

Regulation of the floral repressor gene *FLC*: the complexity of transcription in a chromatin context

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The genetic pathways regulating the floral transition in *Arabidopsis* are becoming increasingly well understood. The ease with which mutant phenotypes can be quantified has led to many suppressor screens and the molecular identification of the underlying genes. One focus has been on the pathways that regulate the gene encoding the floral repressor *FLC*. This has revealed a set of antagonistic pathways comprising evolutionary conserved activities that link chromatin regulation, transcription level and co-transcriptional RNA metabolism. Here we discuss our current understanding of the transcriptional activation of *FLC*, how different activities are integrated at this one locus and why *FLC* regulation seems so sensitive to mutation in these conserved gene regulatory pathways.

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Introduction

One of the most exciting aspects of plant science today is how much it contributes to general concepts in biology. The ability to combine a genetic analysis with detailed molecular and biochemical approaches offers real advantages for mechanistic analysis. Of all the developmental transitions in plants flowering has been the best studied and over the last 15 years a concentrated effort in *Arabidopsis thaliana* has provided a good understanding of how different environmental and endogenous cues are integrated to cause the developmental switch [1]. Several flowering time pathways converge on *FLOWERING LOCUS C (FLC)*, a MADS box transcriptional regulator that represses the floral transition (Figure 1). Vernalization, the acceleration of flowering by prolonged cold, epigenetically silences *FLC* through the action of Polycomb proteins which deposit the repressive histone mark

H3K27me3 [2]. Acting in parallel to vernalization is the autonomous pathway, a series of activities that involve RNA-mediated chromatin silencing of *FLC* [3]. These repressive pathways have received much attention and involve a complex regulation of sense and antisense *FLC* transcripts [4^{**},5^{**}] that we shall review in a future COPB. Here we focus on the transcriptional activating mechanisms and discuss how they might be co-ordinated to regulate *FLC* expression. Some of these activities clearly involve transcriptional processes common to many organisms, whilst others appear to involve proteins with no clear homologues in animals. How these activities can be fine-tuned in response to different inputs is likely to provide an important paradigm for gene regulation in many eukaryotes.

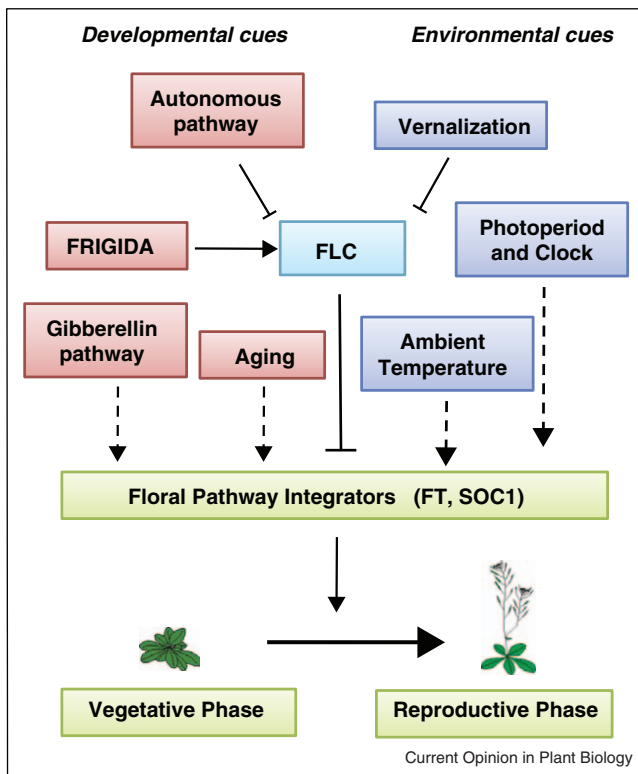
Conserved chromatin complexes required for transcriptional activation of *FLC*

Many genetic screens have identified suppressors of high-level *FLC* expression. Molecular characterization of these has identified a series of activities that link chromatin regulation and transcriptional control, which have been characterized extensively in yeast and metazoans (Table 1). Mutation of these generic factors would be expected to have catastrophic effects for the plant but remarkably many are still viable. A common phenotype is early flowering due to reduced expression of *FLC* and in many cases also of its homologues, the *MAF* genes [1,2]. What are these generic chromatin complexes and what is their function in transcription? The nucleosomal structure of chromatin imposes problems for the large RNA polymerase complex during transcription, necessitating activities by chromatin complexes mediating histone modifications, histone variant exchange and chromatin remodelling [6,7]. At many genes the polymerase engages and then quickly pauses, a state often characterized by accumulation of a peak of H3K4me3, a histone mark associated with active transcription. Transition into the elongation phase requires RNA polymerase-associated factor 1 complex (Paf1C); RAD6-BRE1, important for histone H2B ubiquitination; and Set complexes, important for histone methylation. A schematic overview of their functions in the transcription initiation and elongation is given in Figure 2. Below we describe what we know of these complexes at *FLC* and how their functions are integrated.

Paf1 complex

The Paf1 complex accompanies the RNA polymerase II (PolII) from the promoter to the 3' end of the mRNA. All

Figure 1



Flowering Time. Flowering occurs in response to different developmental and environmental cues. Genetic analysis using the model plant *Arabidopsis thaliana* defined a number of flowering pathways that converge onto the so-called 'floral pathway integrator' genes. *FLC* is a target of several regulatory pathways making this gene a key regulator upstream of the floral pathway integrators.

the components of the *Arabidopsis* Paf1C (Table 1) have emerged from screens for early flowering mutants suggesting flowering time regulation by *FLC* is very sensitive to changes in Paf1C function [8–11,12*,13*]. There is still extensive discussion and research into the precise role it plays but loss of Paf1C in yeast and mammalian cells affects transcriptional elongation, a number of histone modifications/chromatin-remodelling activities and 3' end processing factor recruitment [7]. It is therefore still not clear whether Paf1C is primarily a platform on PolII that coordinates association of many factors, or if the complex plays a more direct role in one or more key steps in transcription.

At the *FLC* locus, *paf1c* mutations cause reduction in transcription, loss of H3K4me3 at the 5' end and H3K36me2/3 in the main body of the gene. This is accompanied by increases in H3K27me3 over the locus [14]. Most of the mutations also caused floral organ phenotypes and reduced plant size, however mutants in *Atcdc73* (also called *PHP*) showed less pleiotropy [12*,13*]. Why *FLC* expression is apparently more sensitive to loss of *AtCDC73* is not clear but the human

homologue, also called Parafibromin, is required for correct transcript processing likely through a direct interaction with the CPSF-CstF RNA processing complex [15]. This may provide an interesting connection to the autonomous pathway function where the same 3' processing complex is important for alternative processing the *FLC* antisense transcript [5**]. Antagonistic effects on co-transcriptional RNA metabolism of *FLC* could account for why *Atcdc73* shows different interactions with mutants of the autonomous pathway [12*].

Histone 2B ubiquitination

Mutations disturbing ubiquitination levels of histone H2B also result in early flowering phenotypes. In yeast H2B monoubiquitination requires RAD6-BRE1 activities; Paf1C is necessary for RAD6-BRE1 dependent ubiquitination and in turn H2Bub1 is necessary for H3K4me3 (Figure 2). *Arabidopsis* has two E3 ubiquitin ligases homologous to BRE1 (HUB1 and HUB2) and three E2 carrier proteins homologous to RAD6 (UBC1 to UBC3) [16*]. *hub1* and *hub2* mutants, and *ubc1 ubc2* double mutant show loss of H2Bub1, reduction of H3K4me3 and H3K36me3, early flowering and misregulation of *FLC* [16*,17,18]. They also show pleiotropic phenotypes and determine genome-wide levels of H2Bub1 but not H3K4me3 [18]. The ubiquitin modification enhances the movement of PolIII through nucleosomes, possibly via Facilitates Chromatin Transcription (FACT) complex dependent removal of an H2A/H2B dimer. In *Arabidopsis*, mutations in the FACT subunits *SSRP1* and *SPT16* also produce severe phenotypes including early bolting due to *FLC* misregulation [19]. In some cases, full transcription requires cycling of both H2B ubiquitination and deubiquitination although the exact function of the later is not well known [6]. *Arabidopsis* UBP26 catalyses the deubiquitination reaction and *ubp26* is also early flowering [20].

Histone K4 and K36 methyltransferase complexes

Arabidopsis genome sequence analysis initially identified 29 SET domain proteins [21] and several have now been shown to function at *FLC*. EFS/SDG8 functions as the homologue of methyltransferase SET2 delivering H3K36 methylation to the 3' end of *FLC* [22,23]. The Trithorax homologues ATX1 and ATX2 deposit H3K4me3/me2 respectively at the 5' end of *FLC* [24*,25]. And mutants in the Set1 homologue ATXR7/SDG25 show reduced *FLC*, decreased H3K4me3, slightly decreased H3K36me2 and increased H3K27me3 [26*,27*]. Interestingly, ATX1, ATX2 and ATXR7 are not fully redundant and have additive roles regulating *FLC* expression. These histone methyltransferases also regulate a number of targets genome wide, for example about 900 genes with changed expression levels were found in *atx1* mutant plants [25]. *FLC* is also target of the *Arabidopsis* homologue of the human WD40 domain-containing protein WDR5, namely *AtWDR5a*, which is a conserved core

Table 1

Conserved and plant specific activities required for *FLC* transcriptional activation

Gene number	Arabidopsis	Yeast/human	Complex	Protein domain	Activity
At4g29830	VIP3	hSki8	Paf1C	WD40 domain	–
At5g61150	VIP4	Leo	Paf1C	Leo-like	–
At1g61040	VIP5	Rtf1	Paf1C	Plus-3 domain	–
At2g06210	VIP6/ELF8	Ctr9	Paf1C	TPR domain	–
At1g79730	ELF7	Paf1	Paf1C	Paf1-like	–
At3g22590	CDC73/PHP	CDC73/HRPT2	Paf1C	CDC73-like	–
At2g44950	HUB1	BRE1	RAD6-BRE1	RING finger	Ubiquitin ligase
At1g55250	HUB2	BRE1	RAD6-BRE1	RING finger	Ubiquitin ligase
At1g14400	UBC1	RAD6	RAD6-BRE1	UBCc domain	Carrier protein
At2g02760	UBC2	RAD6	RAD6-BRE1	UBCc domain	Carrier protein
At5g62540	UBC3	RAD6	RAD6-BRE1	UBCc domain	Carrier protein
At3g49600	UBP26	Ubp/USP	–	USP domain	Ubiquitin protease
At4g10710	AtSpt16	Spt16	FACT complex	FACT-like	Histone chaperone
At3g28730	AtSSRP1	SSRP1	FACT complex	HMG domain	Histone chaperone
At1g66240	ATX1	Trithorax	Compass-like	SET domain	H3K4 methyltransferase
At1g05830	ATX2	Trithorax	Compass-like	SET domain	H3K4 methyltransferase
At5g42400	SDG25/ATXR7	Set1/MLL	Compass-like	SET domain	H3K4 methyltransferase
At3g49660	AtWDR5a	WDR5	Compass-like	WD40 domain	H3K4me binding
At1g77300	EFS/SDG8	Set2	–	SET domain	H3K36 methyltransferase
At3g12810	PIE1	Swr1/SRCAP	SWR1	SNF2 protein	DNA-dependent ATPase
At3g33520	ARP6/SUF3/ESD1	Arp6	SWR1	Actin-related	–
At5g37055	SEF/AtSW6C	Sw6c	SWR1	HIT zinc finger	–
At4g00650	FRIGIDA	–	FRI complex	Coiled coil domain	–
At5g16320	FRIL1	–	FRI complex	Coiled coil domain	–
At1g31814	FRIL2	–	FRI complex	Coiled coil domain	–
At2g33835	FES1	–	FRI complex	CCCH zinc finger	–
At1g30970	SUF4	–	FRI complex	BED zinc finger	DNA binding protein
At2g30120	FLX	–	FRI complex	Leucine zipper	–

component of the H3K4 methyltransferase COMPASS/MLL complex. Recombinant AtWDR5a binds H3K4-methylated peptides and directly interacts with ATX1 [28**]. Current data support that FRIGIDA, a profound up regulator of *FLC* levels (see below), is required for the enrichment of AtWDR5a at the *FLC* locus and increases in H3K4me3 [28**].

SWR1 complex

Another series of early flowering mutants identified a major role for the SWR1 complex in *FLC* expression. The SWR1/SRCAP complex is a chromatin-remodelling complex that has been shown to be involved in substitution of histone H2A by the histone variant H2AZ. Mutations in *PIE1* [29], *ARP6* (also known as *SUF3* and *ESD1* in Arabidopsis) [30–32], *SEF/AtSW6C* [33–35] all gave early flowering and ARP6 and PIE1 were found to be required for H2AZ deposition at *FLC* [36]. H2AZ is frequently enriched at the 5' and 3' end of genes, and is associated with both gene activation and gene repression. It is thought to lead to relative nucleosome instability so aiding in nucleosome displacement by transcription machinery and this could be the mechanism by which it promotes *FLC* transcription.

Requirements for *FLC* activation by FRIGIDA

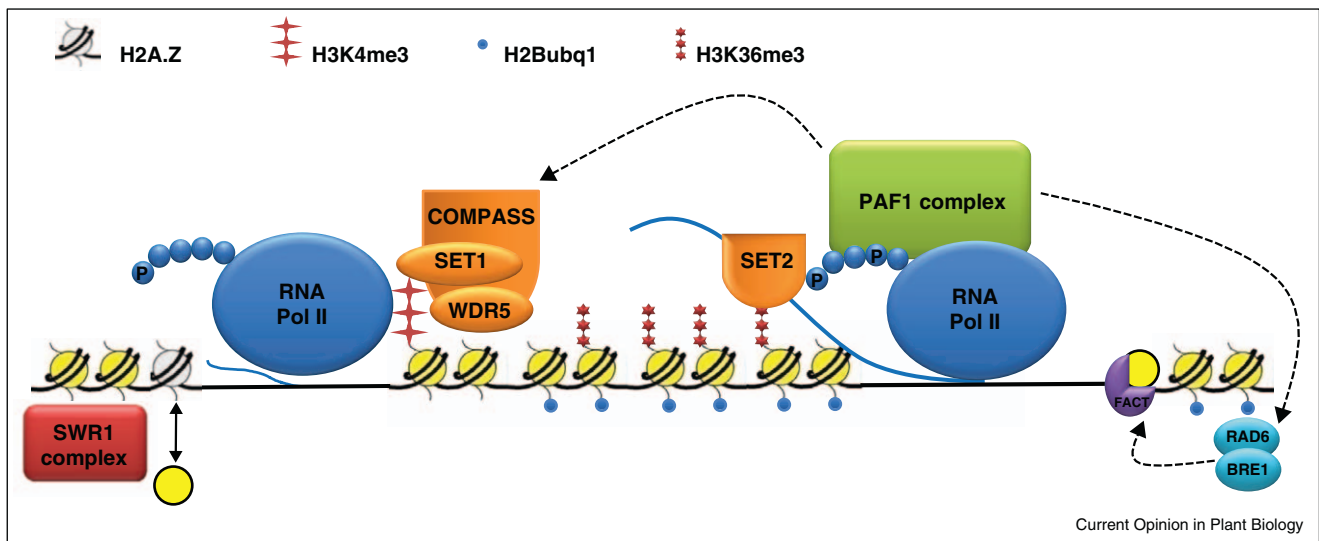
In the 1950s it was established that the overwintering habit of Arabidopsis could be mapped predominantly as a

monogenic trait to the *FRIGIDA* (*FRI*) locus. Arabidopsis laboratory accessions like Columbia and Landsberg *erecta* carry non-functional *fri* alleles, but addition of an active *FRI* very strongly upregulates *FLC* expression so determining a vernalization requirement on plants [2]. The majority of *FRI* suppressors identified components required for high *FLC* expression as described above. Some, however, identified components that had much weaker effects on autonomous pathway mutations or were specifically required for *FRI*-dependent increases. Since *FRI* is a novel protein with coiled-coil domains but little other homology to other known proteins [37] these proteins could represent a plant specific function or perhaps more likely a function with poorly conserved components. Mutations in five genes fall into this group (Table 1).

FRIGIDA-like genes (*FRL1* and *FRL2*)

The Arabidopsis *FRI* family has seven members and is conserved among other plant species [37]. *FRL1* was isolated as an *FRI* suppressor in a mutagenesis screen of a Columbia-*FRI* line [38]. *FRL1* shares only a low level of homology with *FRI* throughout the length of the proteins and they do not function redundantly. The different *FRL* proteins may have specific roles as the *fri1 fri2* double mutant in Columbia flowers earlier than *fri1*. Interestingly, *FRL1-Ler* is non-functional and its function is replaced by a strong *FRL2-Ler* allele [39].

Figure 2



Chromatin-associated factors regulating transcription. Once activators bind at the promoter they trigger recruitment of chromatin-remodelling and histone-modifying complexes to undertake the chromatin changes necessary for accommodation of the transcription machinery. After the preinitiation complex is assembled the carboxy terminal domain (CTD) of RNA polymerase II (PolII) is phosphorylated at serine 5. This targets the H3K4 methyltransferase SET1 (ATX1, ATX2 or ATR7) in a protein complex that includes WDR5 (AtWDR5a), a WD40 protein that recognize H3K4 methylation. SET1 has a basal level of H3K4 methylation activity but requires PAF1 complex association with PolII to produce H3K4me3, an important signalling mark defining the transcription start and enhancing the activity of PolII. The dynamic exchange of H2A.Z with H2A around the transcription start site by the SWR1 complex (PIE1, ARP6 and SEF) is required for full transcription. Transition into the elongation phase is associated with phosphorylation of the PolII CTD at serine 2. This recruits SET2 complex (EFS) and promotes the accumulation of H3K36me3 in the body of the gene. H3K36me3 enhances transcriptional elongation preventing the appearance of cryptic transcripts from within the gene body in the wake of the elongating PolII. The PAF1 complex (VIP3, VIP4, VIP5, VIP6/ELF8, ELF7 and PHP/AtCDC73) is an early PolII elongation associated factor required for H2Bub1, acts downstream of H3K4me3 and has an intrinsic role facilitating transcriptional elongation. The Rad6-Bre1 complex (HUB and UBC) ubiquitinates H2B and requires PAF1 complex and active transcription. H2Bub1 enhances transcription by facilitating assembly and disassembly of nucleosomes in front of the PolII elongation. This requires FACT complex (AtSSRP1 and AtSPT16) dependent removal of an H2A/H2B dimer due to its intrinsic histone chaperone activity.

FRIGIDA ESSENTIAL 1 (FES1)

A CCCH zinc finger protein expressed in shoot and root apices and the vascular system and conserved in other plant species [40]. CCCH zinc finger proteins can bind RNA so FES1 may function co-transcriptionally on *FLC*.

SUPPRESSOR OF FRIGIDA 4 (SUF4)

This contains a BED-ZP207 zinc finger domain (DNA binding) and proline-rich region (often important for protein interactions) [41,42]. SUF4 appears to be nuclear-localized, expressed more widely than *FLC* and the transcript is alternatively spliced [42]. SUF4 interacts with FRI and FRL1 and binds to the *FLC* promoter, and may provide specificity to the putative FRI complex. [41]

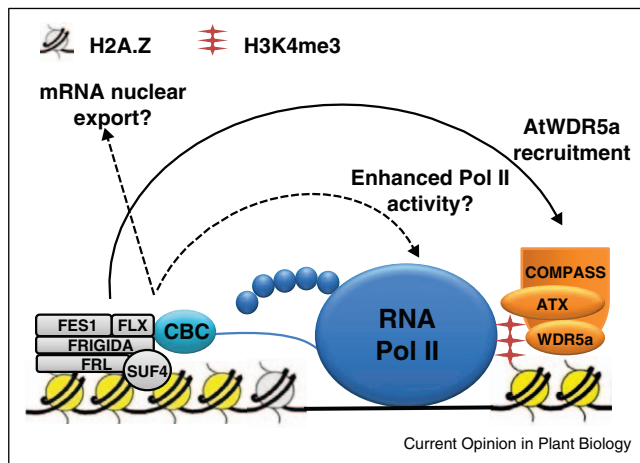
FLC EXPRESSOR (FLX)

An alpha-helix protein that forms part of a small family of related proteins in Arabidopsis [43]. FLX function is unknown but its leucine zipper domain shares homology with the yeast nuclear proteins SMC and NUF1 [43].

All these mutants are genetically epistatic with *fri* suggesting that they function in the same pathway. There

have also been reported protein-protein interactions between FRI or FRL1 with SUF4 [41], and between FRI and FRL1 [41] supporting the idea that they physically interact and form a protein complex (Figure 3). How such a complex might activate *FLC* transcription is still a matter of speculation. However, like the autonomous pathway it could involve co-transcriptional processes. Mutations in the Arabidopsis CAP binding complex (CBC) subunits *CBP80* and *CBP20* suppress FRI, and CBP20 interacts directly with FRI in yeast and *in planta* [44*,45]. The data suggest that FRI upregulates *FLC* expression through a co-transcriptional mechanism involving direct physical interaction with the nuclear CBC with concomitant effects on *FLC* transcription [44*]. This is not the only example of late flowering phenotype suppression linked to RNA metabolism; mutations in HUA2 [46], a putative DExH-box RNA helicase required for the processing of *AGAMOUS* pre-mRNA, and PEPPER [47], a protein with three KH RNA-binding domains, also suppress late flowering due to high *FLC* levels. There are now many examples in yeast and metazoans indicating a tight connection between transcriptional elongation, RNA processing, and export [48].

Figure 3



FRIGIDA complex. The putative FRIGIDA complex would be composed of FRI, SUF4, FES1, FLX, and a FRIGIDA-like protein. The DNA binding protein SUF4 binds to *FLC* promoting FRI complex interaction with CAP binding complex (CBC). This co-transcriptional interaction could facilitate *FLC* mRNA nuclear export and/or enhance RNA polymerase II activity. FRI complex is also required for the enrichment of a WDR5a-containing COMPASS-like complex at the *FLC* locus that methylates H3K4. The result is a strong *FLC* expression up regulation in Arabidopsis accessions expressing an active FRIGIDA allele.

Indeed, *FLC* is sensitive to mutations in the nuclear pore proteins required for maintaining the homeostasis between the nuclear and cytoplasmic RNA pools [49,50]. It is possible that highly transcribed genes are associated with the nuclear pore to facilitate transcript production, quality control and export. A FRIGIDA complex involving FRL proteins, FES, SUF4 and FLX might be important in the interconnection of these co-transcriptional activities.

Reactivation of *FLC* during the late reproductive phase

After Arabidopsis has been vernalized, generally in the vegetative phase, *FLC* expression is silenced and this state is epigenetically maintained throughout most of the rest of the life-cycle. However, to ensure each generation of plants requires vernalization, *FLC* expression is reactivated during gametogenesis and embryo development [51*,52*]. Current data suggest a default program for reactivation of *FLC* expression even in non-vernalized plants [52*] indicating that *FLC* 'resetting' could be part of a genome-wide epigenetic reprogramming at plant embryogenesis. The precise role of all the chromatin regulators and FRI-accompanying proteins in this process is still unknown. But *pie1* mutation impairs *FLC::GUS* transgene expression at all embryonic states whereas FRI and SUF4 are only required to maintain *FLC::GUS* expression during late embryogenesis [52*]. High *FLC* expression during later stages of embryo development has been shown to be crucial for late flowering [51*], so the identity of the regulatory factors

activating *FLC* during embryogenesis remains an important, unresolved question.

Conclusions

Analysis of mutations that activate or silence expression of the floral regulator gene *FLC* have revealed conserved gene regulatory pathways that link chromatin regulation and transcription. This system therefore provides an excellent vehicle to combine genetic with biochemical analysis and fully define those activities whilst exploring the conservation in gene regulatory pathways. An interesting question that emerges from this analysis is why *FLC* is apparently so sensitive to mutations in pathways that probably regulate most of the genome. Could this be due to *FLC* transcriptional output being set by a balance between the antagonistic pathways that promote or repress — a molecular sumo wrestle? The effect of loss of one of the activities is magnified by the increased activity of the opposing effect. Mutations in components leading to loss of H3K4me3 often result in increased H3K27me3, and mutants with high levels of *FLC* and H3K4me3 show reduced H3K27me compared to wild type plants. [53]. Continued analysis of mutants that affect *FLC* expression will allow the interactions of these antagonistic pathways to be understood — what determines which activity is predominant and how does this change in natural variants? How is that interaction influenced by different environmental cues? The conservation of the pathways involved suggests lessons from these studies will be widely relevant to gene regulation generally in plants. Given the importance and remarkable conservation of co-transcriptional mechanisms across eukaryotes, they will keep interest in plant biology strong.

Update

While preparing this manuscript new data addressing EFS role in *FLC* up-regulation has appeared (Ko JH *et al.*, EMBO J 2010). This excellent paper shows that EFS is crucial for FRI recruitment to *FLC* and FRI enhances EFS histone methyltransferase activity. The authors propose a model of how flowering time may be regulated by the balance between different histone methylation and demethylation activities.

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