Role of chromatin modification in flowering-time control

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The regulation of the *FLC* locus provides a plant model of how chromatin-modifying systems have emerged as important components in the control of a major developmental switch, the transition to flowering. Genetic and molecular studies have revealed that three systems of *FLC* regulation (vernalization, FRI and the autonomous pathway) all influence the state of *FLC* chromatin. Histone H3 trimethylation at lysine 4 and histone acetylation are associated with active *FLC* expression, whereas histone deacetylation and histone H3 dimethylation at lysines 9 and 27 are involved in *FLC* repression. These chromatin modifications provide an additional level of regulation of gene expression beyond that of the transcription factors that recruit RNA polymerase to target genes.

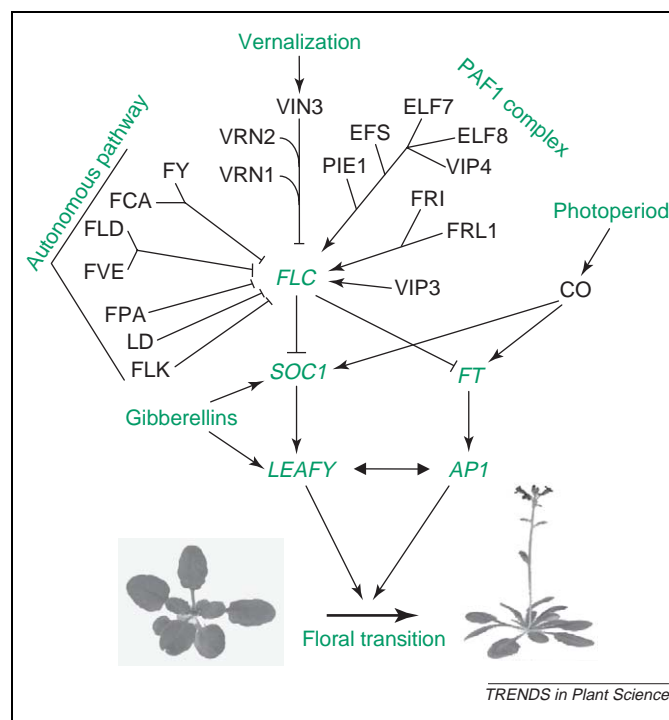## The floral transition in *Arabidopsis*

The 'decision' of a plant to initiate flowering often involves coordinating the perception of environmental cues such as day length, light conditions and temperature with endogenous factors such as plant developmental status and age. This coordination ensures that the transition is properly timed to achieve maximal reproductive success. In this article, we focus on the recent recognition that, in *Arabidopsis*, several components of flowering pathways that have been defined by genetics and physiology are involved in modifying the chromatin of target genes.

Arabidopsis flowering is regulated by four major pathways [1,2] (Figure 1). *Arabidopsis* is a facultative long-day plant and the photoperiod pathway accelerates flowering in response to increasing day length. The vernalization pathway renders *Arabidopsis* competent to flower after long-term cold exposure, the autonomous pathway constitutively represses flowering and gibberellins promote flowering and are required for flowering in non-inductive photoperiods. Inputs from these pathways are integrated to control the expression of a set of common downstream target genes such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FT*, which are called 'flowering-time integrators' [1,2]. SOC1 and FT in turn activate floral-meristem-identity genes such as *LEAFY* and *APETALA 1*, which cause the production of flower primordia [1,2].

There is natural flowering-time variation among *Arabidopsis* accessions. The winter-annual (late flowering without vernalization) versus rapid-cycling (early flowering) habit is determined by allelic variation at *FRIGIDA*

(*FRI*) and *FLOWERING LOCUS C* (*FLC*) [3–5]. Winter annuals have dominant alleles of *FRI* and *FLC*, whereas rapid-cycling types have either a non-functional *fri* allele [3] or a weak *flc* allele [4,5]. FLC is a MADS-box transcription factor that quantitatively blocks the floral transition [6,7], in part by repressing expression of the flowering-time integrators *SOC1* and *FT* (Figure 1) [8]. *FRI* elevates *FLC* expression to levels that inhibit flowering [6,7]. Vernalization represses the *FRI*-mediated increase in *FLC* expression [6,7].

The photoperiod and autonomous pathways were identified in screens for late-flowering mutants in

Figure 1. Pathways regulating flowering time in *Arabidopsis*. The autonomous pathway regulators and vernalization repress *FLC* expression. FRI and FRL1 upregulate *FLC* expression, and the H3-K4 trimethylation mediated by the PAF1 complex (ELF7, ELF8 and VIP4) activates *FLC* expression. FLC represses expression of the 'flowering-time integrators' *SOC1* and *FT*, whereas the photoperiod pathway promotes expression of these integrators. *SOC1* and *FT* expression leads to the induction of floral-meristem-identity genes such as *LEAFY* and *AP1*, and thus of flowering. Lines with arrows indicate upregulation (activation) of gene expression and lines with bars for gene repression. Abbreviations: *AP1, APETALA 1; CO, CONSTANS; EFS, EARLY FLOWERING IN SHORT DAYS; ELF7, EARLY FLOWERING 7; ELF8, EARLY FLOWERING 8; FLC, FLOWERING LOCUS C; FLD, FLOWERING LOCUS D; FLK, FLOWERING LOCUS K; LD, LUMINIDEPENDENS; PIE1, PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1; VIN3, VERNALIZATION INSENSITIVE 3; VIP3, VERNALIZATION INDEPENDENCE 3; VIP4, VERNALIZATION INDEPENDENCE 4; VRN1, VERNALIZATION 1; VRN2, VERNALIZATION 2.*

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mutagenized rapid-cycling backgrounds [e.g. Columbia and Landsberg *erecta* (L*er*)] [9]. The role of autonomous-pathway genes in flowering is to repress *FLC* expression [10]. Many summer annuals are early flowering without vernalization because, in the absence of a functional *FRI* allele, the autonomous pathway keeps *FLC* levels low [10]. In winter annuals, FRI overcomes the autonomous-pathway-mediated repression of *FLC* [6,7]. Therefore, *FLC* regulation is a convergence point for the control of flowering time by vernalization, *FRI* and the autonomous pathway (Figure 1).

The regulatory mechanisms by which *FLC* expression is controlled are of great interest given its central role in controlling flowering time. Recent screening for mutants that render winter-annual, *FRI*-containing lines early flowering have led to the identification of genes that are additional positive regulators of *FLC* expression [11–14]. Some of these are required for the elevated *FLC* expression in both autonomous-pathway mutants and *FRI*-containing lines {e.g. PHOTOPERIOD-INDEPEN-DENT EARLY FLOWERING 1 (PIE1) [13], VERNALIZA-TION INDEPENDENCE 3 (VIP3) [11] and VIP4 [12]}, whereas others are specifically required only for FRI-mediated *FLC* activation (e.g. FRI LIKE 1 [14]).

The recent characterization of *FLC* repressors and activators has shown that some of these regulatory proteins are involved in the covalent modification of *FLC* chromatin [15–17]. Chromatin modifications such as the acetylation and methylation of specific residues of specific histones are typically referred to as the 'histone code' [18,19]. These chromatin modifications provide an additional level of regulation of gene expression beyond that of the transcription factors that recruit RNA polymerase to target genes. Recent studies of *FLC* regulation provide perhaps the best-studied example in plants of how changes in the chromatin environment are used to modify a developmental program.

Activation of *FLC* expression

H3-K4 hyper-trimethylation of FLC *chromatin is associated with the delay of flowering in winter-annual* Arabidopsis

Trimethylation of histone 3 (H3) at lysine 4 (H3-K4) is associated with the chromatin of active genes [20–22]. Lysine-4 methylation of H3 occurs in three states: mono-, di- and trimethyl. In yeast and *Drosophila*, the presence of a trimethylated K4 defines an active state of gene expression [21,22]. Other reports indicate that dimethyla-tion of H3-K4 might also associate with active genes in multicellular eukaryotic organisms [22,23]. Recent studies in yeast have provided an outline of how H3-K4 trimethylation levels are increased and how this chroma-tin modification leads to gene activation. The yeast RNA polymerase II associated factor 1 (PAF1) complex associ-ates with the RNA polymerase II complex during transcription [24–26]. One role of the yeast PAF1 complex is to recruit a H3-K4 methyltransferase known as SET1 to target genes [20,27]. SET1 generates H3-K4 trimethyla-tion predominantly in the 5′ portion of actively transcribed regions [20]. The resulting increase in H3-K4 trimethyla-tion links transcription and chromatin structure, and is

thought to serve as a 'transcriptional memory' that estab-lishes a positive feedback loop of gene activity [20,21].

Screens for mutants in which winter annuals are converted into rapid-flowering types because of attenu-ation of *FLC* expression have led to the identification of several *Arabidopsis* relatives of the yeast PAF1 complex [12,28]. The yeast PAF1 complex consists of PAF1, CTR9, LEO1, CDC73 and RTF1 [24–26]. The *Arabidopsis* relatives of PAF1, CTR9 and LEO1 are present in the genome as single copies [12,28]. Lesions in EARLY FLOWERING 7 (ELF7, the relative of yeast PAF1), EARLY FLOWERING 8 (ELF8, the relative of yeast CTR9) or VERNALIZATION INDEPENDENCE 4 (VIP4, the relative of yeast LEO1) suppress the ability of FRI to increase *FLC* expression and thus to convert winter annuals into rapid-flowering types [12,28]. When tested, these lesions also suppress the increased *FLC* expression in autonomous-pathway mutants [12,28], indicating that the *Arabidopsis* PAF1 complex is generally required for high levels of *FLC* expression.

In a *FRI*-containing winter annual line (in which *FLC* is highly expressed), *FLC* chromatin has increased levels of H3-K4 trimethylation compared with a rapid-flowering line that lacks an active *FRI* allele and has relatively low levels of *FLC* expression [28]. Late-flowering autonomous-pathway mutants also have increased levels of H3-K4 trimethylation compared with the rapid-flowering parental line [28]. Thus, FRI is formally a promoter of H3-K4 trimethylation of *FLC* chromatin, and autonomous-pathway genes are repressors of this chromatin modifi-cation. The increased levels of H3-K4 trimethylation are present in a distinct region of *FLC* chromatin around the transcription start site, the first exon and a 5′ portion of the first intron [28]. In *elf7* and *elf8* mutants, the H3-K4 trimethylation of *FLC* chromatin is greatly reduced [28]. These studies indicate that the PAF1-like complex is necessary for the increased *FLC* expression of *FRI*-containing lines or autonomous-pathway mutants by increasing H3-K4 trimethylation of *FLC* chromatin.

The *elf7* and *elf8* mutants also cause early flowering in non-inductive short days [28]. This is due, at least in part, to a requirement for the *Arabidopsis* relatives of the PAF1 complex components for expression of additional MADS-box genes [28]. In *Arabidopsis*, there are five *FLC* relatives: *FLOWERING LOCUS M* (*FLM*), *MADS AFFECTING FLOWERING 2* (*MAF2*), *MAF3*, *MAF4* and *MAF5* [29–31]. Like FLC, FLM and MAF2 are also floral repressors. However, unlike FLC, FLM and, pos-sibly, MAF2 repress flowering in non-inductive photo-periods [29–31]. In *elf7* and *elf8* mutants, *FLM* and *MAF2* expression is greatly reduced, and *FLM* chromatin has reduced levels of H3-K4 trimethylation [28]. Thus, the *Arabidopsis* PAF1-like complex might be required for the expression of the *FLC* clade of MADS-box genes, and loss of expression of this clade would lead to both early flowering in non-inductive short days and a conversion of winter annuals into rapid-flowering types in inductive long days.

SET1 is the only H3-K4 methyltransferase in yeast [21,32]. During the course of evolution, plants have undergone extensive duplication of SET-domain proteins [33,34]. *Arabidopsis* has several potential H3-K4

methyltransferases, based on sequence and domain similarities to known K4 methyltransferases in other organisms [33,34]. Recent work (S.D. Michaels *et al.*, unpublished) has revealed that a particular *Arabidopsis* relative of yeast SET1 [32] and the *Drosophila* H3-K4 methyltransferase ASH1 [35,36] is necessary for H3-K4 trimethylation in *FLC* chromatin (and probably for other members of the FLC clade). Lesions in the SET-domain gene *EARLY FLOWERING IN SHORT DAYS* (*EFS*; this locus has been genetically characterized before [37]), like those in *ELF7* and *ELF8*, cause early flowering in short days as well as suppression of FRI-mediated late flowering and reduction in trimethylation levels of *FLC* chromatin and suppression *FLC* expression. It thus appears that, similar to the situation in yeast, the *Arabidopsis* PAF1-like complex recruits EFS to methylate target genes.

Connection between H3-K4 trimethylation and ATP-dependent nucleosome remodeling

Recently, it has been shown that ISW1p (a yeast ATP-hydrolyzing, chromatin-remodeling protein) preferentially binds di- and trimethylated H3-K4 and H3-K4 methylation of chromatin of certain actively transcribed genes is required for association with ISW1p *in vivo* [38]. An *Arabidopsis* relative of ISW1p, PIE1, has been found to be necessary for *FLC* expression [13]. Also, the range of phenotypes of the *pie1* mutant, such as early flowering in short days and a suppression of FRI-mediated late flowering, is similar to that of *efs*, *elf7* and *elf8* mutants [13,28,37]. Thus, it is tempting to speculate that PIE1 binds trimethylated K4 and remodels chromatin of *FLC* and its relatives to increase gene expression.

Overall, the studies of the regulation of flowering time and *FLC* expression indicate that plants and yeast use H3-K4 trimethylation in similar ways. A model summarizing the putative roles of several of the players required for *FLC* expression is presented in Figure 2.
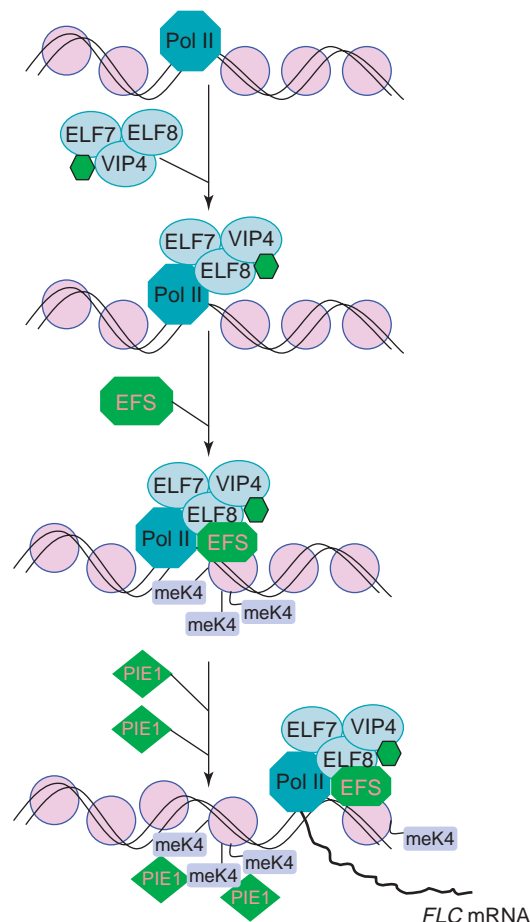
Repression of *FLC* expression

Certain autonomous-pathway components repress FLC expression by histone deacetylation
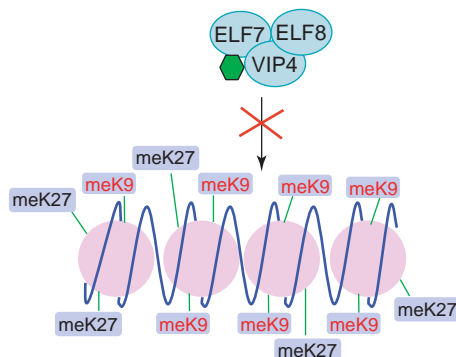
The autonomous pathway consists of seven known genes: *FCA* [39], *FLOWERING LOCUS D* (*FLD*) [15], *FPA* [40], *FVE* [41], *FY* [42], *LUMINIDEPENDENS* (*LD*) [43] and *FLOWERING LOCUS K* [44,45]. The autonomous pathway was originally defined by late-flowering mutations that retained a photoperiod response [9]. Subsequent molecular and genetic studies have defined the autonomous pathway as repressors of *FLC* [10].

Histone acetylation is another chromatin modification that affects gene expression. Generally, hyperacetylation of core histone tails of H3 and H4 is linked to actively expressed genes, whereas hypoacetylation of these histone tails is associated with inactive genes [18,46]. The recent cloning of two autonomous-pathway genes, *FLD* and *FVE*, revealed that these genes encode proteins similar to components of a mammalian histone deacetylase (HDAC) complex: FLD and FVE are relatives of human KIAA0601 and RbAp48/46, respectively [15,41]. Indeed, histone acetylation levels of *FLC* chromatin were

Figure 2. **(a)** Model of *FLC* activation by PAF1-complex-mediated trimethylation of histone H3 at lysine 4 (H3-K4). When the *Arabidopsis* PAF1 complex (whose known members include ELF7, ELF8 and VIP4) associates with RNA polymerase II (Pol II), an H3-K4 methyltransferase (EFS) is recruited to *FLC* chromatin by Pol-II–PAF1 complex, resulting in K4 trimethylation in the 5′ portion of transcribed *FLC* chromatin. PIE1 binds the trimethylated K4 and further remodels *FLC* chromatin to an active state, thus facilitating *FLC* transcription by Pol II. **(b)** Vernalization converts active *FLC* chromatin into a heterochromatin-like state so that the PAF1 complex is not able to access *FLC* locus.

increased in *fld* and *fve* mutants [15,41] but not in other autonomous mutants including *fca*, *fpa* and *ld* [15]. This indicates that FLD and FVE participate in the deacetylation of *FLC* chromatin as components of an HDAC complex. Furthermore, the lack of an effect on the histone

acetylation level of *FLC* chromatin in other autonomous-pathway mutants indicates that the autonomous pathway is not a typical linear pathway, but must be composed of at least two subpathways that repress *FLC* expression by different mechanisms (Figure 1) [15]. This molecular subdivision of the autonomous pathway is consistent with the previous genetic studies that indicated that subpathways exist [47]. Recent studies also indicate that a *cis*-regulatory region in the first intron of *FLC* is necessary for deacetylation (Figure 3) [15]. However, the protein(s) that binds to this regulatory region and recruits the FLD-containing HDAC complex to *FLC* chromatin has not yet been discovered.

H3-K9 methylation in the first intron of FLC directed by short interfering RNAs suppresses FLC expression

Naturally occurring allelic variation at *FLC* contributes to the rapid-flowering behavior of certain *Arabidopsis* accessions such as L*er* and Da (1)-12 [4,5]. Both accessions have a functional FRI but are early flowering owing to lower steady-state mRNA levels of *FLC*; that is, these alleles of *FLC* are not effectively upregulated by FRI [4]. In L*er*, there is a 1.2-kb insertion of a Mutator-like transposable element (TE) in the first intron of *FLC* [4,5]. This TE is clearly responsible for the inability of FRI to upregulate *FLC* because transfer of this TE to a strong *FLC* allele causes it to behave like the L*er* allele [4]. This TE insertion renders L*er* *FLC* subject to repressive chromatin modifications mediated by silencing short interfering RNAs (siRNAs) generated from related TEs elsewhere in the genome [48]. Specifically, the level of H3-K9 dimethylation in a region of L*er* *FLC* chromatin including the TE insertion and an intronic region immediately downstream of TE is elevated [48]. Although methylation of K4 of H3 is associated with active genes, other lysine methylations of H3 (such as methylation of K9 and K27) are characteristic of inactive genes that adopt a repressive, heterochromatin-like conformation [49]. Furthermore, siRNAs are known to direct H3-K9 dimethylation to regions of sequence relatedness [23,50,51]. Thus, it is likely that siRNAs

Figure 3. A 295-bp *cis*-regulatory region in the first intron of *FLC* mediates histone deacetylation of *FLC* locus. Deletion of this region prevents deacetylation of *FLC* chromatin and upregulates *FLC* expression [15], thus causing extremely late flowering (as found in the plant on the right). On the left is an *flc* plant transformed with a wild-type *FLC* transgene (i.e. without the 295-bp deletion), and this transgene is repressed, similar to the native *FLC*, by FLD, FVE and other autonomous regulators.

trigger histone H3-K9 dimethylation, resulting in a heterochromatin 'island' in the middle of *FLC* chromatin, which causes transcriptional gene suppression in L*er* *FLC*. The Da (1)-12 ecotype has a different class (Copia-like) of TE in its first *FLC* intron [4] and, similar to the situation in L*er* *FLC*, there is an island of heterochromatin around the TE in the Da (1)-12 *FLC* allele [48]. Thus, the conversion of a winter annual into a rapid-flowering type by TE insertions in *FLC* provides another example of McClintock's model that insertion of a TE can result in 'the transposition of heterochromatin' [52]. These examples of TEs in L*er* *FLC* and Da *FLC* result in a large effect on flowering time that might have had adaptive value.

Epigenetic memory of winter in Arabidopsis is a result of FLC chromatin modifications

Vernalization-requiring plants such as winter annuals and biennials require exposure to the prolonged cold of a typical winter to acquire the competence to flower [53]. In *Arabidopsis*, vernalization establishes this competence largely by repressing *FLC* expression [53]. Cold-mediated *FLC* repression is stably maintained after plants resume growth in warm conditions. The mitotic stability of the vernalized state in *Arabidopsis* in the absence of the inducing signal (cold exposure) is characteristic of an epigenetic switch, and this switch permits plants to 'remember' winter at a cellular level [53–55]. This memory is not passed to the next generation; loss of this memory is necessary so that each generation will have a vernalization requirement.

Screening for mutants that are not able to become vernalized after a long-term cold exposure has led to identification of three genes involved in vernalization: *VERNALIZATION 1* (*VRN1*) [56], *VRN2* [57] and *VERNALIZATION INSENSITIVE 3* (*VIN3*) [54]. Recent studies have shown that vernalization leads to a series of repressive modifications in *FLC* chromatin, which converts active *FLC* chromatin into a heterochromatin-like state [17,54].

During vernalization, histone tails of *FLC* chromatin are deacetylated [16], followed by an increase in H3-K27 and H3-K9 methylation [16,17]. VIN3, a plant-homeodomain-containing protein, is likely to play a role in the initiation of these modifications because none of these vernalization-mediated modifications are observed in *FLC* chromatin of vernalized *vin3* mutants [16]. VRN2 is a plant relative of the *Drosophila* Polycomb-group protein Suppressor of Zeste-12 [57], and is thought to be a component of a Polycomb repressor complex that mediates H3-K27 methylation of *FLC* chromatin, which probably leads to H3-K9 methylation [16,17]. VRN1, a B3-domain-containing DNA-binding protein [56], is required for H3-K9 methylation of *FLC* chromatin during vernalization [16,17].

Vernalization is dominant over the *FLC* activation caused by FRI and FRL1 or by lesions in autonomous-pathway genes [9]. Recent studies show that, like lesions in *ELF7* and *ELF8*, vernalization causes a reduction of H3-K4 trimethylation in *FLC* chromatin in *FRI*-containing lines (Y. He and R.M. Amasino, unpublished). The reduction of H3-K4 trimethylation does not appear to be

the result of vernalization suppressing the expression of the genes involved in this modification of *FLC* chromatin; expression of *ELF7*, *ELF8*, *VIP4* [13] or *EFS* is not affected by vernalization. One of several possibilities to account for the vernalization-mediated decrease of H3-K4 trimethylation is that after vernalization the PAF1 complex is unable to access *FLC* chromatin owing to its heterochromatin-like nature (Figure 2b).

Conclusions

The study of *FLC* regulation provides a paradigm for the control of an important developmental switch by chromatin modification. The autonomous pathway appears to provide constitutive repression of *FLC*, and one component of this repression involves histone deacetylation. FRI and FRL1 are able to overcome autonomous-pathway repression and cause increased *FLC* expression. The presence of *FRI* or the loss of an autonomous pathway gene leads to increased histone H3 trimethylation at lysine 4, and this modification is characteristic of active chromatin. Vernalization is an environment-sensing pathway that also causes deacetylation as well as an increase in two repressive chromatin modifications: dimethylation of lysines 9 and 27 of histone H3. Vernalization is dominant over the *FLC* activation caused by FRI and FRL1 or by lesions in autonomous-pathway genes. It is important to realize that a broad range of chromatin modifications have been found in eukaryotes and only a subset of these has been evaluated with regard to *FLC* regulation. Thus, it would not be surprising if additional chromatin modifications were found to be associated with the regulation of *FLC* expression.

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