Dissection of the role of FT and FD in the regulation of flowering in *Arabidopsis thaliana*

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To my parents,

Whose aspiration and ordeal this was as much as my purpose.

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1.0 SUMMARY

(SUMMARY IN ENGLISH)

The transition of *Arabidopsis thaliana* from a vegetative growth to a reproductive development is regulated by well defined genetic pathways that integrate exogenous stimuli from the environment such as day length, quality of light, temperature, exposure to winter and endogenous clues such as age of the plant, hormonal levels like gibberellic acid etc. The length of the day or photoperiod is measured in the leaves and a conducive photoperiod is translated into a flowering stimulus that is transduced from the leaves to the shoot meristem by a long distance signal, the florigen. There has been some debate over the nature of the florigen but recent results suggest that the product of the *FLOWERING LOCUS T (FT)* gene plays a prominent role in this process. Several experiments performed independently by different groups have provided circumstantial evidence that the FT protein indeed acts as a florigen, but since all of these experiments relied on the use of strong promoters, certain questions regarding the function of the native FT protein remained.

Here we show that the FT protein does in fact function as the florigen when under the control of its own promoter. To this end, we first rendered the FT protein immobile by fusing it with three copies of YFP. We observed that release of the mature FT protein from the FT-3xYFP precursor by proteolytic cleavage was able to efficiently rescue the late flowering phenotype of the *ft-10* mutant. Second, we engineered a version of the FT protein that was susceptible to protease cleavage and employed this technique to demonstrate that the FT protein synthesized in the leaves is required to induce flowering at the shoot meristem. Since all these experiments were performed in the genomic context, it can be concluded that the results obtained were not an artifact caused by FT overexpression, and nicely show that the movement of the FT protein to the apex is in fact essential for flowering.

At the apex, FT interacts with the bZIP transcription factor FD, which in turn initiates the transcription of homeotic genes required for the formation of a flower.

Since the interaction between FT and FD proteins has only been confirmed by yeast-two-hybrid assays or transient expression assays in plants, we searched to study the interaction of the two proteins in the apex of *A. thaliana.* To deduce the transcriptional network that regulates the transition to flowering at the shoot apex, we first performed a genome wide expression analysis to identify genes that were misexpressed in a FD overexpression line. We recognized several key players of floral transition and flower development such as *APETALA1, SEPALLATA3, SEPALLATA1, FRUITFUL, AGAMOUS* to be targets of FD. Since all these genes encoded transcription factors, it was possible that some of them were only indirectly activated by direct targets of FD. To discriminate between direct and indirect targets, a GR-FD fusion protein was employed followed by whole-transcriptome analysis. This way, several key genes including *APETALA1* and *SEPALLATA3* were identified as direct FD targets and binding to G-box element in the promoter of *SEPALLATA3* was confirmed *in vitro*. Interestingly, the ability of FD to transcriptionally activate its targets was greatly enhanced in the presence of FT, indicating that these two proteins could act cooperatively. As a final step, we analyzed the spatial and temporal expression of the FD protein compared to some of its targets to get a better understanding of the mode of action of the protein at the shoot apex.

Taken together, our results indicate that the FT protein moves to the shoot apex and activates a plethora of genes along with FD. Some of these targets were confirmed as direct targets while others were classified as indirect targets of FD, providing us with a comprehensive overview of the FD-mediated transcriptional network that regulates the transition to flowering at the shoot apex.

1.0 ZUSAMMENFASSUNG (SUMMARY IN GERMAN)

Der Übergang vom vegetativen Wachstum zur reproduktiven Entwicklung wird in *Arabidopsis thaliana* von definierten genetischen Signalwegen reguliert, die exogene Reize wie Tageslänge, die Qualität des Lichts, Temperatur, Überwinterung und endogenen Hinweise wie das Alter der Pflanze, den Hormonspiegel (insbesondere Gibberellinsäure) usw. integrieren. Die Tageslänge (oder Photoperiode) wird in in Pflanzen in den Blättern bestimmt. Unter den richtigen Bedingungen wird dabei in den Blättern ein, das Blühen induzierende, Langsteckensignal, genannt Florigen, gebildet und zum Sprossmeristem transportiert. Die Natur des Florigens wurde in der einschlägigen Literatur lang kontrovers diskutiert, aber neuere Ergebnisse deuten darauf hin, dass das Produkt des *FLOWERING LOCUS T* (*FT*) Gens hierbei eine prominente Rolle spielt. Mehrere Experimente, die von verschiedenen Gruppen unabhängig voneinander durchgeführt wurden, haben, haben Hinweise darauf geliefert, dass das FT Protein in der Tat als Florigen fungiert. Da diese Ergebnisse aber samt und sonders auf Überexpression das FT Proteins beruhen blieben einige Fragen in Bezug auf die Funktion des nativen FT Proteins offen.

Im Rahmen der vorliegenden Arbeit konnte gezeigt werden, dass das FT Protein auch dann als Florigen fungiert wenn es unter der Kontrolle der eigene regulatorischen Sequenzen exprimiert und auf eine Überexpression verzichtet wird. Hierzu wurde FT zum einem als Fusionsprotein mit drei Kopien des YFP fusioniert und somit ein Transport aus den Blättern zum Sprossapex verhindert. Erst nachdem FT mittels proteolytische Spaltung von diesem Fusionsprotein abgetrennt worden war, war es in der Lage das späte Blühen einer *ft-10* Mutante zu korrigieren. Unabhängig hiervon konnte eine Variante des FT Proteins erzeugt werden, die durch eine Protease gespalten und somit inaktiviert wurde. Mittels dieser Variante des FT Proteins gelang der Nachweis, dass das in den Blättern produzierte FT Protein notwendig ist, um das Blühen am Sprossapex zu induzieren. Da alle diese Experimente mit genomischen *FT* Konstrukten durchgsführt und auf ein Überexpression verzichtet wurde, kann man schlussfolgern, dass FT in der Tat als Florigen fungiert und sein Transport zum Sprossapex essentiell für die Induktion es Blühens ist.

Am Sprossapex angekommen interagiert FT vermutlich mit dem bZIP Transkriptionsfaktor FD, der wiederum die Expression von homeotischen Genen, welche für die Bildung der Blüten verantwortlich sind, steuert. Bislang wurde die Interaktion zwischen FT und FD jedoch nur in Hefe-Zwei-Hybrid Untersuchungen und in transienten Assays in Pflanze bestätigt. Eine Bestätigung der Interaktion dieser beiden Proteine am Sprossapex steht noch aus. Um die Rolle von FD innerhalb des transkriptionellen Netzwerks, welches den Übergang zum Blühen am Sprossapex reguliert, besser zu verstehen, wurden in einem ersten Schritt in einer genomweiten Analyse Transkripte identifiziert, die in einer FD – Überexpressionslinie fehlreguliert sind. Dadurch konnten eine bekannte Schlüsselgene der Blühregulation und/oder der Blütenmusterbildung wie *APETALA1, SEPALLATA 3, SEPALLATA1, FRUITFUL* und *AGAMOUS* als möglich Zielgene von FD identifiziert werden. Da es sich bei diesen Genen wiederum um Transkriptionsfaktoren handelte, musste davon ausgegangen werden, dass zumindest einige dieser Gene nur indirekt durch FD reguliert werden. Um direkte von indirekten Zielgene unterscheiden zu können wurden Transkriptomanalysen in GR-FD Pflanzen, in denen FD mit der Ligandenbindedomäne des Glucocorticoidrezeptors (GR) fusioniert worden war, durchgeführt. Hierdurch konnten *APETALA1* und *SEPALLATA3* als direkte FD Zielgene verifiziert werden. Im Falle von SEPALLATE3 konnte darüber hinaus die Bindung von FD an eine G-Box im Promoter *in vitro* bestätigt werden. Ein interessantes Ergebnis dieser Arbeit war darüber hinaus, dass die Fähigkeit von FD die Expression seiner Zielgene zu aktivieren durch FT kooperativ verstärkt wurde. Um eine besser Vorstellung von der Rolle von FD am Sprossapex zu bekommen, wurde die zeitliche und räumliche Expression von FD und einiger seiner Zielgene analysiert.

Zusammenfassend kann festgestellt werden, dass FT als Florigen fungiert und am Sprosspex zusammen mit FD eine Vielzahl von Zielgenen transkriptionell reguliert. Die Identifizierung direkter und indirekter FD Zielgene liefert darüber hinaus einen umfassenden Überblick über das FD-abhängige transkriptionelle Netzwerk, das die Induktion des Blühens am Sprossapex steuert.

2.0 INTRODUCTION

This section of the thesis contains excerpts from:
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2.1. General introduction to the regulation of flowering time

Plants, being sessile organisms, have developed a complex mechanism to recognise a conducive environment that would ensure reproductive success. While the earliest seed-free plants, ancestors of today's mosses and ferns, still required water to reproduce using motile spores, most non-flowering seed plants (gymnosperms), with the notable exceptions of *Ginko biloba* and *Cycadacae*, and flowering seed plants (angiosperms) do not. In particular the angiosperms have evolved specialized organs to further reproduction, the flower. The earliest fossil of a flowering plant, *Archaefructus liaoningensis,* date back about 125 million years. Nowadays, fruits of angiosperm flowers form a major source of the staple diet of people and livestock. Flowers are also appreciated for their aesthetic value, their fragrance and their medicinal properties. Because of their importance, plants have attracted a lot of interested throughout the ages.

Flowering is one of the indispensible events in a plant's life cycle. But the timing of flowering is just as critical as flowering itself. If the timing is correct, it ensures reproductive success of the plant and hence helps the plant to adapt better to its environment. Due to its importance, flowering is tightly regulated by a complex network of genes that may regulate each other or other genes and serve as checkpoints at every step to ensure that the timing of flowering is in perfect accordance with both internal (hormones) and external (temperature, light) cues.

2.1.1 Multiple pathways control flowering.

A major developmental transition in plants is the switch from vegetative to reproductive growth. Plants have hence evolved distinct reproductive strategies such as response to environmental cues. These environmental cues could be day-length, seasonal changes, or simply stress due to overcrowding, nutrition deficiency or drought (Simpson and Dean, 2002).

Arabidopsis thaliana has provided many paradigms for understanding the basics of plant genetics and molecular biology. Flowering time in *A. thaliana* is dependent on the length of the day, with long days (16h light) in general promoting the floral transition when compared to short days (8h). However, *A. thaliana* will eventually flower even under SD and has hence been classified as a facultative LD plant.

Four classical pathways namely photoperiod, vernalization, gibberellic and autonomous have been described to regulate flowering. Recently other pathways that are independent of these classical pathways such as Age pathway have also been described (Fornara et al., 2010). The different pathways involved in flowering have been described in the following sections.

2.1.1.1. The photoperiod pathway

As one moves away from the equator, the length of the day varies significantly between summers and winters. Plants have developed the ability to sense this distinction and use it as an indicator to control the onset of flowering. The cascade of events responsible for measurement of day-length and the subsequent initiation of flowering is referred to as the photoperiod pathway.

Light is perceived by plants at different wavelengths by specialized photoreceptors. Phototropins (blue), cryptochromes (blue) and phytochromes (red/far-red) are the three main classes of plant photoreceptors (Lariguet and Dunand, 2005; Li and Yang, 2007; Quail et al., 1995).

Rédei (1962) was the first to describe mutants that were insensitive to inductive day-length. Among them was the *constans* (*co*) mutant. The *CONSTANS* (*CO*) gene encodes a putative zinc finger transcription factor (Putterill et al., 1995), the temporal and spatial regulation of which turned out to be key to the photoperiod-dependent induction of flowering (An et al., 2004). *CO* expression is under the control of the circadian clock, which causes a basic oscillation of CO expression with a phase of 24 hr, and a maximum approx. 20 hr after dawn under SD conditions (Suárez-López et al., 2001). This phasing of *CO* expression is further modified under LD by the activity of three other proteins: GIGANTEA (GI), FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1), and CYCLING DOF FACTOR1 (CDF1) (Fornara et al., 2009; Imaizumi et al., 2005; Sawa et al., 2007).

Interestingly, these three genes are themselves regulated by the circadian clock. In long days, both the FKF1 and GI proteins follow the same phase with maximum levels being reached 13 hr after dawn (Imaizumi et al., 2003; Sawa et al., 2007). In contrast, under short day conditions, GI peaks at 7 hours after light onset, but FKF1 peaks 10 hours after light onset (Fornara et al., 2009). Interaction assays in yeast showed that FKF1 physically interacts with GI (Sawa et al., 2007). Interestingly, FKF1 protein binds GI only in the presence of blue light, which it perceives through its flavin binding domain. As a result of this, FKF1-GI complexes are formed much more efficiently during long days when there is sufficient exposure of the FKF1 protein to blue light and FKF1 and GI proteins peak at the same time, unlike under short days, where the proteins are in different phase and the light, which is required for FKF1-GI complex formation, is lacking (Sawa et al., 2007)(Fig 1).

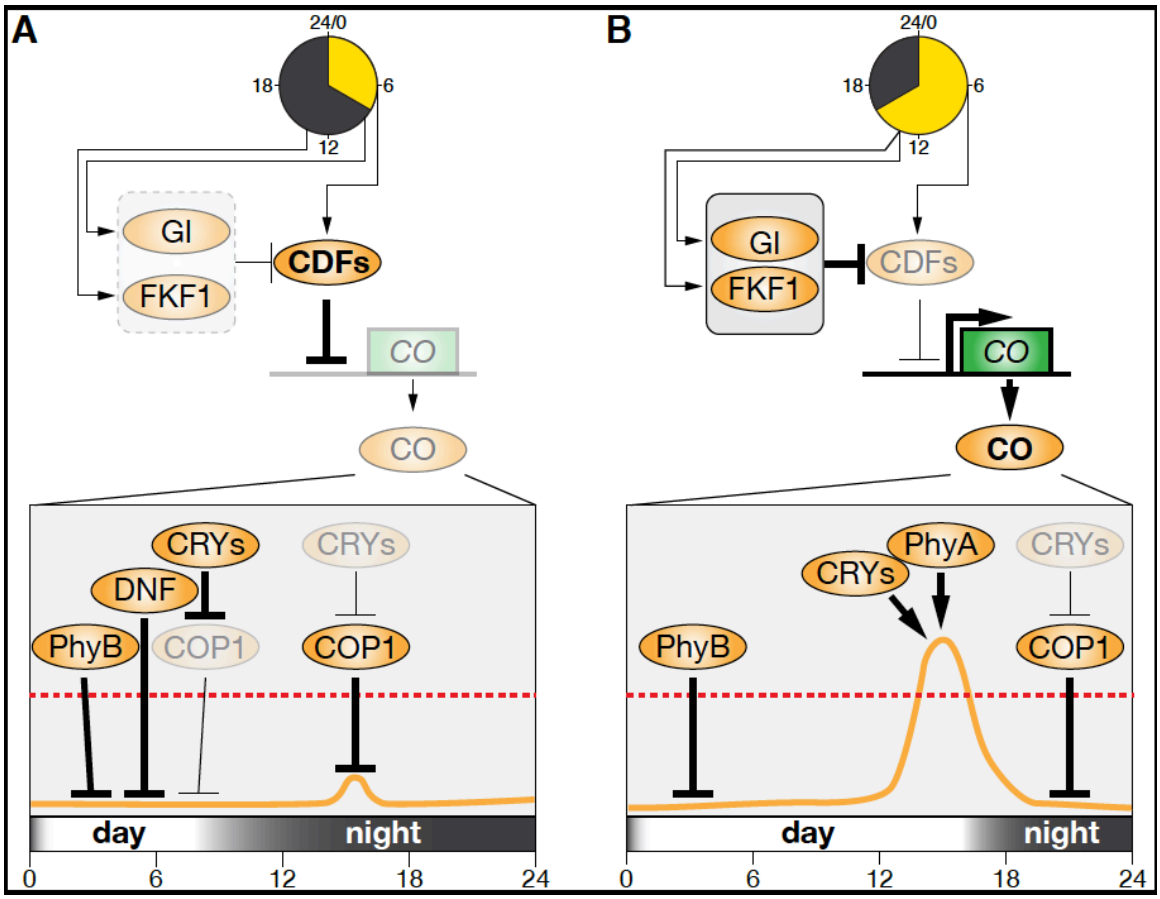


Figure 1. ***Regulation of CONSTANS at the transcriptional and protein level.*** (A) In short days, FKF1 and GI proteins peak at different times and hence are not able to efficiently repress CDF1, a transcriptional inhibitor of *CO.* COP1 protein is exported into the nucleus due to the inactive state of CRY, resulting in the ubiquitination of CO. PHY B is responsible for the early dip in CO levels between 4 and 8 Zeitgeber hours. (B) In long days, both FKF1 and GI peak at approx. 13 Zeitgeber hours, resulting in active repression of CDF1, and thereby, *CO* transcription. The protein levels are regulated by PHY B in the early morning hours, while active CRY and PHYA repress PHYB during the rest of the day. Active CRY protein also binds to and inhibits transport of COP1 into the nucleus, hence preventing it from efficiently ubiquitinating the CO protein. Besides CRY, COP1 also interacts with SPA proteins to regulate CO. Gray lines show weak effects, black lines indicate strong effects. This figure was adapted from Srikanth and Schmid (2011).

FKF1 and GI do not regulate *CO* expression directly but through interactions of FKF1-GI with CYCLING DOF FACTORs (CDF) (Fornara et al., 2009; Imaizumi et al., 2005). The quadruple *cdf* mutant accumulates *CO* mRNA both during the day and night and flowers early both in short and long days. CDF1 has been shown to directly bind to the *CO* regulatory regions and act as a repressor of *CO* transcription (Imaizumi et al., 2005). Chromatin immunoprecipitation (ChIP) showed that the

three proteins bind to similar regions on the *CO* promoter (Sawa et al., 2007). Finally, analysis of the abundance of the three proteins showed that CDF1 peaks first, followed by increasing GI levels and then finally FKF1 peaks in the afternoon in long days (Imaizumi et al., 2005; Sawa et al., 2007). Together these studies suggest that CDF1 protein first binds to the *CO* promoter in the morning. As soon as there is sufficient GI, the CDF1-GI complex is formed that represses on *CO* transcription. Once FKF1 protein peaks, it interacts with the CDF1-GI complex and targets CDF1 for degradation through its F-Box domain to finally activate transcription of the *CO* gene (Fig. 1) (Sawa et al., 2007). Together the activity of FKF1/GI/CDFs results in a second peak of *CO* expression towards the end of the subjective LD at approx. 16 hr after dawn (Fig. 1).

CO, however, is not only regulated at a transcriptional level, but also at the level of its protein stability and accumulation. Central to the posttranslational regulation of CO are CONSTITUTIVELY PHOTOMORPHOGENIC (COP1) and members of the SUPPRESSOR OF *PHYA-105* (SPA) protein family (Fig. 1). COP1 functions as an E3 ubiquitin ligase and has been shown to act downstream of the cryptochrome signalling but upstream of CO. Liu et al., (2008) reported that CO-GST was ubiquitinated specifically by COP1. Furthermore, constitutive overexpression of the CO protein fused to luciferase in *cop1* mutants resulted in a drastic increase in luciferase signal when compared to wild-type, providing evidence that degradation of CO by COP1 also occurred *in vivo.* Finally, assays in yeast (Y2H) and *in vivo* assays (co-localization) confirmed that COP1 interacts with CO (Liu et al., 2008).

Besides COP1, the SPA protein family consisting of 4 members have also been shown to regulate CO (Hoecker and Quail, 2001; Laubinger et al., 2006). While *CO* mRNA levels were found to be unaltered in the *spa1,3,4* triple mutants, CO protein levels were strongly elevated in the triple mutants when compared to wild-type, suggesting that SPA proteins were regulating the CO protein posttranslationally (Laubinger et al., 2006). In agreement with this hypothesis, co-immunoprecipitation studies established that all the 4 SPA proteins indeed interacted with CO through its

CCT domain. Further, the SPA1, SPA3 and SPA4 proteins were shown to physically interact with the coiled coil domain of COP1 (Hoecker and Quail, 2001; Laubinger and Hoecker, 2003). These results suggest that SPA proteins enable degradation of the CO protein by the COP1 mediated ubiquitination (Laubinger et al., 2006).

Analysis under different light conditions showed CO accumulation in plants grown under white, blue and far red but not in plants that had been exposed to red or had been kept in the dark. This indicated that the accumulation of the CO protein was influenced by a photoreceptor (Valverde et al., 2004). Subsequently, *phyB* mutants were shown to exhibit increased levels of CO in the red light and early morning hours, indicating that PHYB plays a major role in regulation of CO in the early hours of the day (Fig. 1) (Jang et al., 2008; Valverde et al., 2004).

In the end, the complex regulation of CO enables the plant to discriminate SD, where CO protein is not being stably produced, from LD, where CO protein accumulates towards the end of the day. An important aspect of this is that regulation of CO happens in the leaves and not at the shoot apex where flowers will eventually be formed (An et al., 2004).

For flowering to occur, the information that a plant experiences inductive photoperiod needs to be transferred from the leaves to the apex. The question arose whether CO itself might constitute a long-distance signal (florigen). However, expression of *CO* mRNA from various tissue specific promoters suggested that CO regulates production of a systemic flower-promoting signal in the leaves, but does not act as a florigen (An et al., 2004; Ayre and Turgeon, 2004).

Instead, several lines of evidence now indicate that a protein called FLOWERING LOCUS T (FT) is contributing to the floral induction by acting as a long distance signal between leaves and the shoot meristem. *FT* was simultaneously cloned by two independent groups using an activation tagging approach (Kardailsky et al., 1999; Weigel et al., 2000) and a large chromosomal deletion mutant caused by a T-

DNA insertion (Kaya et al., 2000; Kobayashi et al., 1999). The *FT* gene encodes a protein with similarities to Raf kinase inhibitory protein (RKIP) and phoshaptidylethanolamine binding protein (PEBP) proteins. These proteins are known to inhibit Raf, and thereby result in signal transduction through the MAP kinase pathway. However, since FT lacks certain key residues conserved in all PEBP and RKIP proteins (Ahn et al., 2006), the molecular function of FT is not entirely clear. Analysis of *FT* expression revealed not only that its expression is much higher in long days, but also that it follows a circadian pattern, peaking in the evening (Harmer et al., 2000; Suárez-López et al., 2001). Promoter GUS constructs showed that the *FT* gene is transcribed in the phloem companion cells, where CO is also present (Takada and Goto, 2003). Recently, GI was shown to independently regulate *FT* bypassing CO. GI was found to bind three FT supressors and ChIP showed that GI bound chromatin in close proximity to one of these supressors (Sawa and Kay, 2011). Temporal and spatial expression of *FT* in the vasculature is controlled by a complex orchestration of activating and repressive inputs. The latter include proteins that regulate chromatin structure (Farrona et al., 2008) and thus accessibility of *FT* locus for transcription factor binding. Several studies have demonstrated that trimethylation of lysine 27 in the amino terminus of histone H3 (H3K27me3) provides an assembly platform for repressive complexes. In this context it is interesting to note that recent genome-wide surveys indicate that all flowering time genes but *CO* are H3K27me3 targets (Exner et al., 2009; Turck et al., 2007; Zhang et al., 2007). H3K27 trimethylation is carried out by the Polycomb Repressive Complex 2 (PRC2) and mutants in a number of PRC2 genes (i.e. *CURLY LEAF (CLF)*, *EMBRYONIC FLOWER 2*, etc.) flower early (Kohler et al., 2003; Schubert et al., 2006b; Yoshida et al., 2001). In these mutants, early flowering was shown to be at least in part due to ectopic expression of *FT*, suggesting that PRC2 complexes play a major role in repressing *FT* during vegetative growth. Chromatin-immunoprecipitation experiments revealed that CLF in fact bound *FT* chromatin, establishing a direct link between PRC2 and *FT* repression (Jiang et al., 2008). While PRC2 components can be identified rather easily in plants, proteins homologous to PRC1 are more elusive. However, it has been suggested that LIKE

HETEROCHRMATIN PROTEIN1 (LHP1) might act as a PRC1-like corepressor (Hennig and Derkacheva, 2009). *lhp1* mutants flower somewhat earlier than wild-type and, similar to mutants in PRC2 components, this early flowering has been attributed to increased *FT* expression. Furthermore, LHP1 is directly associated with the *FT* locus (Adrian et al., 2010), indicating that, like PRC2, LHP1 (PRC1) contributes to *FT* repression.

FT mRNA is not readily detected in short days, but mRNA levels rise rapidly in the leaves upon transfer from short to long days and are detectable even after exposure to a single long day (Corbesier et al., 2007; Imaizumi et al., 2005; Yamaguchi et al., 2005).

Several lines of evidence place *FT* genetically downstream of *CO*. In the phloem of *SUC2::CO* plants, *FT* mRNA abundance was increased and *ft* mutations strongly suppressed the early flowering of *SUC2::CO* (An et al., 2004). Overexpression of *CO in ft-10* plants did not rescue the late flowering phenotype, but *FT,* when expressed from the *SUC2* promoter in *co* mutants, was able to completely rescue the late flowering phenotype (Yoo et al., 2005). Further, *pFT::*GUS was shown to be expressed in a CO-dependent manner (Takada and Goto, 2003). In addition, microarray analysis of plants shifted from short day to long days showed CO-dependent upregulation of *FT* (Wigge et al., 2005). Finally, treatment of *35S::CO:GR* plants with dexamethasone and cycloheximide resulted in an increase of *FT* mRNA within 1 h of induction (Kobayashi et al., 1999; Samach et al., 2000; Yamaguchi et al., 2005). Corbesier et al., (2007) later demonstrated that treating a single leaf from *co* mutant plants carrying a *pCO::GR:CO* rescue construct with dexamethasone was sufficient to induce *FT* mRNA and subsequently flowering. Taken together, these data clearly indicate that *FT* is a primary target of CO in leaves.

Interestingly, there is strong evidence that FT is not acting in leaves but might promote flowering at the shoot meristem. In particular the finding that FT can interact with the meristem specific bZIP transcription factor FD immediately

suggested that FT might play an important role in conveying the information to initiate flowering from the leaves to the apex (Abe et al., 2005; Wigge et al., 2005). However, it should be noted that formation of the FT-FD protein complex at the shoot meristem has yet to be demonstrated.

An artificial microRNA against *FT* driven by the *35S* and *SUC2* promoters delayed flowering, but no change in flowering time was observed when amiRNA-*FT* was expressed at the apex using the *FD* promoter. This indicates that *FT* mRNA was required in the phloem companion cells to induce flowering, but not at the apical meristem (Mathieu et al., 2007). Similarly, Jäger and Wigge (2007) could show that trapping FT protein in the phloem companion cells by fusion with a strong nuclear localization signal prevented FT from inducing flowering. In addition, expression of a translational fusion of FT with three molecules of yellow florescence protein (YFP) from the *SUC2* promoter did not induce flowering. As the FT-3xYFP protein was shown to promote flowering when expressed from a constitutive promoter (Mathieu et al., 2007), this also suggested that FT was functioning by direct movement rather than a relay mechanism. As in this particular experiment FT had been separated from YFP by a Tobacco Etch Virus (TEV) cleavage sequence, it was possible to release the mature FT protein from the FT-3xYFP precursor by *in vivo* cleavage using TEV protease expressed from the *SUC2* promoter. Release of FT protein resulted in very early flowering, demonstrating that FT protein in the phloem companion cells was sufficient to induce flowering. In agreement with this, Corbesier et al. (Corbesier et al., 2007), demonstrated by fluorescence microscopy that a GFP:FT fusion protein was exported from the vasculature to the base of the meristem. Finally, expression of a synthetic *FT* gene with synonymous mutations in every possible triplet (*synFT*) was shown to promote flowering just as well as wild-type FT (Notaguchi et al., 2008). In summary, all of these data strongly suggested that FT protein rather than the mRNA is acting as a long-distance signal in *A. thaliana*.

2.1.1.2. Regulation of flowering by temperature.

Besides light and photoperiod, temperature is a major determinant of flowering time. Temperature effects flowering in two ways: first, many plants require a prolonged period of cold (vernalization) to induce flowering the following spring and second, the ambient temperatures a plant experiences throughout its vegetative growth have a marked effect on the timing of flowering; these mechanisms explain the wide range of flowering time responses in natural accessions of *A. thaliana* (Alonso-Blanco and Koornneef, 2000). Some are rapid-cyclers and flower early, while most late flowering accessions follow a winter-annual life style and require vernalization before they can flower.

Analyses of the genetic differences between rapid-cycling and winter annual varieties of *A. thaliana* revealed that the dominant locus *FRIGIDA* (*FRI*) played a major role in conferring a vernalization requirement to natural accessions of *A. thaliana* (Johanson et al., 2000; Napp-Zinn, 1987). Further studies revealed that another gene, *FLOWERING LOCUS C (FLC),* and *FRI* are both required for vernalization to occur (Koornneef et al., 1994; Lee I et al., 1994; Michaels and Amasino, 1999). FRI functions by upregulating the expression of *FLC*, which is a potent floral repressor (Geraldo et al., 2009). The mechanism by which FRI regulates expression of *FLC* is still not fully understood, although it was recently shown that FRI protein interacts with the cap binding complex (CBC) through its two coiled coil domains, and that this interaction is essential for FRI function (Geraldo et al., 2009).

FLC's mode of action is better characterized than that of *FRI*. *FLC* encodes a MADS box protein that acts to directly repress certain flowering time genes (Koornneef M et al., 1994; Searle et al., 2006; Sheldon et al., 1999). FLC was shown to block the transcriptional activation of *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FT* by directly interacting with CArG boxes on their respective chromatin (Fig. 3) (Helliwell et al., 2006; Hepworth et al., 2002). This binding reduced the effect of photoperiodic activation of these genes. Since the *FLC* null allele was able to

completely suppress the late flowering phenotype of *FRI*, it was concluded that FRI mediates vernalization via *FLC* (Michaels and Amasino, 2001). Together, FRI and FLC are responsible for the winter-annual life history; loss of either of the two genes usually results in early flowering and loss of the vernalization requirement. Interestingly, loss of *FRI* and/or *FLC* have occurred multiple times, indicating that summer annual life histories have evolved independently in different accessions of *A. thaliana* (Alonso-Blanco and Koornneef, 2000).

High levels of *FLC* expression appear to be responsible for the winter-annual behaviour of *FRI*/*FLC* positive accessions. To better understand how expression of *FLC* is regulated in response to vernalization, a genetic screen was performed to identify plants that flowered late even after exposure to long periods of cold. Two important regulators of *FLC*, *VERNALIZATION1 (VRN1) and VERNALIZATION2 (VRN2)* were identified from the screen (Gendall et al., 2001; Levy et al., 2002). These studies demonstrated that *FLC* is epigenetically silenced in response to vernalization. Interestingly, initial silencing of *FLC* was completely normal in both the *vrn1* and *vrn2* mutants, but *FLC* levels increased after plants were returned to higher temperatures, indicating that these genes are required for maintenance, rather than initiation of the *FLC* silencing.

Sung and Amasino (2004a), identified yet another protein, VERNALIZATION INSENSITIVE 3 (*VIN*3), that is required for the initial repression of *FLC* during cold exposure (Fig. 2). *VIN3* encodes a PHD finger protein and is transiently induced by cold temperatures (Bond et al., 2009; Sung and Amasino, 2004a). Lesions in *VRN2* locus were shown to affect the structure of *FLC* chromatin (Gendall et al., 2001), indicating that VRN2 may play a role in *FLC* chromatin remodelling during silencing. Additionally, VIN3 was shown to interact with members of the PRC2 (De Lucia et al., 2008; Wood et al., 2006) that is responsible for tri-methylation of lysine 27 of histone H3 (H3K27me3), a typical sign of gene silencing (Cao and Zhang, 2004; Schubert et al., 2006). This particular methylation mark increases at the transcription start site of *FLC* in response to vernalization (De Lucia et al., 2008;

Finnegan and Dennis, 2007). This results in the recruitment of *VRN1, VRN2* and *LHP1*, which together maintain the repressed state of *FLC*. Thus *VIN3* is induced in response to vernalization and establishes the initial silencing of *FLC*. VRN1 and VRN2 are then required to maintain *FLC* in a silenced state (Fig. 2).

In addition to PRC proteins, noncoding RNAs are emerging as a new family of regulators of gene expression. The production of antisense *FLC* RNA called *COOLAIR* RNA (Cold induced long antisense intragenic RNA) was recently shown to be the first response to cold treatment. The transcription of *COOLAIR* RNA was able to repress sense strand transcription before VIN3 exhibited any effects (Swiezewski et al., 2009). More recently, Heo and Sung (2011) have identified another noncoding RNA from the sense strand of the first intron of *FLC* that is distinct from *COOLAIR* and has been named *COLD ASSISTED INTRONIC NON-CODING RNA (COLDAIR*). *COLDAIR* is temporally correlated with flowering time; its transcript levels were shown to increase within the first 10 days of vernalization. *COLDAIR* is also mechanistically associated with the flowering time pathway. *FLC* contains a cryptic *COLDAIR* promoter, which is activated when *FLC* is repressed. *COLDAIR* was further shown to be necessary for recruitment of CLF to *FLC*. Although CLF is a component of the PRC2 complex, *COLDAIR's* role in maintaining PRC2 association with *FLC* after vernalization is unclear (Heo and Sung, 2011). Despite this final caveat, non-coding RNAs clearly play an important role in the regulation of *FLC* expression. A detailed review of noncoding RNAs and their function in chromatin regulation has recently been published (De Lucia and Dean, 2010) (Fig. 2).

RNA binding proteins and epigenetic regulators have also been shown to play important roles in *FLC* RNA regulation (Simpson, 2004). CstF64 and CstF77 are two 3' RNA processing factors that were shown to be essential for 3' targeting of the antisense transcripts of FLC (Liu et al., 2010).

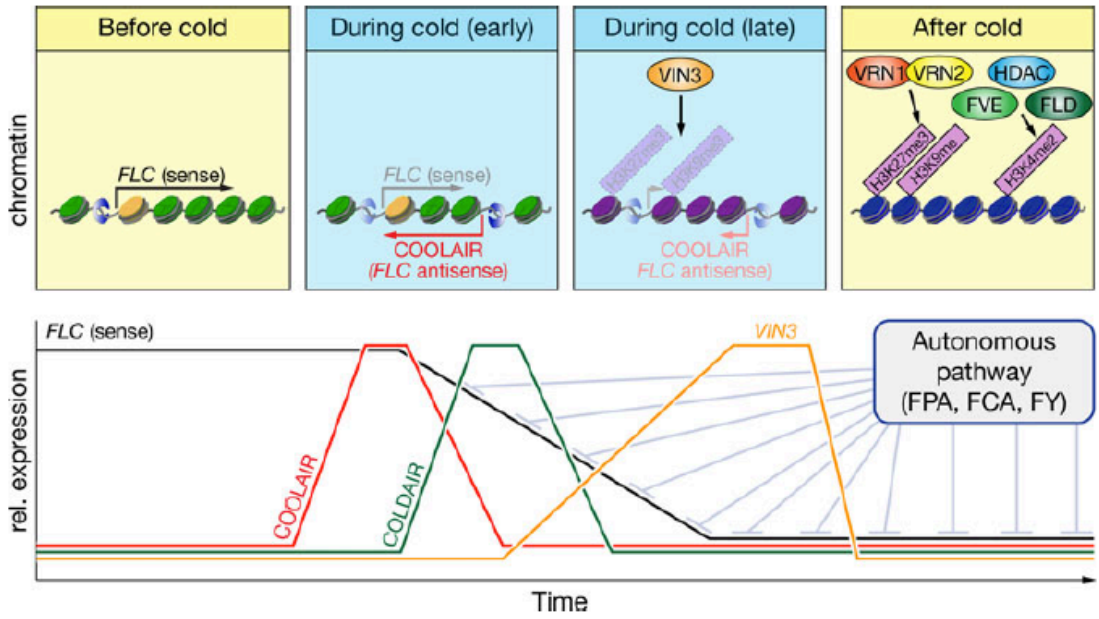


Figure 2. ***Regulation of FLC.*** In plants requiring vernalization, FLC chromatin is acetylated in a nonvernalized state, resulting in active transcription. The first step to negate the effects of FLC is the transcriptional repression of its RNA by COOLAIR, the antisense transcript of FLC during early exposure to cold. Another noncoding RNA called COLDAIR is transcribed from the first intron of FLC and also plays a major role in downregulating FLC transcript levels. Upon initiation of vernalization (late cold), VIN3 methylates lysine residues of histone H3. This vernalized state is maintained by VRN1 and VRN2 upon vernalization, even after the temperatures become warmer. The autonomous pathway regulators FLD and FVE also function by controlling methylation of lysine residues of histone H3. The RNA binding elements Cst64 and Cst77 and the autonomous pathway regulators FPA, FCA, and FY all regulate FLC transcript levels. Levels of FLC RNA (black) are plotted against different stages of cold and compared to levels of COOLAIR RNA (red), COLDAIR RNA (green), and VIN3 protein (orange). This figure has been adapted from Srikanth and Schmid (2011).

Flowering in cultivated beets *Beta vulgaris ssp vulgaris* is regulated by 2 homologs of FT that have evolved antagonistic function. While *BvFT2* is a promoter of flowering in response to an inductive photoperiod, *BvFT1* functions as a repressor of flowering and requires exposure to extended periods of cold to flower (Pin et al., 2010). It is interesting to note how the FT protein (PEBP) homolog has adapted a FLC (MADS) like function.

Flowering is also affected by the ambient temperatures a plant experiences throughout its vegetative development (Blazquez et al., 2003). The flowering response to ambient temperatures is diverse among species, and this diversity

extends to different accessions of *A. thaliana*. Higher temperatures promote flowering. This was demonstrated in *A. thaliana* by growing natural accessions under SD conditions at elevated ambient temperatures (25°C or 27°C) (Balasubramanian et al., 2006). Under these conditions, many accessions flowered as early under SD as they normally would under 23°C LD. Thus, in many accessions, higher temperatures can serve as a substitute inductive LD. Several flowering time mutants show temperature dependence. *phyB* and *cry2* mutants were shown to flower earlier at 23°C compared to 16°C (Blázquez et al., 2003; Halliday et al., 2003). Also, accessions with non-functional *fri* and *flc* alleles responded strongly to higher temperatures and flowered much earlier at 27°C than at 23°C. In contrast, *FRI/FLC* accessions showed a much weaker response to elevated temperatures, indicating that *FLC* plays a role in suppressing thermal induction (Balasubramanian et al., 2006). Further analysis revealed the existence of natural accessions that were unresponsive to thermal induction despite having non-functional *fri/flc* alleles. In the case of Nd-1, the causal gene could be mapped to a deletion at the *FLOWERING LOCUS M (FLM)* locus (Werner et al., 2005). Genes associated with alternative splice site selection were enriched at 27°C compared to 23°C. Temperature-dependent alternative splicing of *FLM* (Balasubramanian et al., 2006) also suggests that splicing is an important regulator of flowering.

Another major regulator of flowering in response to ambient temperatures is *SHORT VEGETATIVE PHASE (SVP).* A MADS box protein, SVP binds to the CArG motifs on the *FT* and *SOC1* promoters and acts as a floral repressor (Fig. 3) (Hartmann et al., 2000; Lee et al., 2007). *SVP* acts downstream of the autonomous pathway mutants *FCA and FVE,* that are known to play a role in ambient temperature sensing in *A. thaliana* (Blázquez et al., 2003). Genetic interactions between *SVP and FLC* indicated that SVP did not regulate FLC. The proteins, however, were shown to co-immunoprecipitate indicating that they may act in parallel. SVP and FLC are mutually dependent and exhibit similar temporal and spatial expression. ChIP analysis of FLC and SVP showed common binding sites in both the flowering integrators *FT* and *SOC1*. SVP may therefore regulate these genes in a FLC dependent manner (Li et al., 2008).

While genetic and molecular analyses have identified several genes that are involved in regulating flowering in response to ambient temperature, the mechanism by which plants detect differences in temperature remains a unknown. Only recently, microarray analyses of plants induced to flower by temperature and photoperiod showed expression of *HEAT SHOCK PROTEIN 70 (HSP70)* to be highly correlated with gradual increases in temperature (Balasubramanian et al., 2006; Kumar and Wigge, 2010). Based on this finding, a genetic screen was designed to identify factors involved in temperature perception. Mutagenesis screens resulted in the identification of the *ACTIN RELATED PROTEIN 6 (ARP6)* as a component in temperature mediated flowering (Kumar and Wigge, 2010). ARP6 is a nuclear protein that acts to repress flowering by maintaining *FLC* expression (Choi et al., 2005; Deal et al., 2005). *arp6* mutants phenocopied warm-grown plants and show a constitutive warm temperature response. ARP6 is part of the SWR1 chromatin remodelling complex and functions by introducing histone H2A.Z rather than H2A into nucleosomes. H2A.Z nucleosomes appear to wrap DNA more tightly than their H2A counterparts. The tight wrapping of DNA by H2A.Z nucleosomes can be overcome by higher temperatures, thereby providing a possible mechanism for temperature-dependent gene regulation (Kumar and Wigge, 2010).

2.1.1.3. Gibberellic acid pathway.

In 1935, Teijiro Yabuta observed that rice seedlings infected with the fungus *Gibberella fugikuroi* grew so quickly that they tipped over. It was later discovered that gibberellins (Giberellic Acids, or GAs) produced by the fungus were regulating growth in the host plants. Numerous GAs have been isolated from plants, but not all of them are biologically active. The first committed step of GA biosynthesis requires the *GA1* gene, which encodes an ent-kaurene synthase (Sun et al., 1992). Flowering of *ga1-3* loss of function mutants was almost normal under LD, but they never flowered in SD unless supplemented with exogenous GA. These results were interpreted as evidence that GA was required for flower initiation in SD, but not in LD. Another genetic screen for mutants that were resistant to the inhibitor of GA

biosynthesis, paclobutrazol, (Jacobsen and Olszewski, 1993) identified *SPINDLY (SPY)* as a negative regulator of GA signalling. *SPY* encodes an *O*-linked *N*-acetylglucosamine transferase. Recent work in rice indicates that SPY regulates the GA pathway by regulating the DELLA proteins (see below) (Shimada et al., 2006). g*a1 spy4* double mutants and wild-type plants flowered similarly, indicating that the *spy4* mutation was able to overcome the late flowering phenotype of *ga1* (Swain et al., 2001).

Bioactive GAs are perceived by plants through a cytoplasmic/nuclear localized receptor, GIBBERELLIC INSENSITIVE DWARF 1 (GID). *A. thaliana* has three functionally redundant copies of the *GID1* receptor (Griffiths et al., 2006; Willige et al., 2007). Interestingly, the *A. thaliana gid1* triple mutant was described to be either moderately late flowering (Griffiths et al., 2006) or extremely late flowering (or not flowering at all) (Willige et al., 2007), even in LD. These findings indicated that contrary to previous results (Wilson et al., 1992), GA signalling contributed to promoting flowering under LD and that their role in regulating flowering time in *A. thaliana* was not limited to SD. The finding that the *ga1-3* mutant accumulates detectable levels of bioactive GAs (King et al., 2001; Silverstone et al., 1998) provides a simple explanation for the observed difference in the severity of phenotypes between *ga1-3* and the *gid* triple mutant.

The *ga1-3 co* double mutant flowered later than both parents, indicating that deficiency in GA biosynthesis has an additive effect on the late-flowering phenotype of *co* mutants in LD (Putterill et al., 1995). It was observed that *FT* mRNA levels increased 15-fold in plants shifted from SD to LD upon application of GA, indicating that *ga1-3* plants required GA in addition to an inductive photoperiod to produce *FT* mRNA (Hisamatsu and King, 2008). Finally, application of paclobutrazol to wild-type plants resulted in delayed flowering in LD, with addition of exogenous GA completely restoring proper flowering (Hisamatsu and King, 2008). Taken together, these results indicate that GAs regulate the expression of *FT* and function in parallel to CO in LD to promote flowering.

GID1 regulates GA signal transduction through interaction with members of the DELLA protein family, named so due to a conserved protein motif starting with the amino acids D, E, L, L and A. They belong to the GRAS family of transcriptional regulators that function as repressors of plant growth and development (Harberd et al., 2009; Sun, 2010). An important role for this protein family was suggested by an experiment showing that a deletion in the DELLA domain resulted in a semi-dwarf phenotype that resembled the GA-deficient mutant that was not rescued by GA supplementation (Koornneef et al., 1985). It was shown recently that the GA-bound form of GID1 induced a conformational change upon binding to the N-terminal region of DELLA proteins (Murase et al., 2008). Because the conformational change promotes ubiquitination by an E3 ubiquitin ligase, the DELLA proteins become susceptible to degradation via the 26S proteasome pathway. DELLA proteins have been shown to be important integrators of GA signalling and play a significant role in many aspects of plant development, in particular photomorphogenesis (Achard et al., 2007; Alvey and Harberd, 2005; Fu and Harberd, 2003). DELLA proteins have been shown to immobilize the *PHYTOCHROME INTERACTING FACTORs (PIFs)* proteins by directly interacting with them, (de Lucas et al., 2008; Schwechheimer and Willige, 2009). Interestingly, Oda et al., (2004) showed that suppression of *PIF3* by antisense RNA induced *CO* and *FT*, resulting in early flowering in LD. Since *PIFs* are regulated by light and GA via the DELLA proteins, they represent a point of convergence of light and GA pathways.

An important point of convergence between the GA pathway and the photoperiod and vernalization pathways are the floral integrators *LEAFY* (*LFY*) and *SOC1* (Fig. 3). Application of GA was shown to increase *LFY* promoter activity in SD (Blázquez et al., 1997). In SD, *LFY* transcription is reduced in *ga1-3* plants. In addition, analysis of the GUS activity in the *spy* mutant indicated an increase in *LFY* promoter activity especially in SD (Blázquez et al., 1998). These findings lead to the conclusion that GA regulates the *LFY* promoter and that at least part of the flower-stimulating activity of GAs is due to an activation of *LFY* expression by GAs (Blázquez et al., 1998).

A more detailed analysis of the *LFY* promoter identified a *cis* regulatory sequence that was required for *LFY* expression in response to GA treatment. This regulatory sequence conforms to the consensus binding site for MYB transcription factors (Blázquez and Weigel, 2000). GAMYBs, a family of R2R3 type MYB transcription factors, have been shown to play an important role during germination in cereals. In *A. thaliana*, At*MYB33,* a potential homolog of GAMYBs, was found to be expressed in the shoot apex as a response to endogenous GAs or application of exogenous GAs (Gocal et al., 2001). In addition, MYB33 protein was shown by EMSA studies to bind to the predicted GA-responsive element in the *LFY* promoter. Analysis of the *Lolium temulentum* homolog of GAMYB, LtGAMYB, showed that the protein is expressed in the shoot apex during floral transition and its levels increased in synchrony with GAs at the apex, indicating that GAs may regulate the floral transition (Gocal et al., 1999; King et al., 2001). Interestingly, *MYB33* and its closest paralogs, *MYB65* and *MYB101*, are predicted targets of the microRNA159 (miR159). Regulation of *MYB33*, *MYB65* and *MYB101* by miR159 has recently been shown to play a major role in regulating the spatial expression of these genes (Palatnik et al., 2003; Park et al., 2002). miR159 was also shown to be downregulated by the DELLA proteins, indicating that GA mediates flowering in response to miR159 by repressing DELLA proteins (Achard et al., 2004).

Another critical gene for promoting flowering in response to GA signalling is *SOC1*. Moon et al. (2003) demonstrated that *SOC1* expression is nearly undetectable in *ga1-3* mutants in SD. These authors further demonstrated that overexpression of *SOC1* was able to overcome the flowering defects of *ga1-3* in SD*.* The exogenous application of GA also resulted in an increased transcript level for *AGAMOUS LIKE 24* (*AGL24*) in a SOC1 dependent manner (Liu et al., 2008; Yu et al., 2002). Finally, *SVP*, a repressor of flowering and a negative regulator of *SOC1*, was also shown to be regulated by GAs. *SVP* levels decreased in GA-treated *wt* plants, while *ga1-3* mutants showed consistently higher levels of *SVP* than their wild-type counterparts. It can be concluded that GA regulates *SOC1* expression at several levels by promoting

expression of *SOC1*-inducing genes (such as *AGL24*) and at the same time down-regulating floral repressors such as *SVP* (Li et al., 2008).

More recently, two GATA-like transcription factors, GNC (GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED) and GNL (GNC-LIKE), have been shown to participate in GA signalling and *gnc* and *gnl* single and double mutants flowered earlier than wild-type in LD. These two genes were shown by ChIP to be direct targets of the PIF transcription factors and thus are regulated by GA in a DELLA-dependent manner (Richter et al., 2010).

2.1.1.4. Autonomous pathway

Autonomous pathway mutants are characterized by delayed flowering irrespective of day length. The autonomous pathway genes include *LD*, *FCA*, *FY*, *FPA*, *FLOWERING LOCUS D* (*FLD*), *FVE*, *FLK,* and *REF6* (Noh et al., 2004; Simpson, 2004). All genes in the autonomous pathway act by repressing *FLC* expression and the late flowering observed in these mutants can largely be explained by elevated *FLC* levels. (Fig. 2).

The proteins encoded by the genes in the autonomous pathway generally fall into two broad functional categories: general chromatin remodelling and maintenance factors and proteins that affect RNA processing. FLOWERING LOCUS D (FLD) was shown to regulate *FLC* by preventing hyperacetylation of the locus, thereby acting as a repressor of *FLC* transcription (He et al., 2003). *fld-3* mutants showed hyperacetylation of histone H4 and a 2-fold increase in histone H3K4 dimethylation (He et al., 2003; Liu et al., 2007). Genetic analyses indicated that *FLD* and *FCA* function in the same genetic pathway, with *FCA* being epistatic to *FLD* with respect to flowering time (Liu et al., 2007). FCA contains two RNA Recognition Motifs (RRM) and a WW domain suggesting a role in post-transcriptional RNA modifications. *FCA* mRNA was shown to be subject to alternative splicing, and different transcripts were expressed at different levels in different tissues (Macknight et al., 1997). FCA was shown to be associated with the *FLC* coding region at exon 6 and intron 6 where it regulates the proximal poly-adenylation site of the antisense RNA (Liu et al., 2007). Another FLC repressor, FVE acts through participation in a histone

deacetylation complex (Ausí-n et al., 2004)(Fig. 2). Interestingly, the loss of *FCA* function was found to be additive with mutations in *FVE*. The *FPA* gene encodes a protein with three RNA Recognition Motifs (RRM). FPA and FCA were shown to act in a partially redundant fashion to control RNA-mediated chromatin silencing of *FLC* (Bäurle et al., 2007; Hornyik et al., 2010). Apart from its participation in chromatin silencing, FPA has also been implicated in alternative cleavage and polyadenylation of RNAs (Hornyik et al., 2010).

In contrast to FPA, FY, an RNA 3' end-processing factor, has been shown to directly interact with FCA (Simpson et al., 2003). FCA/FY interaction is not only required for downregulation of *FLC*, but apparently also plays an important role in the autoregulation of *FCA* expression (Simpson et al., 2003). There exists some natural variation at *FY*, and a mutation in the second PPLPP motif of FY in Bla-6 was recently shown to contribute to the relative insensitivity of this accession to the flower-promoting effects of a reduced red light to far-red light (R/FR) ratio (Adams et al., 2009). *FLK* also encodes a putative RNA binding protein. As is common for autonomous pathway mutants, *flk* flowered late under both LD and SD (Lim et al., 2004). The delayed flowering of *flk* was most likely caused by activation of *FLC* expression, which in turn resulted in the downregulation of *FT* and *SOC1*, which could be overcome by vernalization and application of exogenous gibberellins (Lim et al., 2004).

Finally, *LD* was identified in several genetic screens for late flowering mutants (Koornneef et al., 1991; Rédei, 1962). The gene was eventually cloned by Lee et al., (1994) and was shown to encode a protein with similarities to transcriptional regulators and contains two bipartite nuclear localization domains and a glutamate-rich region. The late flowering phenotype of *ld* mutants was completely suppressed by vernalization. The LD protein was found to localize to the nucleus and regulate the *LFY* promoter (Aukerman et al., 1999). The LD protein also binds to SUPRESSOR OF FRIGIDA 4 (SUF4) preventing its action on the *FLC* locus (Kim et al., 2006a).

2.1.2. Integrators of flowering.

The induction of flowering is a central event in the life cycle of plants. When timed correctly, it helps ensure reproductive success, and therefore has adaptive value. Because of its importance, flowering is under the control of a complex genetic circuitry that integrates environmental and endogenous signals, such as photoperiod, temperature and hormonal status. The different genetic pathways that regulate flowering converge on a relatively small number of common targets, which integrate these endogenous and exogenous signals. *FT* (in leaves) and *SOC1*, *AGL24* and *LFY* (at the shoot apex) are probably the most important genes in this context and have been referred to as central floral pathway integrators (Simpson and Dean, 2002).

Induction of *FT* by CO, which makes up the core of the photoperiod pathway, has been discussed above. However, CO is by no means the only factor to regulate *FT* expression. In particular, several repressors of *FT* have lately been identified. These include AP2-domain containing transcription factors of the RAV family (TEM1, TEM2) and six genes of the euAP2 linage (Kim et al., 2006b) that are targets of miR172 (*AP2*, *SMZ*, *SNZ*, *TOE1*, *TOE2*, *TOE3*). Direct binding of TEM1 and SMZ to regulatory regions of *FT* has been demonstrated by ChIP, indicating that these factors directly regulate *FT* (Castillejo and Pelaz, 2008; Mathieu et al., 2009). *FT* is also a target of FLC, which in itself is repressed in response to vernalization, indicating cross-talk between the photoperiod and vernalization pathways (Fig. 3)(Helliwell et al., 2006; Searle et al., 2006).

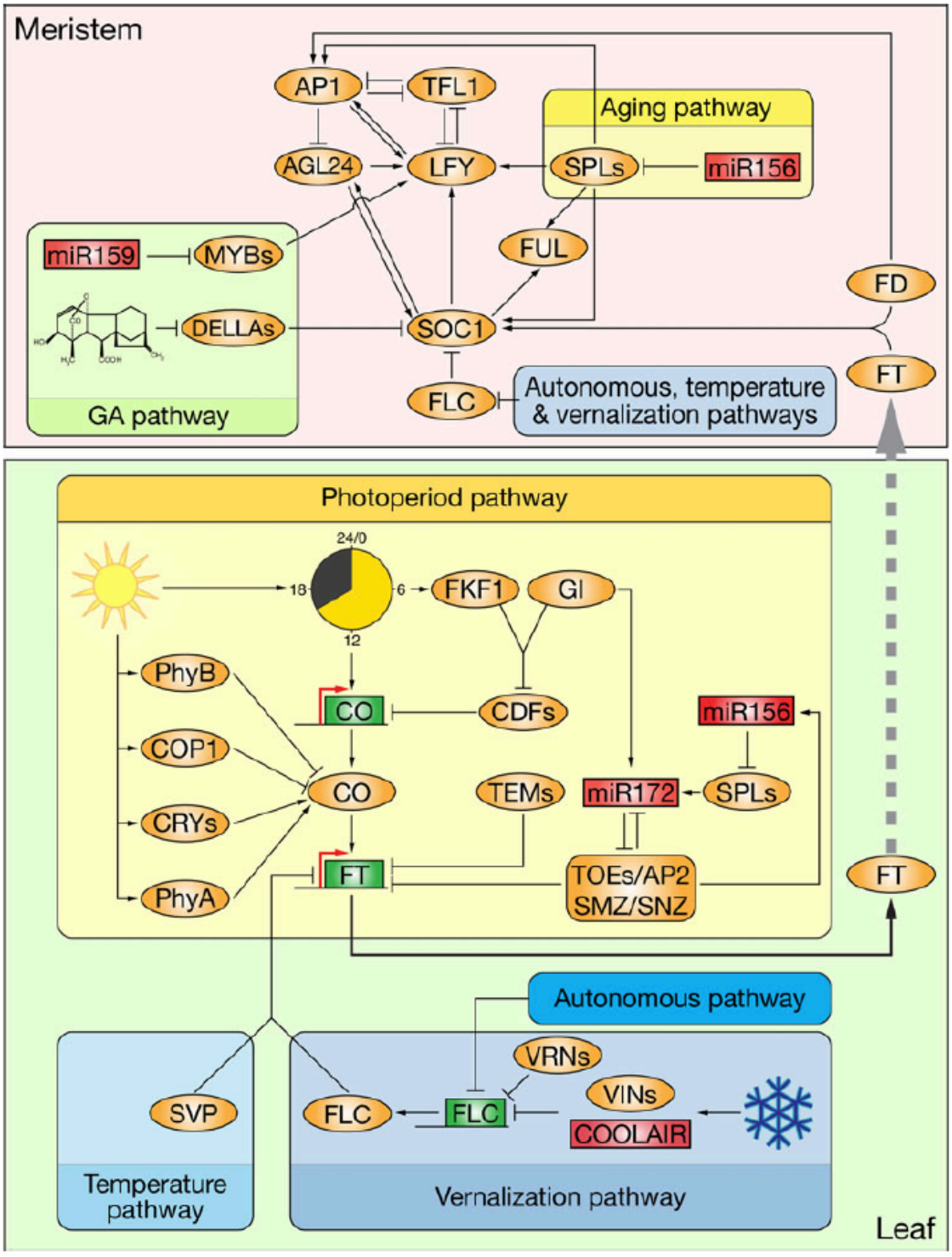


Figure. 3 ***Integration of flowering time pathways***. Light is perceived in the leaves, where it is perceived by photoreceptors such as PHYA, PHYB, CRY1, and CRY2 and regulates expression of genes such as GI, FKF1, and CDF1, all of which have direct or indirect effects on CO expression. CO is a transcriptional activator of FT. miR172 is regulated both by the circadian clock as well as SPLs, which are in turn regulated by miR156. miR172 targets the AP2 family of transcription factors, which play an important role in transcriptional

repression of FT in the leaf. The different autonomous pathway genes regulate FLC, a suppressor of FT and SOC1. Another major environmental factor that affects FLC is temperature. FRI activates FLC, while the histone modification proteins VIN3 and VRN1/2 repress it, thereby promoting flowering. Ambient temperatures affect expression of yet another transcriptional repressor of FT, SVP. As the florigen, FT protein moves from the leaf to the apex, where, with the bZIP transcription factor FD, it activates AP1 and SOC1. In the GA pathway, GA regulates levels of the DELLA proteins, which in turn repress miRNA159, a repressor of MYB. MYBs positively control LFY levels in the meristem. Thus the signals from different pathways integrate at LFY, FT, and/or SOC1. SOC1 and AGL24 regulate each other and act together to activate LFY transcription. TFL1 and LFY repress each other. SOC1 activates FUL, which is also a target of the SPL proteins. Activation of SPLs by miR156 forms a novel pathway for regulation of flowering called the aging pathway. SPL proteins upregulate LFY, AP1, FUL, and SOC1. Hence, the different integrators directly or indirectly activate AP1, which marks the beginning of floral organ formation. All genes are represented in green, microRNAs in red, and proteins in orange. This figure was adapted from Srikanth and Schmid, (2011).

At the shoot apex several MADS-domain proteins have been shown to be regulated by various input signals. *SOC1* for example was initially cloned as a suppressor of CO overexpression but was later shown to also be regulated by GA signalling (Moon et al., 2003). Wang et al., (2009a) have demonstrated that *SOC1* mRNA levels increase in a miR156-SPL dependant manner. Another MADS box protein, AGL24 was also shown to play an important role in flowering. AGL24 was shown to be positively regulated by the vernalization pathway. However, FLC was not shown to affect the *AGL24* mRNA levels, indicating that AGL24 is a target of the vernalization pathway in an FLC independent manner (Michaels et al., 2003). While *agl24* mutants were late flowering, its overexpression resulted in, besides an early flowering phenotype, ectopic floral organ formation that was dosage dependant (Yu et al., 2004), indicating that flowering time and flower development pathways are not clearly separated but are in part controlled by the same factors.

Another gene that is being considered both a meristem identity gene and a flowering time gene is *LEAFY* (*LFY*) (Weigel et al., 1992). *LFY* was among the first genes for which a role in different flower-promoting pathways had been established (Blázquez et al., 1997; Blázquez and Weigel, 2000; Lohmann et al., 2001; Parcy et al., 1998). *LFY* RNA is detectable in young leaf primordia but is most strongly expressed

in floral meristems (Blázquez et al., 1998; Blázquez et al., 1997; Weigel et al., 1992). Overexpression of *LFY* results in an early flowering phenotype, while *lfy* mutants show homeotic transformations with leaf like structures replacing the floral organs (Blázquez et al., 1997; Weigel et al., 1992; Weigel and Nilsson, 1995). How *LFY* is regulated by GA has already been discussed. Besides, *LFY* was shown to be a target of the miR156 regulated SPLs as well (Yamaguchi et al., 2009) Given the time of *LFY* expression in the apex and the phenotypes associated with it, it is clear that *LFY* plays an important role in regulating the transition from vegetative to reproductive development in the plant's life (Liu et al., 2009).

Several of these integrators are vital for transition into flowering. For example*, lfy* mutants bear leaves and associated shoots instead of flowers (Weigel et al., 1992) and *ft* mutants remain in a vegetative state for much longer than their wild-type counterparts (Koornneef et al., 1998). Both mutants eventually flower due to activation *of AP1* in a LEAFY-independent manner or through the close homologues of *FT* such as *TSF* (Yamaguchi et al., 2005).

The above-mentioned genes function predominantly as nodes of integration between the different pathways by functioning downstream of CO (photoperiod), FLC (vernalization and autonomous), in response to GA (GA pathway) and miR156-SPL*s* (age) (Fig. 3). The situation is further complicated by the fact that a feedback regulation exists between several of the integrators (Liu et al., 2008; Lee et al., 2008).

The integrators not only regulate each other, but also the floral organ identity genes. FT moves to the apex where, with FD, it directly or indirectly activates *AP1, SEP3, SOC1* and *FRUITFULL (FUL)* (Abe et al., 2005; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005). SOC1 *and* AGL24 along with SVP repress *SEP3* that, along with LFY, is essential for activation of B and C floral organ identity genes (Liu et al., 2009a). LFY, when fused with the VP16 viral activation domain, has been shown to activate expression of *AP1, APETALA 3 (AP3) and AG* (Parcy et al., 1998). Once

activated by LFY or FT, AP1 activates SEP3. The LFY/SEP3 complex activate *AG*, *AP3* and *PISTILLATA (PI*)(Liu et al., 2009a). This results in a feed forward loop that orchestrates the timing of activation of the floral homeotic genes (Wagner, 2009). The different co-factors of LFY and their effects have been reviewed recently (Liu and Mara, 2010; Moyroud et al., 2010).

The flower development genes not only mark the defined regions of the meristem for organ formation, but also turn off the flowering time genes, ensuring a sharp transition to flower formation. Thus its clear that in the lifecycle of a plant, the transition to flowering is as well orchestrated as the decision to establish fate of the floral primordia by shutting down the flowering time genes.

2.2. bZIP transcription factors in plant development

2.2.1. Introduction to bZIP transcription factors

Transcription factors are proteins involved in the regulation of gene expression that bind to the promoter elements upstream of genes and regulate their expression by either facilitating or inhibiting transcription. The first transcription factor to be discovered was the 66-amino acid Cro repressor protein of bacteriophage lambda that belongs to the Helix turn Helix family of transcription factors (Anderson et al., 1981). The second DNA binding motif to be discovered was the Zinc-finger domain. The founding member of this family was the metalloprotein TFIIIA (Hanas et al., 1983). Landschulz et al., (1988), discovered a third family of transcription factors - the leucine zippers. They described an alpha helix with leucine residues repeating at the same position in the helix. This is termed as the gabcdef heptad model of the leucine zipper family of transcription factors. The repeating leucine residues enclose a hydrophobic region between them, enabling for two identical molecules to 'zip' together. The presence of basic residues such as lysine and argenine results in a 'basic' domain in the transcription factors. These transcription factors are referred to as basic leucine zippers (bZIP).

A comprehensive phylogenetic analysis of bZIP genes from algae, mosses, ferns, gymnosperms and angiosperms suggest that the ancestor of green plants possessed four bZIP genes functionally involved in oxidative stress and in light-dependant regulations. Later, these diverged to evolve multiple functions to survive in changing environments (Correa et al., 2008). Plant bZIP transcription factors specifically identify and bind to either the A-box (T**ACGT**A), G-box (C**ACGT**G) or C-box (G**ACGT**C) (Izawa et al., 1993). Jakoby et al. (2002), identified 75 distinct members of the bZIP protein family in *Arabidopsis* and classified them into 10 different groups (A, B, C, D, E, F, G, H, I and S) based on structural features and functional information such as the number of exons, size, position of the basic domain, number of Leucine repeats, known interactions with other proteins and known binding sites. Deppmann et al., (2004) analysed 67 bZIPs in *Arabidopsis* and compared them with human bZIP proteins and found no bZIPs homologous between the two organisms. Analysis of the 67 *Arabidopsis* bZIPs showed certain conserved patterns that enabled computational prediction of their dimerisation patterns. Three structural properties, namely length of the leucine zipper, the placement of asparagine or a charged amino acid in the hydrophobic interface and the presence of interhelical electrostatic interactions were used as criteria for identification. Several families of bZIPs in *Arabidopsis* tend to homodimerise due to the location of Asparagine at the 'a' position of the gabcdef heptad. Ehlert et al., (2006) showed that the bZIP transcription factors show very specific patterns of dimerization. Group C bZIPs dimerise only with very specific group S bZIPs to regulate the transcription of proline dehydrogenase (Weltmeier et al., 2006).

FD is a bZIP transcription factor that belongs to group A of plant bZIPs along with its paralog *FDP, Early Em. Levels (EEL)* and 7 other members involved in auxin signalling and regulation (Jakoby et al., 2002). While group A bZIP transcription factors have been shown to predominantly homodimerise, the dimerisation patterns for both FD and FDP were not predicted due to their inability to satisfy the criteria of the study (Deppmann et al., 2004).

2.2.2. Role of FD in the regulation of flowering time in Arabidopsis thaliana

While the dimerisation pattern of FD is unclear, it has been shown to interact with the florigen FT. The first evidence of FT-FD interaction was supplied by two laboratories at approx. the same time. Wigge et al., (2005) performed an Y2H screen to identify interaction partners of FT. Two closely related class A bZIP transcription factors, FD and FDP, were found to interact with FT. Of these only FD (*At*4g35900) had available T-DNA insertion lines. Analysis of these lines indicated that the loss of FD function resulted in late flowering, indicating that FD normally promotes flowering. In addition, genetic analyses had indicated that FT and FD belonged to the same class of floral regulators (Koornneef et al., 1991, Koornneef et al., 1998). In contrast to its strong effect on *35S::FT,* the L*er fd-1* loss of function mutant had only a weak effect on a *SOC1* precocious phenotype and *35S::LFY* (Abe et al., 2005). This further clarified that FD function was directly linked to that of FT. Visualization of the FT-EGFP fusion protein indicated that the protein co-existed in the cytoplasm as well as the nucleus. GR fusions of FT promoted flowering on addition of Dexamethasone indicating the need for FT in the nucleus (Abe et. al., 2005). They also performed mutagenesis screens to identify suppressor mutations that suppressed the early flowering of a transgenic *35S::FT* overexpression. In addition, Abe and colleagues tested known late flowering mutants for their ability to suppress the *35S::FT* early flowering phenotype and identified *fd* mutants to be able to do so. The physical interaction between FT and FD, and the their localisation into the nucleus was confirmed by a BiFC assay where the FT and FD were tagged to the two parts of YFP (Abe et al., 2005). Taken together, both laboratories identified that FD functioned downstream of FT, and the two proteins formed a complex in the nucleus that was necessary to trigger flowering.

In contrast to FT, *FD* mRNA was highly expressed in the transition meristem and the floral anlagen. Since FT-FD interaction is necessary for initiation of flowering, the presence of FD in the SAM exclusively can be seen as an indirect evidence entailing the movement of the florigen to the apex. The expression of *FD* RNA declined with the formation of the floral organs and upregulation of *AP1* (Wigge et al., 2005). It

was later confirmed by Kaufmann et al., (2010) that AP1 does in fact downregulate *FD*. Thus FT integrates the environmental cues providing the temporal information while FD, expressed mainly in the apex supplies the spatial specificity transitioning into flowering.

Analysis of loss of function phenotypes of *FT* and *FD* and overexpression phenotypes of *FD* indicated that AP1 is a potential downstream target of FD (Abe et al., 2005; Wigge et al., 2005). Ectopic expression of *AP1* was seen in the leaves of the *35S::FD* plants. *AP1* and *FT* showed similar activation in 35S::FD plants shifted to LD from SD. Further GUS activity was seen only in the part of the *AP1::GUS x 35S:: FD* plant exposed to LD. *In situ Hybridization* showed that *AP1* expression was delayed in *fd-2* plants compared to the wild-type. Taken together, all these data confirm that AP1 is in fact a target of the FT-FD complex.

In wheat, the homolog of FT, *Triticum aestivum FT* (*TaFT*) was shown to interact with homologs of FD expressed in leaves. There seems to be a high conservation of sequences between the *Arabidopsis* and Wheat FD sequences, with 69 amino acids without gaps including the bZIP domain. The TaFD-Like 2 (TaFDL2) protein was further shown to bind the *VRN1* promoter sequence, the homolog of *Arabidopsis AP1.* The protein was shown to preferentially bind to A/G, C/G, A/C box hybrids and a G box on the *VRN1* promoter region but not to a CArG box (Li and Dubcovsky, 2008).

A recent study indicates that the interaction of the rice homolog of FT, Hd3a to OsFD is through the 14-3-3 proteins (Taoka et al., 2011). T-DNA mutation lines of 14-3-3 genes results in a late flowering phenotype in LD but not short day in *Arabidopsis* (Mayfield et al., 2007). The reverse is seen in case of rice where overexpression of the rice GF14c, a 14-3-3 isoform delayed flowering (Purwestri et al., 2009). In tomatoes, overexpression 14-3-3 proteins were shown to overcome the loss of function of *selfprunning (sp)* (Pnueli et al., 2001). Interestingly, Abe et al., (2005) noticed that when the Threonine residue of FD was mutated into an Alanine at the

282nd position, it abolished the interaction between FT and FD. The mutation lies in the recognition motif of the 14-3-3 proteins indicating that in *Arabidopsis* too, the FT-FD interaction might not be direct and via the 14-3-3 proteins instead.

2.3. Objectives

Mathieu et al., (2007) demonstrated that *SUC2::FT-3xYFP* with a TEV cleavage site separating the FT and the three copies of YFP when crossed to plants expressing the TEV protease from the SUC2 promoter resulted in early flowering due to the release of the small mobile FT protein from the immobile FT-3xYFP complex. Two questions can be raised based on this data: Is the SUC2 promoter expressed in the apex? And is the misexpression of the protein by the stronger SUC2 promoter resulting in the observed phenotype? We tried to address these questions by studying the effect of the FT protein movement and cleavage when expressed from the native promoter in a genomic context.

The next step in the photoperiodic pathway is the interaction of FT with the bZIP transcription factor FD. That *AP1* is a downstream target of the FT-FD complex has already been shown (Abe et al., 2005; Wigge et al., 2005). However, it is unlikely that *AP1* is the sole downstream target of this complex, given the intricate network of transcription factors that play a role in formation of floral organs. My second objective is to study the downstream targets of FD.

Given that the MADS box proteins such as AP1 are themselves transcription factors, it is possible that all targets of FD are not direct but a result of transcriptional activation of the direct targets of FD. My third objective is to differentiate the primary and secondary targets of FD and confirm these primary targets by in vitro assays.

The *FD* mRNA is expressed in the transition meristem and fades in the floral primordia. The targets of FD, such as *AP1* are known to be expressed in the floral anlagen and floral organs. The question arises, how does a protein expressed in the meristem activate genes that are not expressed in the meristem? My fourth objective was to compare the expression patterns of mRNA of the targets with that of the FD protein to understand how these targets are regulated at the shoot apical meristem.

In summary, the main focus of my thesis was to study several aspects of flowering time regulation by the photoperiod pathway. My goal was to better understand how the stimulus to induce flowering is transmitted from the leaf to the shoot apex and what occurs at the apex when it stops to form vegetative organs and starts producing flowers instead.

3.0 RESULTS

3.1 Dissecting the role of the FT protein as a long distance signal.

Plants employ environmental and endogenous cues to correctly time developmental processes such as the transition from vegetative growth to flowering throughout the seasons. This ability is of particular importance since plants, as sessile organisms, cannot escape adverse environmental conditions. Several genetically defined pathways that regulate flowering in response to various stimuli have been identified (reviewed in Fornara et al., 2010; Srikanth and Schmid, 2011). One of the key factors that regulate flowering in many plant species is photoperiod, which directly translates to the length of the daily light period. Information on the gradual increase in day-length together with raising temperatures helps plants that grow in the temperate zones of Earth to determine the onset of spring after winter. *A. thaliana* for example is a facultative long day plant, which means that long days have a strong promotive effect on flowering. However, *A. thaliana* will eventually flower even under non-inductive short days.

The photoperiod pathway of flowering starts with the recognition and measurement of day-length in the leaves of plants. This process starts with the perception of light of different wavelength by specific photoreceptors such as phytochromes and cryptochromes, followed by an intricate signalling cascade that involves the GIGANTEA (GI), FLAVIN-CONTAINING F-BOX Protein 1 (FKF1) and the CYCLING DOF FACTOR (CDFs) protein (reviewed in Srikanth and Schmid, 2011). Ultimately, these proteins regulate the expression and protein stability of B-box type zinc finger domain protein CONSTANS (CO). CO has so far shown to have a major direct target, *FLOWERING LOCUS T* (*FT*), which is expressed in leaves in a CO-dependent manner (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Wigge et al., 2005).

The *A. thaliana* FT protein has recently been shown to function as a mobile signal that conveys the information to induce flowering from the leaves to the shoot

meristem (Abe et al., 2005; Wigge et al., 2005; Mathieu et al., 2007; Corbesier et al., 2007). Importantly, the function of FT protein seems to be evolutionary conserved and similar results concerning its function as a florigen have been obtained in a number of plant species (Endo et al., 2005; Igasaki et al., 2008; Kojima et al., 2002; Lifschitz and Eshed, 2006; Lin et al., 2007; Navarro et al., 2011; Tamaki et al., 2007).

In *A. thaliana*, Mathieu et al., (2007) for example used an *in vivo* protease cleavage assay of a FT-3xYFP fusion protein to demonstrate that release of FT protein in the phloem companion cells is sufficient to induce flowering at the shoot apex. In addition, tissue-specific expression of an artificial microRNA against FT mRNA (amiR-*FT*) suggested that RNA movement did not contribute to the induction of flowering (Mathieu et al., 2007). However, since these experiments did not use the endogenous *FT* promoter, it seemed possible that some of the observations made were due to the much stronger and broader expression domain of the *SUC2* promoter used in these studies.

3.1.1 FT protein is required for flowering and functions as the florigen.

To investigate whether FT protein was necessary and sufficient to induce flowering in *A. thaliana* in response to inductive photoperiod when expressed from its own promoter a 13.8 kb genomic FT fragment was used that efficiently rescued the late flowering phenotype of the *ft-10* mutant (M. Schmid, pers. communications). Into this genomic rescue construct, sequences coding for the *tobacco etch virus* (TEV) protease, followed by three copies coding for YFP, to make *gFT-TEV-3xYFP* were introduced. This construct, when introduced into *ft-10* plants, did not rescue the late flowering typically observed in this mutant (Table 3-1, Figure 3-1), presumably because the *FT-TEV-3xYFP* mRNA and the fusion protein were efficiently held back in the phloem companion cells.

Next, the *gFT-TEV-3xYFP ft-10* plants were crossed (see Supplementary Table S3 for list of all crosses) to *ft-10* plants expressing the TEV protease (TEVP) from the *SUC2* promoter (*SUC2*::*TEVP*) and to plants in which TEVP had been translationally fused to two copies of the red fluorescent protein TdTomato (*SUC2*::*TEVP:tdTomato*) and the flowering time was scored in the F1 progeny. The presence of the transgenes was initially confirmed by fluorescence microscopy in the case of *SUC2*::*TEVP:tdTomato* and PCR-based genotyping for the other constructs.

Several F2 lines carrying both the *gFT-TEV-3xYFP* and the *SUC2*::*TEVP:tdTomato* transgene (Fig 3-1 A; Supplementary table S3, crosses #203 and #205) were also analysed by fluorescence microscopy. Interestingly, FT-3xYFP was mostly detected in the peripheral leaf vasculature but was hardly detectable in the main vein (Fig 3-3 A). This pattern is similar to that observed by Adrian et al., (2010) using a 8.1kb *FT* promoter fragment and Takada and Goto, (2003) using a 8.9kb promoter fragment to drive GUS expression. In contrast, the *SUC2::TEVP:tdTomato* expression was observed uniformly throughout the entire vasculature of the leaf (Fig 3-3 B). An overlap of the YFP and TdTomato images indicate that both the *gFT-3xYFP* and

SUC2::TEVP:TdTomato had overlapping domains of expression indicating that TEV protease can cleave the 3x YFP from FT (Fig 3-3 C).

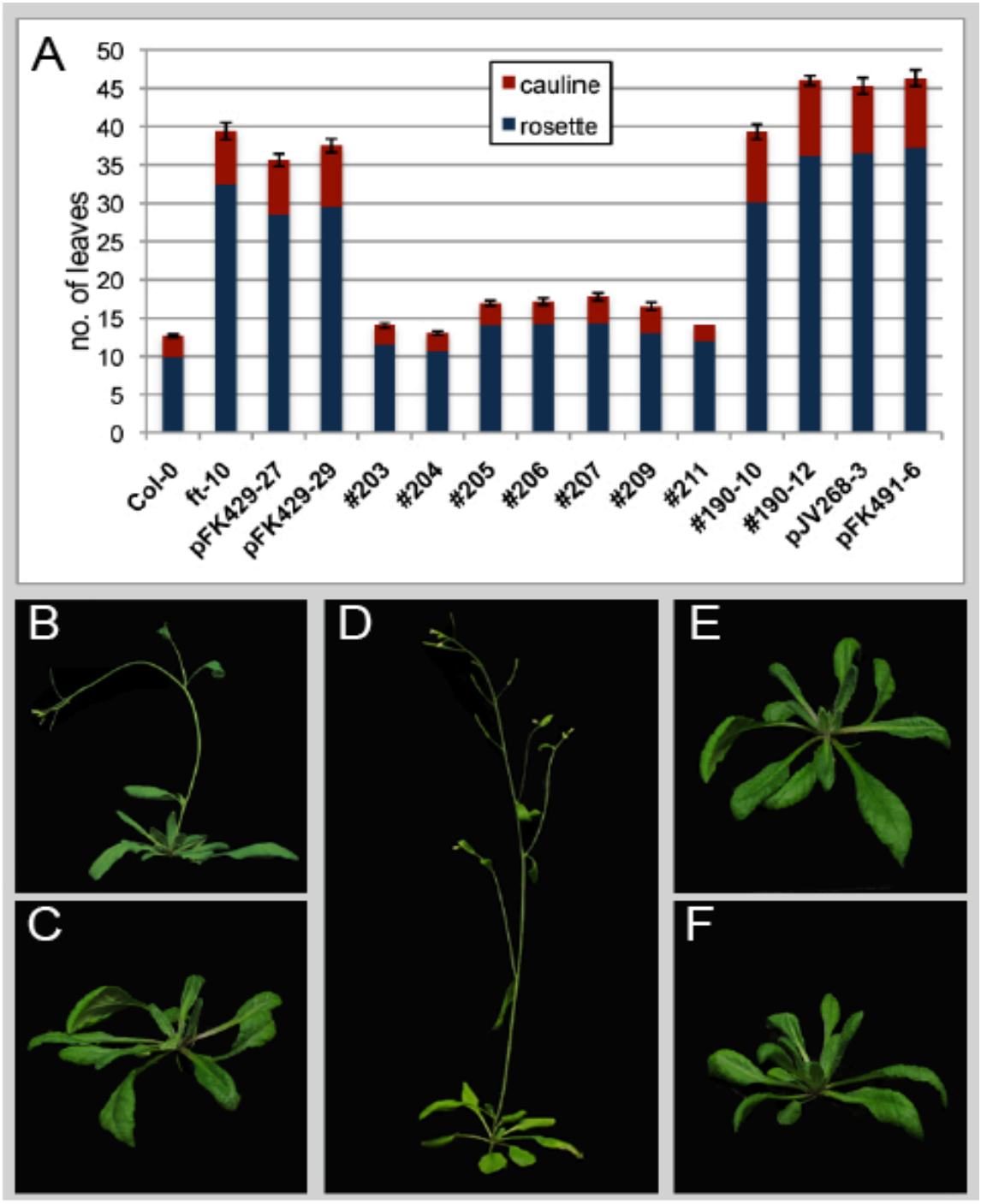


Figure 3-1: *Flowering time data of gFT-TEV-3xYFP* crossed to *SUC2*::*TEVP:tdTomato.*
gFT-TEV-3xYFP and the *SUC2*::*TEVP:tdTomato* plants were crossed to see if the release of FT from the FT-3xYFP complex by the cleavage by Tev Protease was able to enhance flowering. (A) The graph shows the flowering time of different genotypes. pFK429-27 and pFK429-29 represent two independent *gFT-TEV-3xYFP* lines and #190-10, #190-12 and pJV268-3

represent three independent *SUC2*::*TEVP:tdTomato* lines. pFK491-6 represents a *SUC2::TEVP* line without the tdTomato tag. All parental lines were late flowering, similar to or later than the *ft-10* mutant. #203 represents a cross between pFK429-27 and #190-10 and flowers with 14 total leaves, #204 represents a cross between pFK429-27 and #190-12 and flowers with 13 total leaves, #205 and #206 represent crosses between pFK429-29 and #190-10 and #190-12 respectively and flower with 16 and 17 total leaves respectively, #207 and #209 are crosses between pJV268-3 and pFK429-27 and pFK429-29 respectively and flower with a total number of 17 and 18 leaves respectively. #211 is a cross between pFK429-27 and pFK491-6, and flowered with 14 leaves. This experiment shows that the FT protein was released from the bulky FT-3x YFP complex by the TEV protease, restoring the late flowering phenotype of the parents to a wild-type phenotype like Col-0, that flowers with 13 total leaves. X-axis represents the different genotypes while the Y-axis represents the number of leaves. Rosette leaves are represented in blue, and cauline leaves in red. Error bars represent 2xSEM. (B) A wild-type Col-0 plant flowering with 13 total leaves, (C)an *ft-10* mutant plant that has not yet started flowering, (E) a representative of the pFK429-27 parent, (F) a representative of the #190-10 parental line, both of which have not initiated flowering (D) A representative cross #204 that flowers similar to wild-type.

While the parental lines and the F1 progeny that had inherited only one of the two transgenes were late flowering and indistinguishable from the *ft*-10 background (Fig 3-1 A, C, E, F; Table 3-1), F1 plants that carried both transgenes flowered similar to Col-0 (Fig 3-1 B, D; Table 3-1). These results indicate that the TEV protease with or without the TdTomato tag was able to cleave and mobilise the globular FT protein from the immobile FT-3xYFP precursor in the phloem companion cells where the FT protein is normally expressed. As in this experiment FT-3xYFP was under the control of the *FT* promoter, these results further suggest, that vascular-localized FT protein is indeed sufficient to induce flowering at the shoot apex. However, these results do not rule out the possibility that *FT* mRNA movement also contributed to the induction of flowering.

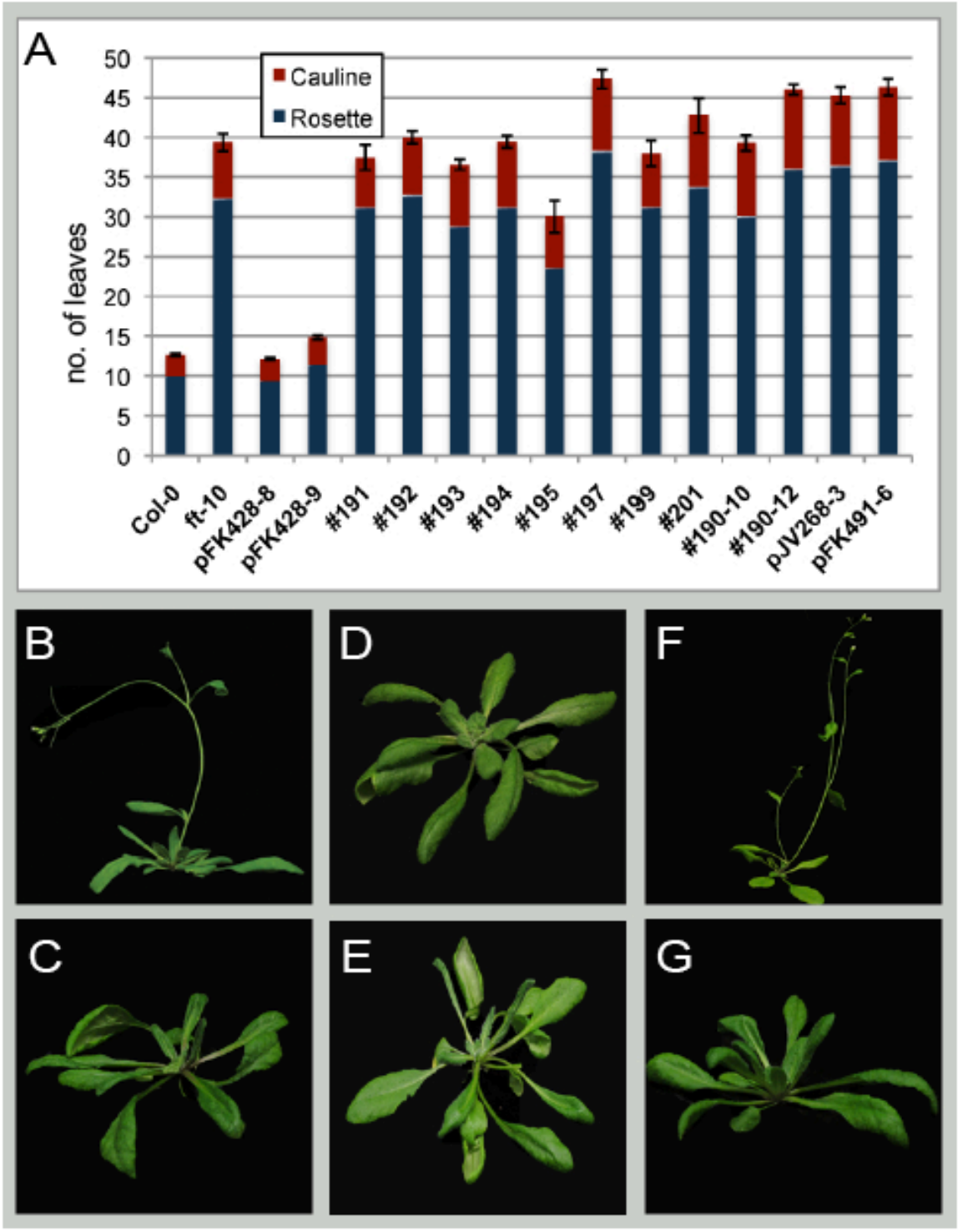


Fig 3-2: ***Flowering time data of gFT-TEVPcs and SUC2::TEVP:tdTomato.***
Plants carrying a TEV protease cleavage site in the FT protein, expressed in a genomic context were crossed with the TEV protease to see if the cleavage of protein was able to delay flowering. (A) pFK428-8 and pFK428-9 are two independent lines expressing the *gFT-TEVPcs* that flowered similar to wild-type with 12-15 total leaves respectively. #190-10, #190-12 and pJV268-3 represent three independent *SUC2*::*TEVP:tdTomato* lines. pFK491-6

represents a *SUC2::TEVP* line without the tdTomato tag. All 4 lines were late flowering, similar to or later than the *ft-10* mutant. #191 and #192 represent crosses of pFK428-8 with #190-10 and #190-12 respectively and flower with 37 and 40 total leaves respectively. #193 and #194 represent crosses of pFK428-9 with #190-10 and #190-12 respectively, and flower with 37 and 40 total leaves respectively. #195 and #197 represent crosses between pJV268-3 with pFK428-8 and pFK429-9 respectively and flowered with 30 and 47 total leaves respectively. #199 and #201 are crosses between pFK491-6 and pFK428-8 and pFK428-9 respectively and flowered with a total leaf number of 38 and 43. X-axis represents the different genotypes while the Y-axis represents the number of leaves. Rosette leaves are represented in blue and cauline leaves in red. Error bars represent 2xSEM. (B) A wild-type Col-0 plant flowering with 13 leaves, (C) an *ft-10* mutant plant that has not initiated flowering (F)an early flowering representative of the pFK428-8 parent (G) a late flowering representative of the #190-10 parental line. (E)representative of # 191 and (F) representative of #194, both plants have not yet initiated flowering due to the cleavage of the FT protein by the TEV protease, thereby delaying flowering.

To address the role of RNA movement in floral induction, the TEVP cleavage site (TEVPcs) flanked by Gly-Ser linker sequence was engineered into an exposed loop of the FT protein after Gly33. Functionality of the modified FT-TEVPcs protein was initially tested by misexpression of the open reading frame from either the *35S*, the *SUC2* or the *FD* promoter, each of which resulted in early flowering of transgenic plants (M. Schmid & V. Costa Galvao, pers. communication). Having demonstrated that the engineered version of the FT protein was functional, the TEVP cleavage site was introduced into the 13.8 kb genomic *FT* fragment.

Table 3-1: ***Flowering time data of the different crosses between SUC2::TEV protease expressing lines and gFT-FTcs or gFT-TEV-3xYFP lines.***

Genotype/ Cross	Total leaf number (Avg)	Range	2x SEM	number of representative plants
Col-0	12.7	12-15	0.2	10
ft-10	39.4	33-44	1.1	10
pFK428-8	12.2	11-14	0.2	20
pFK428-9	14.8	12-16	0.3	20
pFK429-27	35.6	34-41	0.8	10
pFK429-29	37.5	34-42	0.9	10
#190-10	39.3	35-44	1.0	10
#190-12	46	42-48	0.6	10
pJV268-3	45.3	40-52	1.0	10
pFK491-6	46.3	41-51	1.1	10
#191	37.4	32-44	1.6	9
#192	40	33-47	0.8	25
#193	36.6	35-41	0.7	15
#194	39.4	32-43	0.8	18
#195	30.1	25-38	2.0	22
#197	47.3	45-49	1.2	3
#199	38	34-41	1.6	4
#201	42.8	34-56	2.2	50
#203	14	13-16	0.3	10
#204	13	12-15	0.3	14
#205	16.9	16-19	0.3	11
#206	17.1	17-20	0.4	23
#207	17.8	17-19	0.5	4
#209	16.5	16-17	0.5	2
#211	14	14		1

Detailed information on the genotypes used for crosses can be found in Supplementary Table S3.

As expected, the *gFT-TEVPcs* construct was able to rescue the *ft-10* mutant and several transgenic lines that showed partial or complete rescue could be recovered, indicating that the *gFT-TEVPcs* transgene was fully functional (Table 3-1; Fig 3-2 A, F). These plants were then crossed to the established *SUC2::TEVP* and *SUC2*::*TEVP:tdTomato* lines and the progeny was phenotypically screened for TdTomato expression and genotyped for the presence of the *gFT-TEVPcs* constructs. The plants carrying both transgenes flowered with 40 +/- 2 (*SUC2::TEVP*) or 38 +/- 8 (*SUC2*::*TEVP:tdTomato*) leaves and were essentially indistinguishable from the TEVP expressing lines or *ft-10* itself (Fig 3-2 A, D, E). Our results suggest that

degradation of the TEVPcs-tagged FT protein in the phloem companion cells is sufficient to delay flowering.

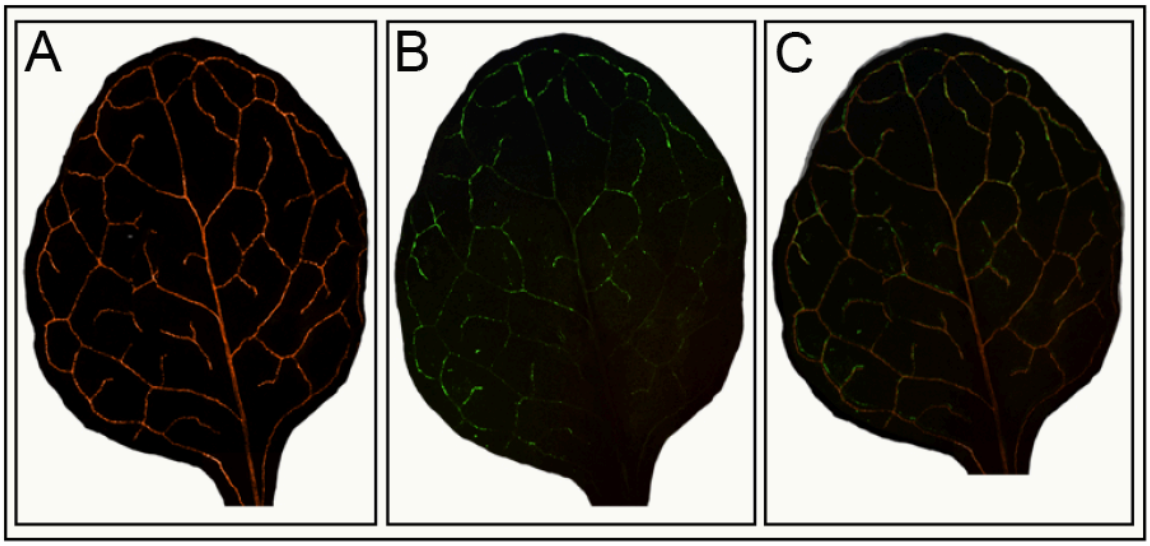


Fig 3-3: ***Expression of the TEV protease TdTomato and FT-3xYFP proteins in the leaf.***
A leaf from #204 expressing both *gFT-TEV-3xYFP* and *SUC2::TEV protease-TdTomato* as observed under a Epifluorescence microscope using RFP and YFP filters. (A) Expression of the *SUC2:: TEVprotease TdTomato* line in the leaf vasculature. The use of the *SUC2* promoter resulted in a stronger expression of the transgene in the vasculature of the leaf. (B) The expression profile of the FT-TEV-3xYFP driven by the native promoter in the vasculature of the leaf showing stronger expression in the peripheral veins than the main vein. (C) Overlap of (A) and (B) to indicate the regions in the leaf vasculature expressing both transgenes.

Taken together, the findings using the genomic *FT* rescue construct indicated that FT protein movement is both necessary and sufficient to induce flowering in A. thaliana and that previous reports to this end (Mathieu et al., 2007) were not caused by the *SUC2* promoter used in these studies. Indirectly our findings also support the idea that *FT* mRNA movement, should it occur at all, is not contributing to the induction of flowering in *A. thaliana*, as mRNA movement should not be affected by vascular-localized TEVP.

3.1.2 Analysis of TEV protease TdTomato expression at the apex.

There exists at least one alternative explanation that potentially explains the findings described above. In this hypothetical scenario, TEVP is able to move from the vasculature into the shoot apex where it would degrade gFT-TEVPcs protein newly synthesized from mRNA that has been transported into the shoot meristem from the vasculature. This scenario would also result in a delay in flowering of the plants expressing gFT-TEVPcs when crossed to either *SUC2::TEVP* or *SUC2*::*TEVP:tdTomato*.

To be able to rule out TEVP movement as a possible cause for the delay in flowering observed in gFT-TEVPcs *SUC2*::*TEVP:tdTomato* crosses we analysed the localization of the TEVP:tdTomato protein. To this end, the apical region of gFT-TEVPcs *SUC2*::*TEVP:tdTomato* plants was dissected and the apical tissue was visualised by confocal microscopy. TdTomato signal could be easily observed to the vasculature under (Fig 3-4 A-C, E-G, I-K) but could not be detected in the shoot meristem (Fig 3-4 D, H, L). These findings make it seem unlikely that a substantial fraction of the TEVP:tdTomato protein is transported into the shoot meristem. In addition, my observations confirm previous reports that the SUC2 promoter is specific to the phloem companion cells and is not active in the shoot meristem. Taken together these findings strengthen the idea that FT protein and nor mRNA act as a florigenic signal in *A. thaliana*.

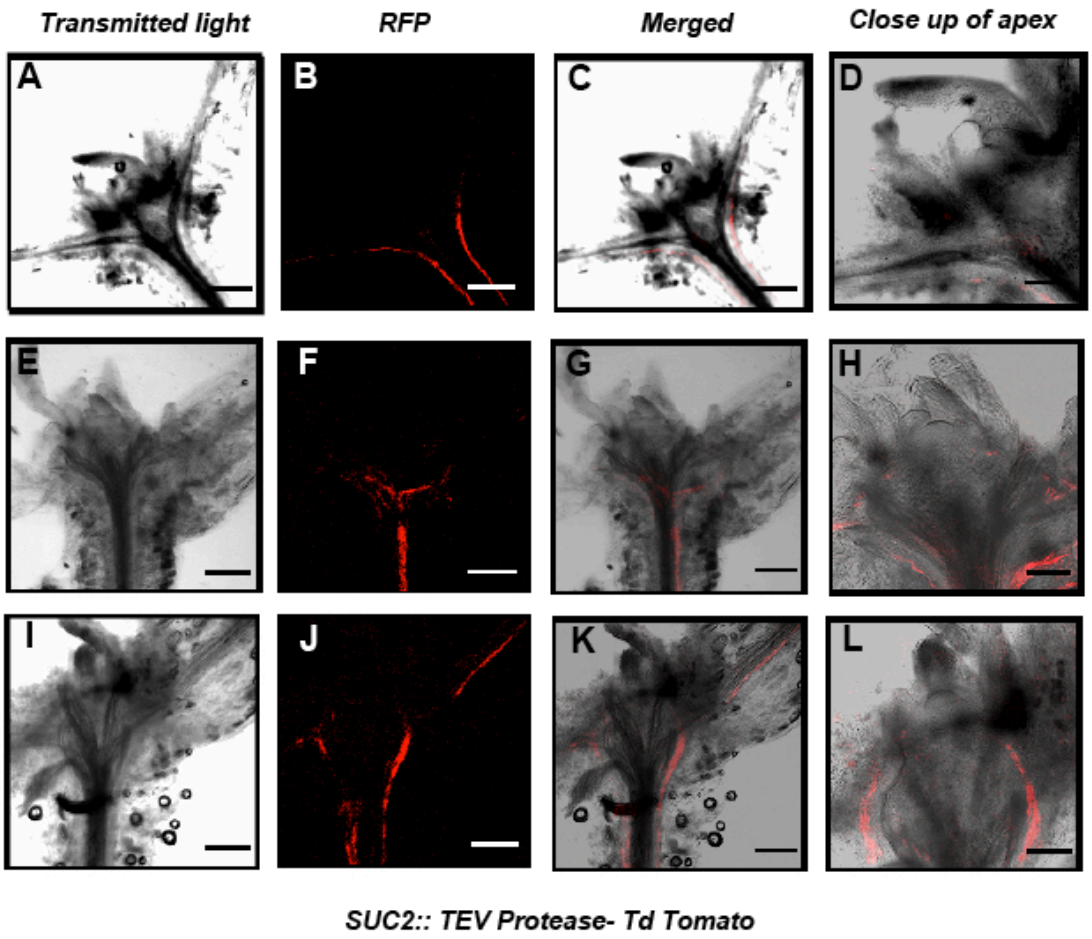


Fig 3-4: ***Analysis of the apex of the SUC2::TEV protease TdTomato plants.***
To test if the SUC2 promoter is active at the apex**,** different plants from #190-10 line were analysed by confocal microscopy. (A), (E) and (I) are three different meristems of the #190 line expressing the TEV protease Td Tomato construct in its vasculature as viewed under transmission light. (B), (F) and (J) represent the same meristems respectively under an RFP channel. (C), (G) and (K) are merged images of the transmission light and RFP channels to indicate that the expression of the TEV protease-TdTomato construct is restricted to the vasculature. (D), (H) and (L) represent a higher magnification of the apex where no expression of the transgene is seen in the apex, but is clearly seen in the vascular tissue. Scale bars in (A), (B), (C), (E), (F), (G), (I), (J), (K) represent 100µm while in (D), (H) and (L) represent 50µm.

3.2 The FT protein interacts with the bZIP transcription factor FD at the apex.

From the results presented above as well as published data it can be deduced that the FT protein functions as the florigen and moves from the leaf to the apex. As outlined above, FT is a small globular protein belonging to the PEBP family (Kardailsky et al., 1999; Kobayashi et al., 1999). Sequence analysis of FT and other similar proteins reveals no nuclear localization signal in the protein. Also, the absence of a DNA binding domain in the FT protein makes it an unlikely candidate to function as a transcription factor. Yet, the protein functions as a florigen that somehow activates a transcription factor cascade at the apex that ultimately result in the induction of flowers. The question arises as to what the molecular function of FT at the shoot apex might be?

Results from yeast-2-hybrid (Y2H) screens indicate that FT can physically interact with the bZIP transcription factor FD (Wigge et al., 2005; Abe et al., 2005). This interaction was further confirmed by BiFC (Abe et al., 2005). Recent studies indicate that FT and FD do not interact directly but that the interaction is instead mediated by 14-3-3 proteins (Pnueli et al., 2001; Purwestri et al., 2009; Taoka et al., 2011). Independently of the exact mechanism by which FT and FD interact, all evidence in support of this interaction originates from studies in heterologous systems such as yeast or from transient assays such as BiFC in tobacco leaves. Surprisingly, no direct evidence exists that the interaction of the two proteins actually takes place at the apex. To address this issue, I initiated an experiment to visualize the FT-FD interaction at the shoot apex of *A. thaliana.*

3.2.1 Generation of the GateWay-compatible vectors for BiFC

As a first step towards testing for interaction between FT and FD at the shoot apex I designed a series of GateWay-compatible destination vectors that would allow easy in frame cloning with split YFP fragments at either the N- or C-terminus of our gene of interest. N- and C-terminal fragments were cloned with or without Myc and HA-tags, respectively, to facilitate easy detection of the fusion proteins by western blot or immunoprecipitation (Fig 6-2).

Next I tested the functionality of my vectors by transient expression of pAS063 (*35S:: FD-nCitrine*) or pAS065 (*35S:: FD-myc-nCitrine*) with pAS067 (*35S:: FT-cCitrine*) or pAS069 (*35S::FT-HA-cCitrine*) in tobacco leaves. I found that, as previously described, FT and FD are able to interact in plant cells (Fig 3-5).

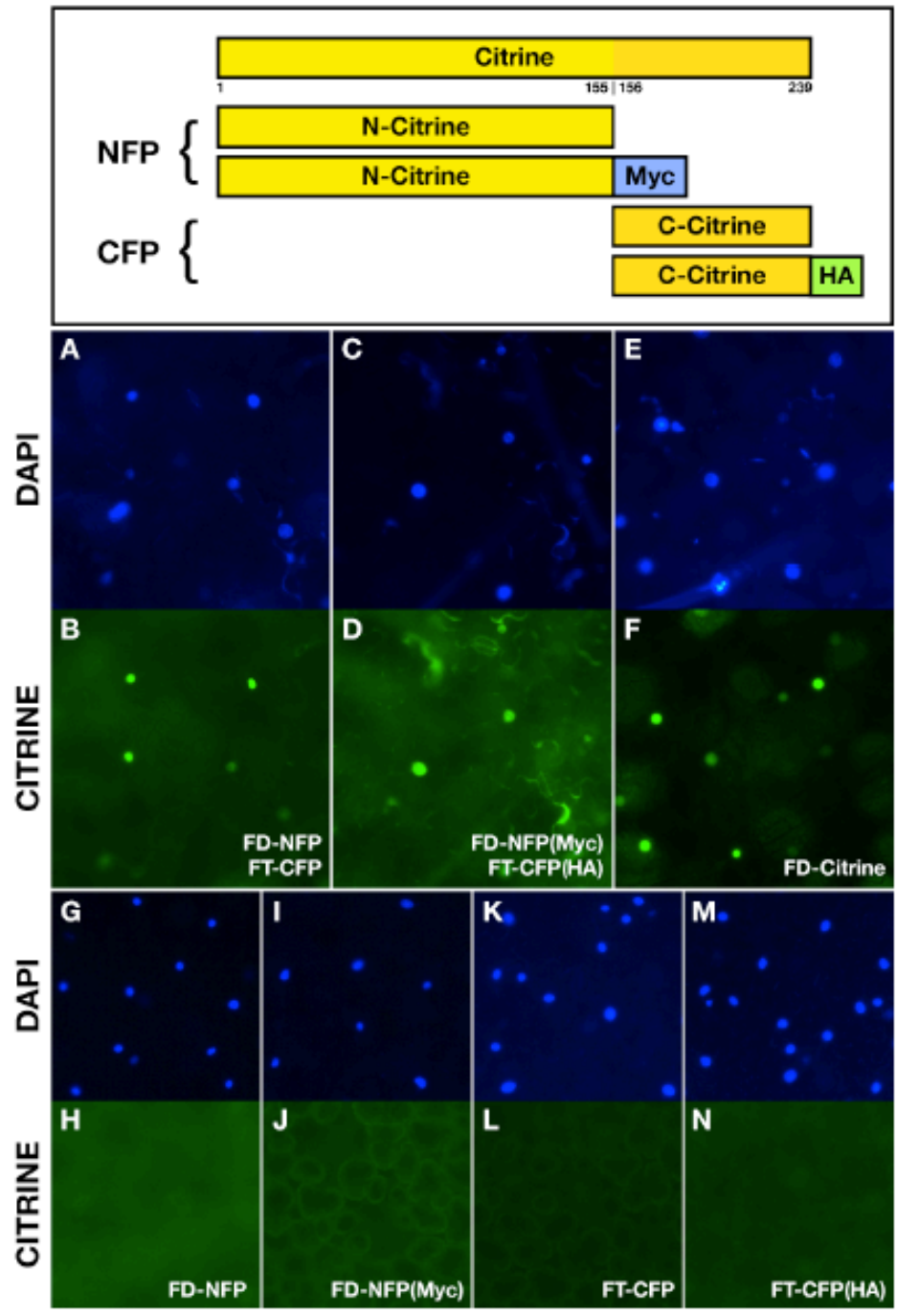


Figure 3-5: ***Interaction between FT and FD proteins in a transcient BiFC assay.***
Tobacco plants were injected with different constructs to study the interaction of FT and FD by BiFC. Panel 1 shows the schematic of splitting the YFP between amino acids 155 and 156 and tagged to FD and FT respectively. Myc and HA tags were added to enable protein immunoprecipitations. Nuclear localisation of the FT-FD complex without (B) or with tags (D), counterstained with DAPI (A) , (C). Nuclear localisation of FD-YFP constructs used as a positive control (C), (F). (H), (J), (L), (N) are single infiltrations indicating that fluorescence is observed only when both constructs are co-infiltrated. DAPI staining outlines nuclei.

Interaction between the two proteins takes place mostly in the nucleus, which is not surprising given that FD is a bZIP transcription factor with a nuclear localization signal. However, I noted that the localization within the nucleus differed depending on whether the tag had been fused to the N- or C-terminal of FD. While N-terminal fusion of FD with YFP resulted in an even labelling of the nucleus, fluorescence was found in speckles in the nucleus in FD-YFP fusion proteins. Similar results were obtained when FD was fused with full-length YFP (Fig 3-6).

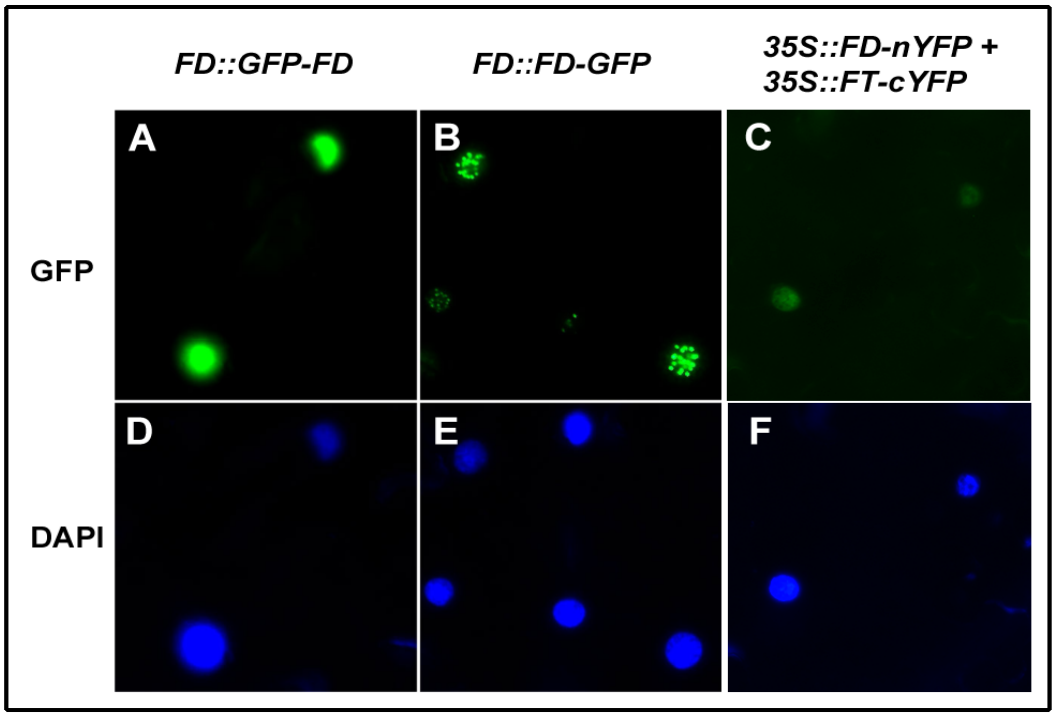


Figure 3-6: ***Localisation of FD-GFP, GFP-FD proteins and FD-nYFP+FT-cYFP complex in tobacco leaves.*** Leaves were infiltrated with the corresponding consructs and the localisation of the protein was studied after 3 days. The nuclei were counter stained with DAPI (D), (E), (F). It was observed that C terminal fusions of FD localised in nuclear speckles (B), (C), while N- terminal fusions seemed to be spread uniformly in the nucleus (A). The complex of FD-nYFP with FT-cYFP (A) was much weaker compared to the full length FD protein fusions (A), (B) since the number of proteins interacting to result in fluorescence would be lesser than proteins expressing the YFP tag from a *35S* promoter.

In this context it should be noted that only a YFP-FD fusion protein is able to rescue the *fd* mutant when expressed from the *FD* promoter, whereas FD-YFP was clearly non-functional (L. Yant; pers. communication). Possibly the nuclear speckles

represent sites of protein degradation as has been suggested for Hy5 by COP1 (Osterlund et al., 2000; Wang et al., 2001).

3.2.2 Testing for FT-FD interaction at the *A. thaliana* shoot apex.

To study the interaction of the FT and FD at the shoot apex, it is essential to maintain the endogenous expression patterns of the proteins whenever possible. Hence the nFP-FD fusion protein was expressed from the *FD* promoter. The FT protein was, however, expressed from the *SUC2* promoter to maximize the number of cFP-FT molecules that would reach the apex to interact with nFP-FD and hopefully provide a fluorescence signal strong enough to be picked up by confocal microscopy. In addition, the two proteins were tagged with a myc and a HA-tag respectively to facilitate pulldowns if an interaction was observed.

To further increase the probability of interaction between the tagged proteins, the *SUC2::cFP-FT* and *FD::nFP-FD* constructs were transformed into the *ft-10 fd-3* double mutant background, which lacks endogenous FT and FD proteins. Since the two constructs carried different resistance markers (Kan and Basta, respectively), the double transgenic plants were selected on plates containing both Kan and Basta. In addition, plants were genotyped by PCR to confirm the presence of both transgenes. From the T1 plants containing both transgenes, only those that flowered early and produced seven or fewer rosette leaves were maintained. It should be noted that even the earliest double transgenic lines flowered later than Col-0 plants carrying an untagged *SUC2::FT* transgene. One possible explanation for this is that even the small, 9.5 KDa C-terminal fragment of YFP might already hinder FT protein movement. Alternatively, single copies of the FT and/or FD transgenes might be insufficient to fully rescue the late flowering of the *ft-10 fd-3* double mutant background. In agreement with the former hypothesis, preliminary examination of vegetative meristems of double transgenic T2 individuals by confocal microscopy failed to detect a specific YFP signal (not shown). However, these preliminary studies need to be repeated in the future once double homozygous transgenic lines have been established.

3.3 Role of FD in orchestration of flowering at the shoot apex.

It is now widely accepted that the FT protein moves from the leaf to the shoot apex where it supposedly interacts with the bZIP transcription factor FD (Amasino, 2010; Kobayashi and Weigel, 2007; Turck et al., 2008).

FD is a bZIP transcription factor that can form homodimers and also heterodimerises with other bZIP transcription factors. The exact dimerisation properties of FD are still unclear (Jakoby et al., 2002). In contrast to FD, which is a bona fide transcription factor, there is so far no evidence that FT itself can bind DNA. However, FT has been shown in a candidate gene approach to weakly associate with the promoter region of *APETALA1 (AP1)*, presumably through its interaction with FD (Wigge et al., 2005).

The binding of FD to the *AP1* promoter has recently been confirmed by ChIP-seq using YFP-FD expressed from the *SUC2* promoter (L. Yant; pers. communication). This study also identified other well characterized flowering time and flower meristem genes such as *FRUITFUL* (*FUL*), *SEPALLATA2* (*SEP2*) and *SEP3* as direct FD targets. However, it is still unclear whether binding of FD to the promoters of these genes does indeed affect their transcription.

To address the question of which of the potential FD targets are transcriptionally regulated by FD, I followed a twofold approach. First I performed a transcriptome analysis of FD gain-of-function lines to identify genes that were differentially expressed in these lines. The drawback of this approach is the individual genotypes flower at different times. As a consequence, most of the differences observed in steady state gene expression are bound to be due to differences of the developmental stage the plants are in. To overcome these limitations, we have in a second step established chemically inducible lines that expressed a translational fusion between the ligand-binding domain of the rat glucocorticoid receptor and FD.

These lines were then used in transcriptome-wide analyses to identify genes that rapidly responded to acute changes in FD availability.

3.3.1 Identification of potential FD targets by expression analysis

To identify genes that are potentially regulated by FT-FD, we performed a microarray analysis of Col-0 and *35S::FD* plants grown under non inductive short day photoperiod (SD; 8h day) for 28 days and shifted to long days (LD; 16h day). This shift facilitated the expression of *FT*, which is required along with FD for activation of its downstream targets. Leaf tissue was collected at the end of every long day for 3 days. RNA was prepared from all the samples and a genome-wide expression analysis was performed using Affymetrix ATH1 expression arrays for samples collected on days 1, 2 and 3 after the shift to LD. The data was normalized using gcRMA implemented in 'R' and genes that were differentially expressed with a percentage false positive (pfp) rate < 0.05 were identified using the RankProducts package, also implemented in 'R' (Breitling et al., 2004). In total, 32 genes were found to be differentially expressed between Col-0 and *35S::FD* two or three days after the shift from non-inductive SD to LD. Out of these, 17 genes were upregulated on both days, whereas 3 and 12 genes were upregulated only on day two and three after the shift, respectively (Fig 3-7).

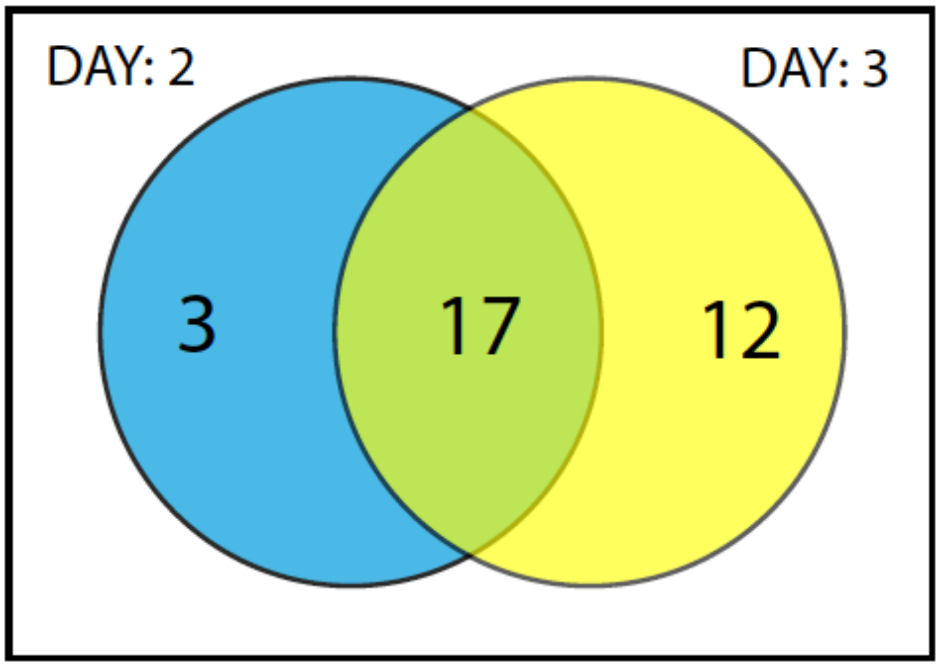


Figure 3-7: ***Venn diagram indicating the number of genes upregulated on days 2 and 3.*** Plants were grown for 28 days in short days and shifted to long days. Plant tissue was collected at the end of every long day. Genes upregulated on days 2 and 3 were analysed by microarray between the *35S::FD* and Col-0 plants. Between days 2 and 3, 17 genes were upregulated on both days, while only 3 genes were upregulated on day 2 and 12 on day 3 exclusively.

The list of 17 genes upregulated by FD in response to inductive photoperiod includes *FRUITFULL, SEPALLATA 3, SEPALLATA 1* and *AGAMOUS*, all of which are known regulators of flowering time and/or flower development (Table 3-2, Fig 3-8 A). *SEP3* and *SEP1* belong to the class E transcription factors that are expressed in all 4 whorls of the flowers. *AG* is a CLASS C gene, expressed in the inner two whorls of the flower, and is essential for the formation of the androecium and gynoecium of the flower. *FUL* is essential for the formation and maturation of the silique and breaking open of the valves for seed dispersal. Interestingly, the *35S::FD* lines, like the *35S::FUL* lines, produced indehiscent fruit and failed to disperse seeds normally (Ferrandiz et al., 2000). Apart from these genes, other genes that were upregulated belonged to the Cytochrome P450 family, metabolism related proteins such as UDP-

Glucose 4-epimerase, UDP-Galactose 4-epimerase, phospholipid/glycerol acyl transferase family protein etc (Table 3-2). The upregulation of these proteins can be explained by the fact that the transition to flowering is a highly energy demanding process and several metabolic reactions are required to supply the sufficient energy required. Another interesting gene upregulated was a seed storage gene belonging to the Lipid Transfer Family (Table 3-2).

Table 3-2: ***Genes upregulated in the 35S:: FD overexpression lines compared to Col-0.***

AGI code	Affymetrix ID	Gene Annotation
At3g14990	[257216_at]	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein, putative
At1g02205	[264146_at]	CER1 protein
At4g35900	[253108_at]	DNA-binding protein-related
At1g24260	[264872_at]	MADS-box protein (AGL9)
At4g10960	[254952_at]	UDP-glucose 4-epimerase, putative / UDP-galactose 4-epimerase, putative
At5g60910	[247553_at]	agamous-like MADS box protein AGL8 / FRUITFULL (AGL8)
At3g51860	[246302_at]	cation exchanger, putative (CAX3)
At3g20100	[257129_at]	cytochrome P450 family protein
At3g03470	[259058_at]	cytochrome P450, putative
At5g15800	[246531_at]	developmental protein SEPALLATA1 / floral homeotic protein (AGL2) (SEP1)
At2g20700	[265430_at]	expressed protein
At4g18960	[254595_at]	floral homeotic protein AGAMOUS (AG)
At1g43590	[262719_at]	hypothetical protein
At1g06520	[262630_at]	phospholipid/glycerol acyltransferase family protein
At1g48750	[256145_at]	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
At1g18830	[261430_at]	transducin family protein / WD-40 repeat family protein
At1g65450	[264160_at]	transferase family protein

Genes that were upregulated in the *35S::FD* lines compared to Col-0 on days 2 and 3 as analysed by gene expression arrays. The columns represent the AGI number, Affimetrix ID and the Gene Annotation as indicated by TAIR 9.0. The *FD* gene which is upregulated in the over-expressor compared to Col-0 is highlighted in dark grey, the light grey bars highlight a list of candidate genes of interest namely *SEP1, SEP3, AG* and *FUL.*

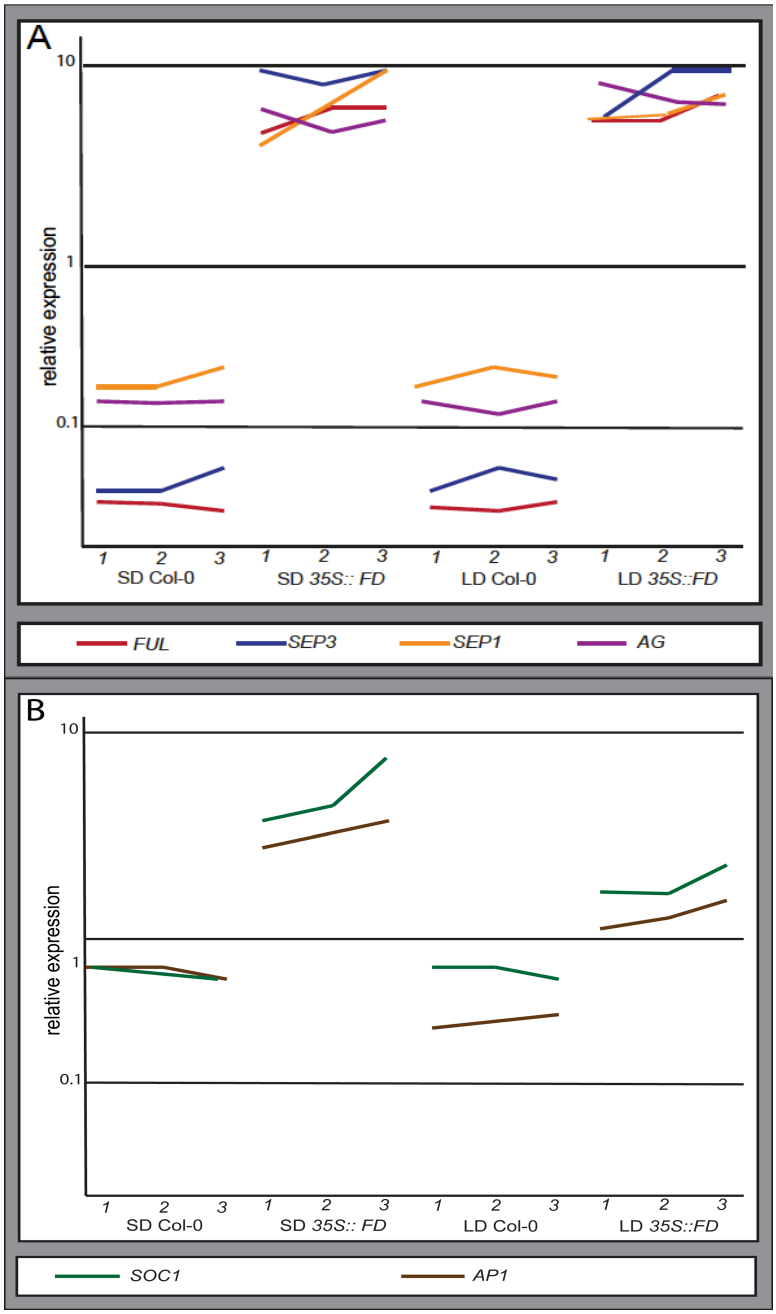


Figure 3-8: ***Expression profiles of some of the interesting candidates from the microarray assay.*** Microarray analysis of the *35S::FD* plants against Col-0 to identify genes upregulated in the overexpression lines resulted in 4 genes that have a significance in flowering (A) namely *FUL, SEP3, SEP1* and *AG.* While *SEP3* and *FUL* showed a 100 fold upregulation, SEP1 and AG showed a 10 fold upregulation. No significant difference was seen between the short day and long day samples. (B) Expression profiles of *AP1* and *SOC1,* that showed a significant upregulation in SD but not LD. X axis shows days 1, 2 and 3 of different photoperiod (short day or long day) for the Col-0 and *35S::FD* samples. Y axis shows relative expression on a logarithmic scale (A) and linear scale (B).

On observation of individual expression profiles of the four genes related to flowering and floral organ formation that were upregulated by FD, it was interesting to note that while *AG, SEP1* were 10 fold upregulated, *SEP3 and FUL* were 100 fold upregulated by FD compared to Col-0 (Fig 3-8 A). Another interesting point was that there was not much difference between the expression profiles of the different genes between short days and long days. Two other genes, namely the known target of FD, *AP1* and the floral integrator *SOC1* were also upregulated by FD (Fig 3-8 B). These genes, however, were not upregulated enough to pass the stringent statistical filters (RankProducts, pfp <0.05) to be considered significantly upregulated. However, it is clear from the expression profiles that both genes show at least a 3-5 fold upregulation in the FD overexpression lines compared to Col-0 (Fig 3-8 B).

However, it should be noted that genes such as AG are expressed in the center of the emerging flower, where FD is not normally expressed. This suggests that at least some of the potential FD targets I have identified by steady-state transcriptome analysis are most likely indirect targets rather than directly regulated by FD.

3.3.2 Differentiating between direct and indirect targets of FD.

Since several genes upregulated in the FD overexpression lines such as *SEP1, SEP3, FUL* and *AG* are transcription factors, it is essential to differentiate the direct targets from the indirect targets i.e. targets of the direct targets. To this end I prepared N-terminal fusions of FD with the rat glucocorticoid receptor, as previous experiments had been shown that C-terminal YFP fusions were unable to rescue the *fd* mutant (L. Yant; pers. communication) and were localized in nuclear speckles (Fig 3-6).

To generate the N-terminal fusion vectors of GR, the ligand binding domain of the rat *GR* gene was amplified by PCR and cloned into the pGREEN-IIS plant binary vector in front of the GateWay rfA* recombination cassette to facilitate easy cloning

by recombination. (Fig 6-1). In addition, vectors were also prepared for the C-terminal tagging of proteins with GR (Fig 6-1).

After transformation of Col-0 with the *35S::GR:FD* construct, 30 transgenic lines were recovered. As constitutive overexpression of *FD* usually results in mild early flowering, I only took into T2 generation those *35S::GR:FD* lines that were phenotypically indistinguishable from wild-type. This ensured that only those lines, in which the GR-FD fusion protein was efficiently trapped in the cytoplasm in the absence of dexamethasone were considered for further experiments. Finally, I performed quantitative RT-PCR on the *GR-FD* transgene to select lines that displayed strong and stable expression of the transgene (Fig 3-9). In the end, progeny of one line, T2-10, was selected to determine the acute responses of the transcriptome to FD using Affymetrix ATH1 microarrays.

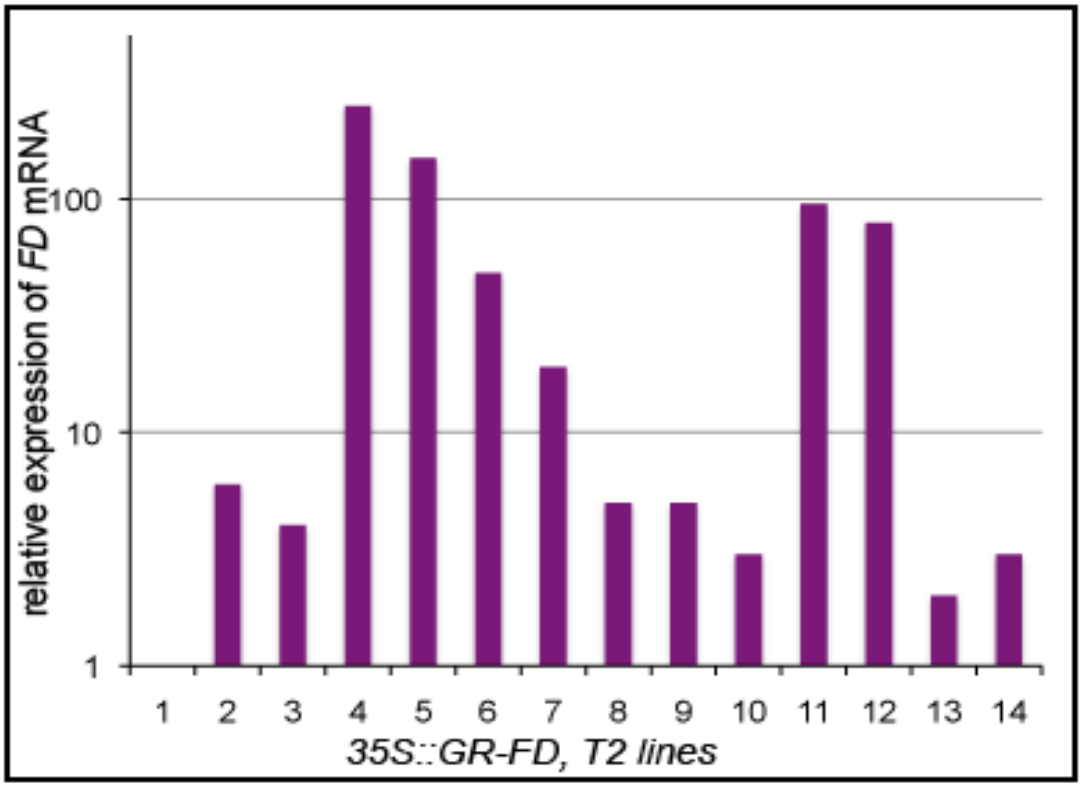


Figure 3-9: ***Expression analysis of FD mRNA in the leaves of 35S::GR-FD plants.***
Leaf tissue was harvested from plants grown in LD for 12 days and the quantity of *FD* mRNA was measured using qPCR. Some lines showed more than a 100 fold increase of *FD* mRNA in the leaves (lines T-4, T-5) while some lines showed no expression of *FD* mRNA (T-1). A line that expressed a moderate level of *FD* mRNA (T-10) in the leaves was chosen for further studies. Scale on Y axis represents relative expression of *FD* mRNA (∂cT_{FD} - $\partial cT_{TUBULIN}$).

For this purpose, plants were either treated with dexamethasone, dexamethasone with cycloheximide, cycloheximide only or a mock treatment with all reagents except dexamethasone and cycloheximide. To minimize the deleterious effects of the protein synthesis inhibitor cycloheximide on plants, short incubation times were chosen. I found that in *35S::GR:FD* (T2-10) upon treatment with 15μM dexamethasone or dexamethasone plus 10μM cycloheximide for 1 hr, expression of genes such as *SEP1, SEP3* and *FUL* was induced less than 3-fold when compared to the controls (Fig 3-10A).

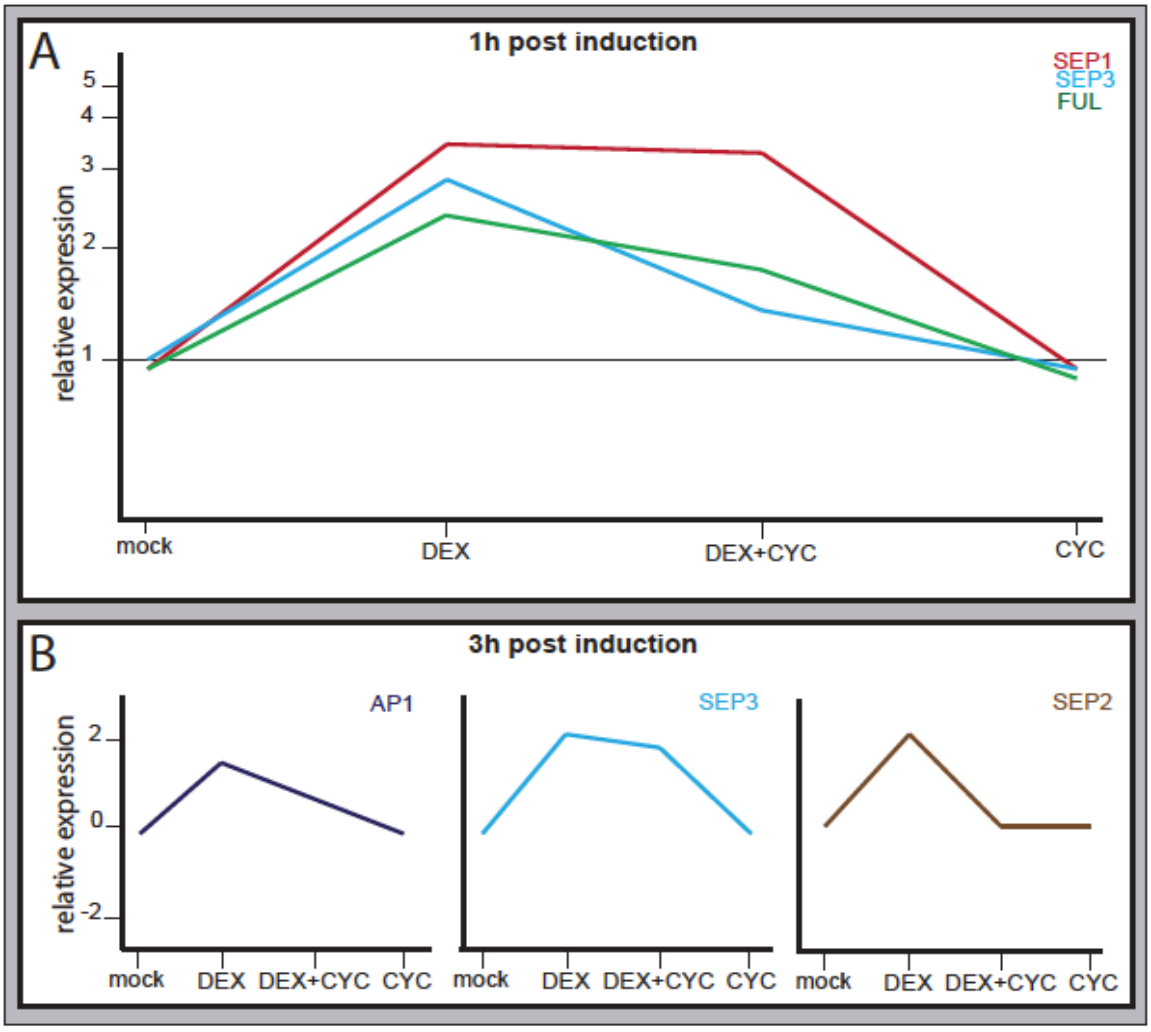


Figure 3-10: ***Expression profiles of some candidate genes on induction of the 35S::GR-FD lines.*** The T-10 plants carrying the *35S::GR-FD* trangene were treated with dexamethasone (DEX), dexamethasone and cycloheximide (DEX+CYC), cycloheximide only (CYC) or a mock treatment (mock) carrying none of the above mentioned chemicals. Leaf samples were collected 1h post induction and the relative expression of target genes induced by the movement of FD protein into the nucleus were analysed by expression arrays. (A) Expression profiles of three candidate genes *SEP1, SEP3, FUL* are shown. All three genes seem to be upregulated on treatment with dexamethasone in the presence or absence of cycloheximide, indicating that they are likely candidates to be direct targets of FD. (B) Expression profiles of *AP1, SEP3, SEP2* after 3h of induction. No striking increase is seen in the expression of any of the candidate genes. *SEP2* however, shows an increase with Dexamethasone treatment but not when cycloheximide is added with dexamethasone, indicating it is an indirect target of FD.

To test if longer exposure to dexamethasone or dexamethasone + cycloheximide had a stronger inductive effect, microarray analysis was also performed on samples

collected 3h after the treatment. Individual profiles of candidates of interest such as *AP1*, an already established target of FD, *SEP3*, a candidate from the previous overexpression screen, and *SEP2,* a candidate that has been shown to be downregulated in the apex of *fd-2* compared to Col-0 (L. Yant; pers. communication) were analyzed (Fig 3-10 B, C, D). None of these genes showed a stronger expression than had been observed after 1h of induction. In contrast, stress related genes displayed extremely high levels of expression in these samples. Induction of these genes was mostly likely the consequence of a combination of factors: the fact that the assays were performed in detached leaves submerged in the chemicals and the effect of the protein synthesis inhibitor cycloheximide present in at least some of the samples. In addition, attempts to improve the induction of the potential target genes by a) increasing the concentration of dexamethasone from 15µM to 30µM and 60µM actually resulted in a decrease of induction. In contrast, increasing the induction time to 6h resulted in enhanced expression of candidate genes in response to dexamethasone treatment, however, the samples treated for 6 hr with cycloheximide suffered from strong RNA degradation and did not perform well. In summary, because of these confounding factors it was not possible to derive a statistically significant list of genes differentially regulated by FD in response to the addition of dexamethasone or dexamethasone and cycloheximide.

3.3.3 FT cooperatively enhances the transcriptional activity of FD

The weak response of confirmed FD targets such as AP1 in *35S::GR:FD* plants to dexamethasone treatment was at first puzzling. However, it should be noted that at the shoot apex, FD is thought to interact with FT to form a transcriptionally active complex (see above). I therefore hypothesized that possibly FT, which is expressed predominantly in the phloem companion cells of the minor veins of leaves, might be limiting in the *35S::GR:FD* plant and prevent a stronger response.

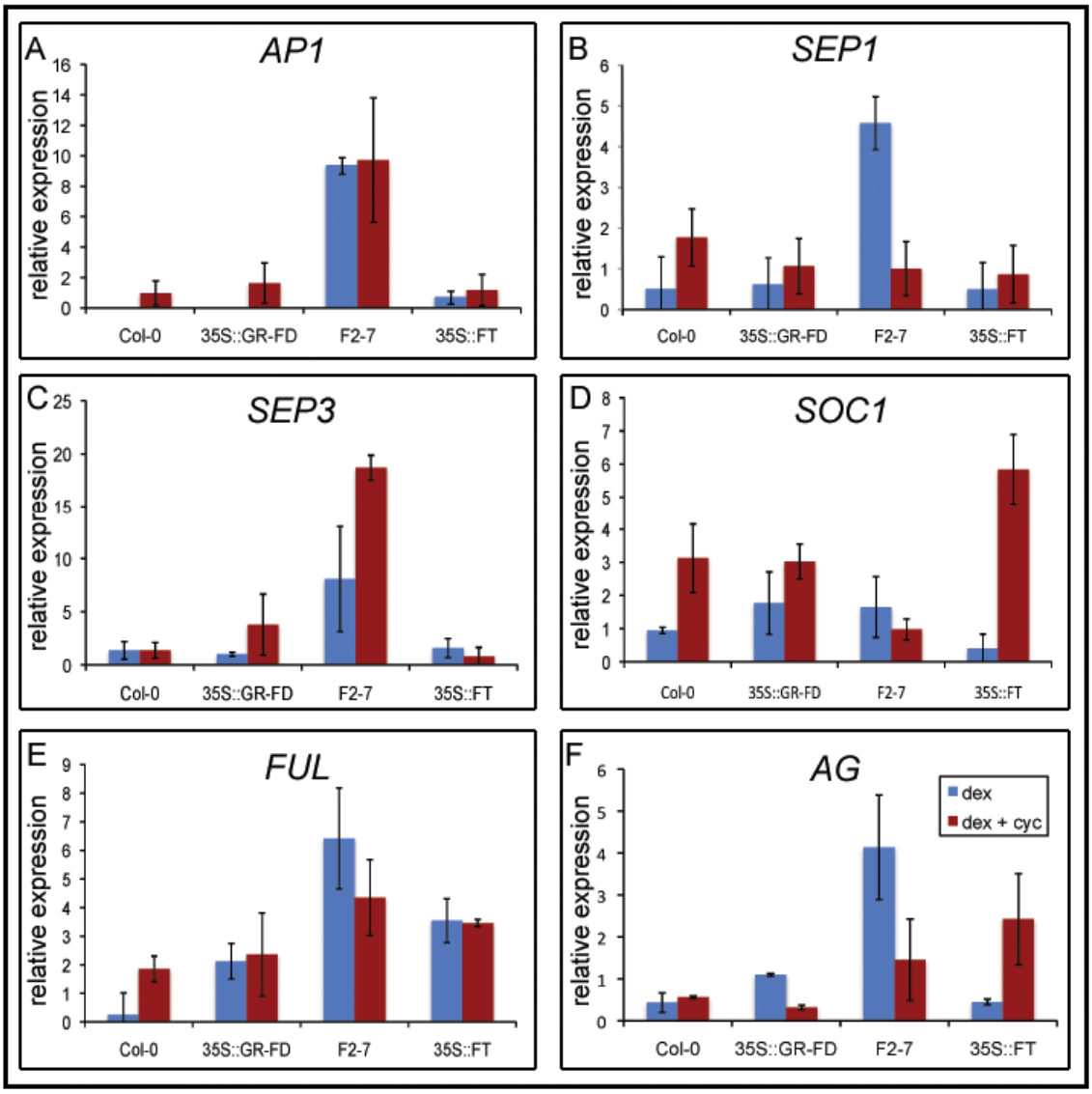


Figure 3-11: ***RNA expression profiles of some candidate genes in the 35S::GR-FDx35S::FT plants compared to parental lines.*** The *35S::GR-FD* T-10 line was crossed to an established *35S::FT* line (JM037) to yield the cross F2-7. qPCRs were performed on 12 day old plants treated with dexamethasone with or without cycloheximide. All samples were normalized to Tubulin. The dexamethasone samples were compared to mock treated plants and dexamethasone+cycloheximide samples to plants treated with cycloheximide only. Expression of the candidate genes in the parental lines ie *35S::GR-FD* and *35S::FT* and the wild-type Col-0 were compared to the cross F2-7. Expression data of (A) *AP1,* (B) *SEP1,* (C) *SEP3,* (D) *SOC1,* (E) *FUL,* and (F) *AG* were compared. Due to a strong induction seen in both dexamethasone and dexamethasone + cycloheximide treated samples, *AP1, SEP3, FUL* were considered direct targets, while *SEP1, AG* were considered indirect targets of FD. Since *SOC1* did not show a nice induction in the F2-7 lines, it is not considered a target of FD. Scale on Y axis represents relative expression of target mRNA (∂cT_{TARGET} - $\partial cT_{TUBULIN}$).

To overcome this possible limitation I crossed the *35S::GR-FD* transgene into an established *FT* overexpression (*35S::FT*) line. The idea behind this experiment was that in such a double-overexpressing background, none of the two proteins (GR:FD and FT) would be limiting as they would both be expressed in the entire leaf and the transcriptional responses to the chemical activation of GR-FD would thus be maximized.

In an initial experiment, qPCR was performed for specific potential targets of FD in the F2 progeny of the *35S::GR-FD 35S::FT* cross (line F2-7) after treatment with dexamethasone in the presence and absence of cycloheximide. Results showed that *AP1, SEP3* and *FUL* were induced in plants treated with dexamethasone and dexamethasone + cycloheximide indicating that these genes are direct targets of FD (Fig 3-11 A, C, E). In contrast, *AG* and *SEP1* showed an induction with dexamethasone only but not in the presence of cycloheximide, indicating that they are indirect targets of FD (Fig 3-11 D, F). *SOC1*, another important integrator of flowering time signals, did not respond to either of the treatments, indicating, that this gene is not regulated by FD.

Comparison of the expression data with results from FD ChIP-Seq experiments generated by L. Yant (pers. communication) indicated that some of the genes induced by the *GR-FD* plants upon treatment with dexamethasone + cycloheximide were also bound by the FD protein. In particular, regions of the *SEP3* and *AP1* promoters had been shown to be strongly bound by FD (L. Yant, pers. communication). In summary, these data suggest that FT and FD act together to activate their target genes. It should be noted that at least for some genes such as AP1, the effect of a combination of the two proteins (GR-FD and FT) substantially exceeded the effect of the individual genes, indicating that the two proteins act synergistically.

3.3.4 Transcriptome-wide identification of FD targets by RNA-seq

To test the effect of FT-FD complex on the entire transcriptome, the plants treated with mock, dexamethasone, were used for preparing RNA libraries. Samples that had been treated with the respective chemicals for 3h were used for analysis. More than 900 genes were differentially regulated in plants treated with dexamethasone compared to mock treated plants. These genes included several interesting candidates such as *AP1, SEP3, SEP1, AG, FUL, SEP2, PI*, that have been known to be involved in flowering. However, no difference was seen in the expression of the integrator gene *SOC1,* or the repressor of flowering *TFL1. SOC1* had been shown to be differentially regulated when plants overexpressing FD in leaves were compared to Col-0 (Fig 3-8 B). Since *TFL1* has been shown to be a repressor of flowering, it was interesting to learn whether the FT-FD module regulate *TFL1* at a transcriptional level. However, it seems unlikely that the interaction between FT-FD and TFL1 is by downregulation of the repressor.

Several genes involved in petal differentiation and expansion such as *MLP-LIKE PROTEIN 168 (MLP168)* were also upregulated in the presence of FD. Other genes such as *VANGUARD 1 (VGD1), VGDH1* that are predominantly expressed in male gametophyte, pollen, pollen tube cells were also shown to be upregulated by FD. These however were speculated to be indirect targets of FD. Several metabolic genes were also upregulated by FD such as *SLR1*, which plays a role in sugar metabolism, and *PGA4*, which has polygalacturonase activity (Hanada et al., 2011; Torki et al., 2000). Genes that are involved in lipid binding such as *LIPID TRANSFER PROTEIN 6 (LTP6)* and lipid storage such as *GLYCINE RICH PROTEIN 17 (GRP17)* were also upregulated in the dexamethasone treated plants. Other upregulated genes were involved in redox reactions, copper binding, cell wall differentiation, cell wall modification etc. Of the approx. 400 genes that were upregulated in the dexamethasone samples, compared to mock treated samples, 25 genes were

selected based on their relevance in flowering, and metabolism. qRT-PCR experiments were performed on plants treated with Dexamethasone or Dexamethosone + cycloheximide to identify the direct targets and differentiate them from the indirect targets.

In order to identify the direct targets, plants that were treated with dexamethasone and cycloheximide were used to compare with plants treated with dexamethasone alone. Of the 25 genes selected, several genes such as *AP1, FUL, SEP3* (Fig 3-11 A, C, E) and *VGD1, VGDH1, POLYGALACTURONASE 4 (PGA4), PROTODERMAL FACTOR 1 (PDF1)* (Fig 3-12 A) were induced in both the dexamethasone and the dexamethasone+ cycloheximide samples, indicating that these are directly regulated by FD. Several other genes such as *AG, SEP1, PI, LTP6* (Fig 3-11 B,F; Fig 3-12 B) were induced well in the dexamethasone samples, but not the dexamethasone + cycloheximide samples, indicating that they are not direct targets of FD.

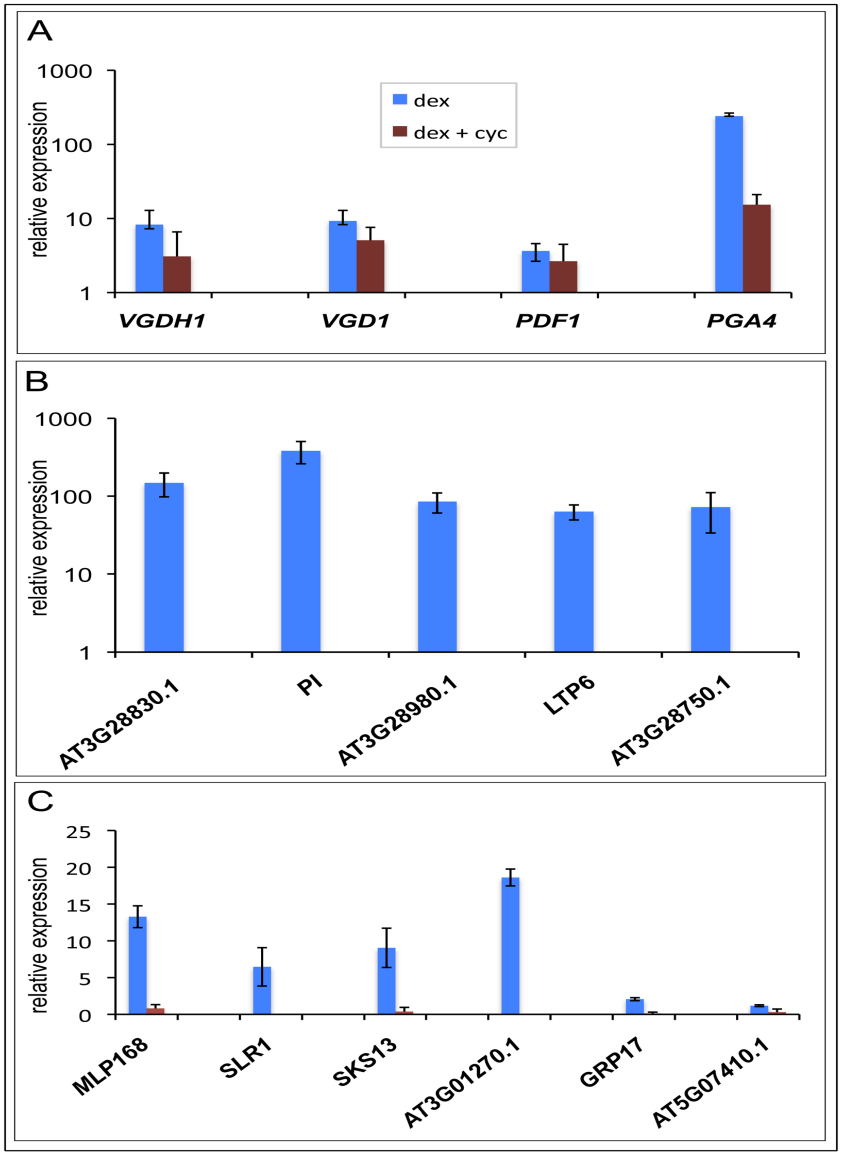


Figure 3-12: ***Expression profiles of genes that were induced in the transcriptome library analysis.*** Several genes were upregulated in the transcriptome library comparison between dexamethasone and mock treated plants. qRT-PCR was performed on samples treated with dexamethasone or dexamethasone+cycloheximide. All samples were normalized to tubulin. (A) Genes that were induced on treatment with dexamethasone in the presence or absence of cycloheximide, most likely direct targets of FD. (B) Genes that were induced nicely with dexamethasone but not on treatment with cycloheximide, most likely indirect targets of FD. (C) Genes that were not strongly induced by dexamethasone and not induced at all on treatment with cycloheximide along with dexamethasone, maybe weak indirect targets. X-axis represents the genes tested, Y-axis represents the relative expression of the mRNA on a logarithmic scale (A, B) or a linear scale (C) normalised to Tubulin. Blue columns represent dexamethasone treated samples, red columns indicate dexamethasone + cycloheximide treated samples.

Further, transcriptome libraries were prepared using the dexamethasone + cycloheximide treated plants with plants treated with cycloheximide alone as controls. These plants did not perform well on cycloheximide treatment, and were extremely stressed. Most of the genes that were induced in these plants compared to the dexamethasone and mock treated plants were mainly stress factors. None of the interesting candidates that were identified by qPCR were induced in the dexamethasone + cycloheximide samples with statistical significance. Hence this dataset generated was not used for drawing conclusions about the direct and indirect targets of FD.

3.3.5 *In vitro* confirmation of *SEP3* as a the direct target of FD

Among the genes identified as potential direct targets of FD, *SEP3* is of particular interest as this transcription factor has been shown to hetero-dimerize with many other MADS-domain proteins and potentially regulates different aspects of flowering time and flower development. *SEP3* had been identified as a FD target in both my GR-FD experiments as well as in the ChIP-Seq analysis carried out by Levi Yant (pers. communication). Furthermore, *de novo* Gibbs sampling of the promoter regions identified by ChIP-seq had identified a conserved G-box element (*CACGTG*) as the preferred binding site of FD (L. Yant; pers. communication).

Interestingly, the region bound by FD in the *SEP3* promoter, approx. 800bp from the transcription start, peaked over a G-box. To verify that FD indeed binds to the *SEP3* promoter, electro-mobility shift assays (EMSA) using His-tagged FD protein expressed in *E. coli* were performed. In a first experiment, I amplified a 200 bp region centred around the G-box of the *SEP3* promoter. A random 200bp fragment of DNA, which did not contain a G-box, was used as a negative control. As expected, a shift was seen when FD protein was added to the G-box containing DNA fragment but not the negative control (Fig 3-13A).

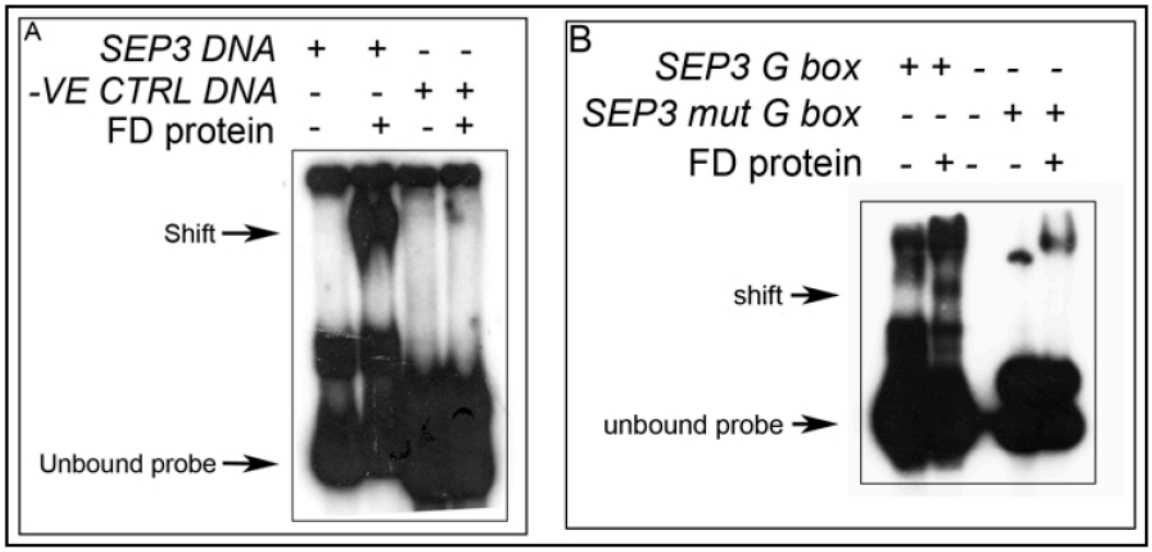


Figure 3-13: ***Electrophoretic mobility shift assay of FD with SEP3 promoter sequence containing G box.*** The DNA binding ability of FD protein was tested in vitro using EMSA. (A) The FD protein was able to specifically bind to a 200bp long *SEP3* promoter fragment harbouring a G-box sequence in it. FD was however unable to shift a random fragment of DNA represented here as the –ve ctrl DNA. (B) To verify if the FD protein specifically bound to the G-box on the *SEP3* promoter, the G-box sequence was mutated to CTCGAG. Binding was abolished in the presence of the mutated G box while a shift was seen when the native G-box was retained.

To verify that the binding of FD to the *SEP3* promoter fragment was indeed due to an interaction with the G-box, the EMSA analysis was repeated using DNA fragments in which two bases had been changed in the core of the G-box from *CACGTG* to *CTCGAG*. This type of mutation has been shown to abolish the G-box function and transcription factors that recognise G-boxes no longer bind to this sequence (Izawa et al., 1993). In agreement with FD binding to G-boxes, I found that the mutated G-box was no longer bound by FD. In contrast, a DNA oligomer representing the wild-type G-box present in the SEP3 promoter was readily bound by FD (Fig 3-13B). In summary my results indicate that a FD homodimer produced in *E. coli* can indeed bind to the G-box present in the *SEP3* promoter *in vitro*, providing further independent confirmation of the results discussed above.

3.4 Spatial and temporal expression analysis of FD and its targets.

As outlined above, FD directly binds to regulatory sequences and regulates expression of genes such as *AP1, SEP3* and *FUL* (see section 3.3). Furthermore, my results using a cross between *35S::GR:FD* and *35S::FT* plants indicates that the two proteins act synergistically to induce these genes. However, *FD* expression is thought to be limited to the meristem but seems to rapidly fade away in the emerging flower primordia (Wigge et al., 2005). Similarly, *FT* is apparently not expressed at the meristem and the FT protein, at least when translationally fused to GFP, is not reaching the flower primordia either (Corbesier et al., 2007). In addition, *FD* is not only an upstream regulator of *AP1* but is also downregulated by the latter in the developing flower primordium (Kaufmann et al., 2010). Taken together, these findings raise the questions how this temporal and spatial conflict between expression of *FD* in the meristem and its targets, i.e. *AP1*, in the emerging flower primordium can be reconciled.

3.4.1 Immunolocalisation of the FD protein

To address this apparent discrepancy between the expression domain of the *FD* *m*RNA and its targets, I have analysed the distribution of the distribution of the FD protein at the shoot meristem. Since there are no antibodies available that specifically recognize FD, these experiments were carried out in the *FD::GFP-FD fd-2* rescue line established by L. Yant. As a control I also examined the *fd-2* mutant and plants which expressed a nuclear localized version of GFP from the *FD* promoter. All plants were grown in short days for 28 days and shifted to long days to synchronously induce flowering. Apical tissue was harvested on the 4th day after shift as the differences in *AP1* expression have previously been shown to be maximal between wild-type plants and *fd* mutant at this time point (Wigge et al., 2005). A FITC labelled anti-GFP antibody was used to localize the GFP-FD protein on thin sections prepared from paraffin-embedded transition meristems.

The expression domain of the protein was found to be largely overlapping with the one described for the *FD* mRNA in wild-type. The protein is detectable in all layers of the meristem (Fig: 3-14). However, in contrast to the mRNA, the GFP-FD protein is also detectable in emerging flower primordia. Possibly the GFP-FD persists in cells emerging from the flanks of the meristem as they differentiate into flower primordia. Alternatively, the GFP-FD protein might be mobile and be able to move a few cell layers within the meristem as has been demonstrated to be the case for several other transcription factors (Wu et al., 2003). However, one can not exclude the possibility that the *FD::GFP:FD* transgene does not faithfully recapitulate the expression domain of the endogenous *FD* gene as attempts to detect both GFP-FD protein and RNA on the same section have so far failed.

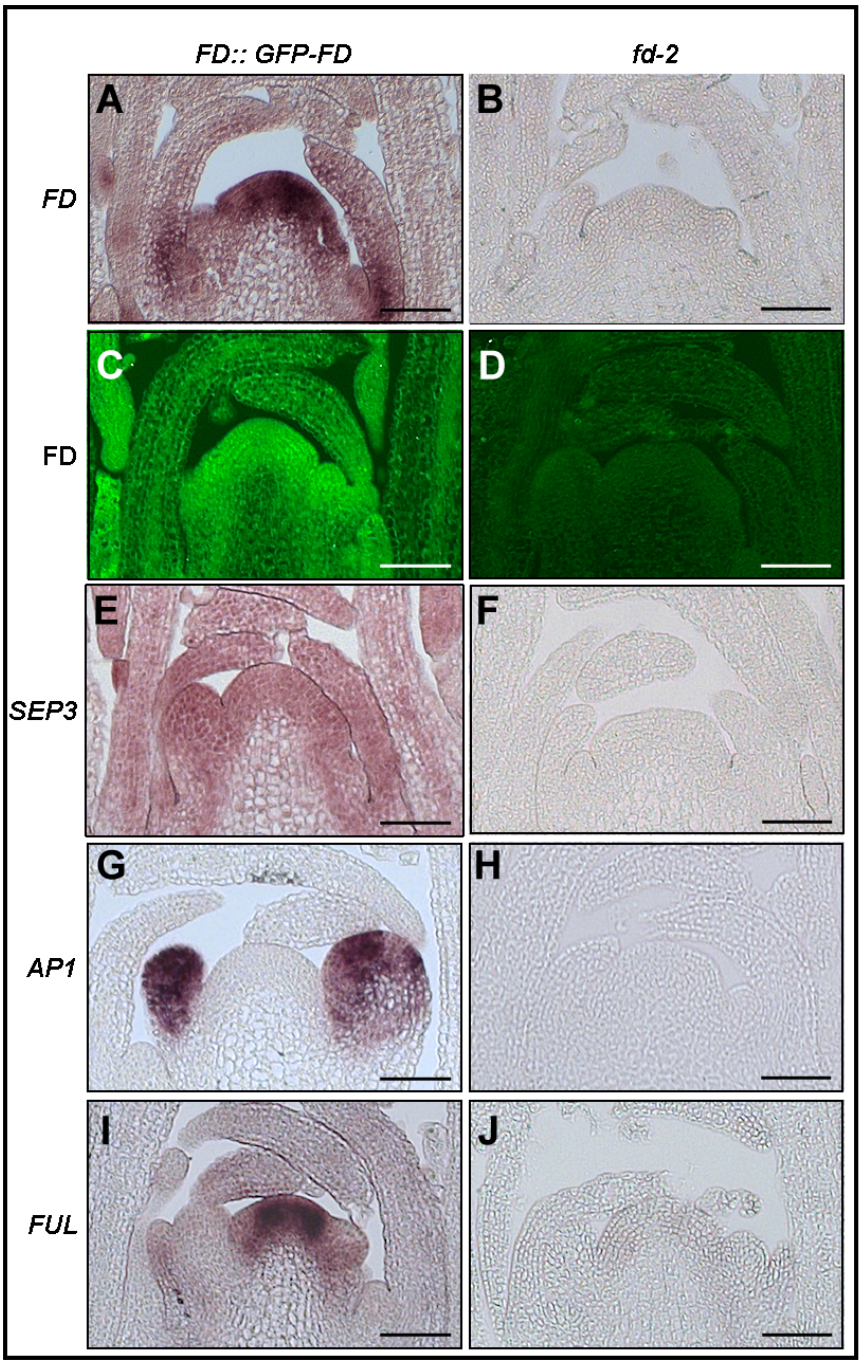


Figure 3-14: ***Comparison of expression patterns of FD protein with FD, AP1, SEP3, FUL RNA.*** Immunolocalisation of GFP-FD protein (B) using FITC labelled α-GFP antibody was performed and compared to *SEP3* (E), *AP1* (G), *FUL* (I) mRNA that are the direct targets of FD on plants carrying the rescue construct *FD::GFP-FD* in a *fd-2* mutants. *In situ* hybridisation was also performed against the *FD* mRNA (A) to compare its expression with that of the protein. *fd-2* plants were used as negative controls for in situ hybridisation (B),(F), (H) and (J) while Col-0 plants were used as a negative control for immunolocalisation (D). Scale bars represents 20μm.

3.4.2 Expression of the FD target genes at the shoot meristem

RNA *in situ* hybridization performed on the fd-2 mutant and the *FD::GFP-FD fd-2* rescue line indicate that genes such as *AP1*, *FUL* and *SEP3* are normally expressed in the rescue line but are substantially delayed in their expression or not detectable at all in the mutant (Fig 3-14), arguing that at least the GFP-FD protein is functional.

To investigate the temporal and spatial activation of FD target genes at the shoot meristem more closely, I next performed a time-course experiment, following the expression of *AP1*, *FUL* and *SEP3*. As previously described, induction of flowering and expression of *AP1* were significantly delayed in the *fd* mutant when compared to wild-type control plants. Similarly, expression of *FUL* was delayed in *fd-2* while SEP3 transcript could not be detected at all (Fig 3-15). Taken together my results provide evidence that the FD protein is required for the induction of a number of flowering time and flower development genes at the shoot apex. In addition, the detection of GFP-FD protein in the emerging flower primordia provides a tentative answer to the question of how FD can possibly activate *AP1* expression in these cells.

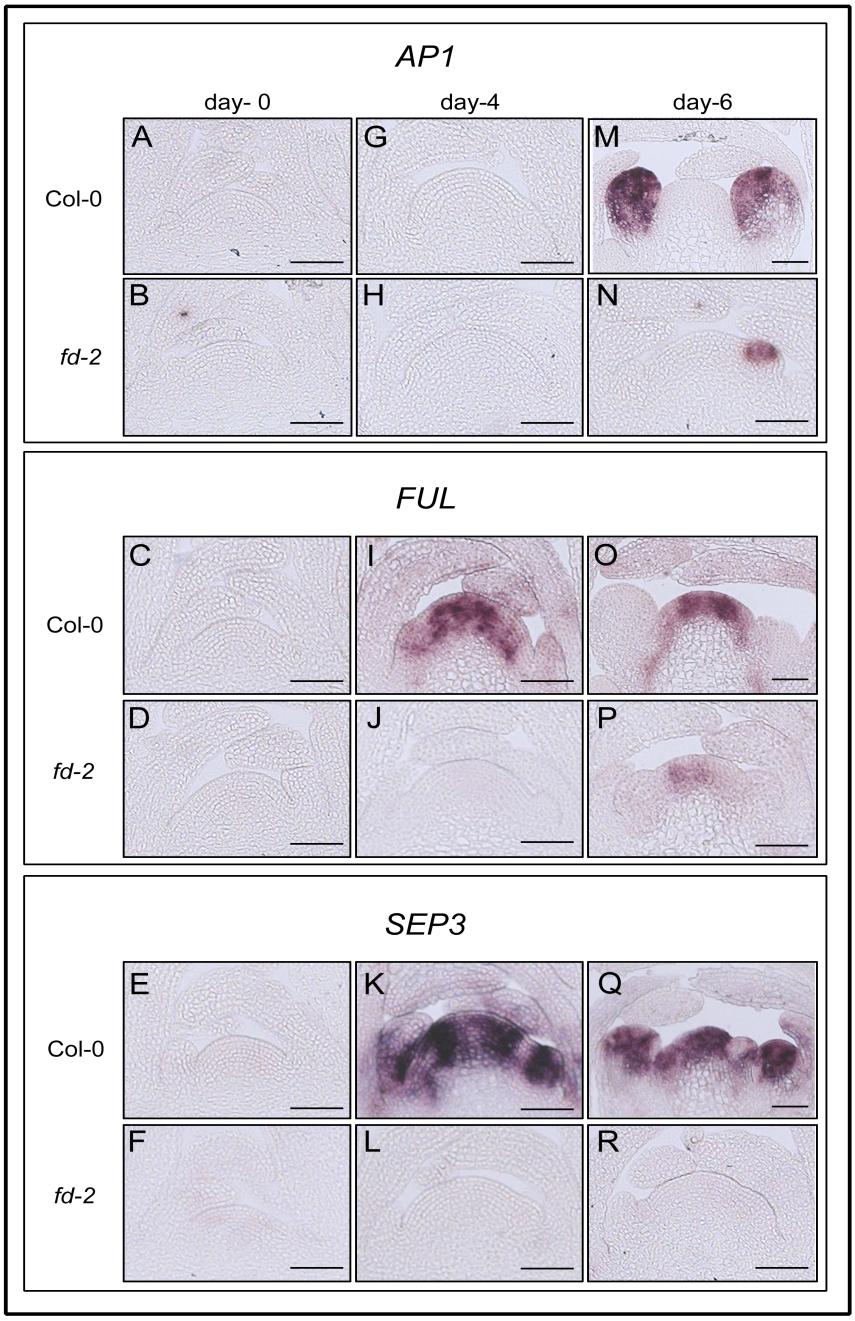


Figure 3-15: ***Time course in situ hybridisation of Col-0 and fd-2 apices.***
Plants were grown in short day for 28 days and shifted to long day and tissue was harvested on every alternate day ie day 0 (A-F), day 2 (G-L), day 4 (M-R). The apices of Col-0 and *fd-2* were fixed, sectioned and tested for the presence of the *AP1* (A,B, G,H, M, N) , *FUL* (C,D, I, J, O, P) and *SEP3* (E,F, K, L, Q, R) RNA by *in situ* hybridisation. Scale bars represent 20 µm.

4.0 DISCUSSION

Being immobile, plants must endure various environmental conditions that might hamper seed set or reproductive success. Further, they must recognise the ideal conditions for seed production to ensure that the future generations do not succumb to harsh environmental conditions that may prove to be fatal to the plants. These environmental conditions include day length, quality of light and temperature. For this reason, plants have developed several mechanisms to measure each of the above mentioned environmental stimuli and regulate its flowering appropriately. Further, flowering is affected by several internal factors such as the hormonal levels, age of the plant etc.

For several years, scientists have recognized that presence of a mobile signal that travels from the leaves to the apex of plants to induce flowering. This mobile signal has recently been identified as the FT protein, thereby bringing this protein into limelight (Kobayashi et al., 1999, Kardailsky et al, 1999, Mathieu et al., 2007, Corbisier et al., 2007). It has been shown that the FT protein interacts with the bZIP transcription factor FD at the apex (Wigge et al., 2005, Abe et al., 2005). The mechanism of interaction between FT and FD has been unclear. Recent studies show that the interaction between FT and FD maybe mediated by the 14-3-3 adaptor proteins (Pnueli et al., 2001, Purwestri et al., 2009, Taoka et al., 2011). This interaction has not only been confirmed in rice, but a protein structural model has been described. This model indicates the need for two molecules of the rice FT homolog Hd3a to form a complex with two molecules of rice FD, OsFD, by interacting with a dimer of the 14-3-3 homolog of rice, GF14c protein (Taoka et al., 2011). FT and FD on interaction are known to activate the downstream target *AP1* (Abe et al., 2005, Wigge et al., 2005).

4.1 FT protein is sufficient to induce flowering.

The nature of the florigen has remained elusive for several years. Sugars were considered a plausible candidate for the florigen (Bernier et al., 1993; Bodson and Outlaw, 1985). While it was established early on that the florigen is not a plant hormone such as Gibberellin (Lang, 1957; Lang, 1960), debate on whether the florigen was a nucleic acid (mRNA) or a protein that moved to the apex has been a heated debate among scientists. Different laboratories used different approaches to address this problem. Artificial miRNA against *FT* expressed from different promoters indicated that the RNA was essential in the leaf vasculature, but not the shoot apex to induce flowering (Mathieu et al., 2007). Further, a synthetic *FT* RNA was created that contained synonymous changes at every base possible. Since these changes had no effect on the ability of *FT* to induce flowering, it was concluded that the RNA sequence and folding were not essential for floral induction (Notaguchi et al., 2008). Corbesier et al., (2007) used GFP tagged versions of the FT protein and tracked its movement to the apex in the early flowering plants. These results were further confirmed by a recent publication, which also provided evidence that the ER membrane protein FT INTERACTING PROTEIN 1 (FTIP1) is required for the export of the FT protein from the phloem companion cells to the sieve elements (Liu et al., 2012). In contrast, Mathieu et al., (2007) used SUC2::FT-TEV-3xYFP constructs to show that while the bulky FT protein when tagged to three copies of YFP could not rescue the late flowering phenotype of *ft-10* mutants, when crossed plants expressing the TEV protease from the same promoter, were able to result in early flowering plants due to the cleavage of the 3xYFP tag from the FT protein.

However, these studies suffer from the use of the strong promoters such as *SUC2* and *35S*, which always result in early flowering phenotypes when used to drive the expression of *FT*, over compensating the original flowering phenotype of a wild-type plant. In order to address this issue, the experiments performed by Mathieu et al., (2007) were repeated using a native *FT* promoter instead of the *SUC2* promoter (Results section 3.1.1). The expression of the FT-TEV-3xYFP was restricted to the

phloem companion cells. Crossing these lines to the TEV Protease expressing lines rescued the late flowering phenotypes of the *ft-10* mutants. This indicated that the endogenous levels of FT protein expressed from the genomic construct was sufficient to restore wild-type pattern of flowering (13-17 total leaf number). It was also confirmed that the TEV protease was not expressed at the apex by tagging the protein with the RFP tandem-dTomato. It may also be noted that some of the early flowering F1 lines generated by crossing the *gFT-TEV-3xYFP* to the *SUC2::TEV* protease were slightly later than the Col-0 controls. However, it is interesting to note that these plants were the progeny of crosses where the parental lines also flowered slightly later than the *ft-10* mutant lines in which they were generated. This could be due to the low expression of the transgene because of an unfavourable integration site into the genome. This however, has to be investigated.

Since the TEV protease can cleave only proteins with a TEV cleavage site and does not alter the mRNA, these results also indicate indirectly that the RNA may not have a role as the florigen. These results further concur with the results already published by Notaguchi et al., (2008) and Corbesier et al., (2007) where the mRNA was unable to move over graft junctions, while the FT protein was able to move from stalks to scions and result in flowering. The tomato ortholog of FT, SFT protein but not the RNA was also shown to move over graft junctions to induce flowering in *sft* shoots (Lifschitz et al., 2006). In rice, *Hd3a* mRNA was shown to accumulate in the inductive short day conditions in leaves, but was virtually undetectable at the apex (Tamaki et al., 2007). Taken together, it can be safely said that the FT protein functions as the florigen. Nevertheless, it is interesting to note that the 5' end of the *FT* mRNA facilitates movement of heterologous RNA in a viral expression system (Li et al., 2011). Whether this contributes to flowering is still unclear. Based on results from RFP fusion proteins, which unlike the GFP fusions of FT are unable to migrate to neighbouring cells, it has recently been suggested that the *FT* RNA play a role in floral regulation after all. The use of RNAi and miRNA against *FT* was able to delay flowering indicating that the RNA may play a role in flowering (Lu et al., 2012).

However, whether the RNA moves or not, it is the FT protein that is finally significant for floral transition.

4.2 FT mRNA does not contribute to floral induction in *Arabidopsis thaliana.*

Recent findings including Lu et al., (2012) imply the requirement of *FT* mRNA movement to the apex for the plant to flower. This however has been widely met with skepticism as they contradict previously published results (Mathieu et al., 2007; Notaguchi et al., 2008). More importantly, while the FT protein homologs have always been shown to move across graft junctions in a variety of plant species such as tomato, tobacco, *Cucurbis* apart from *Arabidopsis*, the RNA has never been detected in the phloem sap of the scion (Corbesier et al., 2007; Notaguchi et al., 2008; Lifschitz et al., 2006; Lin et al., 2007). All the above mentioned experiments were however performed using promoters such as *35S* or *SUC2* which are much stronger than the native *FT* promoter. These strong promoters lead to precoccious phenotypes which cannot be seen in a wild-type plant. To conclude the debate on the requirement of *FT* mRNA movement for flowering, an experiment was devised to knock out the FT protein in a tissue specific manner and study its effect on flowering. The genomic construct of FT was modified to include a TEV cleavage site in the coding frame of the protein. It was confirmed that these constructs were able to rescue the late flowering phenotype of the *ft-10* plants, indicating that the modified FT protein was fully functional. Crossing these plants with plants expressing the TEV protease from the *SUC2* promoter results in late flowering progeny. In other words, the TEV protease renders the FT protein non-functional by cleaving it, resulting in the plants to revert to the late flowering *ft-10* phenotype (Results section 3.1.1). It maybe noted that some crosses of either the *gFT-TEV* to the *SUC2::TEV protease* didn't take well and yielded very few progeny. But several parental lines with the same genotype were used for the crosses to ensure the absence of any bias that maybe caused by a single parent.

Since the TEV protease does not cleave mRNA, it can be concluded that the presence of a functional protein and not the mRNA in the vasculature is essential for flowering. These results also suggest that, the FT protein, which in theory could be produced from mRNA transported to the apex is not sufficient to induce flowering. This is in line with experiments where an amiRNA against *FT* expressed from the *FD* promoter did not delay flowering while the same construct expressed in the vasculature using the *SUC2* promoter resulted in late flowering plants (Mathieu et al., 2007). Taken together with the observations in rice and tomato as discussed above, one can safely conclude that whether *FT* RNA moves to the apex or not, the protein alone is sufficient to induce flowering. The results presented would thus predict that TEV protease at the SAM should be sufficient to delay flowering. However, *pFD::TEV* plants in an *ft* mutant background were not late flowering (M Schmid, pers. communications; data not shown), This could be explained by the fact that the *FD* promoter is only activated during the floral transition, hence the expression of the TEV protease could come late to have an effect on the FT protein to delay flowering. This can be verified by using SAM markers that are active from early on in vegetative development.

FT homologs have been identified namely *PtFT1*, *CiFT*, *Hd3a*, and *SFT* from poplar, citrus, rice, and tomato that cause early flowering of transgenic plants (Bohlenius et al., 2006; Endo et al., 2005; Kojima et al., 2002; Lifschitz et al., 2006). *Arabidopsis thaliana* is a long day plant, while tomato is day-neutral and rice is a short day plant. SFT was able to induce early flowering in both day neutral tomato as well as tobacco, a short day plant. It is further interesting to note that in case of some homologs of FT, the proteins have further evolved to gain new functions. Paralogs *Bvft1* and *Bvft2* belong to the same clade as *AtFT*. While BvFT2 is functionally conserved with AtFT, the BvFT1 acts a floral repressor and is itself regulated by vernalization (Pin et al., 2010). *StSP3D* and *StSP6A,* paralogs of *FT*-like genes in potato play a role in flowering and tuberisation respectively (Navarro et al., 2011). FT codes for a small globular protein belonging to the Phosphatidyl Ethanolamine Binding Protein (PEBP) family that has been conserved among several angiosperms.

This indicates that the presence of a mobile signal that functions as a florigen might have existed in early angiosperm ancestors. While FT proteins are still evolving to acquire new functions (e.g. in beet), the core function as a florigen seems to conserved in angiosperms. Since no concrete evidence occurs for the presence of a florigen in gymnosperms, though several PEBP proteins have been identified whose functions are unknown, it is hard to predict when this regulatory mechanism was invented during the evolution of plants.

Another flowering time gene that shows conservation at least in the Brassicaceae is *FLC* , which plays a major role in the vernalization pathway (Alexandre and Hennig, 2008; Hecht et al., 2005; Michaels and Amasino, 1999, 2001; Schläppi and Patel, 2001; Sung and Amasino, 2004b). *FLC* orthologs such as *PERPETUAL FLOWERING 1 (PEP1)* have acquired additional functions. In *Arabis alpina, PEP1* appears to be important not only for the induction of flowering in response to vernalization but also for the establishment of a perennial life history (Wang et al., 2009b). Since *FLC* and its regulator *FRI* are found only in the Brassicaceae, but vernalisation is common to other plant families as well, it is evident that a different mechanism or a different set of target genes have evolved to measure the exposure to prolonged periods of cold.

In summary, while one important pathway- photoperiod has been conserved throughout angiosperm evolution, another pathway ie vernalization has evolved multiple times through angiosperm evolution, though the proteins involved in these pathways may have diversified in their function.

4.3 FT protein regulates flowering at the shoot meristem.

It has been well established that the FT protein moves to the apex where it induces flowering. The molecular mechanism of how this happens is however unclear. No DNA binding sequence has been identified on the FT protein, making a direct

protein-DNA interaction seem unlikely. FT has, however, been shown to bind to the bZIP transcription factor FD, which activates downstream targets such as *AP1.* Taken together these findings suggest a model in which interaction with FT by an unknown mechanism triggers or enhances the binding of FD to its target sites. In this scenario, FT would act as a cofactor in a transcriptional complex with FD. There however are several loopholes that need to be clarified in this model.

a) Interaction with FD

So far the evidence showing that the FT protein moves from the leaves to the shoot meristem has been mostly indirect (Corbesier et al., 2007; Jaegar and Wigge, 2007; Mathieu et al., 2007; Notaguchi et al., 2008). The closest scientists have got to showing that the FT moves to the shoot apex is by Corbesier et al., (2007). Here again, the authors use the *SUC2* promoter to express the GFP fusion of FT, which is much stronger than the native *FT* promoter. Furthermore, in this study the FT protein could only be detected within a few cell layers close to the vasculature but not in the entire apex. Recently, another group has been able to confirm the presence of an FT-GFP fusion protein at the SAM, when driven by the *SUC2* promoter (Liu et al., 2012). They also show that the presence of a functional FTIP1 protein is necessary for FT movement.

Once the protein reaches the shoot apex, FT has been suggested to interact with the bZIP transcription factor FD. So far, the interaction between FT and FD has only been studied in heterologous systems such as by Yeast-2-Hybrid assay or by transient bimolecular fluorescence assay in tobacco leaves (Abe et al., 2005; Wigge et al., 2005). Since no direct interaction of the FT protein and the FD transcription factor has ever been shown *in planta* at the apex, I tried to address this problem. I generated a reporter system by tagging FT and FD to two parts of the Yellow Fluorescence protein, expressed from the *SUC2* and *FD* promoters respectively. (Results 3.2.2). The rational behind this experiment was to see if the FT protein

tagged to the smaller fragment of YFP was able to move to the apex and interact with the FD protein tagged to the other fragment of YFP. I tested the functionality of the constructs by using *35S* promoter to drive the expression of *FT* and *FD* tagged to the two parts of YFP in a transient BiFC assay in tobacco leaves, for which I got positive results. However, I was unable to visualize this interaction thus far at the *A. thaliana* shoot apex. Initially, my studies were done on Col-0 plants, where I suspected that the native untagged versions of the FT and FD may interfere with the interaction by diluting the titers of tagged proteins at the apex. So this experiment was repeated in a *ft-10/fd-3* double mutant, but preliminary screening did not show any fluorescence at the apex. This could be due to several reasons. There might not be sufficient FT protein reaching the apex, as the *SUC2::cYFP-FT* plants were always later than their *SUC2::FT* counterparts. Hence, though there is some movement of the tagged FT protein to result in an early flowering phenotype (15 total leaves in *ft-10/fd-3* double mutant that flowered with 55 total leaves in the absence of any transgene), the number of molecules reaching the apex may just not be enough to be visualized by standard confocal microscopy. Plants were tested by *in situ* hybridization and qRT-PCR for the presence of the transgenes to ensure that the problem was that of visualization of the fluorescence at the apex and not expression of the transgene (data not shown). Finally, when I tested my constructs in a transient assay in tobacco leaves, I used the same constructs driven by a *35S* promoter. My success with the transcient BiFC assay may not have been mimicked by my constructs expressed in the *Arabidopsis thaliana* apex due to the fact that the *35S* promoter is much stronger and may result in more copies of the proteins that interact efficiently to give strong signals in tobacco leaves that can be easily detected by the microscope. Repeating the experiment using more powerful microscopes that can detect a few molecules of YFP in a few cells of the entire meristem may yield more promising results.

b) Possible Roles of FT at the SAM

It is now widely accepted that the FT proteins moves from the leaf to the shoot apex to induce flowering. In agreement with the role of FT as a florigen, *ft* mutants are very late flowering and misexpression of FT at the shoot apex is sufficient to induce flowering. While the role of FT in flowering is undisputed, the exact molecular mechanism by which this protein facilitates this function is unclear. As such, no DNA binding domain has been found on analyzing the protein sequence of FT, indicating an alternative mechanism of action. The molecule has been classified as belonging to the PEBP family of proteins, which has also not shown to have a DNA binding function. Hence it is very essential to understand the molecular function of this protein that necessitates the long distance travel from leaf to apex.

Heterologous systems have confirmed the interaction between FT and FD, the latter being a transcription factor that can bind DNA. Chromatin-immunoprecipitation followed by microarray analysis or deep sequencing indicates that FD does in fact, bind DNA (data from L. Yant). To complement this data generated by ChIP-Chip and ChIP-seq, I aimed to identify the transcriptional targets of FD. A tripartite approach was devised for this purpose. As a first step, FD was overexpressed in leaves using the viral 35S promoter and the transcriptome was analysed by gene expression microarrays. This experiment identified a handful of potential targets that have already been shown to be involved in flowering and/ or flower formation. Several other genes that were involved in metabolism were also identified. However, the effect of FD overexpression on these potential targets was rather mild. Since the experiment was performed using leaf tissue, it might be possible that certain co-factors that are necessary for the FD function at the apex are missing in the leaf.

In the next step, I attempted to differentiate the targets that were directly regulated by FD from those that were regulated by targets of FD, i.e. indirect targets of FD. For this, I prepared GR fusions of FD and tested the leaves constitutively overexpressing the fusion protein in the presence of dexamethasone for genes expressed when FD is

allowed to translocate into the nucleus. Again, only a small subset of genes (41, data not shown) seemed to be differentially regulated by FD. This concurs with the observation that *fd* mutants have a milder flowering time defect than the *ft* mutants. Since upon DEX treatment no striking upregulation was observed for even known targets such as *AP1*, and altering the dexamethasone concentrations and/or the duration of the treatment did not enhance the induction, it was evident that some cofactor maybe missing that would enhance the induction of targets by FD. The most plausible cofactor was FT, since FT-FD interaction has already been established by Y2H and BiFC. This could also explain the weak induction seen when leaves of the *35S::GR-FD* plants were tested. FT protein has been shown to be expressed in the phloem companion cells of leaves (Takada et al., 2003), which contributes to a very small percentage of the total number of cells on the leaf. Hence, the number of cells expressing both FT and FD is minimal, which could result in the weak induction seen in the experiment.

To overcome this, I crossed the *35S::GR-FD* plants to the *35S::FT* lines to maximize the number of cells expressing both proteins. I tested the theory that FT might act as a cofactor necessary for FD action on a double transgenic line expressing both FT and FD. Induction of the known targets such as *AP1* was substantially increased in the double transgenic lines compared to results seen in plants expressing either FT or the fusion GR-FD protein alone. Further, the induction was not an additive but a cooperative effect of expressing both FD and FT. Genes such as *AP1, SEP3, FUL* were induced several fold more in the double trangenics when compared to the parental lines expressing FD or FT alone (Fig 3-11). This indicates that the presence of FT somehow increases the ability of FD to either bind to or activate expression of its target genes. How this occurs however, is unclear and is being currently investigated.

Abe et al., (2005) showed that mutating the threonine residue at position 282 of FD to alanine or prematurely truncating the protein by removing the last few amino acids including the T282 prevents its interaction with FT. This indicates that this

residue at position 282 plays a critical role in the interaction of FD and FT. Abe et al., (2005) also observed that the binding to FT was not affected when T282 was mutated to serine. This leads to the theory that, since both serine and threonine are residues that can undergo phosphorylation, this may play a role on the binding specificity of FD to FT. Preliminary studies are being undertaken to see if the effect of phosphorylation alters the binding specificity of FD to DNA. Attempts to purify FD protein from eukaryotic sources such as plant extract, wheat germ, and yeast *Pichia pastoris* are being undertaken to see if the protein undergoes the post-translational modification that is not possible when expressed in bacterial cells.

As a third step to understanding the function of FT at the apex, the binding specificity of FD was tested using *in vitro* assays such as EMSA. FD was able to bind to a G-box in the *SEP3* promoter specifically, where as mutating the G-box abolished binding (Fig 3-13). However, the same results could not be replicated in case of the C- box in the *AP1* promoter where FD was shown to bind in the ChIP-seq data. This is in agreement with findings using an ELISA based DNA-binding assay where FD was shown to bind to a doublestranded DNA oligo containing a G-box but not a C-box (A.L Schinke; pers. comunications). This leads us to hypothesize that FT may somehow change the DNA-binding specificity of FD. To test this, plants expressing *SUC2::GFP-FD* are being crossed to the *ft-10* mutant to test the DNA-binding specificity of FD by ChIP in the absence of FT.

Recent studies in rice have indicated that the FT-FD interaction is not direct, but in turn mediated by the 14-3-3 proteins (Taoka et al., 2011, Mylne and Wigge, 2011). It has been observed that the FD protein homologs from monocots such as rice and wheat bind efficiently to the C box containing DNA oligos even in the absence of FT (Li and Dubcovsky, 2008; Taoka et al., 2011). This clearly indicates that there maybe variations in the mechanism of binding and /or function between the AtFD and its monocot counterparts. Closer analysis of the FD protein sequence indicates that AtFD has a STAPF motif in its carboxyl end, compared to the SAPF motif in rice. Further, this site is similar to the site of recognition of the 14-3-3 proteins, also

explaining why no interaction was seen between the truncated FD protein and FT in Y2H (Taoka et al., 2011; Abe et al., 2005). Since both serine and threonine can undergo phosphorylation, the difference between OsFD1 (rice), FDL2 (wheat) and the AtFD protein function may be due to multiple phosphorylations at the 14-3-3 recognition site. This however has to be verified by altering the serine and threonine residues with phospho-mimicking amino acids such as glutamate or phosphorylation abolishing amino acids such as alanine and using these modified proteins to study its DNA-binding and interaction abilities.

c) Spatial control of flower primordium initiation.

FD is expressed in the transition meristem and fades away into the floral anlagen, suggesting that the meristem is the tissue in which FT and FD predominately interact. However, downstream targets such as *AP1* are expressed exclusively in the emerging flowers. Hence there seems to be a discrepancy between the expression domain of the *FD* mRNA and that of its target, *AP1*. The question arises as to how it is possible for a protein to activate genes in parts of the plant where it is not expressed. In order to understand this, I performed immunolocalisation studies on the FD protein tagged to GFP, expressed from a 2kb *FD* promoter. My results show that in these reporter lines, there is a definite overlap between the region of FD protein expression and its target genes. This offers a plausible explanation on how the FD protein may regulate the *AP1* gene in the floral primordia. This also requires the clarification if the FD protein actually moves from the meristematic tissue into the floral primordia. Movement of transcription factors across cell layers is not uncommon in the meristem as has been seen for *LFY* (Wu et al., 2003). Besides, several transcription factors have been shown to move across plasmodesmata between cells (Lee and Zhou, 2012). Alternatively, it is also possible that the FD protein is stably maintained in the cells at the flank of the meristem as they undergo differentiation to take on a flower primordium fate, resulting in the protein, but not the RNA being observed in the floral primordia.

Comparison of *FD* mRNA expressed in the *FD::GFP-FD* rescue lines in the *fd-2* background with Col-0 showed the expression domain of FD in the transgenic line was more broad compared to the wild-type scenario. It was observed that *GFP-FD* mRNA was expressed in the floral anlagen when the 2kb *FD* promoter region was used to express the transgene. This could explain the presence of the protein in the floral anlagen in the immunolocalisation studies. Since efforts to raise an antibody against the FD protein were futile, one cannot rule out the possibility that the overlap seen in the expression domain of the FD protein with that of the target genes is an artifact of the *FD* promoter that may lack some crucial regulatory elements that restrict its expression domain. To overcome this, immunolocalisation studies can be done by generating plants that express the GFP-FD fusion protein in a genomic context.

5.0 CONCLUSION

The transition from vegetative growth to flowering is one of the most important phase changes in a plant's life. The induction of flowering by permissive photoperiod requires the movement of the florigen, which is the protein FT, from leaves to the shoot apex. There it interacts with the bZIP transcription factor FD. The complex thus formed is thought to activate downstream targets such as *AP1, SEP3, SEP1, AG, SOC1, FUL* that are well known for their role in flowering and floral organ formation, and other genes that play a role in metabolism of carbohydrates, lipids etc. in plants. My results demonstrate that of these targets, *AP1, SEP3* and *FUL* are direct FD targets while *SEP1* and *AG* are most likely indirect targets that are regulated by FD-downstream factors. The FD homodimer is able to bind to the G-box element in the promoter region of *SEP3 in vitro*. Similarly, binding of FD to this motif was also observed *in vivo*. In contrast, binding of FD to the C-box in the core *AP1* promoter apparently requires additional factors as binding could only be observed *in planta* but not *in vitro*. One likely candidate for such a cofactor is FT. In agreement with this hypothesis, I found that co-expression of FT and FD have a cooperative effect on FD target gene expression *in vivo*. Taken together my results provide new insights into how FD plays a major role in the regulating the transtion of the vegetative meristem to a reproductive meristem.

6.0 MATERIALS AND METHODS

6.1 Bacterial strains

For general cloning techniques, *Escherichia coli* strains DH5α and DB3.1 (Invitrogen) were used. For stable transformation of *Arabidopsis*, *Agrobacterium tumefaciens* strains ASE with kanamycin and chloramphenicol**.**

6.2 Plasmid construction *

6.2.1 Generating the 35S:: GR-FD constructs.

FD cDNA was amplified from genomic DNA using the oligos G-5636 and G-5637, listed in Supplementary Table: S1. The amplified cDNA was cloned into pGEM-T-Easy (Promega) for *in situ* hybridization or the GATEWAY pJLSMART entry plasmid between the *attL1* and *attL2* recombination sites by digestion of the plasmid with *Sma*I restriction enzyme. The Rat Glucocorticoid (GR) cDNA was amplified using the oligos G-12223, G-12224 listed in Supplementary Table: S1. These oligos introduced a StuI site at the 5' end and an *Stu*I, *Sma*I site at the 3' end. This was then cloned into pGEM-T-Easy (Promega) and sequenced using the SP6 and T7 primers. The rfA cassette was then cloned into the *SmaI* site of the pGEM-T-Easy GR construct. The GR-rfa construct was then cut from the pGEM-T-Easy GF-rfA construct using *Stu*I and cloned into the pPCRSMART vector containing the rbcs cassette at the *Stu*I site. The GR-rfA-rbcs cassette was then cut out using the restriction enzymes *Xba*I and *Sac*I and cloned into a GATEWAY destination vector pGREEN-IIS conferring either Basta or Kanamycin resistance. The CaMV 35S promoter was then added in front of the GR using the *Kpn*I and *Sal*I restriction enzymes for digestion followed by ligation. An LR clonase (Invitrogen) reaction was performed to introduce FD from the pJLSMART entry vector to the destination vector.

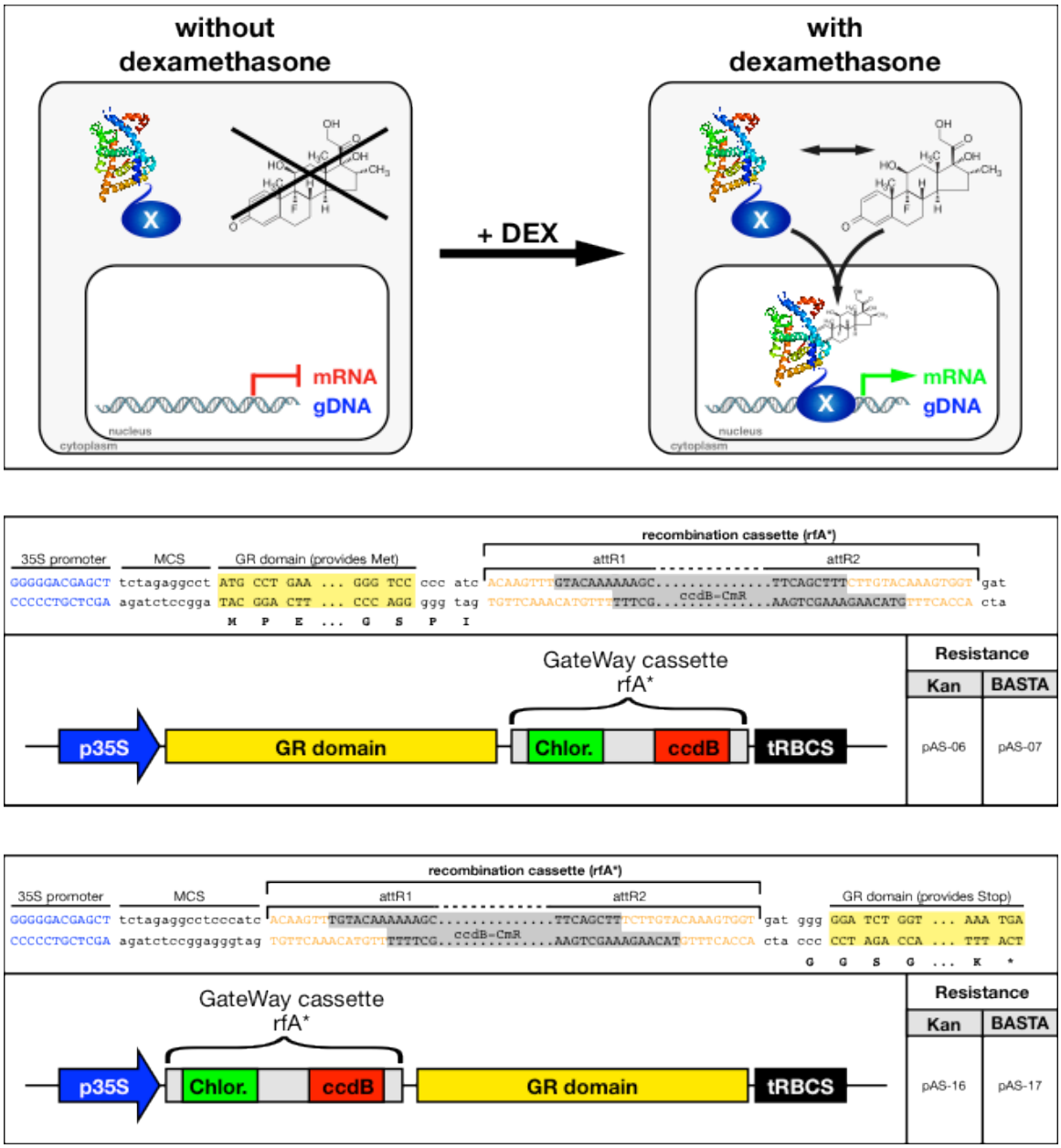
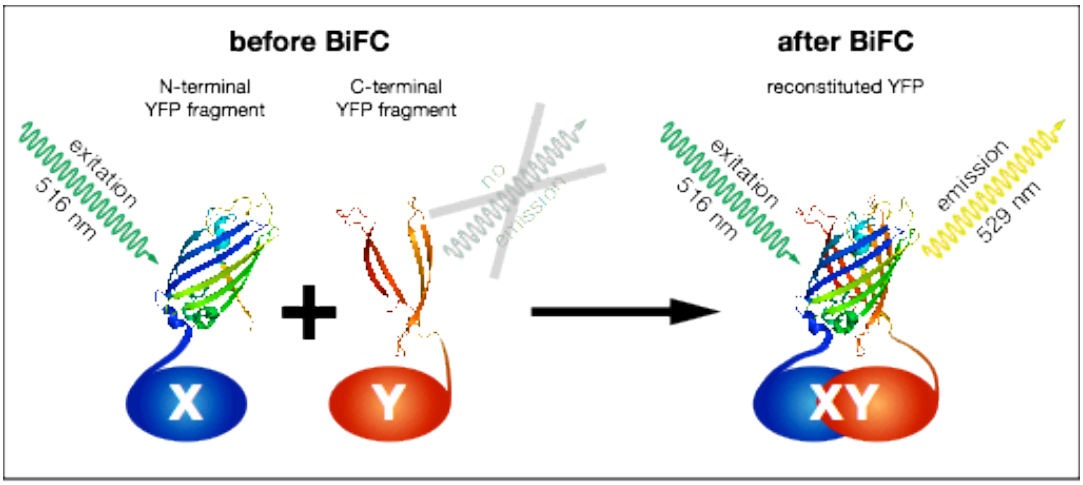


Figure 6-1: ***Schematic showing principle and cloning of GR fusion proteins.*** When tagged to the rat Glucocorticoid Receptor (GR), the protein gets trapped in the cytoplasm by heat-shock proteins. In the presence of Glucocorticoid or its analog Dexamethasone, the complex can move into the nucleus, and if the tagged protein is a transcriptional factor, activate its target genes. This figure was generated by M. Schmid.

A similar protocol was used to generate plasmids with 35S:: rfA-GR- rbcs in the pGREENII vector backbone conferring either Basta or Kanamycin resistance. But since only N- terminal FD fusions were used in the experiments, the details of cloning have not been described.

6.2.2 Generating the BiFC constructs.

The Yellow fluorescence protein was split at amino acid 153. To clone the 2 parts of YFP, oligos were designed to introduce *Eco*RV and *Sma*I sites for cloning. Oligos were also designed to enable introduction of a HA tag (G-17683) and a Myc tag (G-17684) to the C and N terminal fragments respectively (Supplementary table S1). For constructs with out tags, G- 15956 was used for the N- terminal fragment and G-17682 was used for the C terminal fragment. G-17681 was used as the reverse oligo for the the N terminal constructs, and G-20655 for the C- terminal constructs (Supplementary table S1). The different fragments were then cloned into pGEM- T-EASY (Promega). The plasmids were cut with *Sma*I restriction enzyme and the rFA cassette was ligated into it. The rfA- YFP fragments were cut from the pGEM-T-EASY vector using the enzyme *Eco*RV and cloned into the SmaI site of the pGREENII destination vector containing the rbcs terminator fragment conferring either Basta or Kanamycin resistance. The promoter (*35S* or *SUC2* or *FD*) were added between the KpnI and SalI sites.



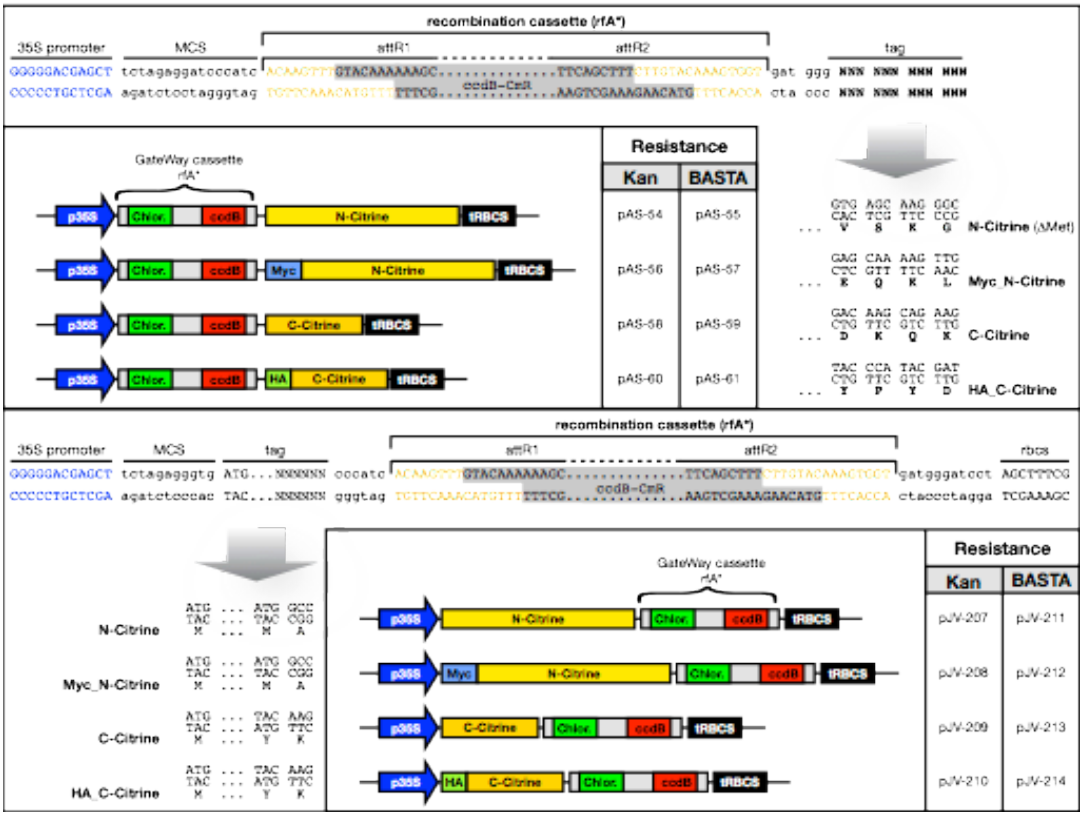


Figure 6-2: Schematic for principle and cloning of the split-YFP constructs. The two parts of YFP split at aa 155 were tagged to different proteins. If these proteins interact, on excitation with 516nm, emission is seen at 529nm. This figure was generated by M. Schmid.

* All restriction enzymes used for cloning were from Fermentas.

6.3 Plant transformation.

cDNA cloned in the GATEWAY destination vector pGREEN-IIS were co-transformed into *Agrobacterium tumefaciens* ASE with the pSOUP helper plasmid (Invitrogen). Positive ASE were selected on LB Agar plates with Kanamycin, Chloramphenicol, Spectinomycin, Tetracyclin and confirmed by colony PCR. Plants were grown till the primary bolt was approx. 5cm and cut back to allow a bushy outgrowth of sideshoots. Plants were transformed by the floral dip method. T1 plants were screened for Basta or Kanamycin resistance.

6.4 Plant material and growth conditions.

Arabidopsis thaliana Columbia (Col-0) accession was used in all the mutant backgrounds. The T-DNA insertion mutants used in this work namely *fd-2* (Abe et al., 2005), *fd-3* (Abe et al., 2005) and *ft-10* (Yoo et al., 2005) have been described before. Mutants were confirmed by PCR based genotyping as described below. Plants were grown at 23º C, either in long days (16h light, 8h dark), or short days (8h light, 16hr dark), 65% relative humidity under a 2:1 mixture of cool white and Gro-Lux Wide Spectrum fluorescence lights at an irradiance of 125 to 175 $\mu mol/m^2/s$. All light bulbs were of the same age.

For growth on soil, seeds were first frozen for 2 days at -20°C and sterilized with 90% ethanol containing 0.1% Triton-X-100 for 15 minutes. Later they were briefly washed in 95% ethanol and dried on sterile filter paper. The sterilized seeds were then stratified at 4°C for 2-3 days in 0.1% agarose and were finally placed on soil. For transgenic seedling selection, Basta treatment was carried out either by direct application to the water when first soaking the soil (1:1000 of 0.25g/l Basta stock solution) or by spraying (1:1000 of Basta stock solution) about 5-7 days after germination.

For growth on sterile 0.5X MS-agar plates (Murashige and Skoog, 1962), seeds were sterilized with 90% ethanol containing 0.1% Triton-X-100 for 15 min, briefly washed in 95% ethanol and dried on sterile filter paper in a flow hood. After spreading on agar plates, seeds were stratified at 4°C for 2-3 days. Kanamycin selection was done on plates by growing plants with 50µg/ml of the antibiotic infused into the media. The plants carrying the transgene were then transferred on to soil.

6.5 Genomic DNA extraction

Isolation of genomic DNA from plants for amplification of transgenes was carried out using the quick-prep protocol (Edwards et al., 1991). One to two young leaves were collected in eppendorf tubes and then frozen in liquid nitrogen. The samples were ground with a mortar and pestle, and homogenized with 400 µl of Edwards buffer (200 mM Tris pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS). The mixture was centrifuged for 3 min at full speed (13000 rpm) at room temperature. The supernatant was transferred to a new tube prefilled with 300 µl isopropanol. The DNA was precipated by centrifugation for 10 min at 13000 rpm. The supernant was discarded and the precipitated DNA was washed with 70% ethanol. The tubes were air dried and DNA was dissolved in 100 µl of nuclease-free distilled water and stored at 4ºC. 1-2 µl of dissolved DNA was used for PCR amplifications.

6.6 Crosses

Anthers were carefully removed prior to the opening of the flowers using sharp forceps. Pollen was then rubbed on the stigma and the flower was tagged and wrapped with thin plastic film. All flowers that were not cross pollinated and young siliques on the shoot were removed. Within two or three days the pollinated siliques turned purple in color, which is an indication of a successful cross. Dried siliques were collected and stored at 16º C. Once completely dry, the seeds were dislodged from the silique and the valves were discarded. The schematics for the crosses are listed in Supplementary Table S3.

6.6.1 Constructs and crosses to study the movement of the FT protein

The constructs for studying the movement of the FT protein were all generated by Ms. Janina Vogt, a technician in the lab. The crosses were performed as listed in Supplementary Table: S3. Rosette and cauline leaves were counted to score the phenotype once the plant exhibited a 2 cm bolt.

6.6.2 Confocal microscopy

In order to study if the *SUC2::TEVprotease-TdTomato* constructs were expressed in the shoot apex as well, confocal microscopy was performed using a Leica SP2 microscope. The samples were prepared by removing all the leaves and dissecting the apex longitudinally while maintaining the meristem and vasculature. The samples were mounted on HBSS buffer with 50% Glycerol and observed under a 10x or 20x magnification.

6.7 Targets of FD

6.7.1 Overexpression of FD in leaves

An established *35S::FD* line generated by Min Chul Kim was used for the overexpression studies. Plants Col-0 and 35S::FD were grown in SD for 28 days and shifted to LD, while some plants were retained in SD. Leaf tissue (3 leaves from 1 plant, 8 plants per sample) was collected at the end of every long day from the plants in both the SD and LD chambers.

5.7.2 Induction of the plants containing the GR-FD fusion protein

To test the effect of FD on the activation of its targets, the plants were treated to the following chemicals prepared in 0.5 strength MS media.

Dexamethasone:
Dexamethasone dissolved in ethanol: 15μM
Silvette: 0.015%
DMSO: 0.001%

Mock:
Absolute Ethanol: 0.3%
Silvette: 0.015%

DMSO: 0.001%

Dexamethasone + Cycloheximide:
Dexamethasone: 15µM
Cycloheximide: 10µM
Silvette: 0.015%

Cycloheximide:
Cycloheximide: 10µM
Silvette: 0.015%
Absolute Ethanol: 0.3%

Plants were sprayed with the chemicals and returned to the growth chambers. Tissue was collected after 3h into 1.5ml microcentrifuge tubes and snap frozen on liquid nitrogen until processing.

RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) following the protocol described by the manufacturers. The RNA quality and quantity were tested using a Nanodrop 2000 (Thermo Scientific) or the RNA 6000 Nano Kit for Bioanalyser (Agilent Technologies).

6.8 quantitative PCR studies.

The RNA purified using the RNeasy Plant Mini Kit was treated with DNAse I (Fermentas) at 37ºC for 1h. The DNase was inactivated by heating the samples to 80ºC and treating with 0.025mM EDTA. The cDNA synthesis was performed using the RevertAid First Strand cDNA synthesis kit (Fermentas). SYBRGreen mix (Invitrogen) was used for the qPCR reaction that is read on an Opticon DNA Engine Continuous Fluorescence detector (MJ Research). The list of oligos used for the qPCR reactions are listed in Supplementary Table: S2.

6.9 Microarray analysis

To prepare the samples for the microarray, the MessageAmp II- Biotin *Enhanced* (Ambion) kit was used and the samples were prepared as per the instructions of the manufacturer. The samples were hybridized on GeneChip *Arabidopsis* ATH1

Genome (Affymetrix) expression arrays and processed using a GeneChip Fluidics station 450 (Affymetrix) and scanned using a GeneChip Scanner (Affymetrix). The samples were analysed using the GeneScript v2 software and the data was processed using the R program.

6.10 Transcriptome library generation and RNA-seq.

Transcriptome libraries were generated using the TruSeq RNA Sample Preparation Kit- v2 (Illumina) using the protocol supplied with the kit. The samples were then analysed using either a Genome Analyzer II (Illumina) or the HiSeq 2000 (Illumina). Reads were aligned using bwa version 0.6.0 with a seed length of 25, allowing for one mismatch in the seed, against the TAIR10 *A. thaliana* annotation. Read counts per gene were generated using a custom-made Perl script. Differential gene expression was analyzed using the DESeq package (v. 1.8.2) in *R* (v. 2.15). Variance was estimated in all possible pair-wise combinations using the *estimateDispersion* function in mode "pooled" with *sharingMode* set to "fit-only" and *fitType* set to "local". Following multiple correction according to Bonferroni, genes with a corrected p-value ≤ 0.01 and an absolute log2 fold change ≥ 1 were classified as differentially expressed.

6.11 Electrophoretic Mobility shift assay

Oligos G-21223 and G-21224 (Supplementary Table: S1) were used to amplify a 200bp region around the G-box on the SEP3 promoter that was bound by FD in the ChIP-seq experiment. To create the G-box mutant, PCRs were performed using oligos G-21223 and G-22808 and G-22809 and G-21224 (Supplementary Table: S1). A fusion PCR was performed by mixing both PCR products and reamplified using oligos G-21223 and G-21224. The 200 bp mutated fragment was cloned into pGEM-T-EASY and sequenced with the SP6 and T7 primers to confirm the presence of mutation at the site of the G-box. The 200bp fragments were end labelled using $^{32}P_{15}$ and purified by gel elution. The labeled DNA fragments were incubated with the protein in the presence of a Binding Buffer (10mM Tris pH 7.5, 50mM NaCl, 1mM DTT, 1mM EDTA, 50% Glycerol), 0.5µg/µl poly dIdC, 1mg/ml BSA at 4ºC. The DNA-

protein complex was resolved on a 5.5% Polyacrylamide Gel using 0.5x TBE buffer. The shift was visualized on a high performance autoradiography film Amersham Hyperfilm MP (GE Healthcare LTD) and developed using a C100 developer (AGFA).

6.12 Immunolocalisation and *in situ* hybridisation

The protocol described by Paciorek et al., (Paciorek et al., 2006) has been used for immunolocalisation studies. The antibody used was a FITC labeled anti GFP antibody (Abcam). The images were taken on an Axioplan II *imaging* microscope (Zeiss) using a GFP filter with an excitation wavelength of 495nm and an emission wavelength of 528nm.

In situ hybridization was performed according to previously published protocols (Balasubramanian and Schneitz, 2000) with certain modifications. For the Paraplast embedding steps, an automated tissue processor was used (Leica ASP 300). Tissue was collected at the same time in parallel, and sections were hybridized at the same time with the same probe preparation.

7.0 REFERENCES

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., and Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science *309*, 1052-1056.

Achard, P., Herr, A., Baulcombe, D., and Harberd, N. (2004). Modulation of floral development by a gibberellin-regulated microRNA. Development *131*, 3357-3365.

Achard, P., Liao, L., Jiang, C., Desnos, T., Bartlett, J., Fu, X., and Harberd, N. (2007). DELLAs contribute to plant photomorphogenesis. Plant Physiol *143*, 1163-1172.

Adams, S., Allen, T., and Whitelam, G.C. (2009). Interaction between the light quality and flowering time pathways in Arabidopsis. Plant J *60*, 257-267.

Adrian, J., Farrona, S., Reimer, J., Albani, M., Coupland, G., and Turck, F. (2010). cis-Regulatory elements and chromatin state coordinately control temporal and spatial expression of FLOWERING LOCUS T in Arabidopsis. Plant Cell.

Ahn, J., Miller, D., Winter, V., Banfield, M., Lee, J., Yoo, S., Henz, S., Brady, R., and Weigel, D. (2006). A divergent external loop confers antagonistic activity on floral regulators FT and TFL1. EMBO J *25*, 605-614.

Alexandre, C., and Hennig, L. (2008). FLC or not FLC: the other side of vernalization. J Exp Bot *59*, 1127-1135.

Alonso-Blanco, C., and Koornneef, M. (2000). Naturally occurring variation in Arabidopsis: an underexploited resource for plant genetics. Trends Plant Sci *5*, 22-29.

Alvey, L., and Harberd, N. (2005). DELLA proteins: integrators of multiple plant growth regulatory inputs? Physiologia Plantarum *123*, 153-160.

Amasino, R. (2010). Seasonal and developmental timing of flowering. Plant J *61*, 1001-1013.

An, H., Roussot, C., Suárez-López, P., Corbesier, L., Vincent, C., Piéeiro, M., Hepworth, S., Mouradov, A., Justin, S., Turnbull, C*., et al.* (2004). CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development *131*, 3615-3626.

Anderson, W.F., Ohlendorf, D.H., Takeda, Y., and Matthews, B.W. (1981). Structure of the cro repressor from bacteriophage lambda and its interaction with DNA. Nature *290*, 754-758.

Aukerman, M., Lee, I., Weigel, D., and Amasino, R. (1999). The Arabidopsis flowering-time gene LUMINIDEPENDENS is expressed primarily in regions of cell proliferation and encodes a nuclear protein that regulates LEAFY expression. Plant J *18*, 195-203.

Ausí-n, I., Alonso-Blanco, C., Jarillo, J., Ruiz-García, L., and Martínez-Zapater, J. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat Genet *36*, 162-166.

Ayre, B.G., and Turgeon, R. (2004). Graft transmission of a floral stimulant derived from CONSTANS. Plant Physiol *135*, 2271-2278.

Balasubramanian, S., and Schneitz, K. (2000). NOZZLE regulates proximal-distal pattern formation, cell proliferation and early sporogenesis during ovule development in Arabidopsis thaliana. Development *127*, 4227-4238.

Balasubramanian, S., Sureshkumar, S., Lempe, J., and Weigel, D. (2006). Potent induction of Arabidopsis thaliana flowering by elevated growth temperature. PLoS Genet *2*, e106.

Bäurle, I., Smith, L., Baulcombe, D., and Dean, C. (2007). Widespread role for the flowering-time regulators FCA and FPA in RNA-mediated chromatin silencing. Science *318*, 109-112.

Bernier, G., Havelange, A., Houssa, C., Petitjean, A., and Lejeune, P. (1993). Physiological Signals That Induce Flowering. Plant Cell *5*, 1147-1155.

Blázquez, M., Ahn, J., and Weigel, D. (2003). A thermosensory pathway controlling flowering time in Arabidopsis thaliana. Nat Genet *33*, 168-171.

Blázquez, M., Green, R., Nilsson, O., Sussman, M., and Weigel, D. (1998). Gibberellins promote flowering of arabidopsis by activating the LEAFY promoter. Plant Cell *10*, 791-800.

Blázquez, M., Soowal, L., Lee, I., and Weigel, D. (1997). LEAFY expression and flower initiation in Arabidopsis. Development *124*, 3835-3844.

Blázquez, M., and Weigel, D. (2000). Integration of floral inductive signals in Arabidopsis. Nature *404*, 889-892.

Blázquez, M.A., Ahn, J.H., and Weigel, D. (2003). A thermosensory pathway controlling flowering time in Arabidopsis thaliana. Nat Genet *33*, 168-171.

Bodson, M., and Outlaw, W.H. (1985). Elevation in the sucrose content of the shoot apical meristem of Sinapis alba at floral evocation. Plant Physiol *79*, 420-424.

Bohlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A.M., Jansson, S., Strauss, S.H., and Nilsson, O. (2006). CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. Science *312*, 1040-1043.

Bond, D., Dennis, E., Pogson, B., and Finnegan, E. (2009). Histone acetylation, VERNALIZATION INSENSITIVE 3, FLOWERING LOCUS C, and the vernalization response. Mol Plant *2*, 724-737.

Breitling, R., Armengaud, P., Amtmann, A., and Herzyk, P. (2004). Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett *573*, 83-92.

Cao, R., and Zhang, Y. (2004). The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev *14*, 155-164.

Castillejo, C., and Pelaz, S. (2008). The balance between CONSTANS and TEMPRANILLO activities determines FT expression to trigger flowering. Curr Biol *18*, 1338-1343.

Choi, K., Kim, S., Kim, S.Y., Kim, M., Hyun, Y., Lee, H., Choe, S., Kim, S.G., Michaels, S., and Lee, I. (2005). SUPPRESSOR OF FRIGIDA3 encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in Arabidopsis. Plant Cell *17*, 2647-2660.

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

Correa, L.G., Riano-Pachon, D.M., Schrago, C.G., dos Santos, R.V., Mueller-Roeber, B., and Vincentz, M. (2008). The role of bZIP transcription factors in green plant evolution: adaptive features emerging from four founder genes. PLoS One *3*, e2944.

de Lucas, M., Davióre, J., Rodriguez-Falcon, M., Pontin, M., Iglesias-Pedraz, J., Lorrain, S., Fankhauser, C., Blázquez, M., Titarenko, E., and Prat, S. (2008). A molecular framework for light and gibberellin control of cell elongation. Nature *451*, 480-484.

De Lucia, F., Crevillen, P., Jones, A.M., Greb, T., and Dean, C. (2008). A PHD-polycomb repressive complex 2 triggers the epigenetic silencing of FLC during vernalization. Proc Natl Acad Sci U S A *105*, 16831-16836.

De Lucia, F., and Dean, C. (2010). Long non-coding RNAs and chromatin regulation. Curr Opin Plant Biol.

Deal, R.B., Kandasamy, M.K., McKinney, E.C., and Meagher, R.B. (2005). The nuclear actin-related protein ARP6 is a pleiotropic developmental regulator required for the maintenance of FLOWERING LOCUS C expression and repression of flowering in Arabidopsis. Plant Cell *17*, 2633-2646.

Deppmann, C.D., Acharya, A., Rishi, V., Wobbes, B., Smeekens, S., Taparowsky, E.J., and Vinson, C. (2004). Dimerization specificity of all 67 B-ZIP motifs in Arabidopsis thaliana: a comparison to Homo sapiens B-ZIP motifs. Nucleic Acids Res *32*, 3435-3445.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res *19*, 1349.

Ehlert, A., Weltmeier, F., Wang, X., Mayer, C.S., Smeekens, S., Vicente-Carbajosa, J., and Droge-Laser, W. (2006). Two-hybrid protein-protein interaction analysis in Arabidopsis protoplasts: establishment of a heterodimerization map of group C and group S bZIP transcription factors. Plant J *46*, 890-900.

Endo, T., Shimada, T., Fujii, H., Kobayashi, Y., Araki, T., and Omura, M. (2005). Ectopic expression of an FT homolog from citrus confers an early flowering phenotype on trifoliate orange (Poncirus trifoliata L. Raf.). Transgenic Res *14*, 703-712.

Exner, V., Aichinger, E., Shu, H., Wildhaber, T., Alfarano, P., Caflisch, A., Gruissem, W., Kohler, C., and Hennig, L. (2009). The chromodomain of LIKE HETEROCHROMATIN PROTEIN 1 is essential for H3K27me3 binding and function during Arabidopsis development. PLoS One *4*, e5335.

Farrona, S., Coupland, G., and Turck, F. (2008). The impact of chromatin regulation on the floral transition. Semin Cell Dev Biol *19*, 560-573.

Ferrandiz, C., Liljegren, S.J., and Yanofsky, M.F. (2000). Negative regulation of the SHATTERPROOF genes by FRUITFULL during Arabidopsis fruit development. Science *289*, 436-438.

Finnegan, E.J., and Dennis, E.S. (2007). Vernalization-induced trimethylation of histone H3 lysine 27 at FLC is not maintained in mitotically quiescent cells. Curr Biol *17*, 1978-1983.

Fornara, F., de Montaigu, A., and Coupland, G. (2010). SnapShot: Control of flowering in Arabidopsis. Cell *141*, 550, 550.e551-552.

Fornara, F., Panigrahi, K., Gissot, L., Sauerbrunn, N., Röhl, M., Jarillo, J., and Coupland, G. (2009). Arabidopsis DOF transcription factors act redundantly to reduce CONSTANS expression and are essential for a photoperiodic flowering response. Dev Cell *17*, 75-86.

Fu, X., and Harberd, N.P. (2003). Auxin promotes Arabidopsis root growth by modulating gibberellin response. Nature *421*, 740-743.

Gendall, A., Levy, Y., Wilson, A., and Dean, C. (2001). The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell *107*, 525-535.

Geraldo, N., Báurle, I., Kidou, S., Hu, X., and Dean, C. (2009). FRIGIDA delays flowering in Arabidopsis via a cotranscriptional mechanism involving direct interaction with the nuclear cap-binding complex. Plant Physiol *150*, 1611-1618.

Gocal, G., Poole, A., Gubler, F., Watts, R., Blundell, C., and King, R. (1999). Long-day up-regulation of a GAMYB gene during Lolium temulentum inflorescence formation. Plant Physiol *119*, 1271-1278.

Gocal, G., Sheldon, C., Gubler, F., Moritz, T., Bagnall, D., MacMillan, C., Li, S., Parish, R., Dennis, E., Weigel, D*., et al.* (2001). GAMYB-like genes, flowering, and gibberellin signaling in Arabidopsis. Plant Physiol *127*, 1682-1693.

Griffiths, J., Murase, K., Rieu, I., Zentella, R., Zhang, Z., Powers, S., Gong, F., Phillips, A., Hedden, P., Sun, T*., et al.* (2006). Genetic characterization and functional analysis of the GID1 gibberellin receptors in Arabidopsis. Plant Cell *18*, 3399-3414.

Halliday, K.J., Salter, M.G., Thingnaes, E., and Whitelam, G.C. (2003). Phytochrome control of flowering is temperature sensitive and correlates with expression of the floral integrator FT. Plant J *33*, 875-885.

Hanada, K., Sawada, Y., Kuromori, T., Klausnitzer, R., Saito, K., Toyoda, T., Shinozaki, K., Li, W.H., and Hirai, M.Y. (2011). Functional compensation of primary and secondary metabolites by duplicate genes in Arabidopsis thaliana. Mol Biol Evol *28*, 377-382.

Hanas, J.S., Hazuda, D.J., Bogenhagen, D.F., Wu, F.Y., and Wu, C.W. (1983). Xenopus transcription factor A requires zinc for binding to the 5 S RNA gene. J Biol Chem *258*, 14120-14125.

Harberd, N., Belfield, E., and Yasumura, Y. (2009). The angiosperm gibberellin-GID1-DELLA growth regulatory mechanism: how an "inhibitor of an inhibitor" enables flexible response to fluctuating environments. Plant Cell *21*, 1328-1339.

Harmer, S., Hogenesch, J., Straume, M., Chang, H., Han, B., Zhu, T., Wang, X., Kreps, J., and Kay, S. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science *290*, 2110-2113.

Hartmann, U., Höhmann, S., Nettesheim, K., Wisman, E., Saedler, H., and Huijser, P. (2000). Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. Plant J *21*, 351-360.

He, Y., Michaels, S., and Amasino, R. (2003). Regulation of flowering time by histone acetylation in Arabidopsis. Science *302*, 1751-1754.

Hecht, V., Foucher, F., Ferrandiz, C., Macknight, R., Navarro, C., Morin, J., Vardy, M.E., Ellis, N., Beltran, J.P., Rameau, C*., et al.* (2005). Conservation of Arabidopsis flowering genes in model legumes. Plant Physiol *137*, 1420-1434.

Helliwell, C., Wood, C., Robertson, M., James Peacock, W., and Dennis, E. (2006). The Arabidopsis FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. Plant J *46*, 183-192.

Hennig, L., and Derkacheva, M. (2009). Diversity of Polycomb group complexes in plants: same rules, different players? Trends Genet *25*, 414-423.

Heo, J., and Sung, S. (2011). Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. Science *331*, 76-79.

Hepworth, S.R., Valverde, F., Ravenscroft, D., Mouradov, A., and Coupland, G. (2002). Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. EMBO J *21*, 4327-4337.

Hisamatsu, T., and King, R. (2008). The nature of floral signals in Arabidopsis. II. Roles for FLOWERING LOCUS T (FT) and gibberellin. J Exp Bot *59*, 3821-3829.

Hoecker, U., and Quail, P. (2001). The phytochrome A-specific signaling intermediate SPA1 interacts directly with COP1, a constitutive repressor of light signaling in Arabidopsis. J Biol Chem *276*, 38173-38178.

Hornyik, C., Terzi, L.C., and Simpson, G.G. (2010). The spen family protein FPA controls alternative cleavage and polyadenylation of RNA. Dev Cell *18*, 203-213.

Igasaki, T., Watanabe, Y., Nishiguchi, M., and Kotoda, N. (2008). The FLOWERING LOCUS T/TERMINAL FLOWER 1 family in Lombardy poplar. Plant Cell Physiol *49*, 291-300.

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

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

Izawa, T., Foster, R., and Chua, N.H. (1993). Plant bZIP protein DNA binding specificity. J Mol Biol *230*, 1131-1144.

Jacobsen, S.E., and Olszewski, N.E. (1993). Mutations at the SPINDLY locus of Arabidopsis alter gibberellin signal transduction. Plant Cell *5*, 887-896.

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

Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T., and Parcy, F. (2002). bZIP transcription factors in Arabidopsis. Trends Plant Sci *7*, 106-111.

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

Jiang, D., Wang, Y., and He, Y. (2008). Repression of FLOWERING LOCUS C and FLOWERING LOCUS T by the Arabidopsis Polycomb repressive complex 2 components. PLoS One *3*, e3404.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. (2000). Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. Science *290*, 344-347.

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

Kaufmann, K., Wellmer, F., Muio, J., Ferrier, T., Wuest, S., Kumar, V., Serrano-Mislata, A., Madueo, F., Krajewski, P., Meyerowitz, E.*, et al.* (2010). Orchestration of floral initiation by APETALA1. Science *328*, 85-89.

Kaya, H., Sato, S., Tabata, S., Kobayashi, Y., Iwabuchi, M., and Araki, T. (2000). hosoba toge toge, a syndrome caused by a large chromosomal deletion associated with a T-DNA insertion in Arabidopsis. Plant Cell Physiol *41*, 1055-1066.

Kim, S., Choi, K., Park, C., Hwang, H.J., and Lee, I. (2006a). SUPPRESSOR OF FRIGIDA4, encoding a C2H2-Type zinc finger protein, represses flowering by transcriptional activation of Arabidopsis FLOWERING LOCUS C. Plant Cell *18*, 2985-2998.

Kim, S., Soltis, P.S., Wall, K., and Soltis, D.E. (2006b). Phylogeny and domain evolution in the APETALA2-like gene family. Mol Biol Evol *23*, 107-120.

King, R.W., Moritz, T., Evans, L.T., Junttila, O., and Herlt, A.J. (2001). Long-day induction of flowering in Lolium temulentum involves sequential increases in specific gibberellins at the shoot apex. Plant Physiol *127*, 624-632.

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

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

Kohler, C., Hennig, L., Bouveret, R., Gheyselinck, J., Grossniklaus, U., and Gruissem, W. (2003). Arabidopsis MSI1 is a component of the MEA/FIE Polycomb group complex and required for seed development. EMBO J *22*, 4804-4814.

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T., and Yano, M. (2002). Hd3a, a rice ortholog of the Arabidopsis FT gene, promotes transition to flowering downstream of Hd1 under short-day conditions. Plant Cell Physiol *43*, 1096-1105.

Koornneef M, Blankestijn-de Vries H, Hanhart C, Soppe W, and T, P. (1994). The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsbergerecta wild-type. . Plant Journal *6*, 911-919.

Koornneef, M., Alonso-Blanco, C., Blankestijn-de Vries, H., Hanhart, C., and Peeters, A. (1998). Genetic interactions among late-flowering mutants of Arabidopsis. Genetics *148*, 885-892.

Koornneef, M., Elgersma, A., Hanhart, C.J., Vanloenenmartinet, E.P., Vanrijn, L., and Zeevaart, J.A.D. (1985). A gibberellin insensitive mutant of Arabidopsis thaliana. Physiologia Plantarum *65*, 33-39.

Koornneef, M., Hanhart, C., and van der Veen, J. (1991). A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana. Mol Gen Genet *229*, 57-66.

Kumar, S., and Wigge, P. (2010). H2A.Z-containing nucleosomes mediate the thermosensory response in Arabidopsis. Cell *140*, 136-147.

Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science *240*, 1759-1764.

Lang, A. (1957). The effect of fibberellin upon flower formation. Proc Natl Acad Sci U S A *43*, 709-717.

Lang, A. (1960). Gibberellin-like substances in photoinduced and vegetative Hyoscyamus plants. Planta *54*, 498-504.

Lariguet, P., and Dunand, C. (2005). Plant photoreceptors: phylogenetic overview. J Mol Evol *61*, 559-569.

Laubinger, S., and Hoecker, U. (2003). The SPA1-like proteins SPA3 and SPA4 repress photomorphogenesis in the light. Plant J *35*, 373-385.

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

Lee I, Michaels SD, Masshardt AS, and RM, A. (1994). The late-flowering phenotype of FRIGIDA and LUMINIDEPENDENS is suppressed in the Landsberg erecta strain of Arabidopsis. Plant Journal *6*, 903-909.

Lee, I., Aukerman, M., Gore, S., Lohman, K., Michaels, S., Weaver, L., John, M., Feldmann, K., and Amasino, R. (1994). Isolation of LUMINIDEPENDENS: a gene involved in the control of flowering time in Arabidopsis. Plant Cell *6*, 75-83.

Lee, J., Oh, M., Park, H., and Lee, I. (2008). SOC1 translocated to the nucleus by interaction with AGL24 directly regulates leafy. Plant J *55*, 832-843.

Lee, J., Yoo, S., Park, S., Hwang, I., Lee, J., and Ahn, J. (2007). Role of SVP in the control of flowering time by ambient temperature in Arabidopsis. Genes Dev *21*, 397-402.

Lee, J.-Y., and Zhou, J. (2012). Function and Identification of Mobile Transcription Factors. Advances in Plant Biology *3*, 61-86.

Levy, Y., Mesnage, S., Mylne, J., Gendall, A., and Dean, C. (2002). Multiple roles of Arabidopsis VRN1 in vernalization and flowering time control. Science *297*, 243-246.

Li, C., and Dubcovsky, J. (2008). Wheat FT protein regulates VRN1 transcription through interactions with FDL2. Plant J *55*, 543-554.

Li, D., Liu, C., Shen, L., Wu, Y., Chen, H., Robertson, M., Helliwell, C., Ito, T., Meyerowitz, E., and Yu, H. (2008). A repressor complex governs the integration of flowering signals in Arabidopsis. Dev Cell *15*, 110-120.

Li, Q., and Yang, H. (2007). Cryptochrome signaling in plants. Photochem Photobiol *83*, 94-101.

Lifschitz, E., and Eshed, Y. (2006). Universal florigenic signals triggered by FT homologues regulate growth and flowering cycles in perennial day-neutral tomato. J Exp Bot *57*, 3405-3414.

Lifschitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z., Alvarez, J., and Eshed, Y. (2006). The tomato FT ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. Proc Natl Acad Sci U S A *103*, 6398-6403.

Lim, M.H., Kim, J., Kim, Y.S., Chung, K.S., Seo, Y.H., Lee, I., Hong, C.B., Kim, H.J., and Park, C.M. (2004). A new Arabidopsis gene, FLK, encodes an RNA binding protein with K homology motifs and regulates flowering time via FLOWERING LOCUS C. Plant Cell *16*, 731-740.

Lin, M., Belanger, H., Lee, Y., Varkonyi-Gasic, E., Taoka, K., Miura, E., Xoconostle-Coares, B., Gendler, K., Jorgensen, R., Phinney, B.*, et al.* (2007). FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. Plant Cell *19*, 1488-1506.

Liu, C., Chen, H., Er, H., Soo, H., Kumar, P., Han, J., Liou, Y., and Yu, H. (2008). Direct interaction of AGL24 and SOC1 integrates flowering signals in Arabidopsis. Development *135*, 1481-1491.

Liu, C., Xi, W., Shen, L., Tan, C., and Yu, H. (2009). Regulation of floral patterning by flowering time genes. Dev Cell *16*, 711-722.

Liu, F., Marquardt, S., Lister, C., Swiezewski, S., and Dean, C. (2010). Targeted 3' processing of antisense transcripts triggers Arabidopsis FLC chromatin silencing. Science *327*, 94-97.

Liu, F., Quesada, V., Crevillón, P., Bäurle, I., Swiezewski, S., and Dean, C. (2007a). The Arabidopsis RNA-binding protein FCA requires a lysine-specific demethylase 1 homolog to downregulate FLC. Mol Cell *28*, 398-407.

Liu, L., Liu, C., Hou, X., Xi, W., Shen, L., Tao, Z., Wang, Y., and Yu, H. (2012). FTIP1 is an essential regulator required for florigen transport. PLoS Biol *10*, e1001313.

Liu, Z., and Mara, C. (2010). Regulatory mechanisms for floral homeotic gene expression. Semin Cell Dev Biol *21*, 80-86.

Lohmann, J.U., Hong, R.L., Hobe, M., Busch, M.A., Parcy, F., Simon, R., and Weigel, D. (2001). A molecular link between stem cell regulation and floral patterning in Arabidopsis. Cell *105*, 793-803.

Lu, K.-J., Huang, N.-C., Liu, Y.-S., Lu, C.-A., and Yu, T.-S. (2012). Long-distance movement of Arabidopsis Flowering Locus T RNA participates in systemic floral regulation. RNA biology *9*, 653-662.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C.*, et al.* (1997). FCA, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. Cell *89*, 737-745.

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

Mathieu, J., Yant, L., Mürdter, F., Küttner, F., and Schmid, M. (2009). Repression of flowering by the miR172 target SMZ. PLoS Biol *7*, e1000148.

Mayfield, J.D., Folta, K.M., Paul, A.L., and Ferl, R.J. (2007). The 14-3-3 Proteins mu and upsilon influence transition to flowering and early phytochrome response. Plant Physiol *145*, 1692-1702.

Michaels, S., and Amasino, R. (1999). FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell *11*, 949-956.

Michaels, S., and Amasino, R. (2001). Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. Plant Cell *13*, 935-941.

Michaels, S.D., Ditta, G., Gustafson-Brown, C., Pelaz, S., Yanofsky, M., and Amasino, R.M. (2003). AGL24 acts as a promoter of flowering in Arabidopsis and is positively regulated by vernalization. Plant J *33*, 867-874.

Moon, J., Suh, S., Lee, H., Choi, K., Hong, C., Paek, N., Kim, S., and Lee, I. (2003). The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. Plant J *35*, 613-623.

Moyroud, E., Kusters, E., Monniaux, M., Koes, R., and Parcy, F. (2010). LEAFY blossoms. Trends Plant Sci *15*, 346-352.

Murase, K., Hirano, Y., Sun, T., and Hakoshima, T. (2008). Gibberellin-induced DELLA recognition by the gibberellin receptor GID1. Nature *456*, 459-463.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and Bio Assays with tobacco tissue cultures. Physiol Plant *15*, 473-497.

Napp-Zinn, K. (1987). Vernalization, Environmental and genetic regulation. In Manipulation of flowering, J.G. Atherton, ed. (Butterworths, London), pp. 123-132.

Navarro, C., Abelenda, J., Cruz-Oró, E., Cu´ollar, C., Tamaki, S., Silva, J., Shimamoto, K., and Prat, S. (2011). Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. Nature *478*, 119-122.

Noh, B., Lee, S.H., Kim, H.J., Yi, G., Shin, E.A., Lee, M., Jung, K.J., Doyle, M.R., Amasino, R.M., and Noh, Y.S. (2004). Divergent roles of a pair of homologous jumonji/zinc-finger-class transcription factor proteins in the regulation of Arabidopsis flowering time. Plant Cell *16*, 2601-2613.

Notaguchi, M., Abe, M., Kimura, T., Daimon, Y., Kobayashi, T., Yamaguchi, A., Tomita, Y., Dohi, K., Mori, M., and Araki, T. (2008). Long-distance, graft-transmissible action of Arabidopsis FLOWERING LOCUS T protein to promote flowering. Plant Cell Physiol *49*, 1645-1658.

Oda, A., Fujiwara, S., Kamada, H., Coupland, G., and Mizoguchi, T. (2004). Antisense suppression of the Arabidopsis PIF3 gene does not affect circadian rhythms but causes early flowering and increases FT expression. FEBS Lett *557*, 259-264.

Osterlund, M.T., Wei, N., and Deng, X.W. (2000). The roles of photoreceptor systems and the COP1-targeted destabilization of HY5 in light control of Arabidopsis seedling development. Plant Physiol *124*, 1520-1524.

Paciorek, T., Sauer, M., Balla, J., Wiõniewska, J., and Friml, J. (2006). Immunocytochemical technique for protein localization in sections of plant tissues. Nat Protoc *1*, 104-107.

Palatnik, J., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature *425*, 257-263.

Parcy, F., Nilsson, O., Busch, M., Lee, I., and Weigel, D. (1998). A genetic framework for floral patterning. Nature *395*, 561-566.

Park, W., Li, J., Song, R., Messing, J., and Chen, X. (2002). CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Curr Biol *12*, 1484-1495.

Pin, P., Benlloch, R., Bonnet, D., Wremerth-Weich, E., Kraft, T., Gielen, J., and Nilsson, O. (2010). An antagonistic pair of FT homologs mediates the control of flowering time in sugar beet. Science *330*, 1397-1400.

Pnueli, L., Gutfinger, T., Hareven, D., Ben-Naim, O., Ron, N., Adir, N., and Lifschitz, E. (2001). Tomato SP-interacting proteins define a conserved signaling system that regulates shoot architecture and flowering. Plant Cell *13*, 2687-2702.

Purwestri, Y., Ogaki, Y., Tamaki, S., Tsuji, H., and Shimamoto, K. (2009). The 14-3-3 protein GF14c acts as a negative regulator of flowering in rice by interacting with the florigen Hd3a. Plant Cell Physiol *50*, 429-438.

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

Quail, P., Boylan, M., Parks, B., Short, T., Xu, Y., and Wagner, D. (1995). Phytochromes: photosensory perception and signal transduction. Science *268*, 675-680.

Rédei, G. (1962). Supervital Mutants of Arabidopsis. Genetics *47*, 443-460.

Richter, R., Behringer, C., Müller, I., and Schwechheimer, C. (2010). The GATA-type transcription factors GNC and GNL/CGA1 repress gibberellin signaling downstream from DELLA proteins and PHYTOCHROME-INTERACTING FACTORS. Genes Dev *24*, 2093-2104.

Samach, A., Onouchi, H., Gold, S., Ditta, G., Schwarz-Sommer, Z., Yanofsky, M., and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science *288*, 1613-1616.

Sawa, M., and Kay, S.A. (2011). GIGANTEA directly activates Flowering Locus T in Arabidopsis thaliana. Proc Natl Acad Sci U S A *108*, 11698-11703.

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

Schläppi, M., and Patel, M. (2001). Biennialism and vernalization-promoted flowering in Hyoscyamus niger: a comparison with Arabidopsis. Flowering Newslet *31*, 25-32.

Schubert, D., Primavesi, L., Bishopp, A., Roberts, G., Doonan, J., Jenuwein, T., and Goodrich, J. (2006). Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. EMBO J *25*, 4638-4649.

Schwechheimer, C., and Willige, B. (2009). Shedding light on gibberellic acid signalling. Curr Opin Plant Biol *12*, 57-62.

Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Krüber, S., Amasino, R., and Coupland, G. (2006). The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. Genes Dev *20*, 898-912.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999). The FLF MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell *11*, 445-458.

Shimada, A., Ueguchi-Tanaka, M., Sakamoto, T., Fujioka, S., Takatsuto, S., Yoshida, S., Sazuka, T., Ashikari, M., and Matsuoka, M. (2006). The rice SPINDLY gene functions as a negative regulator of gibberellin signaling by controlling the suppressive function of the DELLA protein, SLR1, and modulating brassinosteroid synthesis. Plant J *48*, 390-402.

Silverstone, A., Ciampaglio, C., and Sun, T. (1998). The Arabidopsis RGA gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. Plant Cell *10*, 155-169.

Simpson, G. (2004). The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. Curr Opin Plant Biol *7*, 570-574.

Simpson, G., and Dean, C. (2002). Arabidopsis, the Rosetta stone of flowering time? Science *296*, 285-289.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I., and Dean, C. (2003). FY is an RNA 3' end-processing factor that interacts with FCA to control the Arabidopsis floral transition. Cell *113*, 777-787.

Srikanth, A., and Schmid, M. (2011). Regulation of flowering time: all roads lead to Rome. Cell Mol Life Sci *68*, 2013-2037.

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

Sun, T. (2010). Gibberellin-GID1-DELLA: a pivotal regulatory module for plant growth and development. Plant Physiol *154*, 567-570.

Sun, T., Goodman, H., and Ausubel, F. (1992). Cloning the Arabidopsis GA1 Locus by Genomic Subtraction. Plant Cell *4*, 119-128.

Sung, S., and Amasino, R. (2004a). Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature *427*, 159-164.

Sung, S., and Amasino, R.M. (2004b). Vernalization and epigenetics: how plants remember winter. Curr Opin Plant Biol *7*, 4-10.

Swain, S., Tseng , T., and Olszewski, N. (2001). Altered expression of SPINDLY affects gibberellin response and plant development. Plant Physiol *126*, 1174-1185.

Swiezewski, S., Liu, F., Magusin, A., and Dean, C. (2009). Cold-induced silencing by long antisense transcripts of an Arabidopsis Polycomb target. Nature *462*, 799-802.

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

Tamaki, S., Matsuo, S., Wong, H., Yokoi, S., and Shimamoto, K. (2007). Hd3a protein is a mobile flowering signal in rice. Science *316*, 1033-1036.

Taoka, K., Ohki, I., Tsuji, H., Furuita, K., Hayashi, K., Yanase, T., Yamaguchi, M., Nakashima, C., Purwestri, Y., Tamaki, S.*, et al.* (2011). 14-3-3 proteins act as intracellular receptors for rice Hd3a florigen. Nature *476*, 332-335.

Teper-Bamnolker, P., and Samach, A. (2005). The flowering integrator FT regulates SEPALLATA3 and FRUITFULL accumulation in Arabidopsis leaves. Plant Cell *17*, 2661-2675.

Torki, M., Mandaron, P., Mache, R., and Falconet, D. (2000). Characterization of a ubiquitous expressed gene family encoding polygalacturonase in Arabidopsis thaliana. Gene *242*, 427-436.

Turck, F., Fornara, F., and Coupland, G. (2008). Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol *59*, 573-594.

Turck, F., Roudier, F., Farrona, S., Martin-Magniette, M.L., Guillaume, E., Buisine, N., Gagnot, S., Martienssen, R.A., Coupland, G., and Colot, V. (2007). Arabidopsis TFL2/LHP1 specifically associates with genes marked by trimethylation of histone H3 lysine 27. PLoS Genet *3*, e86.

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

Wagner, D. (2009). Flower morphogenesis: timing is key. Dev Cell *16*, 621-622.

Wang, H., Ma, L., Li, J., Zhao, H., and Deng, X. (2001). Direct interaction of Arabidopsis cryptochromes with COP1 in light control development. Science *294*, 154-158.

Wang, J., Czech, B., and Weigel, D. (2009a). miR156-regulated SPL transcription factors define an endogenous flowering pathway in Arabidopsis thaliana. Cell *138*, 738-749.

Wang, R., Farrona, S., Vincent, C., Joecker, A., Schoof, H., Turck, F., Alonso-Blanco, C., Coupland, G., and Albani, M. (2009b). PEP1 regulates perennial flowering in Arabis alpina. Nature *459*, 423-427.

Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M.*, et al.* (2000). Activation tagging in Arabidopsis. Plant Physiol *122*, 1003-1013.

Weigel, D., Alvarez, J., Smyth, D., Yanofsky, M., and Meyerowitz, E. (1992). LEAFY controls floral meristem identity in Arabidopsis. Cell *69*, 843-859.

Weigel, D., and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. Nature *377*, 495-500.

Weltmeier, F., Ehlert, A., Mayer, C.S., Dietrich, K., Wang, X., Schutze, K., Alonso, R., Harter, K., Vicente-Carbajosa, J., and Droge-Laser, W. (2006). Combinatorial control of Arabidopsis proline dehydrogenase transcription by specific heterodimerisation of bZIP transcription factors. EMBO J *25*, 3133-3143.

Werner, J.D., Borevitz, J.O., Uhlenhaut, N.H., Ecker, J.R., Chory, J., and Weigel, D. (2005). FRIGIDA-independent variation in flowering time of natural Arabidopsis thaliana accessions. Genetics *170*, 1197-1207.

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

Willige, B., Ghosh, S., Nill, C., Zourelidou, M., Dohmann, E., Maier, A., and Schwechheimer, C. (2007). The DELLA domain of GA INSENSITIVE mediates the interaction with the GA INSENSITIVE DWARF1A gibberellin receptor of Arabidopsis. Plant Cell *19*, 1209-1220.

Wilson, R., Heckman, J., and Somerville, C. (1992). Gibberellin Is Required for Flowering in Arabidopsis thaliana under Short Days. Plant Physiol *100*, 403-408.

Wood, C.C., Robertson, M., Tanner, G., Peacock, W.J., Dennis, E.S., and Helliwell, C.A. (2006). The Arabidopsis thaliana vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. Proc Natl Acad Sci U S A *103*, 14631-14636.

Wu, X., Dinneny, J.R., Crawford, K.M., Rhee, Y., Citovsky, V., Zambryski, P.C., and Weigel, D. (2003). Modes of intercellular transcription factor movement in the Arabidopsis apex. Development *130*, 3735-3745.

Yamaguchi, A., Kobayashi, Y., Goto, K., Abe, M., and Araki, T. (2005). TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. Plant Cell Physiol *46*, 1175-1189.

Yamaguchi, A., Wu, M.F., Yang, L., Wu, G., Poethig, R.S., and Wagner, D. (2009). The microRNA-regulated SBP-Box transcription factor SPL3 is a direct upstream activator of LEAFY, FRUITFULL, and APETALA1. Dev Cell *17*, 268-278.

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

Yoshida, N., Yanai, Y., Chen, L., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z., and Takahashi, S. (2001). EMBRYONIC FLOWER2, a novel polycomb group protein homolog, mediates shoot development and flowering in Arabidopsis. Plant Cell *13*, 2471-2481.

Yu, H., Ito, T., Wellmer, F., and Meyerowitz, E. (2004). Repression of AGAMOUS-LIKE 24 is a crucial step in promoting flower development. Nat Genet *36*, 157-161.

Yu, H., Xu, Y., Tan, E., and Kumar, P. (2002). AGAMOUS-LIKE 24, a dosage-dependent mediator of the flowering signals. Proc Natl Acad Sci U S A *99*, 16336-16341.

Zhang, X., Clarenz, O., Cokus, S., Bernatavichute, Y.V., Pellegrini, M., Goodrich, J., and Jacobsen, S.E. (2007). Whole-genome analysis of histone H3 lysine 27 trimethylation in Arabidopsis. PLoS Biol *5*, e129.

8.0 SUPPLEMENTARY INFORMATION

Supplementary Table S1: ***Oligos for cloning and EMSA.***

Oligos for cloning		
G-12223	GR-fwd N term	Agg cct ATG CCT GAA GCT CGA AAA ACA AAG
G-12224	GR-rev N term	agg cct ccc ggg gga ccc tga acc aga tcc TTT TTG ATG AAA CAG AAG CTT TTT G
G-12225	GR-fwd C term	Agg cct gga tct ggt tca ggg tcc GAT CCT GAA GCT CGA AAA AC
G-12226	GR-rev C term	Agg cct TCA TTT TTG ATG AAA CAG AAG CTT TTT G
G-17681	Split Citrine	tca cgt gTT AGG CCA TGA TAT AGA CGT TGT G
G-17682	Split Citrine	gcg ata tcc cgg gGA CAA GCA GAA GAA CGG CAT CAA G
G-17683	Split Citrine	gat atc ccg ggG AGC AAA AGT TGA TTT CTG AGG AGG ATC TTG TGA GCA AGG GCG AGG AG
G-17684	Split Citrine	gcg ata tcc cgg gTA CCC ATA CGA TGT TCC AGA TTA CGC TGA CAA GCA GAA GAAC GGC ATC AAG
G-5636	FD fwd	ATG TTG TCA TCA GCT AAG CA
G-5637	FD rev	AAA ATG GAG CTG TGG AAG AC
G-32986	FT rev	CTA AAG TCT TCT TCC TCC GCA GCC
G-32987	FT fwd	ATG TCT ATA AAT ATA AGA GAC CC
Oligos for EMSA		
G-22808	SEP3 mut EMSA	ctc gag tcc aaa ctc att cat tgc
G-22809	SEP3 mut EMSA	ctc gag tcg aca tct cga tgg tag
G-21223	SEP3 promoter	gtt gaa aat gag aga tta ata atg
G-21224	Sep3 promoter rev	ctg aac tcg att tta taa gta aaa

Supplementary Table S2: *Oligos for qPCR*

Oligos for qPCR		
N-0078	*Tubulin*	GAG CCT TAC AAC GCTACT CTG TCT GTC
N-0079	*Tubulin*	ACA CA GAC ATA GTA GCA GAA ATC AAG
G-0626	*FT*	TCC CTG CTA CAA CTG GAA CAA CCT TTG
G-0627	*FT*	CGC AGC CAC TCT CCC TCT GAC AAT TGT
G-0628	*SOC1*	ATA GGA ACA TGC TCA ATC GAG GAG CTG
G-0629	*SOC1*	TTT CTT GAA GAA CAA GGT AAC CCA ATG
G-0634	*AP1*	AGG GAA AAA ATT CTT AGG GCT CAA CAG
G-0635	*AP1*	GCG GCG AAG CAG CCA AGG TTG CAG TTG
G-0640	*AG*	AGA TTA GAG AGA AGT ATT ACC CGA ATC
G-0641	*AG*	GTC TTG GCG ACC CGC GGA TGA GTA ATG
G-0644	*SEP2*	ATG ATT GGT GTG AGA AGT CAT CAT ATG
G-0645	*SEP1*	GAT GTA ACC GTT TCC CTG CTG CGC CTG
G-0646	*SEP2*	ATC AAC AGA ATA TTG CCT ATG GAC ATC
G-0647	*SEP2*	GAT GTA GCC GTT TCC TTG TTG GGA CTG
G-0648	*SEP3*	GGG TAT CAG ATG CCA CTC CAG CTG AAC
G-0649	*SEP3*	AAC CCA ACA TGT AAT TAT TCA CAC TTG
G-0654	*FUL*	TTG CAA GAT CAC AAC AAT TCG CTT CTC
G-0655	*FUL*	GAG AGT TTG GTT CCG TCA ACG ACG ATG
G-34035	*MLP168*	ATGGTAGAGGCAGAGGTTG
G-34036	*MLP168*	GCGATTGATATGAAGATGAA
G-34037	*VGDH1*	TTCGACGGTTATCAAGACAC
G-34038	*VGDH1*	CGACCACCGTGATAATGAGC
G-34039	*SLR1*	TTCTCGTTCAAACTCGGAAC
G-34040	*SLR1*	TATCCAGATGGGATACGAC
G-34041	*AT2G47050.1*	ACCAAAAAGCCGTCGATGG
G-34042	*AT2G47050.1*	GCATCACCGGCTGCGAGAAAC
G-34043	*SKS13*	CTGCTAGACC TAACCCCCAG
G-34044	*SKS13*	CCTCAACATCACTTTCC
G-34045	*AT5G07410.1*	TGGTAAAACTAAGGGAGCAC
G-34046	*AT5G07410.1*	TGGTAAAACTAAGGGAGCAC
G-34047	*PDF1*	AACTCCTATCATTGACCCAG
G-34048	*PDF1*	CGTGAAGGCACAGCTTCTTG
G-34049	*AT3G28830.1*	GTGGGGCAATGGCTATGTCC
G-34050	*AT3G28830.1*	CATGCAAGCTTCCGCAAGCG
G-34051	*PGA4*	ACATGGGGTGGGTCAGACCC

G-34052	*PGA4*	CAAATCGGTGCCGTGCCAAG
G-34053	*AT3G28750.1*	AGACCTCGCTGTTAAGTTGG
G-34054	*AT3G28750.1*	AGTGCAGCACGAAATTCTCT
G-34055	*AT3G28980.1*	GTGAGAGTAAGACTTCCGC
G-34056	*AT3G28980.1*	GTTCTCTAGGATTAAAACTG
G-34057	*AT3G01270.1*	CTCGACTGACGTCGAGGGG
G-34058	*AT3G01270.1*	GCCTGATTGGGCCAGACG
G-34059	*GRP17*	GCAGATTTTTTCCTTCTC
G-34060	*GRP17*	GGCGACAGGATTCACGGCCG
G-34061	*PI*	ATGAGAACCTTAGCAATGAG
G-34062	*PI*	GGAGATGGCTATAGCAAGC
G-34063	*LTP6*	ATGAGATCTCTCTTATTAGC
G-34064	*LTP6*	CTGTCGTTGCATCAAATCTG
G-34065	*VGD1*	GTCTCCCCAAATGAAAGCG
G-34066	*VGD1*	GCTTGAGGATCTCAGCACC

Supplementary Table: S3- ***Summary of crosses***

Cross	Parent1	Genotype	Parent 2	Genotype
pFK428-8	pFK428-8	*gFT-TEV*	pFK428-8	*gFT-TEV*
pFK428-9	pFK428-9	*gFT-TEV*	pFK428-9	*gFT-TEV*
pFK429-27	pFK429-27	*gFT-TEV-3xYFP*	pFK429-27	*gFT-TEV-3xYFP*
pFK429-29	pFK429-29	*gFT-TEV-3xYFP*	pFK429-29	*gFT-TEV-3xYFP*
#190-10	pJV268-1	*SUC2::TEV-TdTomato*	pJV268-1	*SUC2::TEV-TdTomato*
#190-12	pJV268-1	*SUC2::TEV-TdTomato*	pJV268-1	*SUC2::TEV-TdTomato*
pJV268-3	pJV268-3	*SUC2::TEV-TdTomato*	pJV268-3	*SUC2::TEV-TdTomato*
pFK491-6	pFK491-6	*SUC2-TEV*	pFK491-6	*SUC2-TEV*
#191	pFK428-8	*gFT-TEV*	#190-10	*SUC2::TEV-TdTomato*
#192	pFK428-8	*gFT-TEV*	#190-12	*SUC2::TEV-TdTomato*
#193	pFK428-9	*gFT-TEV*	#190-10	*SUC2::TEV-TdTomato*
#194	pFK428-9	*gFT-TEV*	#190-12	*SUC2::TEV-TdTomato*
#195	pFK428-8	*gFT-TEV*	pJV268-3	*SUC2::TEV-TdTomato*
#197	pFK428-9	*gFT-TEV*	pJV268-3	*SUC2::TEV-TdTomato*
#199	pFK428-8	*gFT-TEV*	pFK491-6	*SUC2-TEV*
#201	pFK428-9	*gFT-TEV*	pFK491-6	*SUC2-TEV*
#203	pFK429-27	*gFT-TEV-3xYFP*	#190-10	*SUC2::TEV-TdTomato*
#204	pFK429-27	*gFT-TEV-3xYFP*	#190-12	*SUC2::TEV-TdTomato*
#205	pFK429-29	*gFT-TEV-3xYFP*	#190-10	*SUC2::TEV-TdTomato*
#206	pFK429-29	*gFT-TEV-3xYFP*	#190-12	*SUC2::TEV-TdTomato*
#207	pFK429-27	*gFT-TEV-3xYFP*	pJV268-3	*SUC2::TEV-TdTomato*
#209	pFK429-29	*gFT-TEV-3xYFP*	pJV268-3	*SUC2::TEV-TdTomato*
#211	pFK429-27	*gFT-TEV-3xYFP*	pFK491-6	*SUC2-TEV*
#213	pFK429-29	*gFT-TEV-3xYFP*	pFK491-6	*SUC2-TEV*

Supplementary folder S4 titled 'RNA seq data' (Available only as an electronic copy)

Contains:

- cyc.dc.all.txt
- cyc.dc.q0.01.log2is1.txt
- cyc.dc.q0.05.log2is1.txt
- cyc.dex.all.txt
- cyc.dex.q0.01.log2is1.txt
- cyc.dex.q0.05.log2is1.txt
- cyc.mock.all.txt
- cyc.mock.q0.01.log2is1.txt
- cyc.mock.q0.05.log2is1.txt
- dc.dex.all.txt
- dc.dex.q0.01.log2is1.txt
- dc.dex.q0.05.log2is1.txt
- dc.mock.all.txt
- dc.mock.q0.01.log2is1.txt
- dc.mock.q0.05.log2is1.txt
- DE_genes.q0.01.log2is1.txt
- DE_genes.q0.05.log2is1.txt
- dex.mock.all.txt
- dex.mock.q0.01.log2is1.txt
- dex.mock.q0.05.log2is1.txt

The lists indicate genes that are differentially expressed between the dexamethasone (dex), dexamethasone+cycloheximide (dc), mock (mock) and cycloheximide (cyc) treated samples. These lists were prepared by C. Becker.