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

Sang Yeol Kim and Scott D. Michaels*

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 (Avin 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 (Avis 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 FLC (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 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 (Johnson 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 (Johnson 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
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-anual 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 methyltransferase-containing complex to the chromatin of target genes (Krokan 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., 1999b), f-3 (Michaels and Amasino, 1999), fca-9 (Bezerra et al., 2004), fve-4 (Michaels and Amasino, 2001), fdl-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 μmol/m²sec⁻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 performed as described previously (Kim et al., 2004). Primers used for the detection of FLC (Michaels et al., 2004), FLM (Scortecchi et al., 2003), At5g10150 (Kim et al., 2005) and UBQ (Michaels et al., 2004) have been described previously. For SUF4 (5'-AGGAAATCACCACCATGTCT-TGAC-3' and 5'-CTGGAATTCTCTGCTCTATCCG-3'), At1g77090 (5'-ATGATGGAAACGCTCTGCTCG-3' and 5'-CAAGTCATCTTGCGTCAAC3'), and FRI (5'-TTCTTCTAATGCCTGATC-3' and 5'-CTCAAGCTACAACTTGTCT-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 pZP211 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 pZP211 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 Felio (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.

**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
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

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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 BAD460082 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.

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**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
interesting to notice that the suf4 mutation did not affect all AP mutants equally. ld suf4 and five suf4 flowered much earlier than the ld and five 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).

**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., 2004; Ratcliffe et al., 2004; Scortecci et al., 2004; 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.

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

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<td>35S::SUF4</td>
<td>Early</td>
</tr>
<tr>
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</tr>
<tr>
<td>FRI fes1</td>
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<tr>
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<td>FRI efs</td>
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</tr>
<tr>
<td>FRI elf7</td>
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</table>
SUF4 is required for FRI function

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*, *SUF4*, and *FLC* 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). Thus, mutations in *fri*, *fri1* and *suf4* appear to specifically regulate FLC, whereas *efs* and *elf7* regulate other members of the FLC clade as well.

**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 *fri1* and *efs* 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 c-o-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 increased 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, it 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 (At1g10150 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

References