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The *Arabidopsis* RNA-Binding Protein FCA Requires a Lysine-Specific Demethylase 1 Homolog to Downregulate *FLC*

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SUMMARY

A repressor of the transition to flowering in *Arabidopsis* is the MADS box protein FLOWERING LOCUS C (FLC). FCA, an RNA-binding protein, and FY, a homolog of the yeast RNA 3′ processing factor Pfs2p, downregulate *FLC* expression and therefore promote flowering. FCA/FY physically interact and alter polyadenylation/3′ processing to negatively autoregulate *FCA*. Here, we show that FCA requires FLOWERING LOCUS D (FLD), a homolog of the human lysine-specific demethylase 1 (LSD1) for *FLC* downregulation. FCA also partially depends on DICER-LIKE 3, involved in chromatin silencing. *fca* mutations increased levels of unspliced sense *FLC* transcript, altered processing of antisense *FLC* transcripts, and increased H3K4 dimethylation in the central region of *FLC*. These data support a close association of FCA and FLD in mediating H3K4 demethylation and thus transcriptional silencing of *FLC* and reveal roles for antisense RNA processing and DCL3 function in this regulation.

INTRODUCTION

In *Arabidopsis*, a central component regulating the switch to reproductive development is FLOWERING LOCUS C (FLC), a MADS box protein that functions to quantitatively delay flowering by repressing expression of genes that promote the floral state (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* is itself regulated by multiple pathways, some promoting and others repressing expression. High *FLC* expression, as found in many naturally occurring *Arabidopsis* accessions that over-winter vegetatively, is the result of the activities of FRIGIDA and a range of chromatin regulators promoting active chromatin states (reviewed in Bäurle and Dean, 2006). *FLC* expression is repressed by prolonged cold experienced during winter in a process called vernalization. This silencing is epigenetically stable and involves a Polycomb-like mechanism and histone methylation at *FLC* (Bäurle and Dean, 2006). Acting in parallel with vernalization to downregulate *FLC* is the autonomous pathway involving FCA, FY, FVE, FPA, LD, FLD, and FLK (Bäurle and Dean, 2006). LD is a homeodomain-containing protein (Lee et al., 1994), FVE is a homolog of MSI1 (Ausin et al., 2004), whereas FLD is a homolog of human lysine-specific demethylase (LSD1) (He et al., 2003; Shi et al., 2004). FCA, FPA, and FLK are all RNA-binding proteins (Macknight et al., 1997; Schomburg et al., 2001; Lim et al., 2004; Mockler et al., 2004) although they share no homology. FY is a close homolog of Pfs2p (Simpson et al., 2003), an essential *S. cerevisiae* WD-repeat protein that functions to regulate polyadenylation and 3′ processing of mRNAs (Ohnacker et al., 2000).

FY was cloned through its interaction with FCA (Simpson et al., 2003), and several pieces of evidence support a role for FCA/FY function in RNA processing: increased use of a distal poly(A) site in the *FCA* transcript in *fca* or *fy* mutants; exclusive use of a proximal polyadenylation site within the *FCA* transcript in plants overexpressing FCA (Quesada et al., 2003); and physical interaction of FY with homologs of CPSF100 and CPSF160, two conserved components known to regulate RNA 3′ processing in human cells (Herr et al., 2006). FCA through its interaction with FY may thus recruit specific transcripts or stabilize weak poly(A) site interactions with FY and the other 3′ end-processing machinery. The requirement of an FCA/FY interaction for *FLC* repression as well as *FCA* autoregulation suggests a common mechanism may be involved (Simpson et al., 2003). Mutants of Pfs2p in *S. cerevisiae* are lethal, whereas the original *Arabidopsis fy* mutants were late flowering but viable. These alleles were found to produce a partial protein containing the conserved 5′ WD-repeat region, and isolation of *fy* null alleles showed FY activity is also essential in *Arabidopsis* (Henderson et al., 2005), consistent with CPSF 73-I and 73-II, two other components of the CPSF complex, being essential proteins in *Arabidopsis* (Xu et al., 2006). The interaction of FCA with SWI3b, an *Arabidopsis* homolog of the conserved chromatin remodeling factor SWI3 (Sarnowski et al., 2002), suggests FCA activity may

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occur in the dynamic chromatin context on the nascent transcript.

Processing of the 3′ end of transcripts has been linked to chromatin regulation through the activity of yeast Swd2p (Dichtl et al., 2004) and the demonstration of alternative poly(A) sites and 3′ extended transcripts in yeast mutants defective in Paf1-RNA polymerase II activity (Penheiter et al., 2005). The coincidence of a small RNA-mediated accumulation of histone H3K9 methylation just downstream of the major poly(A) site of *FLC* suggested there may be a link between the autonomous pathway activities of FCA/FY and chromatin silencing, but no direct connection was established (Swiezewski et al., 2007).

Aberrant transcripts, or more generally double-stranded RNA, are thought to trigger cotranscriptional events linking the RNA silencing and chromatin regulation machinery (Martienssen et al., 2005), and small RNAs appear to direct chromatin silencing to complementary sequences in the genome (Verdel et al., 2004). In *Arabidopsis*, two RNA polymerase IV (Pol IV) complexes, Dicer-like 3 (DCL3), RNA-dependent RNA polymerase 2 (RDR2), and Argonaute 4 (AGO4) are major players in chromatin silencing of repeated DNA, and an analysis of their subcellular localization has contributed to an understanding of the order of their activities (Xie et al., 2004; Herr et al., 2005; Kanno et al., 2005; Li et al., 2006; Pontes et al., 2006).

As part of our efforts to determine how the RNA processing activities mediated by *Arabidopsis* FCA and FY result in the downregulation of the single-copy developmental regulator *FLC*, we undertook a suppressor mutagenesis and identified mutations in components required for FCA-mediated *FLC* regulation. These mutants were named *sof* mutants (suppressor of overexpressed *F*CA), and *sof1* was shown to be a mutation in FLD, a homolog of the human histone demethylase—LSD1 (Shi et al., 2004). Genetic analysis and chromatin immunoprecipitation (ChIP) studies suggest that FCA function requires FLD to induce chromatin silencing of *FLC*. Investigations into how an RNA processing activity links with changes in histone modifications revealed the involvement of DCL3-dependent pathways and altered *FLC* antisense transcripts.

RESULTS

FCA Associates with *FLC* Chromatin

FCA/FY physically interact, and their function leads to altered polyadenylation/RNA processing in the *FCA* transcript (Quesada et al., 2003; Simpson et al., 2003). A prediction from these observations is that they function in a similar way on the *FLC* transcript; however, alternative polyadenylated *FLC* transcripts have not been detected by northern analysis. Messenger RNA 3′ end formation and polyadenylation have been shown to occur cotranscriptionally (Proudfoot, 2004) with some transcripts targeted for rapid degradation (Jensen et al., 2003), so we tested whether FCA associates directly with *FLC* chromatin. ChIP analysis showed specific FCA association with *FLC* chromatin at exon 6 and intron 6 (H-2 and H-3 in Figure 1). The enrichment of *FLC* chromatin was even higher when a line overexpressing FCA was used in the ChIP analysis (Figure 1) but was not found in regions further upstream (Figure 1, G-2—exon 2, intron 2) or downstream (Figure 1, U—3′ untranslated region). FCA was not associated with *FLC* chromatin around the major poly(A) site (data not shown) where small RNA-directed histone H3K9 methylation has been found (Swiezewski et al., 2007).

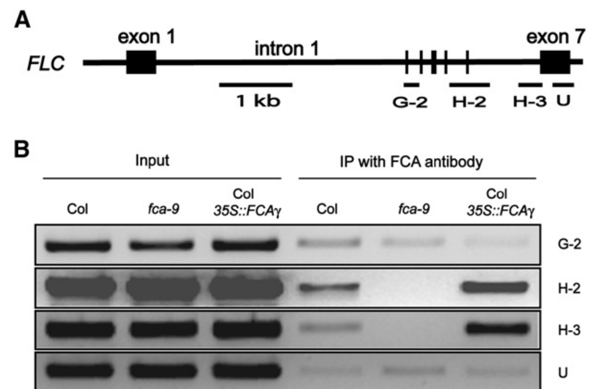

Figure 1. FCA Is Associated with *FLC* Chromatin

(A) *FLC* genomic structure (Col accession) and regions analyzed in the ChIP. Bars represent exons; lines represent promoter and introns; regions amplified by PCR are shown below as bars labeled. One kilobase scale bar is shown.

(B) PCR gel picture of four regions is shown. Different regions were amplified semiquantitatively using a standard curve to define when the PCR amplification was linear (not shown). The ChIP experiment was repeated four times with similar results.

sof1 Suppresses the Downregulation of *FLC* by FCA

To further dissect how FCA/FY repress *FLC*, we undertook a forward genetic analysis to identify mutations that suppressed the ability of FCA to downregulate *FLC*. The progenitor line chosen carried a single insertion of a *35S::FCAγ* transgene in a wild-type Landsberg *erecta* (L*er*) background (L*er*/*35S::FCAγ*). This transgene lacks introns and the 5′ region of *FCA* mRNA and so is not subjected to FCA negative autofeedback control (Quesada et al., 2003). The FCA protein produced is ~10 kDa shorter than the endogenous protein but has been shown to fully rescue the *fca-1* mutant phenotype (Macknight et al., 2002) and enhance polyadenylation in intron 3 of endogenous *FCA* in an FY-dependent manner, and therefore repress accumulation of endogenous FCA protein (Simpson et al., 2003). The *35S::FCAγ* transgene accelerated flowering relative to wild-type plants (Figure 2A), fully reversed the late-flowering phenotype of an active *FRI* allele, but was suppressed by *fy-1* mutation (Figure 2A). We reasoned, therefore, that mutations in loci required for FCA to repress *FLC* expression will lead to higher *FLC* levels and delay flowering of *35S::FCAγ* transgenic line.

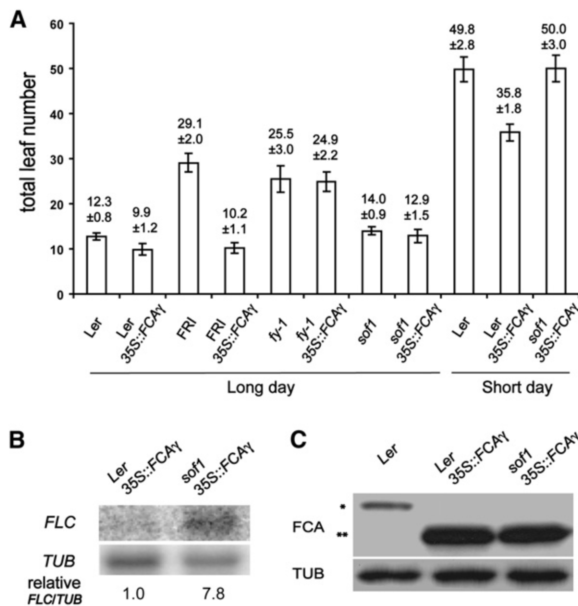

Figure 2. *sof1* Suppresses the Downregulation of *FLC* by FCA

(A) Flowering time of plants (assayed as total leaf number, produced by the apical meristem before it switched to producing flowers) grown in long day and short day photoperiods. Values are means and standard errors for 20 plants.

(B) Northern blot showing *FLC* and *β-TUBULIN* (*TUB*) transcript levels in plants homozygous for *35S::FCAγ* and either wild-type or mutant for *SOF1*.

(C) Western analysis of FCA protein from the endogenous *FCA* gene labeled with asterisk (*), or the *35S::FCAγ* transgene labeled with double asterisk (**). Western of β-TUBULIN (TUB) was shown as loading control.

One hundred and fifty M2 individuals were identified that flowered later than the parent, and a secondary screen of these for higher *FLC* levels identified three mutants in which repression of *FLC* expression by *35S::FCAγ* was suppressed. These mutants were named *suppressor of overexpressed FCA 1* (*sof1*) to *sof3*. The flowering time and *FLC* level in *sof1/35S::FCAγ* are shown in Figures 2A and 2B. The lines carrying *sof1* but not the *35S::FCAγ* transgene flowered earlier than lines carrying an active *FRIGIDA* (*FRI*) allele (Figure 2A) that cannot suppress *35S::FCAγ*. The suppression of *35S::FCAγ* by *sof1* is therefore likely to be specific. The *sof1* mutation delayed flowering only moderately compared to *fy-1*. Protein levels from the *35S::FCAγ* transgene were analyzed in *sof1/35S::FCAγ* to check that the *sof1* phenotype was the result of a *trans*-acting mutation and not a mutation in the transgene (Figure 2C). Expression and sequence of the *FCA* transgene and the feedback regulation on the endogenous FCA were unaffected, indicating that the transgene was fully functional in *sof1* (Figure 2C).

The *FLC* allele in L*er* carries a Mutator-like transposon within intron 1 (Gazzani et al., 2003; Michaels et al., 2003). RNAi-mediated chromatin silencing directed against these transposon sequences results in L*er FLC* being a weaker allele than Columbia (Col) *FLC* (Liu et al., 2004). To check whether the *sof1* phenotype was specific to a L*er FLC* allele, we introduced *sof1/35S::FCAγ* into a background carrying a translational fusion of Col *FLC* gene and firefly luciferase gene (*LUC*) (Figure 3). This *FLC::LUC* transgene mimics the behavior of the endogenous Col *FLC* (Mylne et al., 2004). *FLC::LUC* activity was repressed to low levels by *35S::FCAγ* even in the presence of FRI but was increased by *sof1* (Figure 3), indicating that the *sof1* phenotype is independent of the transposon insertion in the L*er FLC* allele.

sof1 Is a Mutation in FLD, a Known Floral Regulator and Homolog of LSD1

sof1 was mapped to the top arm of chromosome 3 to a physical region of 112 kb covered by BAC clone F3D20. Within this fragment there were 29 annotated genes, one of which was *FLD* (At3g10390; see http://www.arabidopsis.org), a gene previously characterized as functioning in the autonomous floral pathway (Chou and Yang, 1998). Sequence analysis of *FLD* in *sof1* revealed a mutation that changed proline 428 into leucine. FLD is a homolog of the human lysine-specific histone demethylase KIAA0601/LSD1 (He et al., 2003), a protein containing an amine oxidase catalytic domain (Shi et al., 2004). Sequence alignment shows that the proline residue mutated in *sof1* is highly conserved among amine oxidase domains in proteins from a variety of organisms (Figure 3A), indicating that it could be important for the catalytic activity although it is not identified as a residue in either the active site or substrate binding site of LSD1 (Chen et al., 2006; Stavropoulos et al., 2006; Yang et al., 2006). A 4.8 kb Kpn1-Sph1 fragment from BAC F3D20 containing only the wild-type *FLD* gene rescued the mutant phenotype in complementation experiments (Figure 3B). We also did a complementation analysis by crossing *sof1/35S::FCAγ* to *fld-3* and *fld-4* T-DNA insertion knockout lines. The late flowering of the F1 confirmed that *sof1* was allelic to *fld-3* and *fld-4* (see Figure S1 in the Supplemental Data available with this article online). We therefore renamed *sof1* to *fld-6*. Because *fld-6* might be a weak allele we analyzed the effect of strong *fld* mutations on *35S::FCAγ*. The null *fld-3* and *fld-4* Col alleles (He et al., 2003) were combined with the *35S::FCAγ* transgene and were found to largely suppress the early flowering and low *FLC* levels induced by *35S::FCAγ* (Figures 3C and 3D). Thus, FCA requires FLD for most of the downregulation of *FLC* but also functions through FLD-independent pathways.

FLD and FCA Function in the Same Genetic Pathway to Downregulate *FLC*

To test whether endogenous FCA and FLD function in the same genetic pathway to suppress *FLC* normally, complete loss-of-function mutations in *FCA* and *FLD* were combined. *fca-9* was epistatic to *fld-3* and *fld-4* with respect to flowering time, while it was additive with *fve-3*, mutant of another *FLC* repressor, showing that lateness has not been saturated (Figure 4A). The lack of additivity

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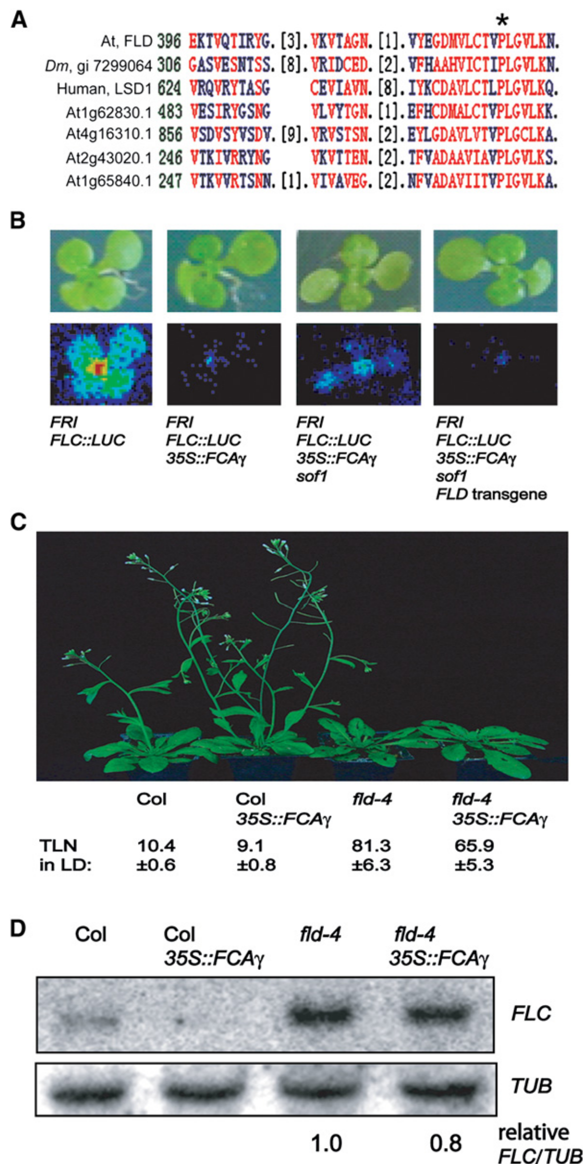


Figure 3. *fld* Mutations Suppress the Downregulation of *FLC* by FCA

(A) Part of the protein sequence of FLD, the closest *Drosophila* (Dm), human, and four *Arabidopsis* homologs. The proline residue (marked with asterisk [*]) in FLD, which is mutated to leucine residue in *sof1* mutant, is conserved.

(B) Individual seedlings imaged by bright field camera, and FLC::LUC signal of the same seedlings taken by CCD camera. Genotype of the seedlings is shown at the bottom.

(C) Flowering time of wild-type and the *fld-4* mutant with or without the *35S::FCAγ* transgene. The total leaf number produced by the plant grown in long day conditions (TLN in LD) is shown below each genotype. TLN of *fld-3* is 90.0 ± 5.5 and of *fld-3/35S::FCAγ* is 72.5 ± 3.5. Values are means and standard errors for 20 plants.

(D) Northern analysis comparing *FLC* and *β-TUBULIN* (*TUB*) expression showing *fld-4* suppresses the ability of *35S::FCAγ* to repress *FLC*. The relative ratio of *FLC/TUB* normalized to *fld-4* is shown.

of *fca* and *fld* supports the conclusion that the corresponding gene products function in the same pathway to downregulate *FLC*. However, the slightly later flowering of *fca-9* compared to *fld-3* and *fld-4* reinforces the conclusion that there are also minor FLD-independent functions of FCA in repressing *FLC*.

fca and *fld* Mutants Show Altered H3K4 Dimethylation in *FLC*

Because the genetic analysis indicates that FCA requires FLD to repress *FLC* and given the association of FCA to *FLC* chromatin (Figure 1) and the fact that *FLD* transcript is not regulated by FCA/FY (Figure S3), we reasoned that FLD might regulate *FLC* directly. A human homolog of FLD (KIAA0601) had been found as a component of histone deacetylase complexes (Humphrey et al., 2001). This led He et al. (2003) to analyze whether *fld* mutations affected histone acetylation at *FLC*, and increased H4 acetylation was found within intron 1–exon 4. Because of that study, the human protein KIAA0601 has been identified as LSD1, initially described as a histone 3 lysine 4 (H3K4) demethylase, specific for monomethylated and dimethylated K4 (Shi et al., 2004). Recent studies have shown that its specificity and activity are regulated by associated protein cofactors (Lee et al., 2005; Metzger et al., 2005; Shi et al., 2005), and at least one of the *S. pombe* LSD1 homologs may function as a K9 demethylase (Lan et al., 2007). We compared global histone modification levels in *fld-3* and Col wild-type, including H3 acetylation, H3K4 dimethylation, and H3K9 dimethylation. *fld* was associated with increased H3K4 dimethylation but H3 acetylation (Figure 4B) and H3K9 dimethylation (data not shown) were unchanged, indicating that dimethylated H3K4 is likely to be a major substrate of FLD histone demethylase activity in *Arabidopsis*. We therefore checked H3K4 dimethylation level of *FLC* in wild-type and *fld* seedlings by ChIP analysis. As shown in Figure 4C, H3K4 dimethylation was reproducibly increased by 1.5- to 2-fold in the central region of the *FLC* locus in *fld* mutant compared to wild-type—including most of intron 1–exon 4. The H3K4 dimethylation status at *FLC* was then analyzed in *fca-9*, and a very similar pattern was found to that in *fld* seedlings (Figure 4C and Figure S2), namely 1.5-fold increase in the central region of FLC, consistent with the hypothesis that FCA functions through FLD in the repression of *FLC*. Increased H3K4 dimethylation is often associated with overall increased transcriptional activity of genes, so we also analyzed H3K4 dimethylation in *fve-3*, another autonomous pathway mutation impaired in *FLC* chromatin histone deacetylation (Ausin et al., 2004). H3K4 dimethylation at *FLC* was not increased in *fve-3* (in region D, shown in Figure 5) as it was in *fca-9*, *fld-3*, or *dcl3*. *dcl3* has a much weaker effect on *FLC* levels than *fve-3* (data not shown), demonstrating that increased H3K4 dimethylation is not simply correlated with *FLC* expression. Thus, FCA appears to repress expression of *FLC* through FLD-directed histone H3K4 demethylation.

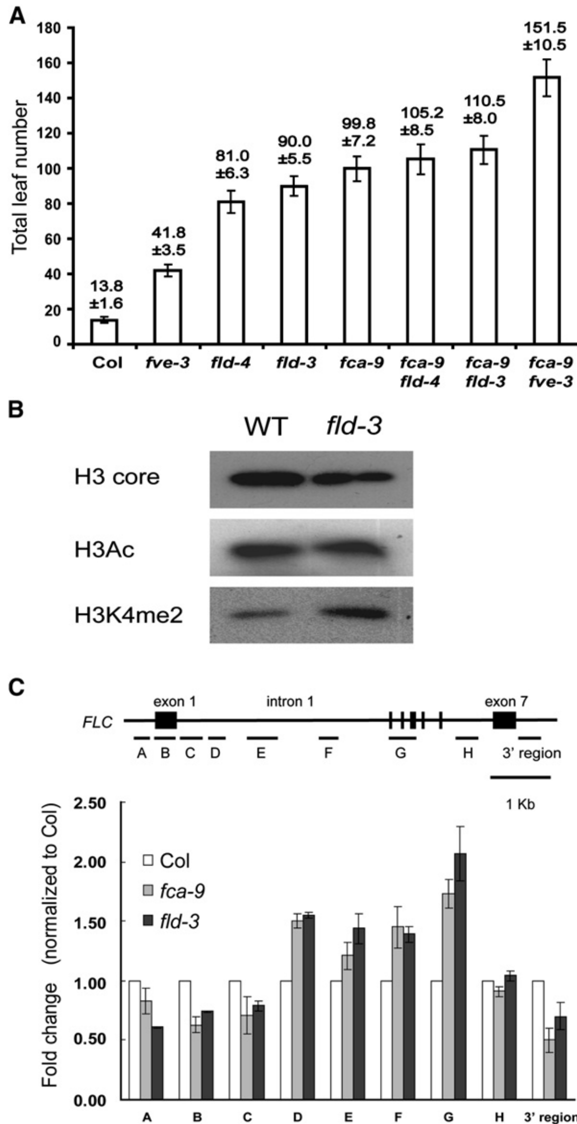


Figure 4. Histone Methylation Status at the *FLC* Locus

(A) Flowering time (assayed by total leaf number) of different genotypes grown side by side in a long day photoperiod. Values are means and standard errors for 20 plants.

(B) Western analysis of total histone fraction extracted from wild-type (WT) and *fld* mutant seedlings. The antibodies used are listed on the left of the panels.

(C) ChIP analysis of *FLC* in Col, *fca-9*, and *fld-3*. Enrichment of H3K4 dimethylation in the different *FLC* regions A–H is normalized to Col. The values are means ± SE from three PCR experiments. The gel picture is shown in the Supplemental Data (Figure S2). *FLC* genomic structure (Col accession) and regions analyzed in the ChIP are shown above. Bars represent exons; lines represent promoter and introns; regions amplified by PCR are shown below as bars labeled as A–H and 3′ region. A 1 kb scale bar is shown.

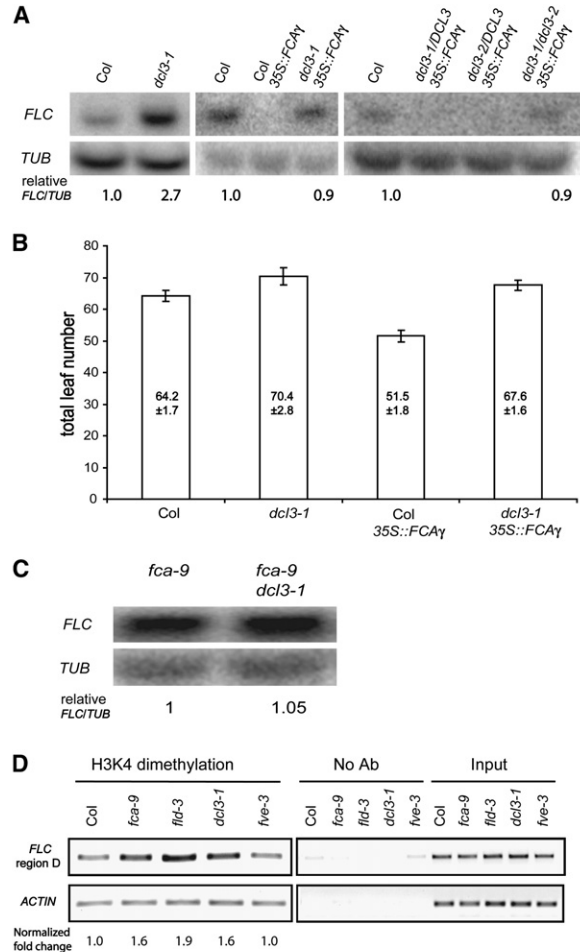


Figure 5. DCL3 Functions in the FCA Pathway by Mediating *FLC* Chromatin Modification

(A) Northern analysis showing upregulation of *FLC* expression, relative to *β-TUBULIN* (*TUB*) in *dcl3* compared to Col wild-type. The relative ratio of *FLC/TUB* normalized to Col wild-type was shown at the bottom. There was no *FLC* signal detected in Col/*35S::FCAγ*, *dcl3-1/DCL3/35S::FCAγ*, and *dcl3-2/DCL3/35S::FCAγ*, so the relative ratio of *FLC/TUB* is not given.

(B) Flowering time of plants (assayed as total leaf number) grown in short day photoperiods. Values are means and standard errors for 20 plants.

(C) Northern analysis showing *FLC* expression in *fca-9* and *fca-9, dcl3-1* double mutant. The relative ratio of *FLC/TUB* normalized to *fca-9* is shown.

(D) ChIP analysis of H3K4 dimethylation in *FLC* region D in a range of genotypes. *ACTIN* was shown as the control for immunoprecipitation efficiency between samples. Enrichment of H3K4 dimethylation in *FLC* region D, normalized to Col wild-type, is shown in the bottom panel. The values are means ± SE from three PCR experiments.

Small RNA Pathways Appear to Link FCA/FY Activity and *FLC* Chromatin Regulation

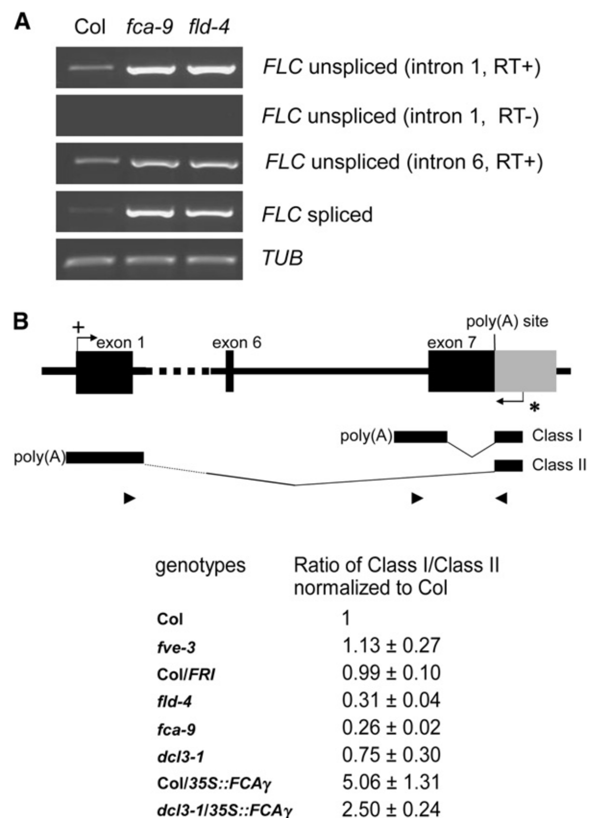
The domains on *FLC* chromatin where FCA localized (Figure 1) and which showed FCA- and FLD-dependent H3K4 demethylation (Figure 4) did not overlap, suggesting that the connection between FCA and FLD activity is not a direct protein-protein interaction. This is consistent with our failure to find protein interaction between FCA and FLD by coimmunoprecipitation analysis (data not shown). RNA-based mechanisms have been shown to direct

different aspects of chromatin regulation in many systems (Bernstein and Allis, 2005). We reasoned that FCA/FY, as demonstrated RNA processing factors for at least the *FCA* transcript, might interact with the RNAi machinery to mediate the FLD-induced histone methylation changes at *FLC*. As a first step to address this, we assayed *FLC* RNA levels in seedlings mutant in various RNAi components. *FLC* was found to be upregulated ~3-fold in two independent *dcl3* alleles, but not in *dcl2-1* (Swiezewski et al., 2007), and *dcl3-1* was found to flower later than Col wild-type (Figure 5). We crossed the *35S::FCA*γ transgene into *dcl3* mutants. Expression of *35S::FCA*γ was not altered in *dcl3* (data not shown), and DCL3 is not required for endogenous *FCA* and *FLD* regulation (Figure S3). Both *dcl3* alleles were found to partially suppress the downregulation of *FLC* by the *35S::FCA*γ transgene (Figure 5). These phenotypic effects are much less than those caused by *fld* mutations (Figure 3D) and may reflect mechanistic redundancy resulting in a minor role for the RNAi machinery or functional redundancy among different Dicers. To understand more about the genetic interaction of DCL3 and FCA, a *dcl3-1, fca-9* double mutant was generated. *FLC* levels were not higher in the double mutant than in *fca-9* (Figure 5C), and similar data were obtained for a *dcl3-1, fld-4* double mutant combination (data not shown). The nonadditivity of the mutations on *FLC* expression suggests that the contribution DCL3 makes to *FLC* repression is in the same genetic pathway as FCA. We then asked whether *dcl3* results in increased H3K4 dimethylation at *FLC*, and ChIP analysis showed that this is the case (Figure 5D and Figure S4). Our genetic and ChIP data therefore suggest that DCL3 contributes to the FCA- and FLD-mediated repression of *FLC*.

We have recently identified small RNAs directing chromatin silencing at the 3′ region of *FLC*, just downstream of the major poly(A) site (Swiezewski et al., 2007). H3K4 dimethylation was decreased in this region in both *fca*, *fld* (Figure 4C), and *dcl3* mutants (data not shown). It will be important to investigate the possible crosstalk between the small RNA-mediated chromatin silencing downstream of the major polyadenylation site and FCA- and FLD-dependent histone H3K4 demethylation in the central part of *FLC*.

fca and *fld* Mutations Increase *FLC* Transcription and Affect Antisense Transcript Processing

If FCA and FLD function as part of a chromatin silencing pathway regulating *FLC*, then the transcription of *FLC* should increase in both *fca* and *fld* mutants. Nuclear run-on transcription experiments were unsuccessful due to the low expression of *FLC*; however, an RT-PCR assay was used to measure levels of the unspliced *FLC* nascent transcript, a proxy for a transcription assay that has been employed previously (Sijen et al., 2001; Sijen and Plasterk, 2003). Levels of unspliced *FLC* nascent transcript (intron 6–exon 7) were increased in *fca* and *fld* mutants compared to Col wild-type (Figure 6A). The increase is not region specific, as *fca* and *fld* also had higher levels of unspliced



genotypes	Ratio of Class I/Class II normalized to Col
Col	1
fve-3	1.13 ± 0.27
Col/*FRI*	0.99 ± 0.10
fld-4	0.31 ± 0.04
fca-9	0.26 ± 0.02
dcl3-1	0.75 ± 0.30
Col/*35S::FCA*γ	5.06 ± 1.31
***dcl3-1/35S::FCA*γ**	2.50 ± 0.24

Figure 6. *FLC* Transcript Analysis

(A) Semiquantitative RT-PCR analysis of unspliced (at intron 1 and intron 6) and spliced sense *FLC* transcripts in seedlings of Col wild-type and *fld* and *fca* mutants. β-*TUBULIN* (*TUB*) was used to control for input of cDNA.

(B) Quantitative RT-PCR analysis of *FLC* antisense transcripts. A schematic drawing of *FLC* is show at the top. Exons 1, 6, and 7 of *FLC* sense transcript are shown as black bars. The region in which small RNAs have been identified (Swiezewski et al., 2007) is shown as a gray bar. Asterisk (*) is transcription start site of antisense transcripts. Plus (+) is transcription start site of *FLC* sense transcript. Splicing and poly(A) site of two *FLC* antisense transcripts (representative of class I and class II antisense transcripts) are shown schematically together with the position of primers (arrowheads) used to amplify the class I and class II type *FLC* antisense transcripts. Ratio of *FLC* antisense transcript abundance was normalized to Col and then expressed as a ratio of class I/class II. Values are means ± standard errors for three individual quantitative RT-PCR experiments, each with triplicate reactions.

FLC nascent transcript when assaying a region spanning exon 1–intron 1. These data reinforce the view that FCA functions with FLD to transcriptionally silence *FLC*.

To begin to dissect with what and how FCA mediates this chromatin silencing, we have used RT-PCR to characterize *FLC* transcripts that are different between wild-type and *fca* mutant plants. We carefully analyzed *FLC* processing around exon 6/intron 6, the location of FCA at *FLC* chromatin as judged by the ChIP experiments, and found no difference between wild-type and *fca* mutants. However, several antisense transcripts of *FLC* identified

by RACE experiments accumulated differently in wild-type and *fca* mutants. Sequence analysis of two of the antisense transcripts (Figure 6B) showed that both initiate downstream of the major sense *FLC* transcript poly(A) site, and that one has a 222 nt intron not found in any sense transcripts (as described previously [Swiezewski et al., 2007]), terminates, and is polyadenylated (with 60–100 A residues) within intron 6. The second has the same intron 5′ splice site but a 3′ acceptor site within intron 1, terminates, and is polyadenylated (with 60–100 A residues) within the *FLC* promoter region. These represent examples of what we call class I or class II *FLC* antisense transcripts, class I terminating around intron 6, and class II terminating around the *FLC* promoter. Primers, chosen to amplify the two classes of transcripts, revealed that the ratio of the two classes of antisense transcripts differed in different genotypes (Figure 6B), class II being relatively more predominant in *fca-9* and *fld-3* compared to Col, *fve-3*, or *FRI*. The relative abundance of the transcripts does not appear to be associated with overall *FLC* expression level. The *35S::FCA*γ transgene resulted in a change in ratio opposite to that of the *fca* mutant, and this was partially dependent on DCL3 supporting data from the suppressor analysis. The processing/polyadenylation site within intron 6 in the class I transcripts corresponds to where FCA was found associated with *FLC* chromatin, so there may be a functional link. The similar effects of *fca* and *fld* mutations on the antisense processing further support the close association of FCA and FLD activities and suggest that either FCA function is involved in RNA processing of antisense transcripts that leads to FLD-induced histone modification changes or that FCA/FLD-induced chromatin modifications in or near the exon 6/intron 6 region lead to altered transcript processing.

DISCUSSION

This study aimed to determine how the activity of the *Arabidopsis* floral regulator FCA, an RNA-binding protein with two RNA recognition motifs (RRM), reduces expression of *FLC*, a transcriptional regulator that delays the transition to flowering. FCA physically interacts with FY, a conserved polyadenylation/3′ RNA processing factor, through domains in their C termini and regulates poly(A) site choice of the *FCA* transcript (Quesada et al., 2003; Simpson et al., 2003). FY is also essential for FCA to downregulate *FLC*, suggesting FCA/FY may also regulate *FLC* RNA processing in a similar way (Simpson et al., 2003). However, alternative sense transcripts for *FLC* do not accumulate as they do for *FCA*. To understand how *FLC* is downregulated by FCA/FY, we undertook a suppressor mutagenesis experiment to identify more components required for FCA repression of *FLC* and identified FLD, a known flowering time regulator functioning in the autonomous pathway (He et al., 2003). We also identified DCL3, the *Arabidopsis* Dicer associated with chromatin modifications, through a candidate gene approach. We show that the primary activity of FLD is likely to be H3K4 demethylation and that FCA and FLD affect levels of nascent (unspliced) and antisense *FLC* transcripts. The simplest model to account for all these data is that FCA functions closely with FLD, and partially with DCL3, to transcriptionally silence *FLC*.

FLD activity had been initially described as regulating *FLC* histone deacetylation (He et al., 2003). A close association between demethylation and deacetylation has been observed previously (Lee et al., 2006) with LSD1 associating with histone deacetylases and their activities on nucleosomal targets stimulated by the repressor complex CoREST (Lee et al., 2005). *fca-9* and *fld-3* both showed 1.5- to 2-fold increases in H3K4 dimethylation (Figure 4), but only *fld-3* showed increased H4 acetylation (He et al., 2003). Loss of the H3K4 demethylation activity of LSD1 did not affect the H3 deacetylation activity of an associated complex when the complex remained intact (Lee et al., 2005). Thus, complete loss of FLD in the null *fld-3* allele may result in complete disruption of the complex perturbing deacetylation and demethylation, whereas loss of FCA may just block one aspect of FLD action while keeping the rest of the complex intact and functional. Another member of the autonomous pathway, FVE, is a homolog of MSI1, a component of HDAC complexes (Ausin et al., 2004). It is therefore possible that an FVE complex is associated with FLD. Further genetic analysis of the interactions of FCA with FVE will be informative in this respect.

The noncorrespondence of FCA association with *FLC* chromatin and the histone demethylation changes observed suggested that FCA and FLD did not physically interact, and this led us to analyze the role of RNAi machinery in FCA suppression of *FLC*. We had previously found RNAi mutants misregulate *FLC* (Swiezewski et al., 2007), so we tested and found *dcl3* mutants partially suppressed the action of FCA on *FLC* regulation (Figure 5). Furthermore, *fca* and *dcl3*, and *fld* and *dcl3* mutations, were not additive in their effects on *FLC* expression, and *dcl3*, like *fca* and *fld*, was associated with elevated H3K4 dimethylation (1.6-fold) at *FLC* (Figures 4 and 5 and Figure S4). No small RNAs have been detected that are homologous to the central region of *FLC*, where FLD- and FCA-dependent changes in H3K4 methylation have so far been found. If *cis*-acting small RNAs are involved, they may not accumulate to high enough levels to be detected in wild-type cells using current techniques (Buhler et al., 2006). Small RNAs homologous to *FLC* have been detected that correspond to the reverse-strand 3′ to the major *FLC* poly(A) site (Swiezewski et al., 2007). These are Pol IVa, RDR2, and DCL3 dependent and appear to direct H3K9 methylation to a localized domain in the 3′ region of *FLC*. Whether and how small RNAs homologous to the *FLC* 3′ region are involved in FCA- and FLD-mediated chromatin changes in *FLC* remains to be dissected, but the correspondence of the small RNA region with the 5′ end of the antisense transcripts is an intriguing coincidence.

The weaker effect of *dcl3* on *FLC* expression relative to *fca* and *fld* suggests other pathways are also involved in

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the suppression of *FLC* by FCA. Redundancy in DICER function is a possibility, as DCL2 and/or DCL4 have been shown to partially substitute for DCL3 function (Gasciolli et al., 2005). Alternatively, the primary activity of FCA/FY might be linked to FLD function by both RNAi- and non-RNAi-mediated pathway(s). Targeting of nascent transcripts to different cellular pathways, for example nuclear export, nucleolar import, or degradation, is an area of great complexity and is likely to be controlled by mechanistically redundant pathways. These could explain why *FLC* levels in *35S::FCAγ dcl3-1* are slightly lower than in *dcl3-1* and why *35S::FCAγ fld-4* is slightly earlier than *fld-4*. Thus, FCA can function in both FLD-dependent and FLD-independent pathways and the FLD-dependent pathway can in turn function through both DCL3-dependent and DCL3-independent pathways, and their predominance might change in different environments and genotypes.

The suppression of FCA function by *fld* and the increase in *FLC* transcription (assayed by unspliced transcript accumulation) in *fca* and *fld* support the view that FCA functions with FLD to regulate H3K4 dimethylation and *FLC* transcription. Many studies have shown that histone H3K4 dimethylation in the body of the gene associates with transcriptional activity, and in *Drosophila* H3K4 demethylation is required for heterochromatin formation (Li et al., 2007; Rudolph et al., 2007; Ruthenburg et al., 2007). How FCA activity triggers FLD-mediated H3K4 demethylation to cause the changes in *FLC* transcription is a key question we now need to address. Our study suggests an association of antisense RNA processing and H3K4 methylation, but similar changes in both *fca* and *fld* mutants mean it is difficult to say which changes first—altered antisense transcript processing leading to changed chromatin modification or altered chromatin affecting RNA polymerase II activity that in turn leads to changed RNA processing (Proudfoot, 2003). DCL3 is required for the FCA promotion of the class I transcripts (processing and termination around intron 6), but its exact role is also unclear at present. In *S. pombe*, loss of one of the LSD1 homologs led to a strong increase in the level of antisense transcripts of many genes (Nicolas et al., 2006), and interestingly this LSD1 homolog was found associated with an RRM protein (Nicolas et al., 2006). Why and how there is this tight association between LSD1 activity and antisense transcripts and whether RNA-binding proteins and RNA processing activities are a feature of this integration in all organisms remain to be determined.

The focus of our analysis has been on FCA/FY regulation of *FLC*, proteins all described as regulators of the *Arabidopsis* floral transition. However, a null allele of *FY* is lethal (Henderson et al., 2005), *fca* mutants have additional phenotypes and targets (Macknight et al., 2002; Marquardt et al., 2006), and given the complex spectrum of transcripts and potential polypeptides that could be produced from *FCA* we cannot be certain we know the phenotype of a true *FCA* null allele. In addition, mechanistic redundancy may well mask the effects of loss of function of these genes on different targets. The focus on *FLC*, however, allows us to dissect these potentially wide-ranging regulatory circuits, and analysis of additional *sof* mutations should help unpick how H3K4me2, antisense transcript processing, and DCL3 function are connected.

EXPERIMENTAL PROCEDURES

Chromatin Immunoprecipitation

ChIP assays were performed as previously described (Gendrel et al., 2002; Bastow et al., 2004) with minor modifications. Chromatin preparations from 20 day old seedlings were immunoprecipitated with specific antibodies against H3K4me2 (Upstate Biotechnology catalog number 07-030, lot number 26-335) or rabbit polyclonal antibody against FCA (Quesada et al., 2003) and magnetic Dynabeads Protein A (Dynal Biotech). ChIP DNA was analyzed by semiquantitative PCR using *FLC* primers and PCR conditions described in Bastow et al. (2004). Sequences of primers used in FCA ChIP analysis are listed in the Supplemental Data. For H3K4 dimethylation ChIP, DNA concentration between different samples was normalized for amplification of *Actin2/7* gene with primers JP1565 and JP1596 (Johnson et al., 2002). Images of PCR products were acquired with AutoChemi System and quantified with LabWorks 4.6 software (UVP Bioimaging Systems). Each ChIP was repeated at least twice.

RT-PCR Analysis of Primary *FLC* Transcript

Total RNA from 17 day old seedlings was isolated as described (Etheridge et al., 1999). After treatment with TURBO DNA-free (Ambion), RT-PCR was performed with the OneStep RT-PCR kit (QIAGEN) using 200 ng RNA in a 20 μl reaction volume with primers and cycle numbers as follows: *FLC* spliced (oIB7/ATGTGAGTATCGATGCTCTTGTTC and oIB8/CTCACACGAATAAGGTACAAAGTTC; 26×); *FLC* unspliced (I1: FLC_RT_F/TTCTCCAAACGTCGCAACGGTCTC and C_R/TCACTCAAC AACATCGAGCAC; 33×); *FLC* unspliced (I6: oIB136/GTGAATAGTG ATTTTGACCTATGA and oIB8; 33×); and *TUB* (primers were decribed [Mathieu et al., 2005]; 26×). To our knowledge, the *FLC* unspliced primer pairs do not detect spliced antisense transcripts.

Antisense *FLC* Transcript Analysis

To clone the 5′ end of *FLC* antisense transcripts, 5′ RACE was performed according to the instructions in the Gene Racer kit (Invitrogen). Gene-specific primers for 5′ RACE (FLC5560F and FLC5575F, see primer sequences in the Supplemental Data) were designed using Primer3 (Rozen and Skaletsky, 2000), according to previously determined *FLC* antisense RNA sequence (Swiezewski et al., 2007). To obtain 3′ sequences of *FLC* antisense transcripts, a DNA oligonucleotide with the first three nucleotides as UUU and the last nucleotide as 3′ inverted deoxythymidine (5′-UUUAACCGCATCCTTCTCTCTACCTACC ATTGACCTGTidT-3′) was ligated using T4 RNA ligase to total RNA isolated from both Col wild-type and *fca-9* mutant. Ligated RNA was converted into cDNA using adaptor primer adp-1 (5′-AACAGGTCA ATGGTAGGTAGAGAGA-3′) by reverse transcriptase. Gene-specific primers (FLC5858R and FLC5855R, see sequences in the Supplemental Data) and adaptor primers (adp-2 and adp-3, see sequences in the Supplemental Data) were used for nested PCR. The PCR products were cloned and sequenced.

Real-time quantitative RT-PCR was used to compare the abundance of *FLC* antisense transcripts in different genotypes: first strand of cDNA was synthesized with oligo(dT) primer. cDNA was analyzed by quantitative PCR on an OPTICON2 instrument and SYBR green JumpStart Taq ReadyMix from Sigma. Primers classI_R and classI/II_F were used to amplify class I while classII_R and classI/II_F were used to amplify class II *FLC* antisense transcripts (see primer sequenced in the Supplemental Data). *Arabidopsis* UBC gene (Czechowski et al., 2005) was used as internal control in the expression analysis.

Supplemental Data

Supplemental Data include four figures, one primer list, and Supplemental Experimental Procedures detailing plant material and growth condition, mutagenesis, map-based cloning of *SOF1*, constructs and genotyping assays, expression analyses, and histone western analysis and can be found with this article online at http://www.molecule.org/cgi/content/full/28/3/398/DC1/.

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