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SUPPRESSOR OF FRI 4 encodes a nuclear-localized protein that is required for delayed flowering in winter-annual *Arabidopsis*

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The floral inhibitor *FLOWERING LOCUS C* (*FLC*) is a crucial regulator of flowering time in *Arabidopsis*, and is positively regulated by the *FRIGIDA* (*FRI*) gene in late-flowering winter-annual accessions. In rapid-cycling accessions, *FLC* expression is suppressed by the autonomous floral-promotion pathway (AP); thus AP mutants contain high levels of *FLC* and are late flowering. Previous work has shown that the upregulation of *FLC* in *FRI*- or AP-mutant backgrounds is correlated to an increase in histone H3 lysine 4 (H3K4) trimethylation at the *FLC* locus. This increase in trimethylation requires a PAF1-like complex and *EARLY FLOWERING IN SHORT DAYS* (*EFS*), a putative histone H3 methyltransferase. We have identified a putative zinc-finger-containing transcription factor, *SUF4*, that is required for the upregulation of *FLC* by *FRI*. *suf4* mutations strongly suppress the late-flowering phenotype of *FRI*, but only weakly suppress AP mutants. As with mutants in *efs* or the PAF1-like complex, *suf4* mutants show reduced H3K4 trimethylation at *FLC*. An interesting distinction between the phenotypes of *suf4* mutants and mutants in *efs* or the PAF1-like complex is observed in the expression of genes that are adjacent to *FLC* or *FLC*-like genes. In *efs* and PAF1-like-complex mutants, the expression of *FLC*, *FLC*-like genes and adjacent genes is suppressed. In *suf4* mutants, however, only *FLC* expression is suppressed. These data are consistent with a model in which *SUF4* may act to specifically recruit *EFS* and the PAF1-like complex to the *FLC* locus.

KEY WORDS: *FLOWERING LOCUS C* (*FLC*), *FRIGIDA* (*FRI*), *EARLY FLOWERING IN SHORT DAYS* (*EFS*), PAF1 complex, Vernalization, Flowering

INTRODUCTION

Nearly all above-ground parts of plants are produced postembryonically by stem cells located in the shoot apical meristem (SAM). In many annual plants, the SAM gives rise to the vegetative structures (e.g. leaves), but later undergoes a developmental transition to produce the reproductive structures (flowers). The timing of this transition is crucial to reproductive success and is regulated by both endogenous pathways and signals from the environment. In *Arabidopsis*, *FLOWERING LOCUS C* (*FLC*) is a crucial regulator of flowering time that is regulated by both endogenous and environmental cues (Michaels and Amasino, 1999; Sheldon et al., 1999; Sung and Amasino, 2005). *FLC* is a MADS-domain-containing transcription factor that acts as a floral repressor. It acts to block flowering, at least in part, by repressing the floral promoters *FT* (Michaels et al., 2005; Searle et al., 2006) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Hepworth et al., 2002; Samach et al., 2000).

In rapid-cycling accessions, *FLC* expression is suppressed by the autonomous floral-promotion pathway (AP); thus AP mutants have high levels of *FLC* expression and are late flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). In total, 8 AP genes have been identified and cloned. Two of these genes, *FLOWERING LOCUS D* (*FLD*) and *FVE*, are predicted to participate in a histone deacetylase complex (Ausin et al., 2004; He et al., 2003; Kim et al., 2004). Consistent with this model, *fld* and *fve* mutants have elevated levels of histone acetylation at the *FLC* locus (He et al., 2003). Thus, the role of these proteins appears to be to repress *FLC* transcription via histone deacetylation at the *FLC* locus (histone deacetylation is associated with transcriptional inactivation of genes). FLD belongs to a class of amine oxidases (He et al., 2003). One member of this class, LSD1 has been shown to repress transcription by acting as a histone H3 lysine 4 demethylase (Shi et al., 2004). Thus, the effect of FLD on histone acetylation may be indirect. *FVE* encodes a protein with similarity to a retinoblastoma-associated protein (Ausin et al., 2004; Kim et al., 2004). Other AP genes include *LUMINIDEPENDENS* (*LD*; a putative homeodomain transcription factor) (Lee et al., 1994a), *FCA* (Macknight et al., 1997), *FPA* (Meier et al., 2001; Schomburg et al., 2001) and *FLK* (Lim et al., 2004; Mockler et al., 2004) (RNA-binding proteins), *FY* (similar to polyadenylation factors) (Simpson et al., 2003), and *RELATIVE OF EARLY FLOWERING 6* (*REF6*; a jumonji-like transcription factor) (Noh, B. et al., 2004); the molecular mechanism of how these genes repress *FLC*, however, is not well understood.

In contrast to rapid-cycling accessions, many naturally occurring *Arabidopsis* are late flowering unless vernalized, and thus behave as winter annuals. These winter-annual accessions contain active alleles of the *FRIGIDA* (*FRI*) gene (Johanson et al., 2000), which act to positively regulate *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999). *FRI* is epistatic to the AP, thus, *FRI*-containing plants have high levels of *FLC* and are late flowering despite having a functional AP. Most rapid-cycling accessions contain naturally occurring loss-of-function mutations in *FRI* (Johanson et al., 2000). The FRI protein shows no significant sequence similarity to proteins of known biochemical function. The mechanism by which FRI upregulates *FLC* expression remains poorly understood, however, histone H3 lysine 4 (H3K4) trimethylation is increased at the *FLC* locus in *FRI*-containing plants. Thus, the regulation of chromatin structure may be important in the regulation of *FLC* by *FRI* (He et al., 2004).

Rapid-cycling accessions with AP mutations and *FRI*-containing winter annuals have nearly indistinguishable flowering behaviors. Both are late flowering and vernalization responsive; after an

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approximately 30-day cold-treatment period as imbibed seeds or young seedlings, the late-flowering phenotype conferred by AP mutations or *FRI* is eliminated (Burn et al., 1993; Koornneef et al., 1991; Lee et al., 1993). Vernalization promotes flowering in these backgrounds by causing an epigenetic repression of *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999). Thus, the repression of *FLC* by vernalization is epistatic to the upregulation of *FLC* by either *FRI* or AP mutants. The epigenetic silencing of *FLC* is associated with repressive histone modifications at the *FLC* locus, such as dimethylation of histone H3 at lysine 9 and lysine 27 (Bastow et al., 2004; Sung and Amasino, 2004). Thus changes in *FLC* chromatin structure have been implicated in the regulation of *FLC* by the AP, *FRI* and vernalization.

Genetic screens for early-flowering mutants in rapid-cycling or winter-annual backgrounds have identified a number of genes that are required for *FLC* expression. These genes can be divided into two classes based on their effects on flowering time and the presence or absence of pleiotropic phenotypes. One class is required for high levels of *FLC* expression in both AP-mutant and *FRI*-containing backgrounds; however, the effects of these genes are not limited to the regulation of *FLC*. In addition to suppressing *FLC* expression, mutations in *PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1* (Noh and Amasino, 2003), *VERNALIZATION INDEPENDENCE 4* (*VIP4*) (Zhang and van Nocker, 2002), *VERNALIZATION INDEPENDENCE 3* (Zhang et al., 2003), *EARLY FLOWERING 5* (Noh, Y. et al., 2004), *EARLY FLOWERING 7* (*ELF7*) (He et al., 2004), *ELF8*/*VIP6* (He et al., 2004; Oh et al., 2004), *VERNALIZATION INDEPENDENCE 5* (*VIP5*) (Oh et al., 2004), *HUA2* (Doyle et al., 2005), *ABA HYPERSENSITIVE 1* (Bezerra et al., 2004), *EARLY FLOWERING IN SHORT DAYS* (*EFS*) (Kim et al., 2005) and *SUPPRESSOR OF FRIGIDA 3*/*ACTIN RELATED PROTEIN 6* (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006) show other pleiotropic phenotypes as well. Although the role of many of these genes in the expression of *FLC* has yet to be determined, it appears that ELF7, ELF8, VIP4 and VIP5 are likely to form a PAF1 (RNA polymerase II associated factor 1)-like complex that promotes *FLC* expression by recruiting the putative histone H3 methyltransferase EFS to the *FLC* locus. In yeast, the PAF1 complex promotes gene expression by recruiting a histone H3K4 methyl transferase-containing complex to the chromatin of target genes (Krogan et al., 2003; Ng et al., 2003). Consistent with this model, mutations in members of the PAF1-like complex or *efs* reduce H3K4 trimethylation of *FLC* chromatin. In addition to suppressing *FLC* expression, mutations in the *efs*/PAF1-like genes also suppress the expression of *FLC*-related genes and adjacent genes at the *FLC* locus (He et al., 2004; Oh et al., 2004).

A second class of genes required for *FLC* expression appear to have more specific roles in the regulation of flowering time by *FRI*. Mutations in *FRIGIDA LIKE 1* (*FRL1*) (Michaels et al., 2004) and *FRIGIDA ESSENTIAL 1* (*FES1*) (Schmitz et al., 2005) strongly suppress *FLC* expression in a *FRI*-containing background, but only weakly suppress *FLC* in an AP-mutant background. In addition, pleiotropic phenotypes have not been reported in these mutants (Michaels et al., 2004; Schmitz et al., 2005). Thus, these genes may define a *FRI*-specific pathway. Here, we report the discovery of an additional gene in the *FRI* pathway, *SUPPRESSOR OF FRIGIDA 4* (*SUF4*). Like *FRL1* and *FES1*, *SUF4* is required for the upregulation of *FLC* by *FRI*. Loss of *SUF4* strongly suppresses *FLC* expression in a *FRI*-containing background and results in increased H3K4 trimethylation in *FLC* chromatin. In contrast to *efs* or PAF1-like complex mutants, which also show reduced H3K4 trimethylation at *FLC*, mutations in *suf4* do not suppress the expression of the genes surrounding *FLC* or of *FLC*-like genes. Thus *SUF4* is specifically required for the expression of *FLC*, whereas the EFS/PAF1-like complex is required for the expression of multiple genes in the regions of *FLC* and *FLC*-like genes. To explain these results, we propose a model in which SUF4 and members of the FRI pathway are specifically required to recruit the EFS/PAF1-like complex to the *FLC* locus.

MATERIALS AND METHODS

Plant material

FRI (Lee et al., 1994b), *flc*-3 (Michaels and Amasino, 1999), *fca*-9 (Bezerra et al., 2004), *fve*-4 (Michaels and Amasino, 2001), *ld-1* (Redei, 1962), *frl1-1* (Michaels et al., 2004), *efs-3* (Kim et al., 2005) and *elf7* (He et al., 2004) are in the Columbia (Col) genetic background and have been described previously. *co* (SAIL24H04) and *suf4-2* (SALK_093449) were obtained from the *Arabidopsis* Biological Resource Center (Columbus, Ohio) and are also in the Col background. The T-DNA population used to identify *SUF4* has also been described previously (Michaels and Amasino, 1999). Plants were grown under cool-white fluorescent light (approximately 100 $\mu mol/m^{-2}sec^{-1}$. Long days consisted of 16 hours light followed by 8 hours darkness; short days consisted of 8 hours light followed by 16 hours darkness.

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), *At5g10150* (Kim et al., 2005) and *UBQ* (Michaels et al., 2004) have been described previously. For *SUF4* (5′-AGGAATTCCACCCCATGTCT-TGAC-3′ and 5′-CTGAGATTCGTCTGTCTATCGC-3′), *At1g77090* (5′-ATGATGGAAACAGCTCTGCTCCG-3′ and 5′-CAAGTCAATC-TCGGTGCCACCAA-3′), and *FRI* (5′-TTCTTCTAATGCCTGATC-GTGG-3′ and 5′-CTCCAAGCTAACAATTTGCTCT-3′) the indicated primers were used. The data shown is representative of at least three independent experiments.

Constructs

To create a *SUF4::GUS* fusion, a genomic fragment containing the entire coding region of *SUF4,* plus an additional 1252 bp 5′ of the predicted translational start site, were fused to *GUS* (Jefferson, 1987) in the pPZP211 vector (Hajdukiewicz et al., 1994). For *SUF4* overexpression, a genomic fragment containing the entire coding region of *SUF4*, plus an additional 832 bp 3′ of the predicted stop codon, was fused to the 35S cauliflower mosaic virus promoter (Odell et al., 1985), also in the pPZP211 vector.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (Kim et al., 2005). Antibody was obtained from Upstate USA (Charlottesville, VA).

RESULTS

SUF4 is required for the winter-annual flowering habit

To increase our understanding of the late-flowering vernalization-responsive habit of winter-annual *Arabidopsis*, we conducted a mutant screen to identify genes required for the upregulation of *FLC* by *FRI*. A winter-annual strain (Col *FRI*) containing the dominant *FRI* allele from the San Feliu (SF2) accession backcrossed into the Col background was mutagenized by T-DNA insertional mutagenesis; subsequently, the T2 generation was screened for early-flowering mutants (Michaels et al., 2004). One mutant, *SUPPRESSOR OF FRI 4* (*SUF4*), strongly suppressed the late-flowering phenotype of Col *FRI* (Fig. 1A,B). To identify the gene affected by the *suf4-1* mutation, thermal asymmetric interlaced PCR was performed to amplify genomic DNA flanking the site of T-DNA insertion (Liu et al., 1995). Sequencing of the resulting PCR product

showed that the *suf4-1* mutant contained a T-DNA insertion in the last intron of *At1g30970*, 2307 bp downstream of the predicted translational start site. To determine whether the insertion in *At1g30970* was responsible for the early-flowering phenotype of *suf4*, the mutant was crossed with wild-type Col and a T-DNA allele of *At1g30970* (*suf4-2*) obtained from the SALK collection (Alonso et al., 2003). When crossed to Col, all F1 plants were late flowering, indicating that the *suf4* mutation behaves recessively. By contrast, all F1 plants resulting from the *suf4-1 suf4-2* cross were early flowering, indicating that the two mutations are allelic. As a final confirmation that the lesion in *At1g30970* is responsible for the early-flowering phenotype of *suf4-1*, the *suf4* mutant was transformed with a genomic fragment containing *At1g30970*. Late flowering was restored in the majority of the T1 plants (data not shown), thus confirming that *At1g30970* is *SUF4*. The effects of *suf4-1* and *suf4-2* on flowering time were indistinguishable and no pleiotropic phenotypes were observed in either mutant. *suf4-1* was used in all subsequent experiments.

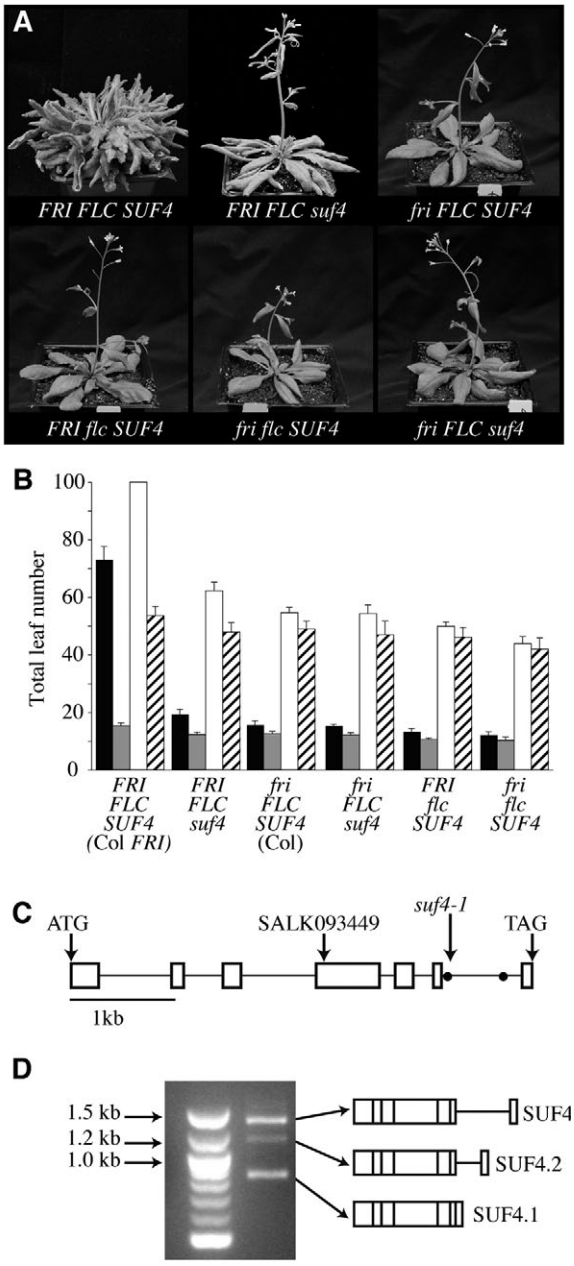

Fig. 1. *SUF4* is required for the late-flowering phenotype of *FRI* and is alternatively spliced. (**A**,**B**) The effect of *suf4* mutations on flowering time in the indicated genetic backgrounds. (A) Plants were photographed at similar stages of development (e.g. at the opening of the first flowers). (B) Bars represent the total number of leaves (rosette and cauline) formed by the primary shoot apical meristem. Black and gray bars represent plants grown under long days; white and cross-hatched bars represent plants grown under short days. Plants represented by gray and cross-hatched bars were cold-treated for 30 days before planting. Error bars represent the s.d. (**C**) Genomic structure of *SUF4*. Exons are indicated by open boxes; filled circles indicate sites of alternative splicing. (**D**) RT-PCR of *SUF4* and a schematic drawing of the three splice variants of *SUF4*.

SUF4 encodes a nuclear-localized zinc-finger protein

The *SUF4* gene is predicted to encode a protein of 368 amino acids, the N-terminal end of which contains a BED-finger domain. The BED domain is named after the *Drosophila* proteins *BEAF* and *DREF*, and contains two C2H2 zinc fingers that are thought to mediate DNA binding (Aravind, 2000). The BED domain from *SUF4* is highly similar to other plant and animal proteins (Fig. 2). Outside the BED domain, the SUF4 protein is proline rich (approximately 20%), suggesting that it may be important for mediating protein-protein interactions (Zarrinpar et al., 2003). Apart from the BED domain, SUF4 shows little relatedness to other proteins in *Arabidopsis* or in other species. Only one protein from rice, BAD460082, shows significant similarity to SUF4 in the C-terminal half of the protein. Most notably, in one region near the C-terminus of SUF4, the sequences of SUF4 and BAD460082 are identical at 30/32 residues (Fig. 2, underlined). Although the biochemical function of this region is unknown, the strong sequence conservation between *Arabidopsis* and rice suggests that this region may be important for protein function.

The presence of the BED domain suggests that SUF4 may bind DNA and act as a transcriptional regulator. This model is supported by the presence of a putative SV40-type nuclear localization signal (Kalderon et al., 1984) at the N-terminus of SUF4 (Fig. 2). To investigate if SUF4 is localized to the nucleus, we created a SUF4::GUS fusion that contained the *SUF4* promoter and full-length coding region fused to the *β-glucuronidase* (*GUS*) gene (Jefferson, 1987). To determine whether the SUF4::GUS fusion would produce a functional SUF4 protein, the construct was transformed into a *suf4*-mutant background. The majority of the resulting T1 plants were late flowering, indicating that the SUF4::GUS fusion was functional (data not shown). GUS staining of lines carrying the SUF4::GUS fusion showed accumulation of SUF4 in the nucleus (Fig. 3A,B). Thus, consistent with its proposed role as a DNA-binding protein, SUF4 is localized to the nucleus.

SUF4 exhibits alternative splicing

The *SUF4* gene is predicted to contain seven exons (Fig. 1C). To verify the annotation of *SUF4*, primers were designed to the predicted 5′ and 3′ ends of the gene and were used to amplify the *SUF4* cDNA via RT-PCR. Three transcripts were detected (Fig. 1D): SUF4.1, SUF4.2 and SUF4.3. Sequence analysis showed that the smallest transcript, SUF4.1, was identical to the predicted cDNA sequence (At1g30970.1). The two larger transcripts were identical to the predicted cDNA with the exception of the last intron. The largest transcript, SUF4.3, contained the entire sequence of intron six (519 bp), whereas the middle transcript, SUF4.2, contained a portion (163 bp) of intron six. Both the donor and acceptor sites used

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SUF4 (At1g30970) M G K K K K R A T E K
Rice (BAD46082) M G K K K K - R V E K
C. briggsae (CAE62050) M G R K K K - K I D K
Human (AAH02372) M G R K K K - K Q L K
Mouse (AAH03715) M G R K K K - K Q L K
Fly (EAL33602) M G R K K K - K A S K
Bee (XP_395716) M G R K K K - K Q S R

V W C Y Y C D R E F D D E K I L V Q H Q
V F C Y Y C D R E F D D E K I L V Q H Q
P W C W Y C N R E F D D E K I L I Q H Q
P W C W Y C N R D F D D E K I L I Q H Q
P W C W Y C N R D F D D E K I L I Q H Q
P W C W Y C N R E F D D E K I L V Q H Q
P W C W Y C N R E F E D E K I L I Q H Q

K A K H F K C H V C H K K L S T A S G M
K A K H F K C H V C H K K L S T A G G M
K A K H F K C H I C H K K L F S G P G L
K A K H F K C H I C H K K L Y T G P G L
K A K H F K C H I C H K K L Y T G P G L
K A K H F K C H I C H K K L Y T G P G L
K A K H F K C H I C H K K L Y T G P G L

V I H V L Q V H K E N V T K V P N A K D
A I H V L Q V H K E S V T K V P N A K P
S I H C M Q V H K E T I D K I P A A V H
A I H C M Q V H K E T I D A V P N A I P
A I H C M Q V H K E T I D A V P N A I P
S I H C M Q V H K E T V D K V P N S L P
S I H C M Q V H K E A I D K V P N S L P

G R D S T D I E I Y G M Q G I P P H V L
E R E S T E I E I F G M Q G I P P D V L
G R D N I H V E I Y G M Q G I P
G R T D I E L E I Y G M E G I P
G R T D I E L E I Y G M E G I P
N R S N I E I E I F G M D G I P
N R S N I E I E I Y G M E G I P

T A H Y G E E E D E P P A K V A K V E I
A A H Y G E E E D - P S S K V A K V E V

P S A P L G G V V P R P Y G M V Y P P Q
P S L R P P - V M P N P A G M V Y P P R

Q V P G A V P A R P M Y Y P G P - P M R
P A Y G - - V A P P M Y N P A L N P L M

H P A P V W Q M P P P R P Q Q W Y P Q N
A R P P I W P A P P P Q P - - W F T Q -

P A L S V P P A A H L G Y R P Q P L F P
P V V S V P Q M A S G L A P Q Q P L F P

V Q N M G M T P T P T S A P A I Q P S -
I Q N M P A P M T S A P A N L L Q T S F

P V T G V T P P G I P T S S P A M P V P
P M A H V G V P - - - - S P V T P Q V S

Q P L F P V V N N S I P S Q A P P F S A
Q P L F P V S T S - - - - - A G N G A V

P L P V G G A Q Q P S H A D A L G S A D
S S P Y V A S V A P - - - - - - G S I P

A Y P P N N S I P G G T N A H S Y A S G
T S S P S V - A P A G V G Y A A T N Q G

P N T S G P S I G P P P V I A N K A P S
- - T G G P A A V P P P A S N N K A P A

N Q P - - N E V Y L V W D D E A M S M E
T Q P G A N E V Y L V W D D E A M S M E

E R R M S L P K Y K V H D E T S Q M N S
E R R L S L P K Y Q V H D E T S Q V S S

I N A A I D R R I S E S R L A G R M A F
D F Y N F I S V I L M I C E K N Q V E V

H G D A L N W N I V
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Fig. 2. Alignment of SUF4 to related proteins. A putative nuclear localization signal is shown in bold (amino acid residues 3-7) and a region of high sequence identity between SUF4 and BAD46082 from rice is underlined. Proteins from *C. briggsae*, human, mouse, *Drosophila* and bee show significant sequence identity to the N-terminal part of SUF4 only; the C-terminal regions of these proteins are, therefore, not shown.

for the splicing of intron six in the SUF4.2 transcript are distinct from those used in SUF4.1. The portion of intron six that is removed is flanked by 7-bp direct repeats (5′-CTTTTTA-3′), one of which is removed during splicing (Fig. 1C). The significance, if any, of these repeats is unknown.

It is interesting to notice that all of the SUF4 splice variants are identical through the end of exon six, which marks the end of the highly conserved region in the C-terminus between SUF4 and BAD460082 (Fig. 2, underlined). The protein sequence encoded for by the seventh exon, by contrast, shows no similarity to BAD460082. Because SUF4.2 and SUF4.3 contain part or all of

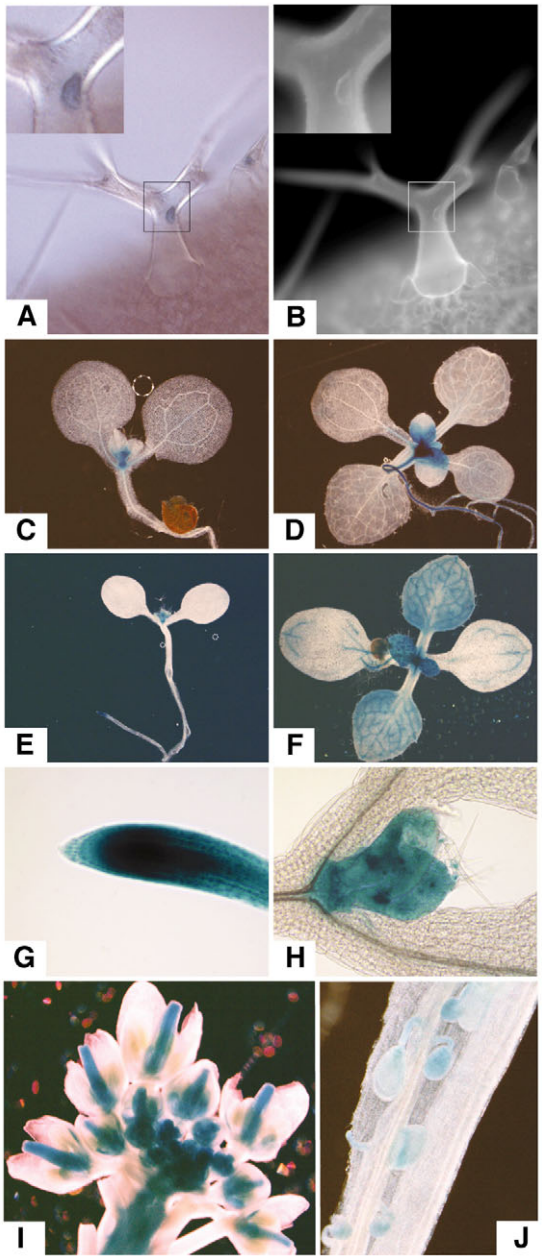

Fig. 3. Spatial expression pattern of *SUF4*. (**A**) Nuclear localization of *SUF4::GUS* in trichomes. (**B**) DAPI-stained image of the same trichome used in A. (**C**,**D**) *FLC::GUS* expression and (**E**,**F**) *SUF4::GUS* expression in seedlings. Staining was performed 4 (C,E) and 10 (D,F) days after germination. *SUF4::GUS* expression in roots (**G**), the shoot apex (**H**), inflorescence (**I**) and developing seeds (**J**).

intron six, they contain stop codons ten- and 47-amino acids after the end of exon six, respectively. Interestingly, the first four amino acids encoded for by the beginning of intron six (VSSD), present in SUF4.2 and SUF4.3, extend the highly conserved region with BAD460082 (Fig. 2, underlined). After these four amino acids, however, there is no further similarity between the C-terminal regions of BAD460082 and SUF4.2 or SUF4.3. To determine whether these four amino acids are crucial for SUF4 function, we placed the SUF4.1 cDNA under control of the constitutive 35S promoter and transformed *FRI suf4* plants. Most T1 plants were late flowering (data not shown), indicating that the SUF4.1 transcript does produce a functional protein.

SUF4 is expressed more widely than *FLC*

RT-PCR and the *SUF4::GUS* fusion were used to examine the expression of *SUF4*. In young seedlings, *SUF4* expression is expressed most highly in the growing regions of the plant (e.g. shoot and root apex) (Fig. 3E,G,H). At this stage of development, the pattern of expression is similar to that observed with *FLC::GUS* (Fig. 3C). Later in development, however, *SUF4::GUS* shows broader expression than *FLC::GUS* and is expressed in expanding leaves, in the vasculature of fully expanded leaves, in the inflorescence, throughout young floral primordia, in the carpels of older flowers and in fertilized ovules (Fig. 3D,F,I,J). These results are consistent with the expression pattern determined by RT-PCR (Fig. 4A). The effect of *FRI*, AP mutations and vernalization on *SUF4* expression was also determined. None of these factors influenced the abundance of the *SUF4* transcript (Fig. 4B). For RT-PCR analysis of *SUF4* expression, primers that spanned the alternatively spliced regions of *SUF4* were used for amplification. This enabled the monitoring of the relative abundance of the three splice forms in each experiment. No consistent difference was observed in *SUF4* splicing as a result of tissue type, genetic background or vernalization treatment.

suf4 mutants strongly suppress *FRI*, but only weakly suppress AP mutants

Mutations in *suf4* strongly suppress the late-flowering phenotype conferred by *FRI* and *FLC* (Fig. 1B). Under long days, *suf4* mutants flower after forming approximately 54 fewer leaves than Col *FRI*. Although *suf4* strongly suppresses the late-flowering phenotype of *FRI* and *FLC*, it should be noticed that this suppression is not complete, as *fri* or *flc* mutants flower with approximately six fewer leaves than *suf4* under long days (Fig. 1B). In the Col background (which contains a naturally occurring null allele of *FRI*), *suf4* had no detectable effect on flowering time (Fig. 1B). Mutations in *suf4* did also not appear to affect the vernalization response under long or short days (Fig. 1B).

Because winter-annual strains of *Arabidopsis* are late flowering because of the upregulation of *FLC* by *FRI*, we investigated whether *SUF4* was required for the expression of *FRI* and/or *FLC*. No detectable difference was found in *FRI* mRNA levels (Fig. 4C); however, *FLC* expression was reduced in the *suf4* mutant (Fig. 4D). Thus, *SUF4* is required for the upregulation of *FLC* by *FRI*. As in *FRI*-containing winter annuals, AP mutants are also late flowering because of elevated levels of *FLC* expression. To determine whether *SUF4* is also required for high levels of *FLC* expression in AP-mutant backgrounds, double mutants were created between *suf4* and *ld*, *fve* or *fca*. With each of the AP mutants tested, the double with *suf4* flowered earlier than the single mutant. The early-flowering phenotypes in the AP-mutant backgrounds, however, were less pronounced than in the *FRI*-containing background (Fig. 4E). It is

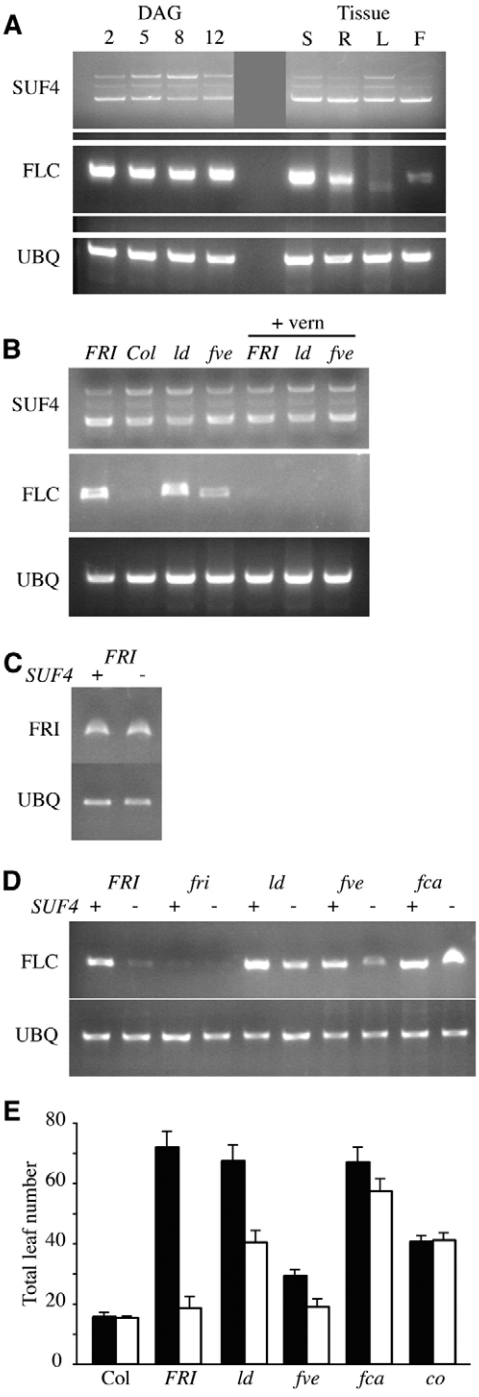

Fig. 4. *SUF4* expression, and its effect on flowering time and gene expression in *FRI* and AP-mutant backgrounds. (**A**) RT-PCR analysis of *SUF4* in the early stages of development and in various tissues. Expression of *SUF4* and *FLC* at 2-12 days after germination (DAG), and in shoots (S), roots (R), leaves (L) and flowers (F). (**B**) Effect of genotype and vernalization on *SUF4* and *FLC* expression, as determined by RT-PCR. (**C**) RT-PCR analysis of *FRI* expression in wild-type (+) and *suf4*-mutant (–) backgrounds. (**D**) RT-PCR analysis of *FLC* expression in the indicated backgrounds with wild-type *SUF4* (+) or mutant *suf4* (–). (**E**) Effect of *suf4* mutations on flowering time. Bars represent the total number of leaves (rosette and cauline) formed by the primary shoot apical meristem. Black bars represent the indicated genotypes with wild-type *SUF4*; white bars represent the indicated genotypes with the *suf4* mutation. Error bars represent the s.d.

interesting to notice that the *suf4* mutation did not affect all AP mutants equally. *ld suf4* and *fve suf4* flowered much earlier than the *ld* and *fve* singles; however, the difference in flowering time between *fca suf4* and *fca* was much smaller. Consistent with the weaker effect of *suf4* on flowering time in the AP-mutant backgrounds, the suppression in *FLC* expression in these lines was reduced compared with that seen in Col *FRI* (Fig. 4D). A double mutant was also created between *suf4* and the photoperiod-pathway mutant *constans* (*co*). The late-flowering phenotype of *co* mutants does not depend on *FLC* expression (Michaels and Amasino, 2001) and, consistent with *SUF4* acting as a regulator of *FLC*, *suf4* had no effect on flowering time in the *co*-mutant background (Fig. 4E).

SUF4, FRI, FRL1 and *FES1* are required to delay flowering

The result that loss-of-function mutations in *suf4*, *frl1* and *fes1* strongly suppress the late-flowering phenotype of *FRI*, but have only a relatively weak effect on the flowering time of AP mutants, suggests that they may comprise a *FRI*-specific pathway. The role of these genes in the regulation of flowering time was further investigated using overexpression analysis. Overexpression constructs for *FRI*, *FRL1* and *FES1* have been described previously (Michaels et al., 2004; Schmitz et al., 2005). A *SUF4* overexpression construct was created by placing a genomic copy of the *SUF4* gene under control of the strong 35S Cauliflower mosaic virus promoter (Odell et al., 1985). To ensure that the *35S::SUF4* fusion is functional, it was used to transform *suf4* mutants in the Col *FRI* background. Late-flowering plants were obtained in the T1, indicating that the *35S::SUF4* construct is able to restore *SUF4* function (Table 1, Fig. S1 in the supplementary material). Similar to plants overexpressing *FRI*, *FRL1* (Michaels et al., 2004) or *FES1* (Schmitz et al., 2005), *35S::SUF4* plants are vernalization responsive (data not shown); thus, *SUF4* overexpression does not interfere with suppression of *FLC* by vernalization.

35S::SUF4 was transformed into the Col background to determine whether *SUF4* overexpression is sufficient to delay flowering in the absence of *FRI*; however, only early-flowering plants were obtained in the T1, indicating that *SUF4* requires *FRI* in order to upregulate *FLC*. Similar results were obtained when *35S::SUF4* was transformed into *frl1*- and *fes1*-mutant backgrounds; all T1 plants were early flowering (Table 1, Fig. S1 in the supplementary material). Thus, *SUF4* requires *FRI*, *FRL1* and *FES1* in order to upregulate *FLC*. This result is consistent with a model in which SUF4 acts upstream of, or in a complex with, FRI, FRL1 and FES1. In an attempt to clarify the genetic relationships between these genes, *FRI*, *FRL1* and *FES1* were overexpressed in a *suf4*-mutant background. If *FRI*, *FRL1* and *FES1* act downstream of *SUF4*, then overexpression of these genes may restore late flowering in a *suf4* mutant. In the T1, however, only early flowering plants were obtained (Table 1, Fig. S1 in the supplementary material). Thus *FRI*, *FRL1* and *FES1* require *SUF4* in order to upregulate *FLC* and delay flowering. This observation suggests that these proteins might function as part of a complex. To investigate this possibility, SUF4.1 was used as bait and FRI, FRL1 and FES1 were each used as prey in the yeast-two-hybrid assay; however, no interactions were detected (data not shown).

Table 1. Effect of *SUF4, FRL1, FES1* and *FRI* overexpression

Background	Transgene	Phenotype
FRI suf4	None	Early
FRI suf4	*35S::SUF4*	Late
FRI suf4	*35S::FRL1*	Early
FRI suf4	*35S::FES1*	Early
fri SUF4 (Col)	None	Early
fri SUF4 (Col)	*35S::FRI*	Late
fri SUF4 (Col)	*35S::SUF4*	Early
fri SUF4	None	Early
fri SUF4	*35S::FRI*	Early
FRI frl1	None	Early
FRI frl1	*35S::SUF4*	Early
FRI fes1	None	Early
FRI fes1	*35S::SUF4*	Early
FRI efs	None	Early
FRI efs	*35S::SUF4*	Early
FRI elf7	None	Early
FRI elf7	*35S::SUF4*	Early

SUF4 is required for H3K4 trimethylation of *FLC* in a *FRI*-containing background

Previous work has shown that genes encoding members of a PAF1-like complex are required for elevated expression of *FLC* in *FRI* or AP-mutant backgrounds (He et al., 2004; Oh et al., 2004; Zhang and van Nocker, 2002). In yeast, the PAF1 complex acts to promote transcription of target genes by recruiting a histone H3K4 methyltransferase (H3K4 trimethylation is often associated with actively transcribed genes) (Krogan et al., 2003; Ng et al., 2003). In *Arabidopsis*, the PAF1-like complex may recruit the putative histone H3 methyltransferase EFS, as mutations in *efs* or members of the PAF1-like complex result in reduced histone H3 trimethylation at the *FLC* locus and in reduced *FLC* transcription (He et al., 2004; Kim et al., 2005; Oh et al., 2004; Zhao et al., 2005). To investigate whether SUF4 also affects histone H3 trimethylation at the *FLC* locus, H3K4 trimethylation was determined by ChIP analysis. At positions in both the *FLC* promoter and at the beginning of intron I, *suf4* mutants showed reduced H3K4 trimethylation compared with Col *FRI* (Fig. 5A,B). These two regions are identical to those examined in previous studies of histone modification at the *FLC* locus (He et al., 2003; Kim et al., 2005). The reduction in H3K4 trimethylation was similar to that observed in *fri* mutants (Fig. 5B). Thus, *suf4* mutations prevent the increased H3K4 trimethylation of *FLC* that is normally conferred by *FRI*. Consistent with this result, *SUF4* overexpression in *FRI*-containing *efs* or *elf7* mutants had no effect on flowering time (Table 1).

The effect of *SUF4, FRL1* and *FRI* on gene expression is more localized than that of *EFS* or the PAF1-like complex

The genes that are required for high levels of *FLC* expression can be divided into two categories based on pleiotropic effects and their effects on flowering time. Genes such as *FRI*, *SUF4*, *FRL1* and *FES1* appear to function predominantly to regulate *FLC* in a *FRI*-containing background. Mutations in these genes are not associated with pleiotropic phenotypes and strongly block the upregulation of *FLC* by *FRI*, but have little or no effect on *FLC* expression in an AP-mutant background (Michaels et al., 2004; Schmitz et al., 2005). Mutations in genes such as *efs* or the PAF1-like complex genes, by contrast, suppress *FLC* expression in both *FRI*-containing and AP-mutant backgrounds, and also cause pleiotropic phenotypes, such as reduced plant size and reduced fertility (He et al., 2004; Oh et al., 2004; Zhang and van Nocker, 2002). In addition to suppressing *FLC* expression, *efs* and PAF1-like complex mutations also show reduced H3K4 trimethylation (He et al., 2004) and reduced expression (He et al., 2004; Kim et al., 2005; Oh et al., 2004) of other members of the *FLC* clade, such as *FLOWERING LOCUS M* (*FLM*)/*MADS AFFECTING FLOWERING 1* (Ratcliffe et al., 2001; Scortecci et al.,

2001). *efs* mutations have also been shown to suppress the expression of the genes that flank *FLC* (Kim et al., 2005); thus, the role of these genes is not limited to the regulation of *FLC*. Interestingly, the coordinate regulation of genes at the *FLC* locus have also been reported in response to vernalization and in the autonomous-pathway mutant *fca* (Finnegan et al., 2004).

Given the effects of mutations in *efs* and PAF1-like complex genes on the expression of *FLC*-clade members and neighboring genes at the *FLC* locus, we investigated whether mutations in *FRI*, *FRL1* and *SUF4* would show similar effects on gene expression. As expected, mutations in *fri*, *frl1*, *suf4*, *efs* and the PAF1-like complex member *elf7* all suppress *FLC* expression (Fig. 5C). The expression of FLM, which is the *FLC*-clade gene that is most similar to *FLC*, however, was only suppressed in *efs* and *elf7* backgrounds (Fig. 5C). Thus, mutations in *fri*, *frl1* and *suf4* appear to specifically regulate *FLC*, whereas *efs* and *elf7* regulate other members of the *FLC* clade as well.

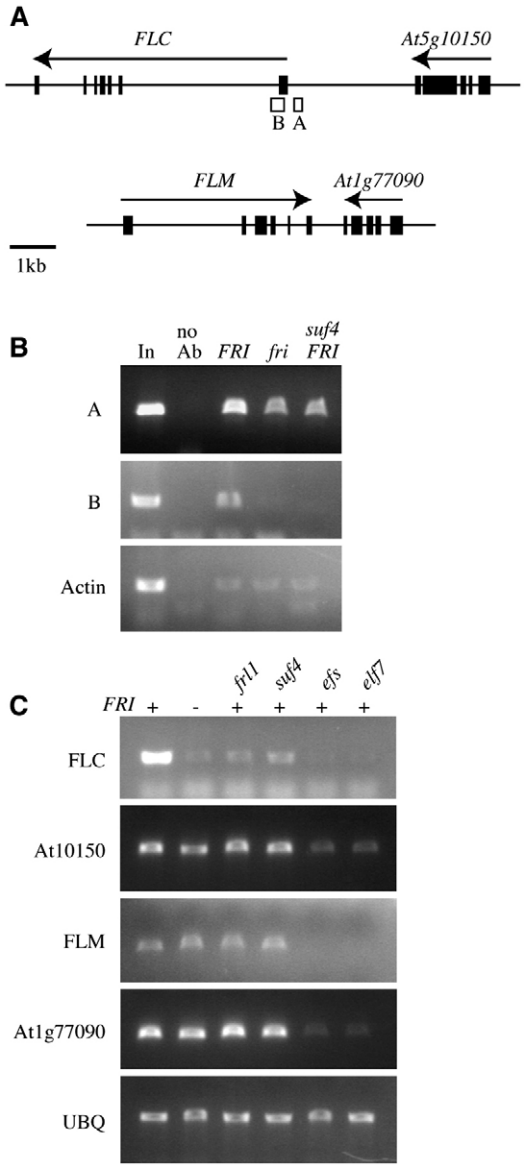

Fig. 5. Effect of *SUF4* on H3K4 trimethylation and gene expression at the *FLC* and *FLM* loci. (**A**) Schematic drawing of the *FLC* and *FLM* loci. White boxes represent the regions of *FLC* amplified in ChIP analysis (B and A), black boxes represent exons. (**B**) ChIP analysis of the histone H3-K4 trimethylation state of *FLC* chromatin in *suf4* and related lines. The input is Col *FRI* chromatin before immunoprecipitation. 'No AB' refers to the control sample lacking the anti-trimethyl H3-K4 antibody. 'A' and 'B' refer to the regions of *FLC* indicated in 5A. *ACTIN* served as an internal control. The results shown are representative of three replicates. (**C**) Effect of various mutations on the expression of *FLC*, *FLM* and neighboring genes, as determined by RT-PCR.

This distinction between *FRI*, *FRL1*, *SUF4* and EFS/PAF1-like complex genes was also apparent in the regulation of other genes at the *FLC* and *FLM* loci (Fig. 5A). As previously reported, the expression of a gene adjacent to *FLC*, *At5g10150*, is suppressed in an *efs*-mutant background (Kim et al., 2005) (Fig. 5C). Consistent with the model that the PAF1-like complex recruits *EFS*, mutations in *elf7* show a similar repression of *At5g10150* transcript levels. To determine whether coordinated changes in gene expression are also observed at the *FLM* locus in *efs*/PAF1-like complex mutants, we investigated the expression of *At1g77090* (Fig. 5A). Similar to *At5g10150* at the *FLC* locus, expression of *At1g77090* is suppressed by mutations in *efs* or *elf7*. Thus, at both the *FLC* and *FLM* loci, mutations in *efs* or the PAF1-like complex genes suppress the expression of adjacent genes. By contrast, mutations in *fri*, *frl1* or *suf4* only suppress the expression of *FLC* (Fig. 5C). Therefore, although mutations in *suf4*, *efs* or members of the PAF1-like complex all block the increased H3K4 trimethylation of *FLC* chromatin conferred by *FRI*, the effects of *SUF4* are relatively *FLC*-specific, whereas *EFS* and members of the PAF1-like complex are required for the expression of multiple genes at the *FLC* and *FLM* loci.

DISCUSSION

FLC is a central regulator of flowering time in *Arabidopsis* and is regulated by three major pathways; the *FRI* pathway positively regulates *FLC*, whereas the AP and vernalization negatively regulate *FLC*. Here, we report the identification of *SUF4*, a gene that is required for the upregulation of *FLC* by *FRI*. Recently, screens for early-flowering mutants in *FRI*-containing winter-annual or rapid-cycling backgrounds have identified a number of genes that are required for the proper expression of *FLC*. The function of most of these genes, however, is not limited to the regulation of *FLC*. In addition to reducing levels of *FLC* in either *FRI*-containing or AP-mutant backgrounds, mutations in members of the PAF1-like complex – *EFS*, *PIE1*, *VIP3*, *ELF5*, *SUF3*, *HUA2* and *ABH1* – all lead to various pleiotropic phenotypes. By contrast, *FRL1* and *FES* appear to play more specific roles in the upregulation of *FLC*, as obvious pleiotropic phenotypes have not been reported in *frl1* and *fes* mutants. The role of *SUF4* appears to be most similar to that of *FRL1* and *FES1*; *suf4* mutants strongly suppress the late-flowering phenotype conferred by *FRI*, but only weakly suppress AP mutants. Also, similar to mutations in *FRL1* and *FES1*, *SUF4* mutations do not affect flowering under short days or in a *co*-mutant background. Although it is not yet understood at a molecular level how *FRI*, *FRL1*, *FES1* and *SUF4* lead to increased *FLC* expression, it is interesting to notice that, because these genes are not essential for elevated expression of *FLC* in an AP-mutant background, they appear to comprise a *FRI*-specific pathway.

Although loss-of-function mutations in *suf4* strongly suppress the late-flowering phenotype of *FRI*, *FRI suf4* plants still flower approximately six leaves later than plants that lack *fri* (i.e. Col). Thus, *FRI* function is largely, but not completely, dependent on *SUF4*. One explanation for the residual late flowering of *FRI* in a *suf4* mutant is that there may be another gene whose function is

partially redundant to *SUF4*. Because ancient large-scale duplication events have occurred in the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000), many genes exist in families in which the members may have related functions. *SUF4*, however, does not have significant sequence similarity to other proteins in *Arabidopsis*. Thus, the residual late-flowering phenotype observed in the absence of *SUF4* may be due to the action of unrelated proteins.

SUF4 is likely to function as a transcriptional regulator. The N-terminal portion of SUF4 contains a putative nuclear-localization signal sequence and a BED DNA-binding domain that is highly similar (approximately 70% identity) to BED domains from animal proteins. SUF4 appears to be a unique gene in *Arabidopsis*, but is highly similar to BAD460082 from rice. Similarity is highest in the BED domain and regions adjacent to this, and in a highly conserved sequence at the C-terminal end of the proteins. We have detected three alternatively spliced forms of *SUF4*. Interestingly, all three mRNAs are predicted to encode proteins that contain all of the conserved domains between SUF4 and BAD460082. Therefore, is seems possible that all three transcripts may encode functional proteins. The relative abundances of the splice forms of SUF4 do not vary with developmental stage, tissue, genetic background or in response to vernalization; thus, alternative splicing does not appear to play a major role in the regulation of SUF4 activity. Although pleiotropic phenotypes were not observed in *suf4* mutants, the expression pattern of *SUF4* suggests that it has functions other than in the regulation of *FLC*. Early in development, *SUF4* and *FLC* show similar patterns of expression; both genes are expressed at highest levels in the shoot and root apex. Later in development, *FLC* expression remains largely restricted to the growing regions of the plant, whereas *SUF4* shows a broader expression pattern and is expressed, in addition to the apical regions, in both leaves and flowers.

Although the molecular mechanism by which the *FRI* pathway acts is not understood, it is known that the upregulation of *FLC* by *FRI* is accompanied by an increase in H3K4 trimethylation. Mutations in *efs* or members of the PAF1-like complex have been shown to suppress *FLC* expression and decrease H3K4 trimethylation of the *FLC* locus. Here, we have shown that mutations in the *FRI*-pathway genes *SUF4* and *FRL1* also suppress H3K4 trimethylation and *FLC* expression. Interestingly, the suppression of *FLC* expression by mutations in *efs* or members of the PAF1-like complex is stronger than mutations in genes of the *FRI* pathway. *efs* and *elf7* mutants contain levels of *FLC* mRNA that are significantly lower than in *fri*, *frl1* or *suf4* mutants (Fig. 5).

In addition to having stronger effects on *FLC* expression, EFS and the PAF1-like complex also have a broader role in the regulation of other members of the *FLC*-clade and adjacent genes. In *efs* or *elf7* mutants, the expression of *FLC* and *FLM* (the *FLC*-clade member most similar to *FLC*) are both suppressed. The expression of genes adjacent to *FLC* and *FLM* (*At5g10150* and *At1g77090*, respectively) are, likewise, suppressed. Therefore EFS and the PAF1-like complex are required for the proper expression of multiple genes at the *FLC* and *FLM* loci. The effects on the expression of adjacent genes may be indirect, due to changes in H3K4 trimethylation state of *FLC* and *FLM*, or alternatively, EFS and the PAF1-like complex may be responsible for maintaining the H3K4 trimethylation state of other genes at the *FLC* and *FLM* loci as well. The effects of the *FRI* pathway, by contrast, appear to be specific to *FLC* regulation. Mutations in *fri*, *frl1* or *suf4* did not affect *FLM* expression and did not affect the transcript levels of the genes adjacent to *FLC* or *FLM*.

Despite the fact that both the *FRI* pathway and EFS/PAF1-like complex both regulate *FLC* expression and H3K4 trimethylation at the *FLC* locus, these two groups of genes have distinct effects on gene expression. The *FRI* pathway appears to specifically target *FLC*, whereas EFS and the PAF1-like complex also regulate *FLC*-like genes and the neighbors of these genes. A possible model to explain the relationship between these two groups of genes is that the *FRI*-pathway genes are required to recruit the EFS/PAF1-like complex to *FLC*, whereas other, more general, factors target the EFS/PAF1-like complex to *FLM* and the genes surrounding *FLC* and *FLM*. Thus, in *FRI*-pathway mutants, such as *fri*, *frl1* or *suf4*, only *FLC* expression is suppressed. By contrast, in an *efs* mutant or PAF1-like-complex mutant, the effects on gene expression are broader.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/23/4699/DC1

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