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Establishment of the Vernalization-Responsive, Winter-Annual Habit in *Arabidopsis* Requires a Putative Histone H3 Methyl Transferase [W]

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Winter-annual accessions of *Arabidopsis thaliana* are often characterized by a requirement for exposure to the cold of winter to initiate flowering in the spring. The block to flowering prior to cold exposure is due to high levels of the flowering repressor *FLOWERING LOCUS C* (*FLC*). Exposure to cold promotes flowering through a process known as vernalization that epigenetically represses *FLC* expression. Rapid-cycling accessions typically have low levels of *FLC* expression and therefore do not require vernalization. A screen for mutants in which a winter-annual *Arabidopsis* is converted to a rapid-cycling type has identified a putative histone H3 methyl transferase that is required for *FLC* expression. Lesions in this methyl transferase, *EARLY FLOWERING IN SHORT DAYS* (*EFS*), result in reduced levels of histone H3 Lys 4 trimethylation in *FLC* chromatin. *EFS* is also required for expression of other genes in the *FLC* clade, such as *MADS AFFECTING FLOWERING2* and *FLOWERING LOCUS M.* The requirement for *EFS* to permit expression of several *FLC* clade genes accounts for the ability of *efs* lesions to suppress delayed flowering due to the presence of *FRIGIDA*, autonomous pathway mutations, or growth in noninductive photoperiods. *efs* mutants exhibit pleiotropic phenotypes, indicating that the role of *EFS* is not limited to the regulation of flowering time.

INTRODUCTION

In plants, successful reproduction is dependent on flowering at the correct time. Plants monitor both environmental and internal signals in order to ensure that reproduction occurs at the appropriate time of year and stage of development. Many species have evolved pathways that sense environmental cues, such as daylength and temperature, and endogenous signals, such as plant age, to regulate the timing of the floral transition. In *Arabidopsis thaliana*, a facultative long-day plant, flowering is accelerated by environmental factors such as long days (LD) and prolonged exposure to cold (the process by which exposure to cold promotes flowering is known as vernalization). In addition, the autonomous floral promotion pathway and the plant hormone gibberellin promote flowering largely in response to developmental signals (reviewed in Boss et al., 2004; Putterill et al., 2004; He and Amasino, 2005).

Much natural variation in flowering habit exists among *Arabidopsis* accessions. One component of this variation is the degree to which vernalization promotes flowering. Many winter-annual accessions are late flowering unless vernalized, whereas rapid-cycling accessions flower rapidly in the absence of cold treatment. The genetic difference between these vernalization responses is often due to allelic variation at *FRIGIDA* (*FRI*) and/or *FLOWERING LOCUS C* (*FLC*) (Burn et al., 1993; Lee et al., 1993, 1994; Clarke and Dean, 1994; Koornneef et al., 1994). Both *FRI* and *FLC* activity are required for late flowering. *FLC*, a MADS box transcription factor, is a floral repressor (Michaels and Amasino, 1999; Sheldon et al., 1999), and *FRI* upregulates *FLC* expression to a level that inhibits flowering (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). Most winter-annual accessions have dominant alleles of *FRI* and *FLC*, whereas most rapid-cycling accessions that have been examined contain mutations that eliminate *FRI* or *FLC* activity (Johanson et al., 2000; Gazzani et al., 2003; Michaels et al., 2003).

In addition to the positive regulation of *FLC* by *FRI*, a group of seven genes known collectively as the autonomous pathway act to repress *FLC* expression. In rapid-cycling accessions that lack *FRI* activity, the autonomous pathway genes promote flowering by suppressing *FLC* expression. Thus, in rapid-cycling backgrounds, mutations in autonomous pathway genes lead to elevated *FLC* levels and a late-flowering phenotype. In winter-annual accessions, the repression of *FLC* by the autonomous pathway is overridden by *FRI* (i.e., *FRI* is epistatic to the autonomous pathway). Vernalization results in a permanent epigenetic repression of *FLC* expression despite the presence of autonomous pathway mutations or *FRI*; therefore, winter annuals or rapid-cycling accessions containing autonomous pathway mutations flower rapidly after vernalization (Michaels

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and Amasino, 1999; Sheldon et al., 1999). Hence, the regulation of *FLC* expression is the convergence point of *FRI*, the autonomous pathway, and vernalization.

Recent studies have begun to reveal the molecular mechanisms that control *FLC* expression. Genetic and molecular studies have shown that *FRI*, the autonomous pathway, and vernalization all influence the state of *FLC* chromatin (reviewed in He and Amasino, 2005). In *FRI*-containing winter annuals, the level of trimethylation of histone H3 at Lys 4 (H3-K4) of *FLC* chromatin is elevated (He et al., 2004). The autonomous pathway repressors *FLD* and *FVE* are involved in deacetylating *FLC* chromatin (He et al., 2003; Ausin et al., 2004; Kim et al., 2004). Vernalization leads to repressive histone modifications, such as dimethylation of histone H3 at Lys 9 and Lys 27 of *FLC* chromatin (Bastow et al., 2004; Sung and Amasino, 2004). Thus, chromatin modification is emerging as a major regulator of *FLC* expression.

Genetic analyses of mutations that render a *FRI*-containing winter-annual line early flowering have led to the identification of several loci that are required for *FLC* expression: *PHOTOPERIOD INDEPENDENT EARLY FLOWERING1* (*PIE1*) (Noh and Amasino, 2003), *VERNALIZATION INDEPENDENCE4* (*VIP4*) (Zhang and van Nocker, 2002), *VIP3* (Zhang et al., 2003), *EARLY FLOWERING7* (*ELF7*) (He et al., 2004), *ELF8*/*VIP6* (He et al., 2004; Oh et al., 2004), and *VIP5* (Oh et al., 2004). ELF7, ELF8, VIP4, and VIP5 are likely to form an RNA Polymerase II Associated Factor 1 (PAF1)-like complex that promotes *FLC* expression (He et al., 2004; Oh et al., 2004). In yeast, the PAF1 complex promotes gene expression in part by recruiting a histone H3-K4 methyl transferase–containing complex to target gene chromatin (Krogan et al., 2003a; Ng et al., 2003). Increased levels of histone H3-K4 trimethylation is often associated with actively transcribed genes (Krogan et al., 2003a; Ng et al., 2003). Similar to the yeast PAF1 complex, the PAF1-like complex in *Arabidopsis* may also recruit an H3-K4 methyl transferase to *FLC* to regulate its expression.

In this report, we present the identification and characterization of a putative histone H3-K4 methyl transferase involved in modulating *FLC* expression: *EARLY FLOWERING IN SHORT DAYS* (*EFS*), a relative of the *Drosophila melanogaster* H3-K4 methyl transferase *ABSENT SMALL HOMEOTIC DISCS1* (*ASH1*). *efs* mutations suppress *FLC* expression in *FRI*-containing or autonomous pathway mutant backgrounds. Lesions in *EFS* also reduce the level of histone H3-K4 trimethylation in *FLC* chromatin.

RESULTS

Identification of *efs* Alleles as Suppressors of *FRI*

Extensive genetic screens have been effective in identifying a large number of genes that regulate flowering in rapid-cycling *Arabidopsis* accessions. Less is known regarding the genes that are responsible for the creation of the winter-annual habit because the rapid-cycling accessions do not exhibit elevated *FLC* expression; thus, mutations that prevent *FRI* from elevating *FLC* expression are difficult to identify in screens of such accessions. To identify genes required for the late-flowering habit of winter annuals, a line containing *FRI* introgressed into the Columbia background (*FRI*-Col) was mutagenized by fast-neutron radiation and random T-DNA insertions (Michaels and Amasino, 1999). The resulting M2 generations were screened for early-flowering mutants.

One group of six mutants identified in this screen strongly suppressed the late-flowering phenotype of the *FRI*-Col line (see below). In addition, these mutants also showed a number of other distinctive phenotypes, such as reduced plant size (the rosette diameter of the mutants was ~80% of the wild type), leaves that are rounder and slightly paler than the wild type, and reduced fertility (~80% of wild-type seed set when self-pollinated). Pairwise crosses between these mutants produced early-flowering F1 and F2 plants, indicating that these mutations were allelic. Because the phenotypes of these mutants are similar to that of the previously described *efs* mutant (Soppe et al., 1999), allelism tests were performed between a fast-neutron allele of this complementation group (*fn210*) and *efs-1*, which is in the Landsberg *erecta* (L*er*) genetic background. The F1 plants resulting from the *fn210*×*efs* cross were early flowering, indicating allelism between this complementation group and *efs* (Figure 1A). Allelism was also confirmed in the F2 generation (all plants were early flowering; data not shown). Thus, these mutants are alleles of *efs* and were designed as *efs-3* through *efs-8. efs-3* (*fn210*) was used in all subsequent experiments.

EFS Is Required for *FLC* Expression in Backgrounds Containing *FRI* or Autonomous Pathway Mutations

efs mutations result in a large reduction in the number of leaves formed prior to flowering in the *FRI*-Col background (Figures 1B and 1C). Because *FRI* delays flowering in winter-annual accessions by increasing *FLC* expression, a possible explanation for the early-flowering phenotype of *efs* in the *FRI*-Col background is that *EFS* is required for elevated *FLC* expression. To test this hypothesis, *FLC* mRNA levels were determined in wild-type *FRI*-Col and *efs* mutant seedlings. In *FRI*-Col, as expected, *FLC* is highly expressed (Figure 2A). In the *efs* mutant, however, *FLC* expression is greatly reduced. A previously described flowering-time gene, *PIE1*, has been shown to be required for the expression of *FLC* in shoots only (i.e., in *pie1* mutants, *FLC* expression is reduced in the shoot apex, but not in the root apex). To determine if *EFS* is required for *FLC* expression in both the shoot and root, *FLC*:β-glucuronidase (*GUS*) expression was examined in *FRI*-Col and *efs* mutant backgrounds. In the *efs* mutant, GUS staining is reduced in both the shoot and root apex (Figures 3A and 3B). Thus, unlike *PIE1*, *EFS* expression is required for elevated *FLC* expression throughout the plant. Consistent with *EFS* acting as a positive regulator of *FLC*, expression of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), a promoter of flowering that is negatively regulated by *FLC*, is also affected by *efs* mutations. In a *FRI*-containing background, *SOC1* expression is suppressed by high levels of *FLC* (Figure 3C); however, in a *FRI efs* background, *SOC1* is highly expressed.

Like *FRI*-containing winter annuals, rapid-cycling accessions that contain loss-of-function mutations in autonomous pathway genes are also late flowering due to elevated levels of *FLC*

expression. Previous work has shown that *efs* mutations effectively suppress the late-flowering phenotype of the autonomous pathway mutations *fca* and *fve* in the L*er* genetic background (Soppe et al., 1999). Because L*er* contains an atypical weak allele of *FLC* (Michaels et al., 2003), we investigated the ability of *efs* mutations to suppress the late-flowering phenotype of *fca* and *fve* in the Col background, which contains a strong *FLC* allele. *efs* was crossed to *fve-4* and *fca-9*, and in the resulting F2 populations, *efs fca* and *efs fve* double mutants were isolated that were homozygous for the Col allele of *fri* (genotypes were verified using molecular markers). In the double mutants, *efs* strongly

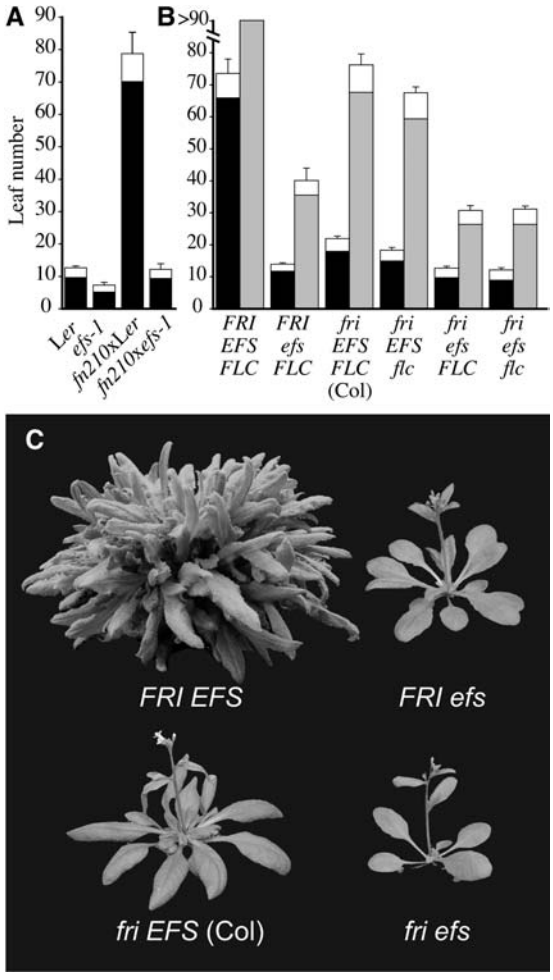

Figure 1. *efs* Mutations Suppress the Late-Flowering Phenotype of *FRI*.

(A) Allelism tests between *efs-1* in the L*er* background and *fn210* in the *FRI*-Col background. The closed portion of the bars indicates the number of rosette leaves formed by the primary shoot apical meristem prior to flowering. The open portion of the bars indicates the number of cauline leaves. Error bars indicate 1 SD.

(B) Number of leaves formed prior to flowering for the indicated genotypes. Black and gray bars represent plants grown in LD and SD, respectively. Error bars indicate 1 SD.

(C) The effect of *efs* mutations on flowering time in *FRI*-Col background (top) or Col background (bottom). Plants were grown in LD.

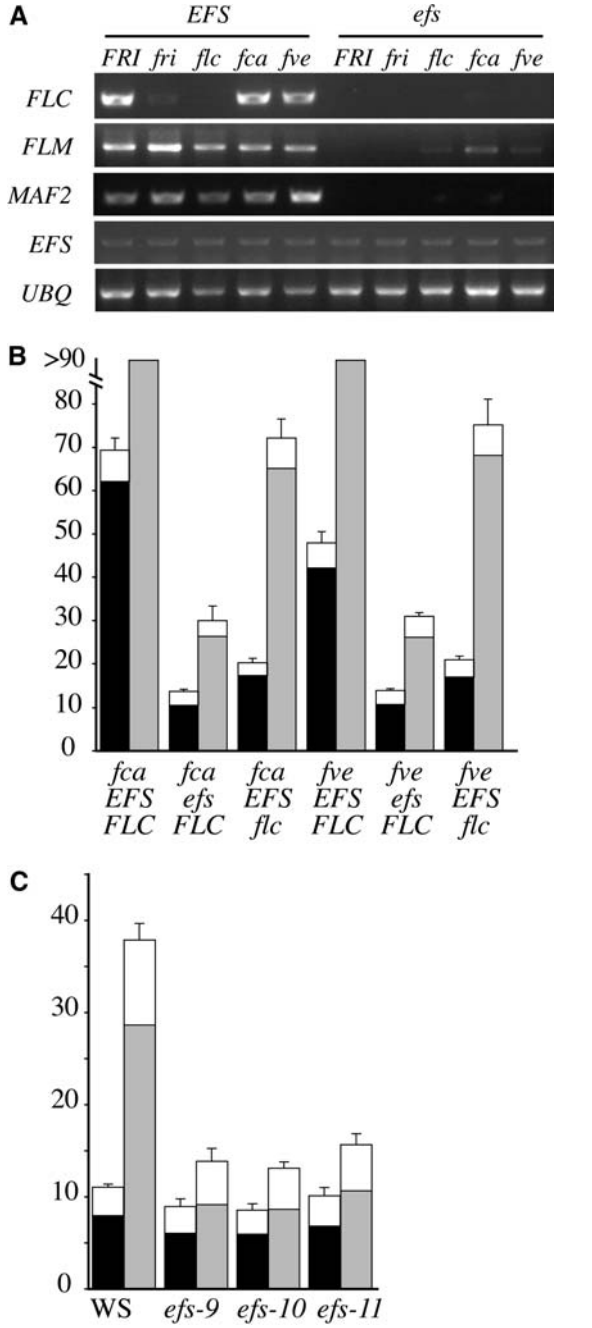

Figure 2. Effect of *EFS* on Gene Expression and Flowering Time of Autonomous Pathway Mutants.

(A) RT-PCR analysis of flowering time gene expression in wild-type and *efs* mutant backgrounds. RNA was isolated from 14-d-old seedlings grown in LD. Tissue was harvested 4 h after lights on. *UBIQUITIN* (*UBQ*) was used as a control for loading.

(B) and **(C)** Effect of *efs* on the flowering time of *fca* and *fve* mutants **(B)** and the effect of *efs* alleles in the Ws genetic background **(C)**. The closed portion of the bar indicates the number of rosette leaves formed by the primary shoot apical meristem prior to flowering, while the open portion indicates the number of cauline leaves. Black and gray bars represent plants grown in LD and SD, respectively. Error bars indicate 1 SD.

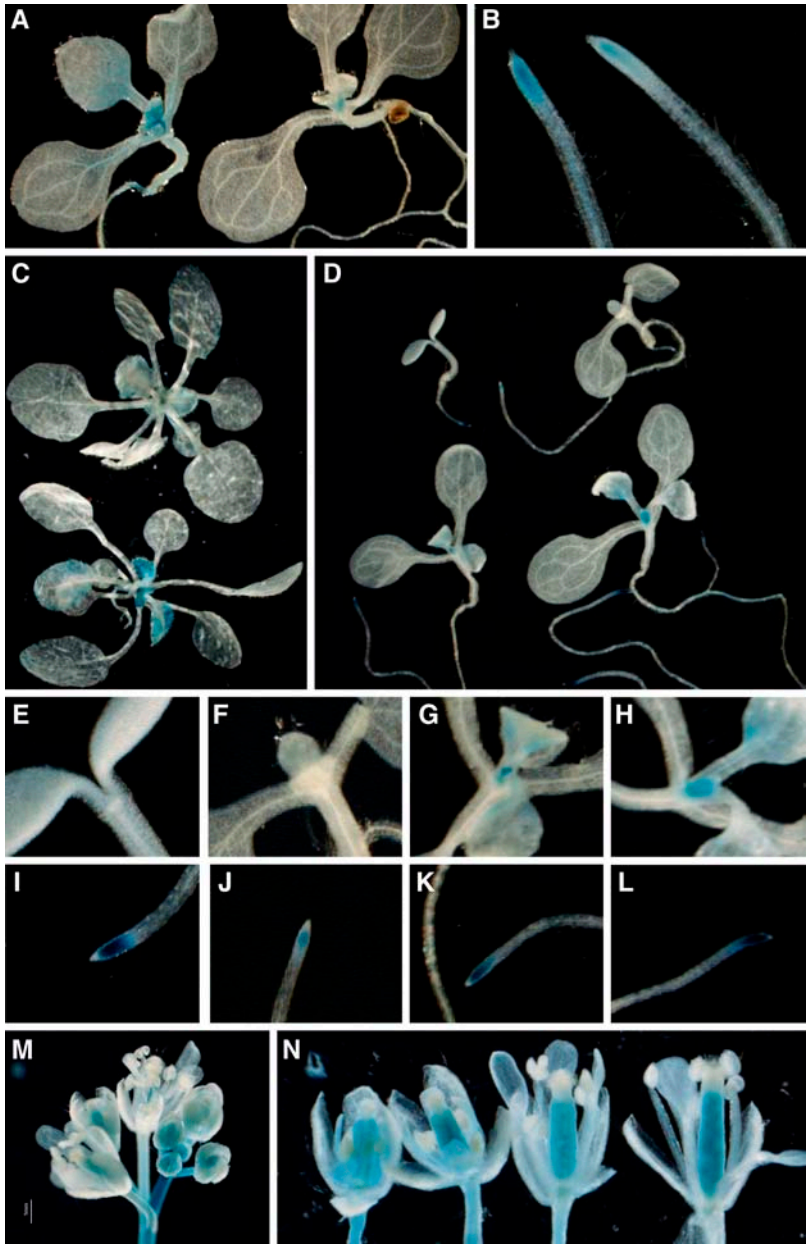

Figure 3. Histochemical Analysis of Gene Expression.

To minimize variation in GUS staining, plants that appear in the same panel were stained in parallel. Unless otherwise mentioned, all plants were grown in LD and are in the *FRI*-Col background.

(A) and **(B)** *FLC*:*GUS* expression in shoots and roots in the wild-type *FRI*-Col (left) or *efs* mutant background (right).

(C) SOC1:GUS expression in wild-type *FRI*-Col (top) or *efs* mutant background (bottom).

(D) *EFS*:*GUS* expression during vegetative development. Plants were harvested 2 (top left), 4 (top right), 6 (bottom left), and 8 DAG.

(E) to **(L)** Higher magnification images of the shoot apex and root apex of the plants shown in **(D)**. 2 DAG (**[E]** and **[I]**), 4 DAG (**[F]** and **[J]**), 6 DAG (**[G]** and **[K]**), and 8 DAG (**[H]** and **[L]**).

(M) *EFS*:*GUS* expression in the inflorescence.

(N) *EFS*:*GUS* expression in flowers.

suppressed the late-flowering phenotype of *fca* and *fve* (Figure 2B). To determine if, as in the *FRI*-containing background, the *efs* lesion blocks the expression of *FLC*, we examined *FLC* mRNA expression in the *efs fca* and *efs fve* double mutants (Figure 2A). In the *fca* and *fve* single mutants, *FLC* mRNA is expressed at high levels (similar to that seen with *FRI*). In the *efs fca* and *efs fve* double mutants, however, *FLC* expression is strongly suppressed (Figure 2A). Thus, *EFS* is required for high levels of *FLC* expression in both *FRI*-containing lines and autonomous pathway mutants. We also found that *efs* could not suppress *FLC* expression from the constitutive cauliflower mosaic virus 35S promoter (data not shown; the construct tested was the complete 35S promoter and 5′ untranslated region joined to start codon of an *FLC* genomic clone).

FLC-Independent Effects of *efs* on Flowering Time

To determine if the early-flowering phenotype of *efs* is solely due to the suppression of *FLC* expression, the phenotypes of *FRI* and autonomous pathway mutations in *efs* and *flc* mutant backgrounds were compared. If the effect of *efs* on flowering time is entirely due to the suppression of *FLC* expression, the flowering time of *FRI* and autonomous pathway mutations should be similar in the *efs* and *flc* mutant backgrounds. In the *FRI*-Col background, however, *efs* mutants flower significantly earlier than either a *fri* null or a *fri flc* double mutant (Figure 1B). Similarly, *efs* mutations in the *fca* and *fve* mutant backgrounds flower earlier than *fca flc* and *fve flc* double mutants (Figure 2B). Thus, the early-flowering phenotype of the *efs* mutation in *FRI* and autonomous pathway mutant backgrounds cannot be fully explained by the suppression of *FLC* expression. This *FLC*-independent acceleration of flowering is also observed in the absence of *FRI* or autonomous pathway mutations. Plants containing *efs* in the Col background (which lacks *FRI* activity) or in an *fl-3* mutant background (which lacks both *FRI* and *FLC* activity) flower earlier than wild-type Col or an *flc* mutant (Figure 1B).

In addition to the *efs* alleles that were isolated as suppressors of the late-flowering phenotype conferred by *FRI* (described above), we also isolated three alleles of *efs* by screening a T-DNA–mutagenized population in the Wassilewskija (Ws) background for early flowering in short days (SD). Like the original *efs* alleles that were isolated from a similar screen performed in the L*er* genetic background, these lines have a strong early-flowering phenotype in SD (Figure 2C). *flc*-null mutants have not been reported in the L*er* or Ws background; therefore, the contribution of the loss of *FLC* expression to the early flowering in SD cannot be determined in these genetic backgrounds. *flc*-null mutants in the Col background, however, have only a modest early-flowering phenotype in SD (Michaels and Amasino, 2001), suggesting that the strong early-flowering phenotype of *efs* in SD cannot be entirely explained by the suppression of *FLC*. To investigate this possibility, we compared the effects of *efs* and *flc* mutations on flowering in SD in the same genetic background. In the Col background, the *flc* mutant flowered with approximately eight fewer leaves than the wild type, whereas the *efs* mutant flowered much earlier, forming approximately 40 fewer leaves than the wild type (Figure 1B). Indeed, in all genetic backgrounds tested (Col, *FRI*-Col, *fve*, and *fca*), mutations in *efs* caused earlier flowering than mutations in *flc* (Figures 1B and 2B) in SD. Thus, the suppression of *FLC* by *efs* accounts for only part of the early-flowering phenotype of the *efs* mutant in SD.

EFS Is Required for the Expression of Additional MADS Box Transcription Factors in the *FLC* Clade

Recent work has shown that the genes *ELF7* and *ELF8*/*VIP6* (He et al., 2004; Oh et al., 2004) are required for the late-flowering phenotype of winter-annual *Arabidopsis*, and like *efs*, *elf7*/*8* mutants are also early flowering in SD (He et al., 2004). *elf7*/*8* mutations lead to decreased H3-K4 trimethylation of *FLC* chromatin and reduced *FLC* mRNA levels (He et al., 2004). Interestingly, in *elf7*/*8* mutants, the expression of two other MADS box transcription factors from the *FLC* clade are also suppressed (He et al., 2004). Loss-of-function mutations in these genes, *FLM*/*MADS AFFECTING FLOWERING1* (*MAF1*) and *MAF2*, are early flowering in SD (Ratcliffe et al., 2001, 2003; Scortecci et al., 2001). Thus, it appears that the early-flowering phenotype of *elf7*/*8* mutants in SD may be due to the cumulative effect of suppression of *FLC*, *FLM*, and *MAF2* (He et al., 2004). To investigate whether *EFS* might also be required for the expression of *FLM* and *MAF2*, mRNA levels were determined in the presence or absence of *EFS* activity. In all genetic backgrounds tested (*FRI*-Col, Col, *flc*, *fca*, and *fve*), the *efs* mutation suppressed the expression of both *FLM* and *MAF2* in addition to *FLC* (Figure 2A). Thus, like *ELF7*/*8*, *EFS* is required for the expression of a group of related flowering repressors in the *FLC* clade.

EFS Encodes a SET Domain–Containing Transcription Factor

Because the *efs-4* through *efs-8* mutations were created by T-DNA mutagenesis, these lines were used to identify the *EFS* gene by isolating genomic DNA flanking the sites of T-DNA integration. Flanking DNA from the *efs* T-DNA alleles mapped to the predicted coding region of *At1g77300* (Figure 4A). *At1g77300* is located on the bottom of chromosome I, and its position is consistent with the previously published mapping data for *EFS* (Soppe et al., 1999). Analysis of the *efs-3* allele (generated by fast-neutron radiation) identified a 23-bp deletion (TATTAGAG-TATCTTGCCACAAGG) in *At1g77300*, which creates a frame shift (Figure 4A). As additional proof that lesions in *At1g77300* are responsible for the mutant phenotype of *efs*, *efs-3* was transformed with a 12.2-kb genomic DNA fragment that is not predicted to contain any genes other than *At1g77300.* The resulting T1 plants were late flowering and did not display any of the pleiotropic phenotypes associated with the *efs* mutation. Thus, *At1g77300* encodes EFS. It should also be noted that this gene has also been referred to as *SDG8* (Springer et al., 2003).

The Arabidopsis Genome Initiative (AGI) annotation predicts that the *EFS* gene contains 17 exons with 5280 bp of coding sequence (www.arabidopsis.org). To verify the predicted cDNA sequence, the *EFS* cDNA was amplified by RT-PCR and sequenced. Due to the large size of the transcript, we were unable to amplify the entire transcript in one reaction. Therefore, the *EFS* cDNA was amplified as a series of overlapping RT-PCR

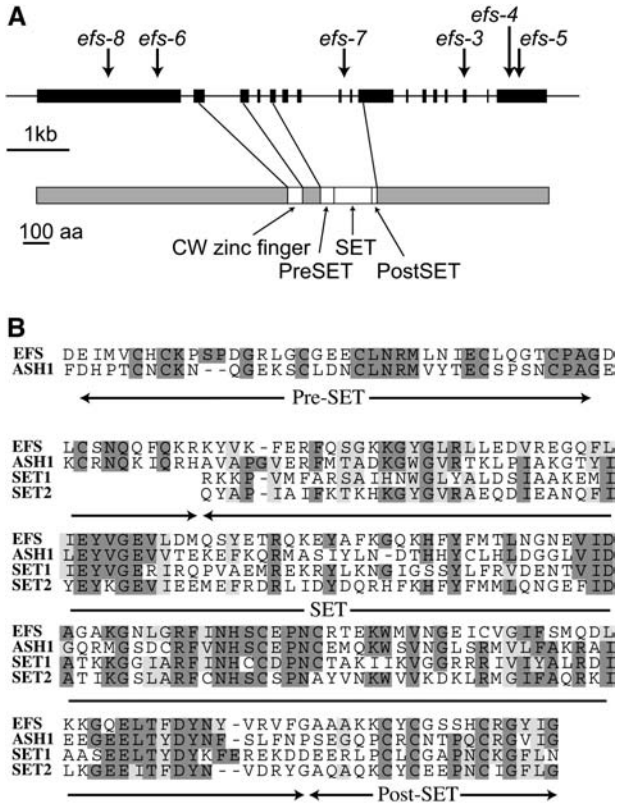

Figure 4. The *EFS* Gene and Predicted Protein.

(A) Schematic representation of the *EFS* gene (top; black boxes indicate exons) and protein (bottom). Positions of lesions in *efs* mutants are indicated.

(B) Clustal alignment of the SET, pre-SET, and post-SET domains of EFS, ASH1, SET1, and SET2. Identical residues are shaded in dark gray and similar residues in light gray.

products. The empirically determined cDNA sequence was identical to that predicted by AGI.

The predicted EFS protein is 1759 amino acids in length. BLAST and InterProScan searches identified several domains indicating that EFS is likely to play a role in regulating gene activity by modifying chromatin structure. EFS contains a SET [for Su(var)3-9, Enhancer-of-zeste, Trithorax] domain and two other domains often found with SET domains, a Cys-rich post-SET domain and a pre-SET domain (Figure 4A) (Trievel et al., 2002; Wilson et al., 2002). Many SET domain proteins have been shown to act as histone methyltransferases (e.g., Rea et al., 2000; Nakayama et al., 2001), and the SET domain itself appears to comprise the catalytic site (Xiao et al., 2003). The set domain of EFS is similar to ASH1 in *Drosophila* and SET1 and SET2 in *Saccharomyces cerevisiae* (Figure 4B; data not shown). ASH1 and SET1 have been biochemically shown to methylate Lys 4 of histone H3 (Briggs et al., 2001; Roguev et al., 2001; Beisel et al., 2002; Byrd and Shearn, 2003), and SET2 has been shown to methylate Lys 36 of histone H3 (Krogan et al., 2003b). EFS also contains a CW domain, which is predicted to be a four-Cys zinc-finger motif (Perry and Zhao, 2003). CW domains are found in a number of proteins that contain other domains that are involved in binding DNA or chromatin (Perry and Zhao, 2003). These domains suggest a role for EFS in the regulation of gene expression through changes in chromatin structure via histone modifications (see below).

EFS Is Not Regulated by *FRI*, by Autonomous Pathway Genes, or by Vernalization

Flowering inputs from *FRI*, the autonomous pathway, and vernalization converge at the level of *FLC* regulation. *FLC* is positively regulated by *FRI* and repressed by autonomous pathway genes and vernalization. Because *EFS* acts as a positive regulator of *FLC*, it is possible that one or more of these flowering

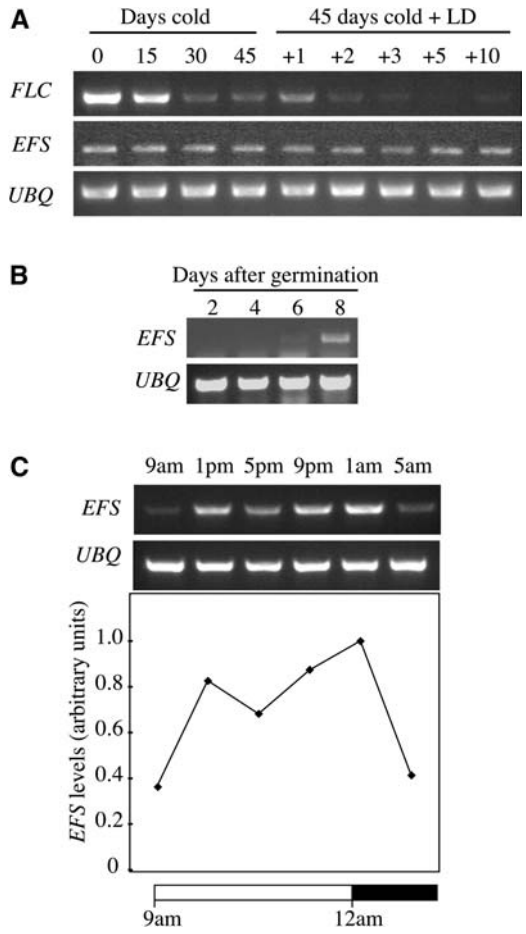

Figure 5. RT-PCR Analysis of *EFS* Expression.

(A) *EFS* and *FLC* expression during vernalization. Eight-day-old seedlings were exposed to cold for 0, 15, 30, or 45 d and harvested for RNA isolation. Also, seedlings exposed to cold for 45 d were grown at 22°C for 1, 2, 3, 5, or 10 d prior to harvest.

(B) *EFS* expression during vegetative development. Shoots were harvested at 2, 4, 6, and 8 DAG.

(C) Daily fluctuations in *EFS* expression. Eight-day-old seedlings were harvested at the indicated times. *UBQ* was used as a control for loading in all experiments.

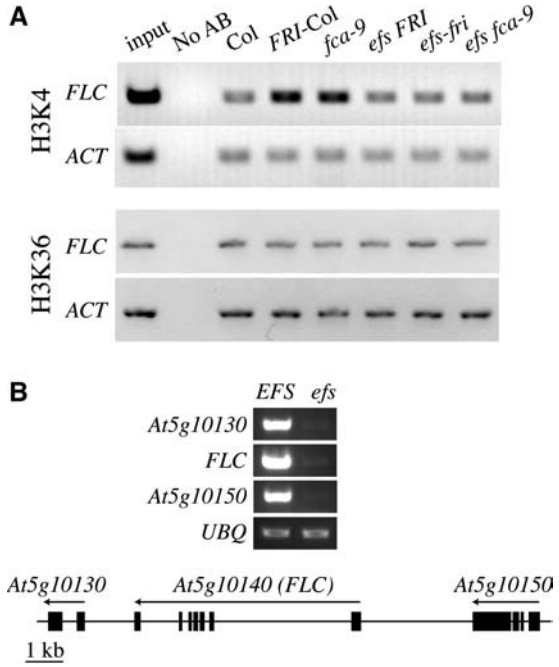

Figure 6. *efs* Mutations Affect Histone H3-K4 Trimethylation and Gene Expression around the *FLC* Locus.

(A) Chromatin immunoprecipitation analysis of histone H3-K4 trimethylation and H3-K36 dimethylation state of *FLC* chromatin in *efs* and related lines. The input is Col chromatin before immunoprecipitation. ''No AB'' refers to the control sample lacking the antitrimethyl H3-K4 or antidimethyl H3-K36 antibody. *ACTIN* (*ACT*) served as an internal control.

(B) RT-PCR analysis of genes flanking *FLC* in wild-type *FRI*-Col and *efs* mutant backgrounds. *UBQ* was used as a control for loading.

inputs could regulate *FLC* levels by modulating *EFS* expression. To test this model, we examined the *EFS* mRNA levels in various genetic backgrounds and in response to vernalization. *EFS* steady state mRNA levels were not affected by *FRI*, by the autonomous pathway, or by vernalization (Figures 2A and 5A). We also examined the effect of *EFS* overexpression by placing the *EFS* gene under control of the strong 35S cauliflower mosaic virus promoter. When placed into *efs* mutant plants in the *FRI* background, the *35S*:*EFS* construct restored a late-flowering phenotype, demonstrating that the construct is functional. When placed into the Col background, however, *35S*:*EFS* construct did not cause late flowering (data not shown). Thus, it seems that *EFS* expression alone is insufficient to activate *FLC* expression and delay flowering.

EFS Is Developmentally Regulated and Is Preferentially Expressed in the Dividing Cells of Apical Regions

Whereas *FLC* levels are relatively constant throughout vegetative development (see Supplemental Figure 1 online; Michaels and Amasino, 1999; Sheldon et al., 1999), *EFS* mRNA levels were found to be developmentally regulated. *EFS* mRNA levels were low immediately following germination and increased over the first 8 d after germination (DAG) (Figure 5B). *EFS* mRNA levels were also found to fluctuate over the course of a day, being lower before dawn (Figure 5C).

An *EFS*:*GUS* fusion was constructed to investigate the developmental and spatial expression pattern of *EFS*. Similar to *FLC*:*GUS*, *EFS*:*GUS* activity was strongest in the shoot and root apex (Figures 3D to 3H) (this expression pattern was verified by RT-PCR; data not shown). Consistent with the RT-PCR data (Figure 5B), *EFS*:*GUS* activity was lower in the shoot apex of seedlings 2 and 4 DAG (Figures 3E and 3F) but was detected 6 and 8 DAG (Figures 3G and 3H). Interestingly, however, *EFS*:*GUS* activity was detected in the root at all stages tested (Figures 3D and 3I to 3L). *EFS*:*GUS* activity was also detected in the inflorescence and in the carpels (Figures 3M and 3N).

EFS Is Required for Elevated Trimethylation of H3-K4 in *FLC* Chromatin

The SET domain of EFS shows amino acid similarity with both SET2, which acts as an H3-K36 methyltransferase, and with ASH1 and SET1, which act as H3-K4 methyltransferases. This suggests that the suppression of *FLC* expression in *efs* mutants might be due to a reduction in H3-K4 or H3-K36 methylation. In support of this model, we have recently shown that H3-K4 hypertrimethylation is associated with actively transcribed *FLC* chromatin (He et al., 2004). To determine if *EFS* has an effect on histone methylation at the *FLC* locus, we examined the H3-K4 trimethylation and H3-K36 dimethylation status of *FLC* chromatin in wild-type and *efs* mutants. Compared with Col, the trimethylated H3-K4 levels were elevated in a *FRI*-containing line (*FRI*-Col) as reported previously (He et al., 2004) and in the *fca* mutant in which *FLC* is actively transcribed (Figure 6A); introduction of *efs* into *FRI*-Col and *fca* eliminated the H3-K4 trimethylation increase in *FLC* chromatin associated with *FRI* and

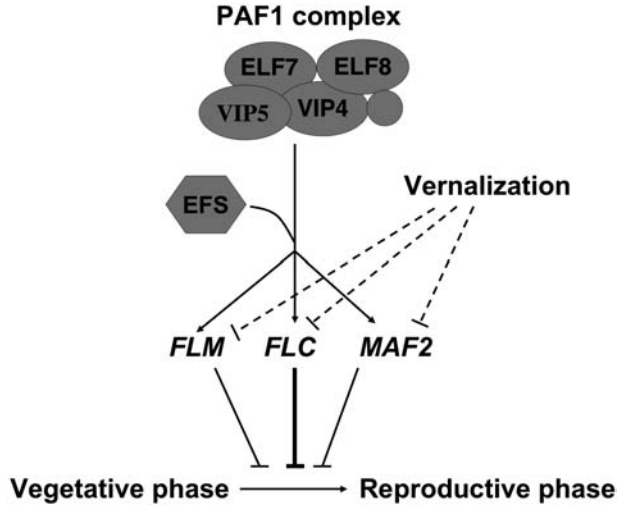

Figure 7. Model of Activation of the *FLC* Clade Genes by *EFS*.

EFS protein is recruited to the *FLC* clade loci by the PAF-like complex and presumably methylates H3-K4, resulting in activating expression of these genes. Vernalization represses expression of the *FLC* clade genes to accelerate floral transition. Lines with arrows indicate upregulation/activation of gene expression, and lines with bars indicate repression.

fca (Figure 6A). These data indicate that EFS is necessary for elevated *FLC* expression and elevated H3-K4 trimethylation levels. By contrast, no significant changes were observed in H3-K36 dimethylation status in any of the genotypes tested (Figure 6A). Thus, EFS is required for the hypertrimethylation of H3-K4 in *FLC* chromatin.

efs Mutations Suppress the Expression of Genes Flanking *FLC*

Recent studies have shown that vernalization lead to repressive histone modifications in *FLC* chromatin (deacetylation of core histones and methylation of H3 at Lys 9 and Lys 27), resulting in epigenetic suppression of *FLC* (Bastow et al., 2004; Sung and Amasino, 2004). The recent observation that the expression of several genes around the *FLC* locus is suppressed by vernalization suggests that the vernalization-induced histone modifications may suppress gene expression in a domain surrounding *FLC* (Finnegan et al., 2004). This model is supported by the fact that the expression of foreign genes inserted into the *FLC* locus is suppressed by vernalization (Finnegan et al., 2004). We investigated whether an *efs* mutation would suppress the expression of other genes around the *FLC* locus. The mRNA levels of *At5g10130*, *FLC* (*At5g10140*), and *At5g10150* were determined in wild-type *FRI*-Col and *efs* mutant seedlings by RT-PCR. The expression of all three genes is suppressed by the lack of *EFS* activity. Thus, *EFS* is also required for the expression of genes flanking *FLC*.

DISCUSSION

In this study, we have identified and characterized a putative histone H3-K4 methyl transferase that is required for elevated *FLC* expression in *FRI*-containing lines and in autonomous pathway mutants. Thus, *EFS* is required for the vernalization-responsive delayed flowering characteristic of the winter-annual habit. Lesions in *EFS* suppress the H3-K4 hypertrimethylation of *FLC* chromatin and prevent *FLC* expression. In addition to the strong suppression of *FLC* expression, *efs* mutants also display *FLC*-independent effects on flowering time. Under LD or SD, *efs* mutants flower earlier than *flc* null mutants; thus, *efs* mutations must promote flowering through other mechanisms in addition to *FLC* suppression. In *Arabidopsis*, there are five *FLC* relatives, *FLM*/*MAF1*, *MAF2*, *MAF3*, *MAF4*, and *MAF5* (Ratcliffe et al., 2001, 2003; Scortecci et al., 2001), and two of these, *FLM* and *MAF2*, have also been shown to play a role in the regulation of flowering time. Like *FLC*, *FLM* and *MAF2* act as inhibitors of flowering; mutations in *FLM* and *MAF2* lead to early flowering in both LD and SD (Ratcliffe et al., 2001, 2003; Scortecci et al., 2001; Y. He and R. Amasino, unpublished data). We found that *EFS* is also required for expression of *FLM* and *MAF2*. Thus, it seems likely that EFS may also affect H3-K4 methylation in *FLM* and *MAF2* chromatin to activate expression of these *FLC*-related genes.

Previously, we have shown that genes encoding components of the PAF1-like complex are also required for the expression and elevated histone H3-K4 trimethylation of *FLC* clade genes (He et al., 2004). Lesions in *ELF7* (encoding the relative of yeast PAF1) and *ELF8* (encoding the relative of yeast CTR9) suppress H3-K4 hypertrimethylation in *FLC* and *FLM* chromatin and prevent their expression (He et al., 2004). The yeast PAF1 complex is a five-member complex consisting of PAF1, CTR9, LEO1, RTF1, and CDC73 (Krogan et al., 2002; Squazzo et al., 2002). *Arabidopsis* relatives of yeast LEO1 and RTF1 also have been identified and characterized (Zhang and van Nocker, 2002; Oh et al., 2004). Loss of function of *VIP5* (encoding the relative of yeast RTF1) also prevents expression of the *FLC* clade genes (Oh et al., 2004), and lesions in *VIP4* (encoding the relative of yeast LEO1) were shown to prevent *FLC* expression (Zhang and van Nocker, 2002). Furthermore, it has been shown that VIP4 protein physically interacts with VIP6/ELF8 protein in vivo (Oh et al., 2004). In yeast, the PAF1 complex is required to recruit the SET1 complex (SET1 is a H3-K4 methyltransferase; Santos-Rosa et al., 2002) and SET2 (an H3-K36 methyltransferase; Strahl et al., 2002) to target gene chromatin, resulting in coordinated methylation of H3-K4 and H3-K36 and the promotion of target gene expression (Krogan et al., 2003b; Ng et al., 2003). Thus, it is possible that EFS is a histone methyltransferase recruited by a PAF1 complex to chromatin of *FLC* clade genes (Figure 7).

Over the entire length of the protein, the closest known relative of EFS in other organisms is *Drosophila* ASH1, a protein that can methylate H3-K4 (Beisel et al., 2002; Byrd and Shearn, 2003). We have shown that loss of EFS activity leads to reduced levels of H3-K4 trimethylation at *FLC*. Thus, EFS may act as an H3-K4 methyltransferase at FLC. We cannot rule out the possibility, however, that the primary target(s) of EFS is located elsewhere in the genome and the reduction in H3-K4 methylation seen at the *FLC* locus in *efs* mutants might be an indirect effect. Alternatively, the biochemical activity of EFS may be to methylate histones at a position other than H3K4 (ASH1 has also been shown to catalyze H4K20 and H3K9 methylation as well as H3K4), and the changes in H3K4 methylation observed in *efs* mutants may be a result of coordinated changes in chromatin modifications.

Interestingly, we observed that *EFS* expression appears to be developmentally regulated. *EFS* mRNA levels were low immediately following germination and increased over the first 8 DAG, whereas *FLC* mRNA levels are relatively constant throughout vegetative development. One possibility to account for the different developmental profiles of *FLC* and *EFS* is that although *EFS* expression was lower during early-stage development of seedlings, there still is sufficient EFS protein for full *FLC* expression. Another possibility is gene redundancy; *Arabidopsis* has at least nine putative histone H3-K4 methyl transferases (Baumbusch et al., 2001), and additional methyl transferases may be involved in methylating *FLC* chromatin during early stages of seedling development. Finally, *FLC* expression during early development may be independent of H3-K4 methyl transferase activity.

METHODS

Plant Materials and Growth Conditions

FRI (Lee et al., 1994), *flc*-3 (Michaels and Amasino, 1999), *fca*-9 (Bezerra et al., 2004), and *fve*-4 (Michaels and Amasino, 2001) are in the Col background and have been described previously. The T-DNA insertional and fast-neutron mutagenized populations used to identify *efs* alleles

have also been described previously (Michaels and Amasino, 1999). Plants were grown under cool-white fluorescent light (~100 μmol m^{-2} s^{-1}. LD consisted of 16 h light followed by 8 h darkness, and SD consisted of 8 h light followed by 16 h darkness. A minimum of eight plants per genotype was under each experimental condition.

Gene Expression Analysis

For RT-PCR analysis, RNA isolation, reverse transcription, and PCR were preformed as described previously (Michaels et al., 2004). Primers used for the detection of *FLC* (Michaels et al., 2004), *FLM* (Scortecci et al., 2003), *MAF2* (He et al., 2004), and *UBQ* (Michaels et al., 2004) have been described previously. For *EFS* (5′-CATCAAGTGAAAGTGCCGTGG-3′ and 5′-AGAGGATTTTCTCAGATGGCGAG-3′), *At5g10130* (5′-CGTTGG-CGTAGTGACGAGTA-3′ and 5′-CGCTTTGGATTCGAGACAAT-3′), and *At5g10150* (5′-CCCATTTTCCGAAGAGTCCAAG-3′ and 5′-CGTCGTGG-AAGATGTGTAACTC-3′), the indicated primers were used. For all experiments, the data shown are representative of at least three independent experiments.

Constructs

A translational EFS:GUS fusion was created by inserting the GUS gene into the *Bsp*EI site in the 14th exon of a genomic EFS clone. The clone contained 3027 bp upstream of the predicted translational start site and 545 bp downstream of the predicted stop codon. A 35S:EFS fusion was created by fusing the 35S cauliflower mosaic virus promoter to the predicted start site of a genomic *EFS* clone. The construct contained the full-length *EFS* gene plus 545 bp downstream of the predicted stop codon.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation experiments were performed as described by Johnson et al. (2002) and He et al. (2003) using 10-d-old seedlings. The primer pair CH2 and CH12 as described by He et al. (2003) was used to amplify *FLC*. Antibodies were obtained from Upstate USA.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *At1g77300* (*EFS*), *At5g10140* (*FLC*), *At4g00650* (*FRI*), *At1g77080* (*FLM*), *At5g65050* (*MAF2*), *At4g16280* (*FCA*), *At2g19520* (*FVE*), and *At2g45660* (*SOC1*).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure 1. Expression of FLC:GUS in a FRI-Containing Background 2 and 6 DAG.

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