PAF1-complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter-annual habit in *Arabidopsis*

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The winter-annual habit (which typically involves a requirement for exposure to the cold of winter to flower in the spring) in *Arabidopsis thaliana* is mainly due to the repression of flowering by relatively high levels of *FLC* expression. Exposure to prolonged cold attenuates *FLC* expression through a process known as vernalization and thus permits flowering to occur in the spring. Here we show that the elevated *FLC* expression characteristic of nonvernalized winter annuals requires two genes, *EARLY FLOWERING 7* (*ELF7*) and *EARLY FLOWERING 8* (*ELF8*), that are homologs of components of the PAF1 complex of *Saccharomyces cerevisiae*. Furthermore, *ELF7* and *ELF8* are also required for the expression of other genes in the *FLC* clade of flowering repressors such as *MAF2* and *FLM*. *FLC*, *FLM*, and *MAF2* are involved in multiple flowering pathways that account for the broad effects of *elf7* and *elf8* mutations on flowering behavior. *ELF7* and *ELF8* are required for the enhancement of histone 3 trimethylation at Lys 4 in *FLC* chromatin. This modification of *FLC* chromatin appears to be required to elevate *FLC* expression to levels that can delay flowering in plants that have not been vernalized. A model of the role of *ELF7*, *ELF8*, and other previously described genes in the modification of the chromatin of flowering repressors is presented.

[*Keywords*: FLC; PAF1 complex; EARLY FLOWERING 7; EARLY FLOWERING 8; MADS-box genes; histone methylation]

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The floral transition is a major developmental switch in the plant life cycle that must be properly timed to ensure maximal reproductive success. This transition involves coordinating responses to environmental cues such as day length, light quality, and temperature with endogenous factors such as developmental status and age. In *Arabidopsis thaliana*, a facultative long-day plant, flowering time is regulated by five distinct, yet linked pathways (Boss et al. 2004; Putterill et al. 2004). The photoperiod and vernalization pathways accelerate flowering in response to day length and long-term cold exposure, respectively. A thermosensory pathway promotes flowering in response to increased ambient temperature (Blazquez et al. 2003). The autonomous pathway and a gibberellin-dependent pathway promote flowering largely in response to developmental signals. Outputs from these pathways are integrated by a set of common downstream target genes, "flowering-time integrators"; expression of these integrators leads to the induction of floral-meristem-identity genes and thus flowering (Boss et al. 2004; Putterill et al. 2004).

Flowering-time variation exists among *Arabidopsis* accessions. The genetic difference between a winter-annual habit (late flowering without vernalization) and a rapid-flowering habit (rapid flowering without vernalization) is often determined by allelic variation at *FRIGIDA* (*FRI*) or *FLOWERING LOCUS C* (*FLC*) (Burn et al. 1993; Lee et al. 1993, 1994; Clarke and Dean 1994; Koornneef et al. 1994). FLC is a MADS-box transcriptional regulator (Michaels and Amasino 1999; Sheldon et al. 1999) that inhibits the floral transition largely by reducing the expression of flowering-time integrators such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* and *FT* (Hepworth et al. 2002). The role of *FRI* is to up-regulate

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Table 1. *Primary leaf number at bolting of* elf7 *mutants*

Day length	Ws	*elf7-1*	Col	*elf7-2*	*elf7-3*
Short days	36.9 ± 4.4	14.4 ± 1.4	66.9 ± 3.9	19.2 ± 2.6	21.2 ± 2.9
	(7.3 ± 0.8)	(5.1 ± 0.6)	(10.7 ± 1.5)	(4.1 ± 0.6)	5.6 ± 0.8
Long days	9.9 ± 1.1	6.8 ± 0.7	12.2 ± 1.2	6.6 ± 0.7	ND[a]
	(2.9 ± 0.4)	(3.4 ± 0.5)	(3.2 ± 0.7)	(2.8 ± 0.7)	ND[a]

Values shown are mean number ± standard deviation of rosette and cauline leaves (in parentheses); 10 plants were scored for each line.
[a]ND, not determined.

FLC expression to levels that inhibit flowering (Michaels and Amasino 1999; Sheldon et al. 1999). Winter annuals have dominant alleles of *FRI* and *FLC*, whereas many rapid-flowering accessions have a nonfunctional *fri* allele (Johanson et al. 2000) or an allele of *FLC* that is not up-regulated by *FRI* (Gazzani et al. 2003; Michaels et al. 2003).

Autonomous-pathway mutations delay the onset of flowering in rapid-flowering accessions (Boss et al. 2004). In fact, such mutations cause otherwise rapid-flowering accessions to behave like *FRI*-containing winter annuals. Both *FRI* and mutations in autonomous-pathway genes result in increased *FLC* expression, and loss-of-function *flc* mutations suppress the delayed flowering of *FRI*-containing lines or autonomous-pathway mutants (Michaels and Amasino 2001). Thus, the normal role of autonomous-pathway genes is to repress *FLC*, and *FRI* can overcome this suppression (Michaels and Amasino 2001). The increased *FLC* expression and delayed flowering in both *FRI* and autonomous-pathway mutants can be reversed by exposure to the prolonged cold typical of a winter season by a process known as vernalization (Michaels and Amasino 2000; Boss et al. 2004).

Recent studies have begun to reveal the molecular mechanisms involved in *FLC* repression. Two genes in the autonomous pathway, *FLOWERING LOCUS D* (*FLD*) and *FVE*, encode proteins involved in deacetylating core histone tails of *FLC* chromatin (He et al. 2003; Ausin et al. 2004). Vernalization leads to repressive histone modifications in *FLC* chromatin including deacetylation, and increased methylation of Lys 9 and Lys 27 of histone 3 (Bastow et al. 2004; Sung and Amasino 2004). These modifications are characteristic of a stable heterochromatin conformation.

Given the central role of *FLC* in controlling flowering time, understanding its regulation at a molecular level is of great interest. Genetic analyses have revealed many of the components involved in the regulatory network governing *FLC* expression. The study of mutants that render a *FRI*-containing winter-annual line early flowering has led to the identification of several loci that are required for *FLC* expression: *VERNALIZATION INDEPENDENCE 4* (*VIP4*) (Zhang and van Nocker 2002), *VIP3* (Zhang et al. 2003), and *PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1* (*PIE1*) (Noh and Amasino 2003). Also, mutations in *EARLY IN SHORT DAYS 4* (*ESD4*) cause a modest decrease in *FLC* levels (Reeves et al. 2002; Murtas et al. 2003). However, the molecular mechanism by which these activators regulate *FLC* expression is not known.

In this report, we present the identification of two genes involved in *FLC* activation: *ELF7*, which encodes the *Arabidopsis* relative of yeast RNA polymerase II (Pol II) Associated Factor 1 (PAF1), and *ELF8*, which encodes the *Arabidopsis* relative of yeast CLN Three Requiring 9 (CTR9). Lesions in *ELF7* and *ELF8* cause early flowering in an otherwise late-flowering, *FRI*-containing line by suppressing *FLC* expression, and also cause early flowering in noninductive photoperiods by suppressing expression of the MADS-box genes *FLOWERING LOCUS M* (*FLM*) and *MADS AFFECTING FLOWERING 2* (*MAF2*). The expression of these MADS-box flowering repressors is associated with increased histone 3 trimethylation at Lys 4, and this chromatin modification requires ELF7 and ELF8.

Results

Mutations in ELF7 *result in early flowering*

We screened a T-DNA mutagenized population in the Wassilewskija (Ws) accession of *Arabidopsis* for mutants that flowered more rapidly than wild type in noninductive short days (SD). One mutant, referred to as *early-*

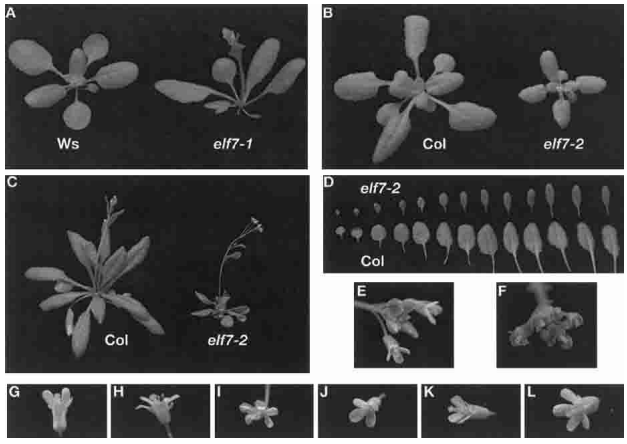

Figure 1. *elf7* mutants are early flowering and display additional accession-dependent phenotypes. (*A–C*) *elf7* mutants grown under LD. (*D*) The first 13 rosette leaves formed on representative *elf7-2* and Col plants grown in SD. (*E*) Flower buds in Col. (*F*) Flower buds in *elf7-2*. (*G–L*) Representative flowers from *elf7* and wild types: Col (*G*), *elf7-2* (*H,I*), Ws (*J*), *elf7-1* (*K,L*). *elf7-1* and *elf7-2* display altered petal number in a fraction of flowers (*I,J*); in addition, in *elf7-2* flowers (*F*), but not in *elf 7-1* flowers, the sepals do not enclose the flower buds.

flowering 7 (*elf7*), flowered earlier than wild type in both SD and also in inductive long days (LD) (Table 1; Fig. 1A). A backcross to wild type revealed that the *elf7* phenotype was recessive. Analysis of the F_2 population indicated that the *elf7* phenotype segregated as a single Mendelian trait, and that the *elf7* line contained a single T-DNA locus that cosegregated with the mutant phenotype (data not shown). The sequence of a genomic fragment flanking the left border of the T-DNA indicated that the T-DNA was inserted in the 5′-UTR of *At1g79730* (http://www.arabidopsis.org; Fig. 2A).

To confirm that the early-flowering phenotype of *elf7* is due to a lesion in *At1g79730*, two additional T-DNA insertional alleles, *elf7-2* and *elf7-3*, were identified in the Columbia (Col) genetic background from the SALK T-DNA collection (Alonso et al. 2003). Both *elf7-2* and *elf7-3* flowered earlier than wild-type Col (Table 1). Furthermore, when *elf7-1* and *elf7-3* were crossed to *elf7-2*, the F_1 progeny flowered early in both SD and LD (data not shown). Finally, a 4.3-kb genomic fragment containing the *At1g79730* coding region plus 1.0 kb of promoter sequence was introduced into *elf7-2*. This fragment was able to rescue the mutant phenotype (data not shown).

In addition to early flowering, *elf7* mutants display other phenotypes, some of which are only found in the Col genetic background. One Col-specific phenotype is that rosette leaves of *elf7-2* and *elf7-3* are smaller and slightly more curled than wild type (Fig. 1B–D; *elf7-2* is shown). There was no difference in the leaves of *elf7-1* compared to wild-type Ws (Fig. 1A). Another Col-specific phenotype is that the sepals often fail to enclose flower buds in the latest stages of development (Fig. 1F). The phenotypic difference between *elf7* in Col and Ws was analyzed genetically. F_1 plants from a cross between *elf7-1* in Ws and *elf7-2* in Col did not display the Col-specific phenotypes (data not shown). F_2 plants displayed these phenotypes in a ratio of 3:1, respectively, suggesting that the additional phenotypes of *elf7* mutations in Col are the result of a single recessive locus in the Col genetic background.

A phenotype of *elf7* mutants in both the Col and Ws backgrounds is that petal number per flower often varied from three to five (the wild-type flower has four petals) (Fig. 1I,L). For example, 16% of flowers had three petals and 17% had five petals in the *elf7-1* mutant in Ws grown in LD, whereas in the same growth conditions <6% of wild-type Ws flowers displayed altered petal number.

ELF7 *encodes the plant relative of yeast PAF1*

ELF7 is a single-copy gene in the *Arabidopsis* genome that consists of 12 exons encoding a predicted 589-amino acid protein. The T-DNA in *elf7-1* was inserted immediately upstream of the translation start codon (Fig. 2A); T-DNAs in *elf7-2* and *elf7-3* were inserted in the eighth and the last intron, respectively (Fig. 2A). The full-length *ELF7* mRNA was not detectable in the *elf7* mutants (Fig. 2C).

Except for the N-terminal region, the ELF7 protein is similar (~30%) over its entire length to PAF1, which was first described in *Saccharomyces cerevisiae* (Fig. 2B; the

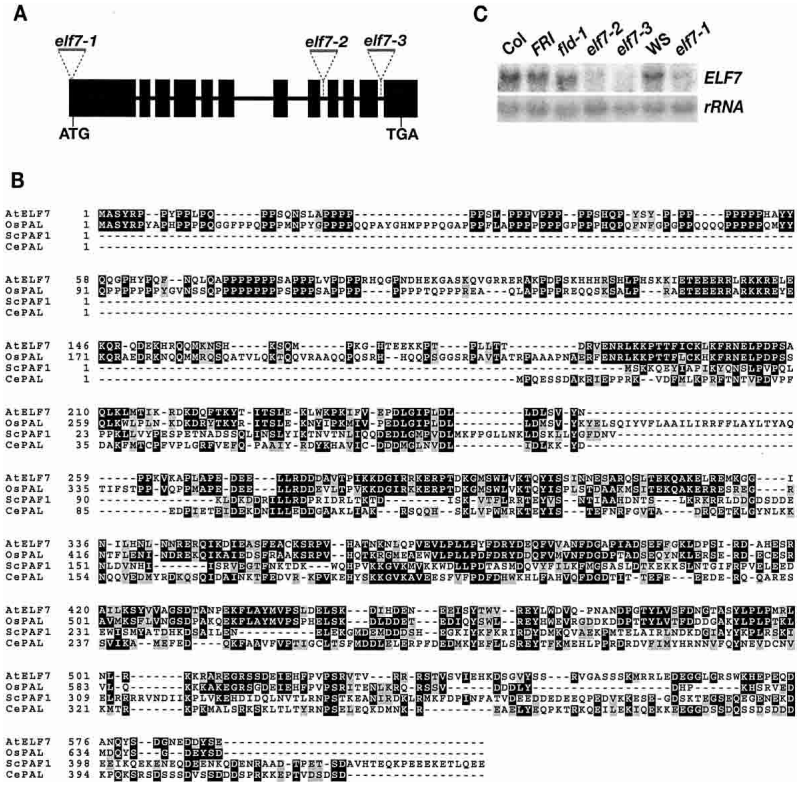

Figure 2. *ELF7* encodes the *Arabidopsis* homolog of yeast PAF1. (*A*) *ELF7* gene structure. Exons are represented by filled boxes, and introns by lines. Triangles indicate *T-DNA* insertions. (*B*) Amino acid sequence alignment of *A. thaliana* ELF7 (AtELF7) with *S. cerevisiae* PAF1 (ScPAF1), *C. elegans* PAF1 like (CePAL), and *Oryza sativa* PAF1 like (OsPAL). Numbers refer to amino acid residues; identical residues are shaded with black, while similar residues are shaded with gray. (*C*) *ELF7* steady-state mRNA levels in seedlings determined by RNA blot analysis. About 15 μg of total RNA extracted from 10-d-old seedlings was loaded in each lane.

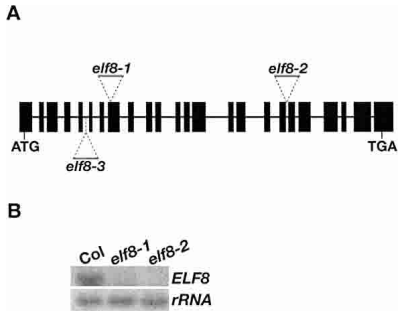

Figure 3. Structure and expression of *ELF8*. (*A*) *ELF8* gene structure. (*B*) RNA blot analysis of *ELF8* steady-state mRNA levels in seedlings.

Caenorhabditis elegans PAF1 is also included for comparison; Shi et al. 1996). The *Arabidopsis* PAF1 homolog has an N-terminal extension that is highly proline-rich (46 prolines in the 94-amino acid region); this region is not found in PAF1s from yeast, *C. elegans*, *Drosophila*, or mammals. This N-terminal extension is also present in the rice PAF1 homolog (Fig. 2B), indicating that this extension may be a unique feature of PAF1 proteins in higher plants.

Lesions in ELF8, *a homolog of another PAF1 complex component, also cause early flowering*

In *S. cerevisiae* (Sc), PAF1 is an integral component of a five-member complex consisting of PAF1, CTR9, LEO1, RTF1, and CDC73 called the PAF1 complex (Krogan et al. 2002; Squazzo et al. 2002). This complex associates with RNA polymerase II (Pol II) to regulate expression of a subset of the yeast genome (Betz et al. 2002; Ng et al. 2003). Searching the *Arabidopsis* protein sequence database revealed that *Arabidopsis* contains genes encoding proteins highly related to ScCTR9 and ScLEO1, but not to ScRTF1 and ScCDC73. At2g06210 (http://www.arabidopsis.org) is the *Arabidopsis* homolog of ScCTR9 (Supplementary Fig. S1).

We obtained three lines with T-DNA insertions in *At2g06210* from the SALK T-DNA collection (Fig. 3A; Alonso et al. 2003). In both SD and LD, these three alleles caused early flowering similar to that of *elf7* (Tables 2, 3). *At2g06210* was therefore designated as *ELF8*. These *elf8* alleles also caused the leaf and flower phenotypes in the Col background described above for *elf7* (Fig. 4; data not shown). F_1 complementation tests among the putative *elf8* alleles showed that all were allelic (data not shown), confirming the involvement of *ELF8*/*At2g06210* in flowering-time regulation.

ELF8 is a single-copy gene in *Arabidopsis* that consists of 24 exons encoding a predicted 1064-amino acid protein. In *elf8-1*, the T-DNA was inserted into the eighth exon, and the T-DNAs in *elf8-2* and *elf8-3* were inserted into the eighteenth and the fifth intron, respectively (Fig. 3A). The full-length *ELF8* mRNA was not detected in either *elf8-1* or *elf8-2* (Fig. 3B) (*elf8-3* was not analyzed).

The *Arabidopsis* homolog of ScLEO1 is At5g61150. At5g61150 has been previously characterized as VIP4 (Zhang and van Nocker 2002). We isolated two *vip4* alleles from the SALK T-DNA collection (Alonso et al. 2003; see Materials and Methods). When these two *vip4* mutants are grown alongside *e1f7*, the phenotypes are identical (and similar to that previously described for other *vip4* alleles) (Zhang and van Nocker 2002): the two SALK *vip4* mutations cause early flowering in both LD and SD, and in the Col genetic background also cause the leaf and flower phenotypes that are observed in *elf7* and *elf8* (data not shown).

The similar phenotypes resulting from lesions in three genes that encode proteins that are homologous to constituents of the PAF1 complex in yeast suggests that the *Arabidopsis* proteins may be components of a similar complex in plants. Genetic evidence is consistent with this hypothesis: a double mutant between *elf7* and *elf8* flowers at the same time as the single mutants (Table 2). In addition, the double mutant was morphologically indistinguishable from either the *elf7-3* or *elf8-1* single mutants with respect to the leaf and flower development phenotypes (Fig. 4; data not shown).

Lesions in elf7 *and* elf8 *suppress FLC-dependent late flowering*

FLC, a MADS-box transcriptional factor, is a central regulator of the floral transition in *Arabidopsis* (Michaels and Amasino 1999; Sheldon et al. 1999). *FLC* is up-regulated by *FRI* and repressed by genes in the autonomous pathway (Michaels and Amasino 2001). Thus, either the presence of *FRI* or a lesion in an autonomous-pathway gene causes delayed flowering due to elevated *FLC* expression. To evaluate the genetic interaction of *elf7* with *FRI*, *elf7-2* was introduced into Col-*FRI*^{sf2} (Lee et al. 1994), and to evaluate the interaction with an autonomous-pathway gene, *elf7-2* was introduced into a line with a mutation in *FLD* (*fld-1*) (He et al. 2003). The late-flowering phenotypes of Col-*FRI*^{sf2} and *fld-1* are completely suppressed by *elf7-2* (Table 3). We also determined *FLC* transcript levels in Col, *FRI*, *fld-1*, *elf7*, and double mutants. *FLC* was expressed at a low level in Col, and, as previously shown, up-regulated in *FRI* and *fld-1* (Fig. 5A,B). However, in *elf7-2*, *elf7-3*, *elf7-2 FRI*, and

Table 2. *Primary leaf number at bolting of* elf7, elf8, *and* elf7 elf8 *mutants in short days*

Col	*elf8-1*	*elf8-2*	*elf8-3*	*elf7-3*	*elf8-1 elf7-3*
66.9 ± 3.9	15.2 ± 1.6	16.3 ± 2.1	17.1 ± 1.7	21.2 ± 2.9	15.0 ± 0.9
(10.7 ± 1.5)	(4.3 ± 0.7)	(4.0 ± 0.7)	(4.1 ± 0.7)	(5.6 ± 0.8)	(4.3 ± 0.8)

Values shown are mean number ± standard deviation of rosette and cauline leaves (in parentheses); 10 plants were scored for each line.

Figure 4. The phenotypes of *elf7*, *elf8*, and *elf7 elf8* mutants in short days.

elf7-2 fld-1, *FLC* transcripts were not detectable (Fig. 5A,B). Thus, ELF7 activity is required for *FLC* expression. We also examined whether *ELF7* expression was influenced by *FRI* or the autonomous pathway. As shown in Figure 2C, *ELF7* transcript levels were not regulated by *FRI* or *FLD*.

To evaluate whether the activity of ELF8 is also required for *FLC* expression, we introduced *elf8-1* into Col-*FRI*^{sf2} and another autonomous mutant, *fve-4* (Michaels and Amasino 2001; Ausin et al. 2004). The late-flowering phenotypes of *FRI* and *fve-4* were completely suppressed by *elf8-1* (Table 3). RNA blot analysis showed that *FLC* transcripts were not detectable in *elf8-1*, *elf8-2*, *elf8-1 FRI*, and *elf8-1 fve-4* (Fig. 5C). Thus, ELF8, like ELF7, is required for *FLC* expression.

Lesions in elf7 *and* elf8 *also affect flowering via other MADS-box genes*

The *elf7* and *elf8* mutants flower earlier in both LD and SD than plants containing an *flc*-null mutant, *flc-3* (Fig. 6A). Thus, ELF7 and ELF8 must participate in at least one *FLC*-independent flowering pathway in addition to their role in *FLC* expression. The early flowering of *elf7* and *elf8* mutants in SD is reminiscent of the phenotype caused by lesions in another MADS-box gene, *FLM* (Ratcliffe et al. 2001; Scortecci et al. 2001). *FLM* is closely related to *FLC* and, like *FLC, FLM* also acts as a repressor of flowering. However, unlike *FLC*, *FLM* is involved in the photoperiod regulation of flowering (Scortecci et al. 2003). We examined *FLM* expression in both *elf7-2* and *elf8-1* plants, and found that *FLM* expression was suppressed in both mutants compared to wild type (Fig. 6B). Therefore, ELF7 and ELF8 are required for expression of both *FLC* and *FLM*. A MADS-box gene that is likely to encode a partner of FLM is *SUPPRESSOR OF VEGETATIVE PHASE* (*SVP*) (Scortecci et al. 2003). *SVP* is in a different clade of MADS-box genes from *FLC* and *FLM*. Lesions in *elf7* or *elf8* had no effect on *SVP* expression (Fig. 6B). We also examined expression of several MADS-box genes that do not affect flowering as additional controls. As in the situation for *SVP*, the mRNA levels of *AGAMOUS LIKE 16* (*AGL16*), *AGL18*, and *AGL30* are not affected by lesions in *elf7* or *elf8* (Fig. 6C). Thus, ELF7 and ELF8 may regulate select clades of MADS-box genes. Another MADS-box gene in the *FLC/FLM* clade is *MAF2*, a repressor of floral transition (Ratcliffe et al. 2003), and consistent with the common regulation of this clade, *MAF2* expression is also suppressed in *elf7* and *elf8* mutants (Fig. 6B).

Table 3. *Primary leaf number at bolting of* elf7, elf8, *and double mutants in long days*

Lines	Rosette leaf number	Cauline leaf number
Col	12.2 ± 1.2	3.2 ± 0.7
flc-3	10.3 ± 1.6	2.4 ± 0.5
elf7-2	6.6 ± 0.7	2.8 ± 0.7
elf8-1	6.5 ± 0.5	3.5 ± 0.5
FRI	67.0 ± 7.8	9.8 ± 2.1
elf7-2 FRI	6.7 ± 0.9	3.3 ± 0.7
elf8-1 FRI	6.9 ± 0.7	3.3 ± 0.5
fld-1	52.6 ± 11	9.0 ± 1.9
fld-1 elf7-2	7.0 ± 0.7	3.1 ± 0.8
fve-4	44.0 ± 4.1	8.5 ± 1.3
fve-4 elf8-1	7.4 ± 0.9	3.6 ± 0.7

Values shown are mean number ± standard deviation; 10 plants were scored for each line.

ELF7 *and* ELF8 *are expressed in the shoot apex*

Given that *ELF7* and *ELF8* are both required for *FLC* and *FLM* expression, it was of interest to compare the spatial expression patterns of these genes to that of *FLC* and *FLM*. Accordingly, fusions to the reporter gene β-*glucuronidase* (*GUS*) were made for each gene (see Materials and Methods). *ELF7* expression is highest in regions that contain dividing cells in the shoot and root apex; this pattern is the same as that of *FLC* (Fig. 7A, panels a,b; also Michaels and Amasino 2000) and *FLM* (Scortecci et al. 2001). The *ELF8* construct produced an expression pattern that overlapped with that of *ELF7* and *FLC*, but it was also readily detected in all root tissues and in regions around hydathodes at the tips of leaves (Fig. 7A, panel c). We examined the steady-state mRNA levels of *ELF7*, *ELF8*, and *FLC* in seedlings by RT–PCR.

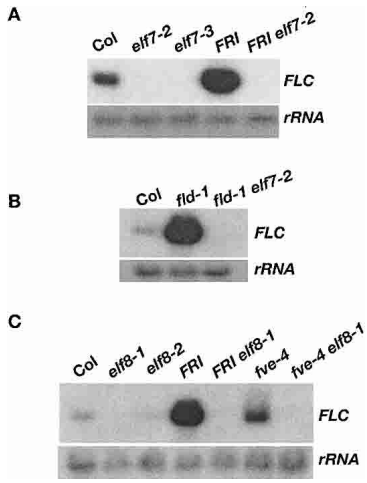

Figure 5. *elf7* and *elf8* suppress *FLC* expression. (*A*) Steady-state *FLC* mRNA levels in *elf7 fri* and *elf7 FRI* seedlings (10 d old) as determined by RNA blot. (*B*) RNA blot analysis of *FLC* mRNA levels in *elf7 fld-1* seedlings. (*C*) Steady-state *FLC* mRNA levels in *elf8*, *elf8 FRI*, and *elf8 fve-4* seedlings as determined by RNA blot.

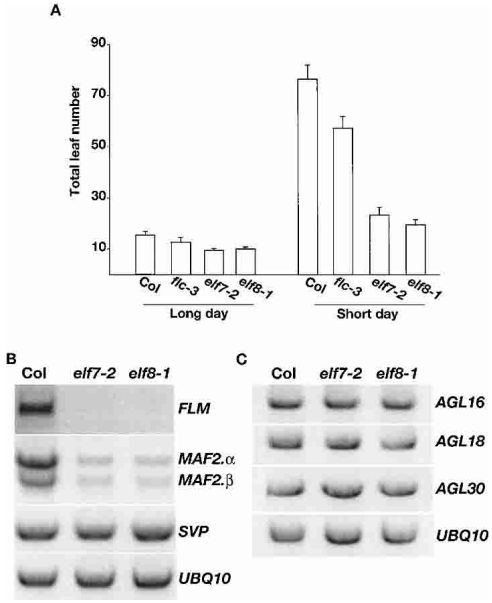

Figure 6. *FLC*-independent early flowering in *elf7* and *elf8*. (*A*) Flowering times of *flc*, *elf7*, and *elf8* in long days and short days. Bars represent mean values ± standard deviation of total leaf number at bolting. For each line, 10 plants were scored. (*B*) RT–PCR analysis of expression of *FLC* relatives in *elf7* and *elf8*. Two variants of *MAF2* were detected. *UBQ10* was used as a control. (*C*) RT–PCR analysis of expression of additional MADS-box genes in *elf7* and *elf8*.

Consistent with the reporter gene patterns, all three genes are expressed in the shoot apex, where the floral transition occurs (Fig. 7B).

Histone 3 is hypermethylated at Lys 4 in actively transcribed FLC *chromatin*

The yeast PAF1 complex associates with RNA Pol II and is thought to regulate gene expression by recruiting SET1 (a histone 3 Lys 4 [H3-K4] methyl transferase) to the initially transcribed (5′) regions of target-gene chromatin (Krogan et al. 2003; Ng et al. 2003). This generates H3-K4 trimethylation predominantly in the 5′ portion of transcribed regions (Ng et al. 2003), and this chromatin modification is a hallmark of actively transcribed genes in *S. cerevisiae* (Santos-Rosa et al. 2002).

Because ELF7 and ELF8 are relatives of yeast PAF1 and CTR9, respectively, we evaluated whether a mechanism similar to that mediated by the yeast PAF1 complex might be involved in *FLC* expression. The H3-K4 trimethylation state of *FLC* chromatin in an *FRI*-containing line (in which *FLC* is actively expressed) was compared to that in Col (which lacks *FRI* and does not express *FLC* at high levels) by chromatin immunoprecipitation (ChIP) assays using an antibody directed against trimethylated K4 of histone H3. Similar to the situation observed for PAF1-regulated genes in yeast, a region of *FLC* chromatin around the start site of transcription is enriched in H3-K4 trimethylation when *FLC* is up-regulated by *FRI* (Fig. 8). The *FRI*-mediated increase in H3-K4 trimethylation of *FLC* chromatin did not occur in *elf7* or *elf8* mutants (Fig. 9A,B). Therefore, the *FRI*-enhanced H3-K4 trimethylation of *FLC* chromatin is dependent on these two relatives of PAF1 complex components. The H3-K4 trimethylation level of *FLC* chromatin is also increased in the autonomous-pathway mutant *fld* (Fig. 9A,B).

H3-K4 trimethylation in the 5′ portion of the transcribed region of *FLM* chromatin was also greatly reduced in *elf7-2* and *elf8-1* mutants compared to wild-type Col (Fig. 9C,D), indicating that ELF7 and ELF8 are involved in H3-K4 trimethylation of two members of this clade of MADS-box genes.

A conserved region located in the 5′-UTR of MADS-box genes is required for FLC *expression*

Because the enhanced trimethylation occurred in the 5′-UTR of *FLC*, it was of interest to determine whether this region plays a role in *FLC* transcription. We first compared the sequence of this region of *FLC* with that of other members of the *FLC* clade: *FLM*, *MAF2*, and *Brassica napus FLC1* (an *Arabidopsis FLC* ortholog) (Tadege et al. 2001), and found a conserved 34-bp motif at the same 5′ location in all of these genes (Fig. 10A). This motif was not found in *SVP*, a gene that is likely to be a partner of *FLM* but that is in a different clade of MADS-box genes (Scortecci et al. 2003), nor was it found in other MADS-box genes that affect flowering. We created an internal 80-bp deletion (Δ80) that spanned this 34-bp motif within the 5′-UTR of *FLC* (Fig. 10B; –4 to –83, A of the start codon ATG is +1). This Δ80 construct preserves a 26-bp region immediately downstream of the transcription start point and a 7-bp region immediately upstream of the translation start codon. The Δ80 construct and the

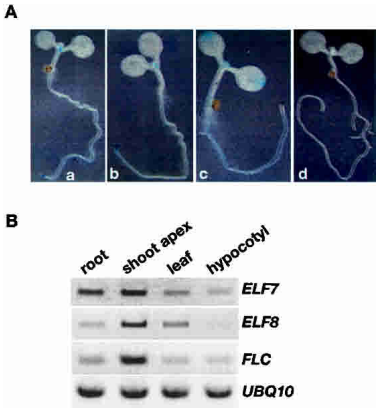

Figure 7. Spatial expression pattern of *FLC*, *ELF7*, and *ELF8* in seedlings. (*A*) Spatial expression pattern of *GUS* reporter gene fused to *FLC*, *ELF7*, or *ELF8* in seedlings. (Panel *a*) FLC:GUS in Col *FRI*^{sf2}. (Panel *b*) ELF7:GUS in Col. (Panel *c*) ELF8:GUS in Col. (Panel *d*) FLC:GUS in *elf7-2 FRI*. (*B*) RT–PCR analysis of expression of *FLC*, *ELF7*, and *ELF8* in various tissues from seedlings.

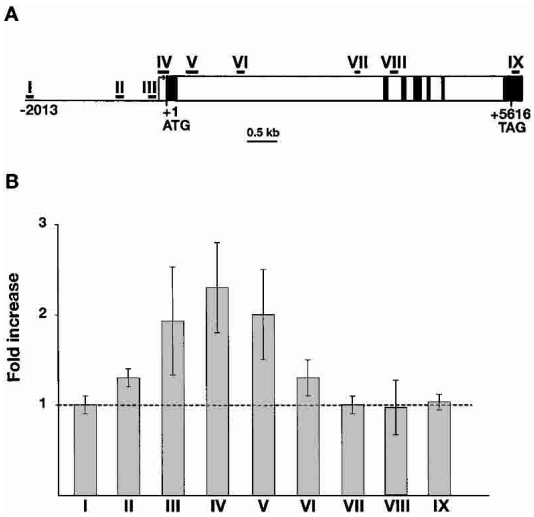

Figure 8. ChIP analysis of the H3-K4 trimethylation level of *FLC* chromatin. (*A*) Schematic structure of *FLC*. I to IX represent the regions in which K4 trimethylation states were examined by ChIP. The translation initiation point is +1. The filled boxes represent exons, and open boxes represent introns. The arrow indicates transcription start point. (*B*) Relative levels of trimethylated K4 at various genomic *FLC* regions in Col FRI^{sf2}. Three independent immunoprecipitations were performed; the immunoprecipitated DNA was quantified using PCR. The fold enrichments of Col FRI^{sf2} over Col at indicated regions are shown. The values shown are mean ± standard deviation.

wild-type *FLC* construct (–1167) were introduced into *flc-3*, *flc-3 FRI*, and *flc-3 fpa* (*FPA* is a repressor of *FLC* expression in the autonomous pathway). The wild-type (–1167) transgene behaved as expected: in *FRI* and *fpa* backgrounds, T_1 transformants harboring this transgene were late flowering (Fig. 10C) and had high levels of *FLC* expression (Fig. 10D). However, the Δ80 transgene did not repress flowering, nor was it up-regulated in *FRI* or *fpa* (Fig. 10C,D). Therefore, the region containing the conserved motif within the 5′-UTR of *FLC* is required for *FLC* expression, although these data do not address whether this region is directly required for ELF7 and ELF8 to promote *FLC* expression.

Discussion

In this study, we identified mutations in two genes, *elf7* and *elf8*, that cause rapid flowering in several situations in which flowering is otherwise delayed. One situation is that *elf7* and *elf8* suppress the delayed flowering of both *FRI*-containing winter annuals and autonomous-pathway mutants. The presence of *FRI* or autonomous-pathway mutations delays flowering by increasing *FLC* expression, and we have shown that *ELF7* and *ELF8* are required for this increase. Thus, *ELF7* and *ELF8* are required to elevate *FLC* expression to a level that creates the vernalization-responsive, winter-annual habit (Michaels and Amasino 2000).

The *elf7* and *elf8* lesions also suppress the delayed flowering that results from growth under noninductive photoperiods. Because loss of *FLC* activity has only a minor effect on flowering in noninductive photoperiods (Michaels and Amasino 2001), other genes must be involved in this aspect of the *elf7* and *elf8* flowering phenotype. *elf7* and *elf8* flower early in noninductive photoperiods, at least in part because *FLM* and *MAF2* expression also requires *ELF7* and *ELF8*. *FLM* is the closest relative of *FLC* in *Arabidopsis* (Ratcliffe et al. 2001; Scortecci et al. 2001); both are flowering repressors, but, unlike *FLC*, *FLM* acts in the photoperiod pathway (Scortecci et al. 2003). *MAF2* is another MADS-box gene in which mutations affect photoperiod-dependent flowering (Ratcliffe et al. 2003). Thus, we have a molecular explanation for the broad effects of *elf7* and *elf8* lesions on flowering behavior: *ELF7* and *ELF8* are required for the expression of members of a clade of MADS-box genes that are involved in several flowering pathways.

ELF7 encodes the *Arabidopsis* homolog of yeast PAF1, and *ELF8* encodes the relative of yeast CTR9. In yeast,

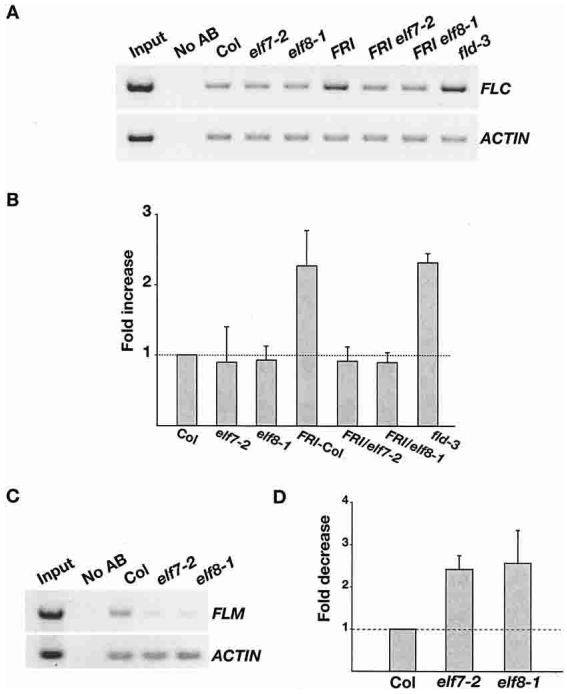

Figure 9. ELF7 and ELF8 are required for H3-K4 hypertrimethylation in *FLC* and *FLM* chromatin. (*A*) ChIP analysis of H3-K4 trimethylation state of Region IV (see Fig. 8A) in *FLC* chromatin of *elf7*, *elf8*, and related double mutants. A representative ChIP–PCR is shown. The input is Col chromatin before immunoprecipitation. "No AB" refers to the control sample lacking antibody. *ACTIN* served as an internal control. (*B*) Relative levels of trimethylated K4 at Region IV in *FLC* chromatin of *elf7*, *elf8*, and related double mutants. Three independent immunoprecipitations were performed; the immunoprecipitated DNA was quantified using PCR. The level of trimethylated K4 in each mutant or FRI-containing line was normalized to that in Col (the level of trimethylated K4 in Col was arbitrarily set at 1.0). The values shown are mean ± standard deviation. (*C*) ChIP analysis of H3-K4 trimethylation state of the 5′-transcribed region of *FLM* chromatin in *elf7* and *elf8*. (*D*) Relative levels of trimethylated K4 at the 5′ region of *FLM* chromatin in *elf7* and *elf8*. The relative levels were calculated as described in *B*.

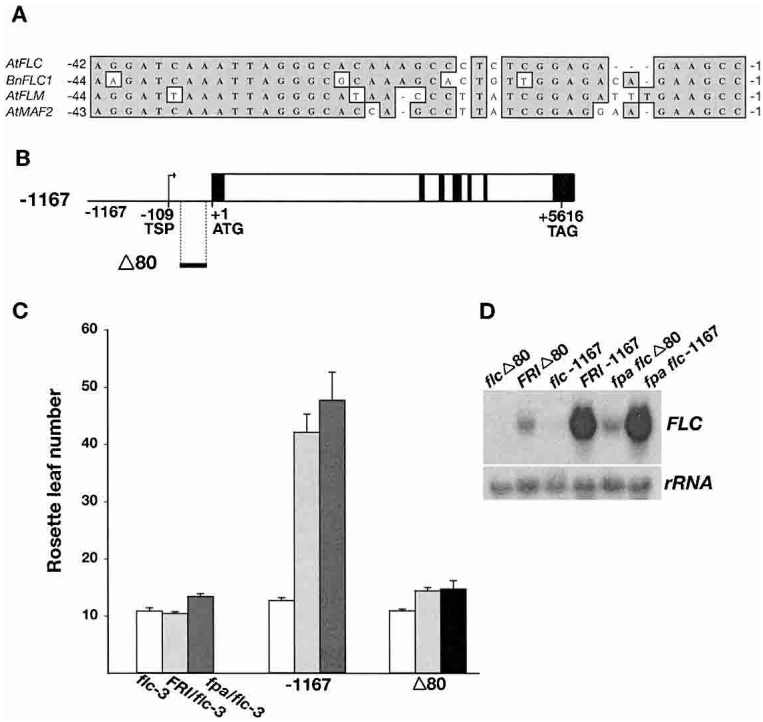

Figure 10. An 80-bp deletion (Δ80) in the 5′-untranslated region (UTR) of *FLC* suppresses its expression. (*A*) 5′-UTR sequence alignment of *A. thaliana FLC* (*AtFLC*) with *B. napus FLC 1* (*BnFLC1*), *AtFLM*, and *AtMAF2*. The translation initiation point is +1. (*B*) Schematic structure of a functional *FLC* transgene (–1167) and the Δ80 transgene. The translation initiation point is +1. The filled boxes represent exons, and open boxes represent introns. The arrow indicates transcription start point. (*C*) Flowering times of T_1 transgenic plants derived from transgenes –1167 and Δ80. For each transgenic line, 15–33 T_1 plants were scored. The values shown are mean ± standard error. White, gray, and black boxes represent *fri flc-3*, *FRI flc-3*, and *fpa flc-3* genetic backgrounds, respectively. (*D*) Abundance of steady-state *FLC* mRNA levels in transgenic seedlings determined by RNA blot.

PAF1 and CTR9 are members of a complex of five proteins, called the PAF1 complex, that associates with RNA Pol II (Squazzo et al. 2002; Krogan et al. 2003; Ng et al. 2003). The other members of this complex are LEO1, RTF1, and CDC73 (Squazzo et al. 2002). To date, a homolog of only one other complex member, LEO1, has been identified in *Arabidopsis* (Zhang and van Nocker 2002). Lesions in this homolog, *VIP4*, create a phenotype similar to that of *elf7* and *elf8*. The similar phenotype of single mutants in three single-copy plant homologs of components of the yeast PAF1 complex is consistent with ELF7, ELF8, and VIP4 operating in a similar complex in plants. The observation that a double mutant between *elf7* and *elf8* is identical to the *elf8* single mutant provides further support for this model (as does the effect on target-gene chromatin discussed below). The closest *Arabidopsis* relatives of RTF1 and CDC73 are At1g61040 and At3g22590, respectively. We have examined a T-DNA insertion mutant in *At3g22590*, and the insertion in this gene does not cause any effect on flowering, nor does it cause any of the other phenotypes of *elf7*, *elf8*, or *vip4* mutants. One possibility is that At3g22590 is not a part of the PAF1 complex in *Arabidopsis*. Another possibility is that this protein is a part of the complex, but its loss of function in *Arabidopsis* does not impair the ability of the complex to regulate flowering. In this regard, it is interesting to note that the plant PAF1 proteins are predicted to encode a proline-rich N-terminal region that is not present in PAF1 proteins from animals and yeast. Perhaps this N-terminal region may permit interaction with other components of the complex that are unique to plants.

Recently it has been demonstrated that the yeast PAF1 complex is involved in recruiting SET1 to the RNA Pol II elongation complex (Krogan et al. 2003; Ng et al. 2003). SET1 is a methyltransferase that catalyzes methylation of histone 3 (H3) on Lys 4 (K4) to produce trimethylated H3-K4 (Roguev et al. 2001; Santos-Rosa et al. 2002). Typically the regions of chromatin in a transcription unit that are transcribed early become enriched in H3-K4 trimethylation, and this chromatin modification is thought to provide a positive epigenetic mark to maintain a gene in an actively transcribed state (Santos-Rosa et al. 2002; Ng et al. 2003). We have shown that ELF7 and ELF8 are required for H3-K4 trimethylation of the chromatin of *FLC* and *FLM*, and that *elf7* and *elf8* lesions attenuate *FLC* and *FLM* expression. Furthermore, we have identified an *Arabidopsis* relative of the *Drosophila* ASH1 (Beisel et al. 2002; Byrd and Shearn 2003) and yeast SET1 H3-K4 methyltransferases (Roguev et al. 2001; Santos-Rosa et al. 2002). Mutations in this gene, *EARLY FLOWERING IN SHORT DAYS* (*EFS*) (Soppe et al. 1999), also reduce H3-K4 trimethylation in *FLC* chromatin and cause an early-flowering phenotype similar to that in *elf7*, *elf8*, and *vip4* (S.D. Michaels, Y. He, and R.M. Amasino, unpubl.). Thus, members of both the yeast and the plant PAF1 complexes appear to regulate target genes by a similar mechanism.

In the Ws genetic background, *elf7* mutants developed normally except for early flowering and altered petal number, which suggests that only a small number of genes were strongly affected by loss of ELF7 activity. In Col, *elf7* mutants exhibit other developmental abnormalities including smaller leaves and sepals, although the Col mutants complete the normal plant life cycle. The lack of lethality in *Arabidopsis* plants that have lost

ELF7 or ELF8 activity, despite the fact that ELF7 and ELF8 are encoded by single-copy genes, is similar to the phenotype of lesions in the corresponding genes in yeast. Disruption of the PAF1 complex in yeast is not lethal, and only the abundance of a small subset of mRNAs is affected (Betz et al. 2002).

Little is known about why the expression of certain genes is affected more strongly than others by loss of PAF1 complex activity. In the examples we have studied, the clade of MADS-box genes appears to be the key feature for ELF7 and ELF8 involvement rather than the flowering pathway in which the MADS-box gene participates. Specifically, *FLM* and *SVP* are in the same flowering pathway (Scortecci et al. 2003) but are in different clades of MADS-box genes, and *FLM* but not *SVP* expression is affected by lesions in *elf7* and *elf8*. However, expression of *FLC*, *FLM*, and *MAF2*, which are in the same clade but affect different flowering pathways, is attenuated by lesions in *elf7* and *elf8*. Members of the *FLC* clade, including a *Brassica napus* gene, have a conserved nucleotide sequence in the 5′-UTR that is within the region of the highest level of H3-K4 trimethylation. This conserved region is not present in *SVP* or in MADS-box genes in other clades that are involved in flowering time regulation. A deletion of this conserved region prevents *FLC* up-regulation by *FRI* or autonomous-pathway mutations. These data are consistent with this conserved region playing a role in the ELF7/ELF8-mediated activation of the *FLC* clade of MADS-box genes, but a direct demonstration of this will require further experiments.

Covalent modifications of chromatin proteins constitute a "histone code" for maintaining states of gene activation or repression (Iizuka and Smith 2003). These modifications recruit transcriptional activators and repressors involved in the regulation of gene expression and the maintenance of certain histone modifications. Trimethylation of H3-K4 in the 5′ regions of genes is thought to promote gene expression in yeast by recruiting ISW1p (Santos-Rosa et al. 2003), a yeast ATP-hydrolyzing, chromatin-remodeling protein that may be involved in nucleosome positioning (Tsukiyama et al. 1999). An *Arabidopsis* relative of ISW1p, PIE1, is a candidate for the plant ATP-dependent chromatin-remodeling protein that is required for expression of genes regulated by ELF7 and ELF8: mutations in *PIE1* suppress *FLC* expression and cause early flowering in both LD and SD and altered petal number, and *pie* alleles in the Col genetic background display additional phenotypes similar to those of *elf7* and *elf8* such as small leaves (Noh and Amasino 2003). The phenotypic similarity among *pie1*, *elf7*, *elf8*, and *vip4* mutants indicates that all of these genes are in the same pathway and regulate similar target genes. We propose that PIE1 binds the 5′ region of *FLC*, *FLM*, and *MAF2* chromatin when these chromatin regions are enriched for H3-K4 trimethylation resulting from ELF7/ELF8-dependent recruiting of the EFS methyltransferase.

This study provides the first molecular correlation of a specific *FLC* chromatin modification and the increased *FLC* expression that occurs in lines that behave as winter annuals because of the presence of *FRI* or autonomous-pathway mutations. In Col, which has a nonfunctional *fri* allele (Johanson et al. 2000), *FLC* is not highly expressed, nor is *FLC* chromatin highly trimethylated at H3-K4. The loss of an autonomous-pathway negative regulator of *FLC* such as *FLD* permits *FLC* up-regulation and increased H3-K4 trimethylation in an *fri* background. Thus autonomous-pathway components act, at least in part, to restrict the ability of ELF7 and ELF8 to activate *FLC* and increase H3-K4 trimethylation. In the presence of a dominant allele of *FRI*, the ability of the autonomous pathway to prevent *FLC* up-regulation and increased H3-K4 trimethylation is blocked. Additional studies are required to determine whether the antagonistic activities of FRI and the autonomous pathway converge on the ELF7/ELF8-mediated H3-K4 methylation of *FLC* chromatin, or whether this *FLC* chromatin modification is downstream of FRI and the autonomous pathway.

Materials and methods

Plant materials and growth conditions

flc-3 (Michaels and Amasino 1999), Col *FRI*^{sf2} (the functional *FRI* from *Arabidopsis* accession San Feliu-2 introgressed into Col) (Lee et al. 1994), *fve-4* (Ausin et al. 2004), *fld-1* (Sanda and Amasino 1996), *fld-3* (He et al. 2003), *FRI flc-3* (Michaels and Amasino 2001), and *fpa flc-3* (Michaels and Amasino 2001) were described previously. The *vip4* alleles were isolated from the SALK T-DNA collection (SALK_122755 and SALK_039374) (Alonso et al. 2003). Plants were grown either under white fluorescent light in short days (8 h light/16 h night) or in long days (16 h light/8 h night) at 22°C.

Plasmid construction

To construct *ELF7:GUS*, a 3.2-kb *ELF7* genomic fragment including a 1.0-kb region 5′ of the transcription start point and 2.2 kb of coding sequence was fused to the *GUS* coding sequence. To construct *ELF8:GUS*, a 2.0-kb region 5′ of the transcription start point was fused to the *GUS* coding region. The 80-bp internal deletion of the 5′-UTR of *FLC* was made by a PCR-mediated fusion strategy.

RNA blot analyses

Total RNA was isolated from 10-d-old seedlings using TRI Reagent as recommended by the manufacturer (Sigma). About 15 µg of total RNA was loaded in each lane, separated in a denaturing formaldehyde agarose gel, and transferred to a Hybond N$^+$ membrane (Amersham Pharmacia). The *FLC* 3′-UTR fragment was used as a probe for *FLC* detection. Blots were subsequently probed with an 18S rDNA probe for comparison of the quantity of RNA loaded among samples.

RT–PCR

cDNAs were synthesized from 2.0 µg of total RNA with Superscript Reverse Transcriptase (Life Technologies). The *FLM*, *SVP*, and *UBQ10* cDNAs were amplified as described previously (Scortecci et al. 2003). The cDNAs of *AGL16*, *AGL18*, and *AGL30* were amplified as described previously by Parenicova et al. (2003). The *MAF2* cDNA was amplified with the primer pair

MAF2f (5′-GGGTAGAAAAAAAGTCGAGATCAAGCGA-3′) and MAF2r (5′-CTTGAGCAGCGGAAGAGTCTCC-3′). The primer pair ELF7f (5′-TCTCAGATGCCCAAGGGACAC-3′) and ELF7r (5′-GGATGCTTCAATATCCTTGATTTGT-3′) was used to amplify *ELF7* cDNA; the primer pair ELF8f (5′-CGGGT ACAAGGACACGGGCAT-3′) and ELF8r (5′-CAAGGCTCA ATCCAAATAACCCAAAATACA-3′) was used to amplify *ELF8* cDNA; the primer pair FLCf (5′-TTCTCCAAACGTCG CAACGGTCTC-3′) and FLCr (5′-GAGCTTTTGACTGATGA TCCAAGGCTTTA-3′) was used to amplify *FLC* cDNA.

β-*Glucuronidase staining in plants*

Histochemical β-glucuronidase staining was performed as described previously (Schomburg et al. 2001). FLC:GUS-, ELF7:GUS- and ELF8:GUS-containing seedlings were stained for 3 h at 37°C.

ChIP assays

The chromatin immunoprecipitation experiments were performed as described by Johnson et al. (2002) using 10-d-old seedlings. The anti-trimethylated K4 histone H3 antibodies and the ChIP assay kit were from Upstate Biotechnology. Each of the immunoprecipitations was replicated three times. The amounts of genomic DNA immunoprecipitated were determined by quantitative PCR. The primer pair CH2 (5′-GTTCTCAATTC GCTTGATTTCTAGT-3′) and CH12 (5′-GGCCCGACGA AGAAAAAGTAGATAGGC-3′) was used to amplify Region IV of *FLC*; the primer pair FLMf (5′-CCGGAGATTAGGATTA AATTAGGGCATA-3′) and FLMr (5′-CTAAGCAATACAAA GATTACTTAAACAACATC-3′) was used to amplify *FLM*; the primer pair used to amplify *ACTIN 2/7* was described previously (Johnson et al. 2002). Aliquots of the PCR reactions were resolved by electrophoresis in 1.5% agarose gels, and quantified with ImageQuant software (Molecular Dynamics, Inc).

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