 and ZTL unique among the various photoreceptors, and their photocycle syndrome is certainly one aspect of it (Ito *et al.*, 2012). Although we will need to perform additional experiments to get at the heart of whether photocycle rate is an all important feature that divides FKF1 and ZTL in terms of function, I believe our evidence points in that direction. An attractive hypothesis for the origins of ZTL and FKF1 (considering non-vascular plants have only a single copy) is that the two copies were able to diverge in function and neofunctionalize partially through changes in the photocycle rate (Pudasaini *et al.*, 2017). If this is indeed the case, then our study of the underlying molecular mechanisms behind the photocycle rate kinetics and its effect on interactions might be quite useful in the future for tailoring the rate more towards a desired flowering outcome in crop species.

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