Hypomorphic Alleles Reveal *FCA*-Independent Roles for *FY* in the Regulation of *FLOWERING LOCUS C*[1][C][W][OA]

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The autonomous floral promotion pathway plays a key role in the regulation of flowering in rapid-cycling Arabidopsis (*Arabidopsis thaliana*) by providing constitutive repression of the floral inhibitor *FLOWERING LOCUS C* (*FLC*). As a result, autonomous pathway mutants contain elevated levels of *FLC* and are late flowering. Winter annual Arabidopsis, in contrast, contain functional alleles of *FRIGIDA* (*FRI*), which acts epistatically to the autonomous pathway to up-regulate *FLC* and delay flowering. To further explore the relationship between *FRI* and the autonomous pathway, we placed autonomous pathway mutants in a *FRI*-containing background. Unexpectedly, we found that a hypomorphic allele of the autonomous pathway gene *fy* (*fy* null alleles are embryo lethal) displayed background-specific effects on *FLC* expression and flowering time; in a rapid-cycling background *fy* mutants contained elevated levels of *FLC* and were late flowering, whereas in a winter annual background *fy* decreased *FLC* levels and partially suppressed the late-flowering phenotype conferred by *FRI*. Because FY has been shown to have homology to polyadenylation factors, we examined polyadenylation site selection in *FLC* transcripts. In wild type, two polyadenylation sites were detected and used at similar levels. In *fy* mutant backgrounds, however, the ratio of products was shifted to favor the distally polyadenylated form. FY has previously been shown to physically interact with another member of the autonomous pathway, FCA. Interestingly, we found that *fy* can partially suppress *FLC* expression in an *fca* null background and promote proximal polyadenylation site selection usage in the absence of FCA. Taken together, these results indicate novel and FCA-independent roles for FY in the regulation of *FLC*.

The timing of flowering is a crucial decision and directly affects the probability of successful reproduction. In Arabidopsis (*Arabidopsis thaliana*), flowering is regulated by pathways that are responsive to both environmental and developmental cues (Michaels, 2009). Several of these pathways converge at the floral repressor *FLOWERING LOCUS C* (*FLC*; Michaels and Amasino, 1999; Sheldon et al., 1999; Crevillén and Dean, 2010). In rapid-cycling accessions, a group of genes known collectively as the autonomous pathway acts to promote flowering by repressing *FLC* expression. Therefore mutations in autonomous pathway genes result in high levels of *FLC* and delayed flowering. In contrast to rapid-cycling strains, many naturally occurring accessions of Arabidopsis are late flowering unless flowering is promoted by a prolonged period of cold treatment known as vernalization. These winter annual accessions contain functional alleles of the *FRIGIDA* (*FRI*) gene, which act epistatically to the autonomous pathway to up-regulate *FLC* mRNA levels and delay flowering (most rapid-cycling accessions contain null mutations in *FRI*; Johanson et al., 2000). Vernalization can promote flowering in *FRI*-containing strains or autonomous pathway mutants by epigenetically suppressing *FLC* through a mechanism involving repressive histone modifications at the *FLC* locus (Bastow et al., 2004; Sung and Amasino, 2004).

Currently, little is known about the mechanism by which the autonomous pathway represses *FLC* expression; however, the known/predicted functions of the autonomous pathway genes suggest a link between RNA and chromatin structure. The most extensively characterized members of the autonomous pathway include three RNA-binding proteins (FCA, FPA, and FLOWERING LOCUS K [FLK]; Macknight et al., 1997; Meier et al., 2001; Schomburg et al., 2001; Lim et al., 2004; Mockler et al., 2004), a homolog of the yeast polyadenylation factor Pfs2p (FY; Simpson et al., 2003), a putative transcription factor that contains a divergent homeodomain (LUMINIDEPENDENS [LD]; Lee et al., 1994a), and two chromatin remodeling proteins (FLOWERING LOCUS D [FLD] and FVE; He et al.,

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2003; Ausín et al., 2004; Kim et al., 2004). Both FCA and FPA have been shown to be involved in alternative processing of their own transcripts (Quesada et al., 2003; Hornyik et al., 2010). In the case of FCA, this autoregulation involves a physical interaction with FY (Simpson et al., 2003). Hypomorphic alleles of *fy* are late flowering, however, because FY is likely an essential component of the polyadenylation complex involved in RNA 3′ end processing, null mutations in *fy* are lethal (Ohnacker et al., 2000; Henderson et al., 2005). It has been suggested that FCA/FY may regulate *FLC* mRNA levels through alternative polyadenylation, although, to date, alternatively polyadenylated *FLC* sense transcripts have not been described. Interestingly, an *FLC* antisense transcript was identified and shown to have two alternative polyadenylation sites; however, the significance of this differential processing of the antisense transcript on *FLC* mRNA levels is unclear (Liu et al., 2007).

Polyadenylation is an important and highly regulated step in pre-mRNA processing. It has become increasingly apparent that this process is tightly interconnected with transcription and translation (Moore and Proudfoot, 2009). Bioinformatic analyses have revealed a large number of alternative polyadenylation sites in the mouse, human, and plant transcriptomes (Tian et al., 2005; Ji et al., 2007; Shen et al., 2008). In some cases, differential polyadenylation can occur in the coding region of transcripts and result in truncated proteins with reduced or novel function. More commonly, however, alternative polyadenylation occurs in the 3′ untranslated region (UTR) of transcripts. 3′ UTRs have been demonstrated to play important roles in regulating mRNA stability, localization, and translational activity, and are in many cases, the targets of regulatory small RNAs. For example, there are examples of alternative polyadenylation in 3′ UTRs in different tissue types or during different stages of development, which correlate with gene expression levels (Beaudoing and Gautheret, 2001; Zhang et al., 2005; Ji et al., 2009).

In addition to *FY*, changes in the expression level of other polyadenylation complex-related genes, including *CstF64*, *symplekin/PTA1*, *CPSF100*, and *PCFS4*, can cause changes in flowering time in Arabidopsis (Herr et al., 2006; Xing et al., 2008). For example, *pcsf4* mutants are late flowering and contain high levels of *FLC* expression (Xing et al., 2008). Likewise, CstF64 and CstF77, two factors in a 3′ end-processing complex, were shown to regulate flowering time through *FLC* (Liu et al., 2010). Interestingly, instead of affecting the polyadenylation of the sense *FLC* transcript, CstF64 and CstF77 appear to promote the 3′ end processing of the *FLC* antisense transcript in both distal and proximal polyA sites.

Here we show that FY promotes usage of a proximal polyadenylation site of the sense *FLC* transcript. In addition, we demonstrate that at least some of the effects of FY on flowering time, *FLC* expression, and *FLC* polyadenylation occur independently of FCA. Thus FY is likely to modulate *FLC* expression through both *FCA*-dependent and *FCA*-independent mechanisms.

RESULTS AND DISCUSSION

Genetic Interactions between *FRI* and the Autonomous Pathway

In an effort to better understand the relationship between *FRI* and the autonomous pathway, *FRI* was introduced into autonomous pathway mutant backgrounds (Table I). If FRI activates *FLC* by preventing the repression of *FLC* by the autonomous pathway, then one would predict that the flowering time of a *FRI* autonomous pathway mutant line would be similar to that of the autonomous pathway mutant alone. If, however, FRI acts through a separate antagonistic pathway, then lines containing both *FRI* and an autonomous pathway mutant should flower significantly later than either *FRI* or the autonomous pathway mutant. When grown without vernalization, the autonomous pathway mutants used in this study vary significantly in their late-flowering phenotypes. *fca-9*, *fld-3*, *fpa-7*, and *ld-1* mutants show a strong late-flowering phenotype, flowering with >65 leaves, whereas *fve-4*, *flk-4*, and *fy-5* mutants show a weaker phenotype (<35 leaves; Fig. 1A). With the exception of *FRI fy-5* (discussed below), *FRI* autonomous pathway lines flowered similarly to the later-flowering parent (Fig. 1A). The vernalization response in the *FRI* autonomous pathway lines was similar to that of the *FRI* or the autonomous pathway mutant parental lines (Fig. 1A). Only a line containing *FRI* and the photoperiod pathway mutant *constans* (*co*) showed an attenuated vernalization response (Fig. 1A). This is expected, as the late-flowering phenotype of *co* mutants is not due to elevated *FLC* expression (Michaels, 2009). These results indicate that FRI is not able to further delay flowering in autonomous pathway mutant backgrounds. This suggests that the repression of flowering by FRI and the promotion of flowering by the autonomous pathway may occur through a common mechanism.

The late-flowering phenotype of both *FRI* and autonomous pathway mutants are due to elevated levels of the floral repressor *FLC* (Michaels and Amasino, 2001). Therefore we also investigated the relationship between *FLC* expression and flowering time in the *FRI* autonomous pathway lines. The level of *FLC* transcript was strongly correlated with flowering time (Fig. 2, A and B; Supplemental Table S1). As expected, levels of the floral integrators *SOC1* and *FT*, which are negatively regulated by *FLC*, are inversely correlated with *FLC* expression (Fig. 2B). The tight correlation between *FLC* expression and flowering time suggests that, similar to *FRI* or autonomous pathway mutants, the late-flowering phenotype of *FRI* autonomous pathway mutant lines is due to *FLC*. To confirm this hypothesis, we created *FRI* autonomous pathway mutants in an *flc-3* mutant background. As predicted, the late-

Table I. *Flowering time mutants and introgressions used in this study*

Line	Gene	Lesion/Introgression	Reference
co	*At5g15840*	T-DNA insertion in exon 1, SAIL24H04 (Syngenta)	Kim and Michaels (2006)
fca T-DNA	*At4g16280*	T-DNA insertion in third intron, SALK_057540	Alonso et al. (2003)
fca-9	*At4g16280*	1-bp deletion, 109 bp 3′ of translational start (atgaga**G**gtt)	Bezerra et al. (2004), C. Dean, personal communication
flc-3	*At5g10140*	104-bp deletion that includes the translational start site	Michaels and Amasino (1999)
fld-3	*At3g10390*	T-DNA insertion in first intron, SALK_075401	Alonso et al. (2003), He et al. (2003)
flk-4	*At3g04610*	T-DNA insertion in first intron, SALK_112850	Alonso et al. (2003), Mockler et al. (2004)
fpa-7	*At2g43410*	T-DNA insertion 3,174 bp 3′ of translational start	Michaels and Amasino (2001)
FRI-Col	*At4g00650*	Dominant *FRI* allele from San Feliu-2 backcrossed 10× into Col	Lee et al. (1994b)
fve-4	*At2g19520*	G to A nonsense mutation 575 bp 3′ of translational start (attt**G**ggatg)	Michaels and Amasino (2001)
fy-2	*At5g13480*	T-DNA insertion in exon 16, SAIL 657D4 (Syngenta)	Simpson et al. (2003)
fy-5	*At5g13480*	T-DNA insertion in last intron, SALK_005697	Alonso et al. (2003)
ld-1	*At4g02560*	G to T mutation at splice donor site at intron 7 (tcct**G**taagt)	Aukerman and Amasino (1996)

flowering phenotype of *FRI* autonomous pathway mutant lines is eliminated by the *flc* null mutation (Fig. 2C).

fy-5 Shows Background-Specific Effects on Flowering Time and *FLC* Expression

In general, *FRI* autonomous pathway mutant lines flowered similarly to the latest flowering parent (Fig. 1A). A notable exception to this trend was *FRI fy-5*. Alone, *fy-5* shows a modest, but significant ($P < 0.0001$), late-flowering phenotype, flowering approximately nine leaves later than Columbia (Col; Fig. 1A). Unexpectedly, *FRI fy-5* plants flowered significantly earlier than *FRI* ($P < 0.01$), forming approximately 30 fewer leaves (Fig. 1). To determine if these variable effects are due to changes in *FLC* levels, we examined *FLC* expression in Col and *FRI* backgrounds ± *fy-5*. Consistent with the effects on flowering time, *fy-5* caused a 90% increase in *FLC* transcript in the Col background, in comparison to a 28% decrease in the *FRI* background (Fig. 2A; Supplemental Table S1). Thus, depending on genetic background, *fy-5* can increase or decrease *FLC* expression and flowering time.

Given the partial suppression of *FRI* by *fy-5*, we also investigated the effect of *fy-5* mutations in other autonomous pathway mutant backgrounds. Interestingly, the double mutants could be grouped into three classes based on flowering time. The first class, which includes *fld* and *ld*, shows an additive delay in flowering with *fy-5*. *fld-3 fy-5* and *ld-1 fy-5* plants flowered approximately 10 leaves later than the *fld-3* and *ld-1* single mutants, which is similar to the delay in flowering caused by *fy-5* in Col (Fig. 3A). The second class, which includes *flk* and *fve*, showed a synergistic delay in flowering. *flk-4 fy-5* and *fve-4 fy-5* flowered much later (47 and 31 additional leaves, respectively) than the *flk-4* and *fve-4* single mutants (Fig. 3, A and B; Koornneef et al., 1998). *fca* belongs to the third class and, similar to *FRI*, the late-flowering phenotype of *fca-9* is partially suppressed by *fy-5* (Fig. 3, A and C). This result is particularly significant because it demonstrates that *FY* can influence flowering time in the absence of *FCA*. As with autonomous pathway mutants containing *FRI*, the flowering time of *fy-5* autonomous pathway double mutants is well correlated with *FLC* expression (Fig. 2, D–F).

Because null alleles of *fy* are embryo lethal, hypomorphic alleles must be used for flowering time analysis. In prior work, a variety of lesions have been shown to cause delayed flowering: *fy-1* contains a base change that blocks splicing of intron 15, *fy-2* contains a T-DNA insertion in exon 16, and *fy-3* contains a G to S substitution in the WD 40 domain (Fig. 3D; Simpson et al., 2003; Henderson et al., 2005). To determine if the observed interactions between *fy* and *FRI*/the autonomous pathway are specific to *fy-5* or are more general, we created double mutants between *fy-2* and *FRI*/autonomous pathway mutants. *fy-2* was chosen because it has the strongest late-flowering phenotype of the described *fy* alleles (Henderson et al., 2005). Overall, the results were similar to those obtained with *fy-5* (Fig. 3A); *flk* and *fve* showed a greater delay in flowering when combined with *fy-2* than *fld-3* and *ld-1*. Although *FRI fy-2* and *fca-9 fy-2* did not flower earlier than *FRI* or *fca-9* alone, it was nonetheless interesting that *fy-2*, which delays flowering by approximately 40 leaves in Col, had no effect on flowering in the *FRI* or *fca* mutant backgrounds (Fig. 3A).

FVE and *FLK* Show Genetic Redundancy in the Repression of *FLC*

As shown above, *fy* alleles show a stronger late-flowering phenotype in *flk-4* and *fve-4* backgrounds than other autonomous pathway backgrounds (Fig. 3A). This is particularly interesting given that the late-flowering phenotype of *flk-4* and *fve-4* single mutants is weaker than that of *fld-3*, *fca-9*, *fpa-7*, or *ld-1* (Figs. 1A and 3A). Because of the relatively weak late-flowering phenotype of *fve-4* and *flk-4*, we wondered if *FVE* and

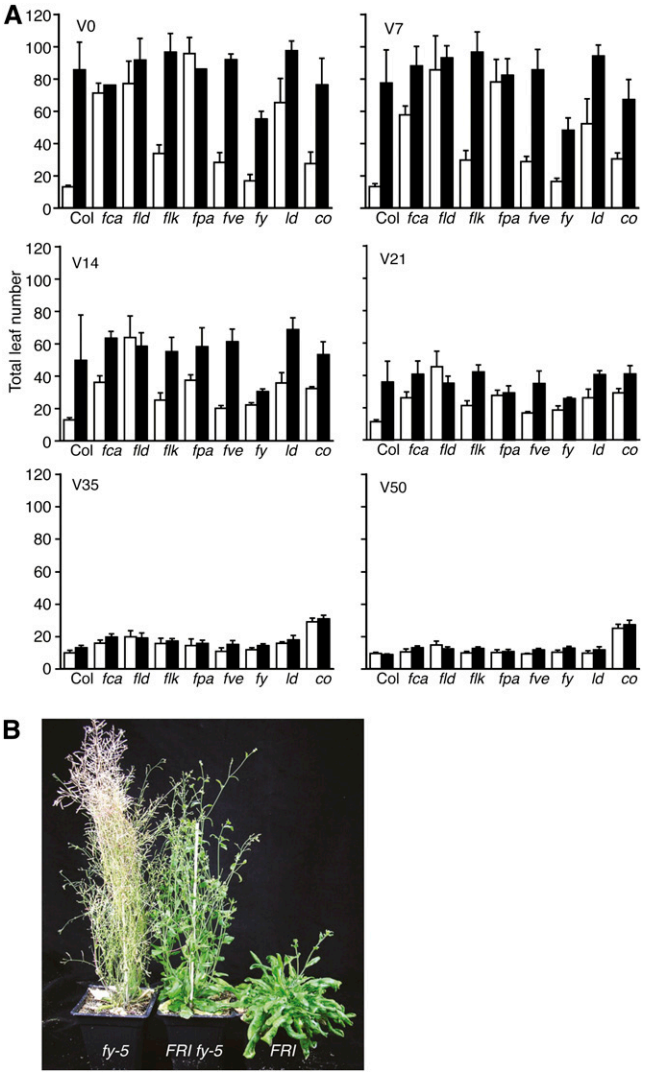

Figure 1. Genetic interactions between *FRI* and the autonomous pathway. A, Bars represent the total number of rosette leaves formed prior to flowering under long days. Genotypes indicated with white bars contain the nonfunctional Col allele of *fri*, whereas black bars contain the active *FRI*-SF2 allele. V indicates days of cold treatment. Error bars indicate 1 SD. B, *fy-5* partially suppresses the late-flowering phenotype of *FRI*. Plants were grown under long days. [See online article for color version of this figure.]

FLK might be partially functionally redundant in repressing *FLC*. Because the protein sequences of FVE and FLK are unrelated, it seems unlikely that the two proteins would have similar biochemical activities. It is possible, however, that they function in redundant pathways that repress *FLC*. Consistent with this model, we found that *fve-4 flk-4* double mutants are much later flowering than either single mutant (Fig. 3E). In fact, the flowering time of *fve-4 flk-4* is similar to, or later than, *FRI* or strong autonomous pathway mutants (e.g. *fld*, *fca*, *fpa*, or *ld*; Figs. 1A and 3, A and E). Thus the three earliest flowering autonomous pathway mutants (*fy*, *fve*, and *flk*) all show strong synergistic delays in flowering time as double mutants. These results suggest that FY, FVE, and FLK have partially redundant roles in the repression of *FLC*.

fpa fy-5 Double Mutants Are Viable

Previous double mutant analysis, performed in the Landsberg *erecta* background, failed to recover double mutants between *fpa* and *fy-1*, suggesting that *fpa fy* mutants are inviable (Koornneef et al., 1998). To de-

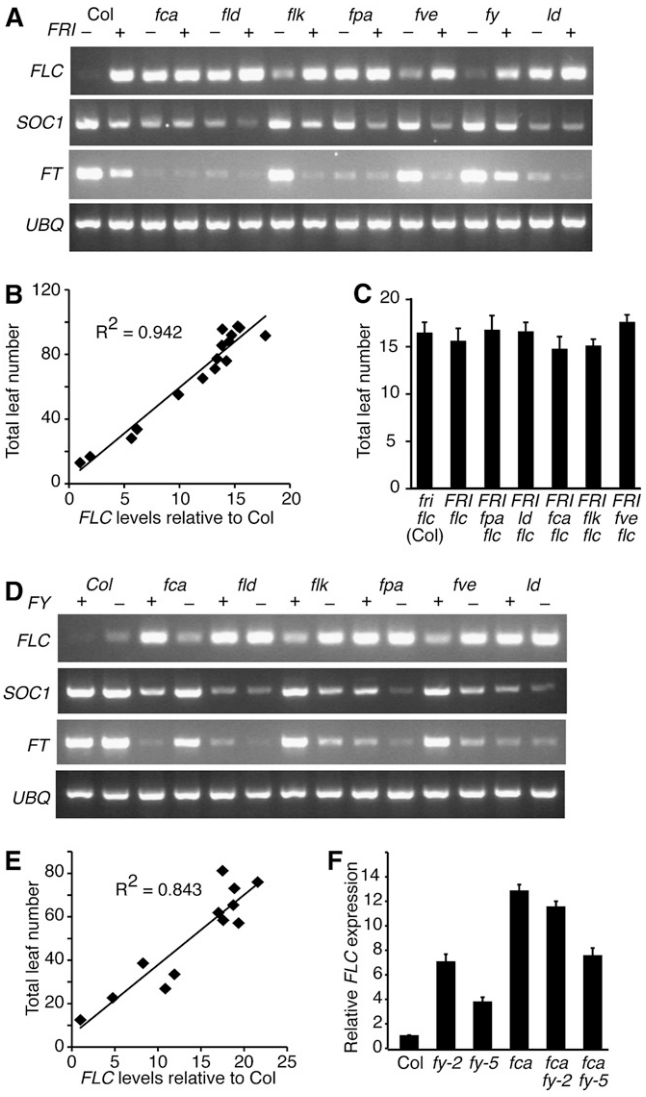

Figure 2. *FLC* expression is correlated with flowering time. A, Semiquantitative RT-PCR analysis of flowering time gene expression in lines ± *FRI*. B, Correlation of *FLC* mRNA expression levels (A) with flowering time (Fig. 1A, V0). C, The late-flowering phenotype of *FRI* autonomous pathway mutant lines is eliminated in an *flc* null background. Plants were grown under long days. D, Semiquantitative RT-PCR analysis of flowering time gene expression in lines ± *fy-5*. E, Correlation of *FLC* mRNA expression levels (D) with flowering time (Fig. 3A). F, Quantitative RT-PCR analysis of *FLC* mRNA levels. For RT-PCR analysis, RNA was extracted from 12-d-old, long-day-grown seedlings. Error bars indicated 1 SD.

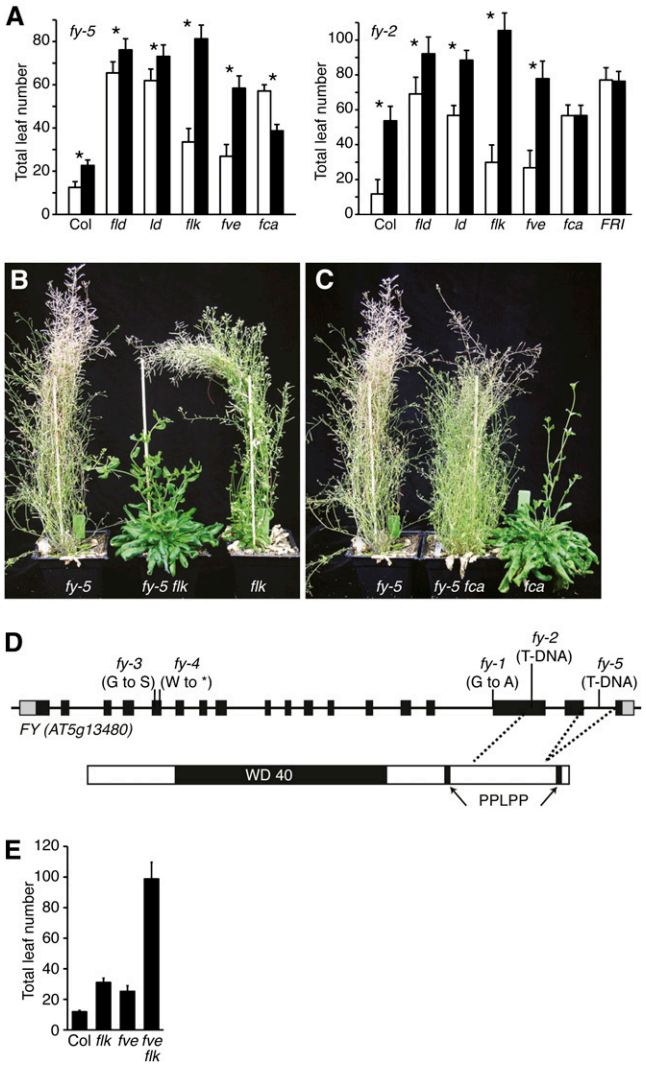

Figure 3. Interactions between *fy* and other the autonomous pathway mutants. A, Bars represent the total number of rosette leaves formed prior to flowering under long days. White bars represent the indicated genotypes in the Col background. Black bars represent lines that also contain the indicated mutations in *fy*. Asterisks indicate differences that are significantly different ($P < 0.01$) between lines ± *FY*. B and C, Differing effects of *fy* on flowering time in *flk* and *fca* backgrounds. Plants were grown under long days. D, Schematic representation of the *FY* gene (top) and protein (bottom). E, Number of rosette leaves formed prior to flowering under long days for *fve flk* single and double mutants. Error bars indicate 1 SD. [See online article for color version of this figure.]

termine if it might be possible to construct an *fpa fy* mutant in the Col background, we crossed *fpa-7* to *fy-2*. In the F2 population, we failed to identify double mutants. We were able, however, to recover plants that were homozygous for one mutation and heterozygous for the other (i.e. *FPA fpa-7 fy-2 fy-2* or *fpa-7 fpa-7 FY fy-2*). In the F3 generation, one-fourth of the progeny should be homozygous for both mutations. Similar to the previous report, however, no double mutants were identified, indicating that *fpa-7 fy-2* plants are likely inviable. It is interesting to note that other autonomous pathway double mutants containing *fpa* (*fpa ld*, *fpa fld*, and *fpa fve*) have also shown reduced viability and pleiotropic developmental defects (Veley and Michaels, 2008).

Because the *fy-5* allele has a weaker flowering time phenotype than *fy-2*, we speculated that an *fpa fy-5* plant might be viable. Indeed, an F2 population between *fpa-7* and *fy-5* yielded viable double mutants (Fig. 4, A and B). The growth rate of *fpa-7 fy-5* plants was similar to Col, *fpa-7*, and *fy-5* (Fig. 4C); however, the double mutants showed other developmental abnormalities. Most notably, *fpa-7 fy-5* plants exhibited reduced apical dominance during vegetative development (Fig. 4, A and B). When grown under long days, the majority of double mutant plants underwent senescence without flowering. It should be noted, however, that *fpa-7 fy-5* mutants typically underwent senescence before *fpa-7* plants flowered (Fig. 4B), thus the lack of flowering cannot be attributed solely to a stronger late-flowering phenotype. In fact, *FLC* levels were similar in *fpa-7* and *fpa-7 fy-5* (Fig. 2D), suggesting that, if *fpa-7 fy-5* plants lived long enough, they would flower similarly to *fpa-7*. To test this hypothesis, we vernalized *fpa-7 fy-5* plants for varying lengths of time to accelerate flowering so that it would take place prior to senescence. Double mutants vernalized for 7 or more days did indeed flower similarly to the *fpa-7* single mutant (Fig. 4D). Thus, with regards to flowering time, the interaction between *fy* and *fpa* appears to be similar to that between *fy* and *fld* or *ld*.

FY Can Affect Flowering Time and *FLC* Levels Independently of *FCA*

A number of experiments indicate that FCA and FY act together in the promotion of flowering. *fca fy* double mutants (in the Landsberg *erecta* background) do not flower later than *fca* single mutants, suggesting they function in a common pathway (Koornneef et al., 1998). More recently, the physical interaction between FY and FCA has also been shown to be important for the regulation of *FLC*. In vitro, the WW protein interaction domain of FCA interacts with the PPLPP domains of FY (Simpson et al., 2003; Henderson et al., 2005) and, in vivo, the repression of *FLC* by *FCA* overexpression (*35S::FCA*) is suppressed by mutations in one of the PPLPP domains of FY (Manzano et al., 2009). Interestingly, our data indicates that FY can also affect flowering time in the absence of FCA. Consistent with previous genetic data (Koornneef et al., 1998), the *fca-9 fy-2* does not flower later than the *fca-9* single mutant (Fig. 2B). The *fy-5* allele, however, causes a reduction in *FLC* expression and earlier flowering in the *fca* mutant background (Figs. 2, D and F, and 3, A and C). This indicates that *fy-5* has effects on *FLC* expression and flowering time that do not require the presence of *FCA*. Of course, this interpretation is predicated on a total lack of functional protein in the *fca* mutant. *fca-9* contains a frameshift mutation in the 37th codon (full-length FCA gamma contains 505

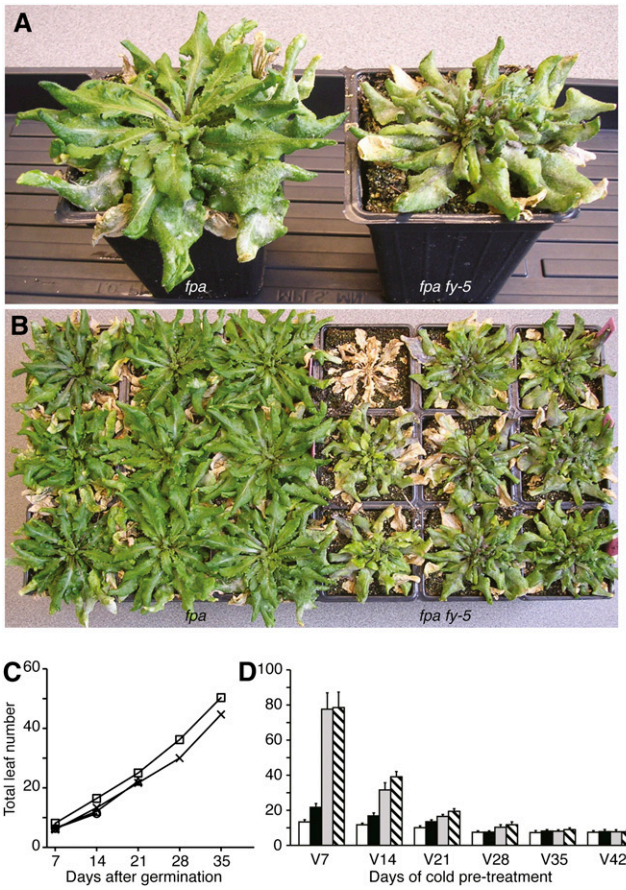

Figure 4. *fpa fy-5* double mutants are viable. A and B, *fpa* and *fpa fy-5* plants grown under long days. *fpa fy-5* plants show a loss in apical dominance and usually senesce without flowering in the absence of vernalization. C, Growth rate of Col (circles), *fy* (triangles), *fpa* (squares), and *fpa fy* (X) plants grown under long days. D, Bars represent the total number of rosette leaves formed prior to flowering under long days for Col (white), *fy-5* (black), *fpa* (gray), and *fpa fy-5* (crosshatched). V indicates days of cold treatment. Error bars indicate1 SD. [See online article for color version of this figure.]

amino acids) and is therefore unlikely to produce a functional protein. To confirm that *fy-5* confers early flowering in the absence of *FCA*, we obtained a second *fca* allele (SALK_057540) that contains a T-DNA insertion. Similar to *fca-9*, the *fca* T-DNA allele *fy-5* double mutant flowers significantly earlier than *fca* alone (Fig. 5A), confirming that *fy-5* can promote flowering and repress *FLC* in the absence of *FCA*.

fy-5 Is Likely to Be a Hypomorphic Allele

In addition to its effect on flowering time, *fy-5* mutants showed abnormal floral development including alteration of floral organ number (Fig. 5B), reduced petal development (Fig. 5, C and D), and incomplete closure of the sepals around the developing floral buds (Fig. 5, F and G). Some of these phenotypes, such as reduced petal development and incomplete sepal closure, were more severe in *fca-9 fy-5* than in *fy-5* alone (Fig. 5E). The organization and appearance of epidermal sepal cells were also abnormal in *fy-5* plants (Fig. 5, H and I). Cellular organization was more random in *fy-5* and the cell surface showed a denser and more random arrangement of ridges (Fig. 5, H and I). Interestingly, a similar change in cell surface morphol-

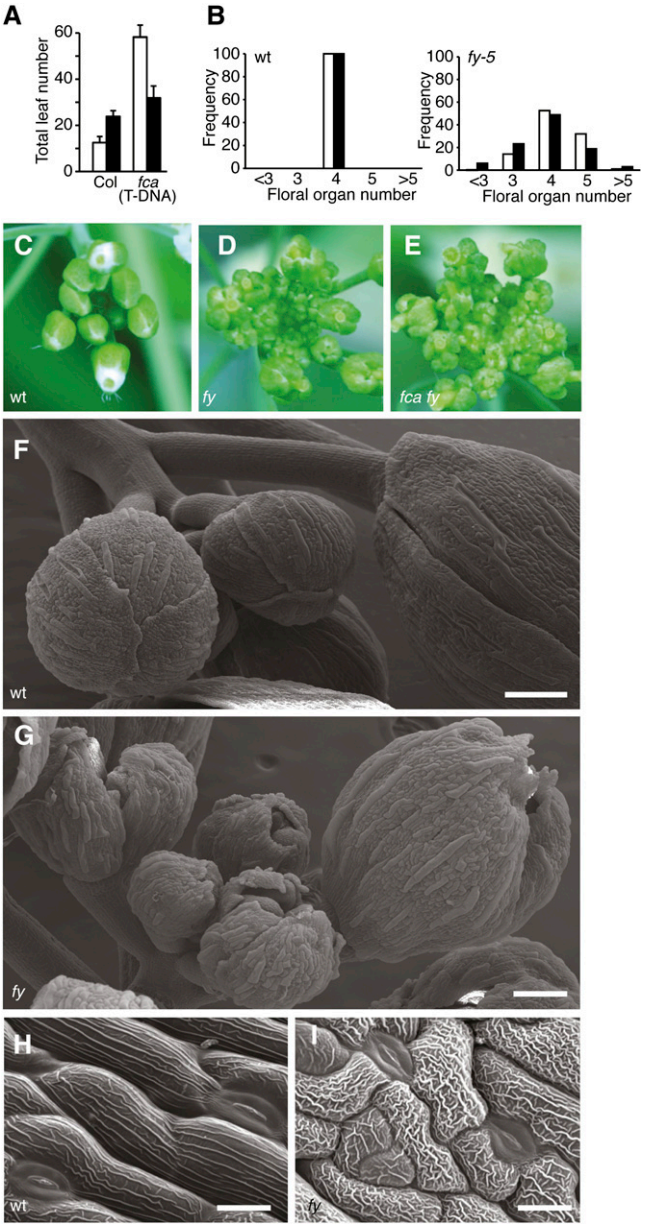

Figure 5. Effect of *fy-5* on flowering time and floral development. A, Bars represent the total number of rosette leaves formed prior to flowering for the indicated genotypes alone (white) or with *fy-5* (black). Error bars indicate 1 SD. B, Distribution of sepal (white bars) and petal (black bars) numbers in wild-type and *fy-5* plants. C to E, Inflorescences of Col (C), *fy-5* (D), and *fca fy-5* (E). F and G, Scanning electron micrographs of developing flower buds in Col (F) or *fy-5* (G). Scale bars represent 100 μm. H and I, Scanning electron micrographs of sepal epidermis in Col (H) or *fy-5* (I). Scale bars represent 10 μm. All plants were grown under long days. [See online article for color version of this figure.]

ogy has been previously observed in other pleiotropic autonomous pathway double mutants (Veley and Michaels, 2008).

The interpretation of the phenotypes of *fy* mutant plants is complicated by the fact that *fy* null alleles are lethal (Henderson et al., 2005). Both *fy-2* and *fy-5* contain truncations of the 3′ end of the *FY* gene (Henderson et al., 2005; Fig. 3D). Truncations could affect the activity of FY in multiple ways, including a partial loss of function (e.g. a hypomorphic allele) or the creation of a protein with a novel function (e.g. a neomorphic allele). In an attempt to distinguish between these two possibilities, we created a genomic clone of *FY*, including the endogenous promoter sequences, that was truncated at a position near the T-DNA insertion in *fy-5* and transformed the construct into Col and *fy-5*. If the truncation creates a neomorphic allele, it may act dominantly to delay flowering, whereas if the truncation produces a hypomorphic allele, it would be predicted to show no phenotype. Consistent with the later model, the truncated *FY* construct had no effect on flowering time in Col or *fy-5* backgrounds (Fig. 6A). We also transformed a full-length FY genomic clone under control of its own promoter into *fy-5*. The majority of the T1 plants flowered earlier than *fy-5*, with many flowering similarly to Col (Fig. 6B). The fact that a wild-type *FY* genomic clone can rescue the late-flowering phenotype of the *fy-5* mutant also suggests that the late-flowering phenotype of *fy-5* is due to a partial loss of *FY* function and not due to a novel function imparted by the truncation. Finally, we transformed the full-length genomic *FY* construct into *flk-4 fy-5* and *fve-4 fy-5* double mutants, which flowered much later than *flk-4* and *fve-4* single mutants, and *fca-9 fy-5* double mutants, which flower earlier than *fca-9* (Fig. 3A). Consistent with the results in Col, the *FY* genomic clone restored earlier flowering in *flk-4 fy-5* and *fve-4 fy-5* and later flowering in *fca-9 fy-5* (Fig. 6B). These experiments show that the flowering time effects of the *fy-5* allele are recessive (Fig. 6, A and B). Nonetheless, it is interesting to note that *FY* transcript levels were significantly elevated in *fy-5* (Fig. 6C). These results support the hypothesis that *fy-5* is likely a hypomorphic allele and that, depending on genetic background, *FY* can act to either promote or repress *FLC* expression.

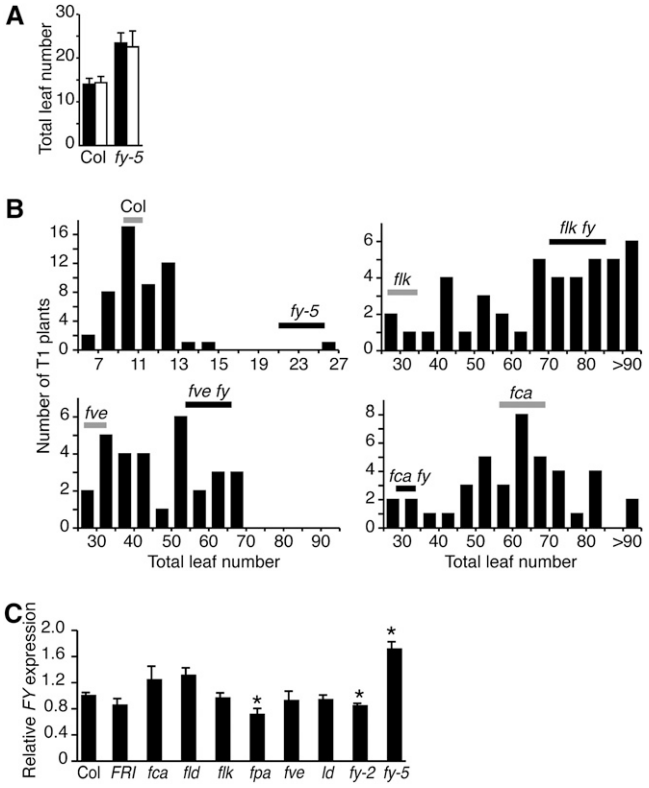

Figure 6. The flowering behavior of *fy-5* is consistent with a hypomorphic allele. A, Total leaf number at flowering for the indicated genotypes alone (black bars) or transformed with a genomic *FY* clone containing a similar truncation to that found in *fy-5* (white bars). B, Rescue of *fy-5* flowering time phenotypes by transformation with a genomic *FY* clone. *fy-5*, *flk fy-5*, *fve fy-5*, and *fca fy-5* were transformed with genomic *FY* under its own promoter. Vertical bars indicated the number of plants that flowered with the indicated number of leaves. Horizontal black bars indicate the flowering time of the untransformed parental line and horizontal gray bars indicate the flowering time of the corresponding line with wild-type *FY*. Note that in *fy-5*, *flk fy-5*, and *fve fy-5* transformation with *FY* causes earlier flowering, but in *fca fy-5* restoration of *FY* leads to later flowering. Error bars indicate 1 SD. All plants were grown under long days. C, Quantitative RT-PCR analysis of *FY* mRNA levels. Asterisks indicate differences that are significantly different ($P < 0.01$) from Col.

FY Promotes Proximal Site Polyadenylation in *FLC* Sense Transcripts Independently of FCA

Previous work has shown that FY promotes usage of a proximal polyadenylation site preference in *FLC* antisense transcripts (Liu et al., 2010). In the course of sequencing *FLC* cDNA clones, we noticed multiple polyadenylation sites. We therefore wondered if FY might also play a role in polyadenylation site selection in *FLC* sense transcripts. We performed 3′ RACE from Col and found two major products, a proximally polyadenylated form (191 bp 3′ of stop codon) and a distally polyadenylated form (250 bp 3′ of stop codon; Fig. 7, A and B). The two forms were present in approximately equal amounts (Fig. 7B). To determine if polyadenylation site selection might play a role in the regulation of *FLC*, we examined distal/proximal ratios in lines containing *FRI* or autonomous pathway mutations. In *FRI*, *fca-9*, *fld-3*, *flk-4*, *fpa-7*, *fve-4*, or *ld-1* backgrounds, distal/proximal ratios were similar to Col (Fig. 7B), thus in these backgrounds, increased *FLC* expression does not correlate with altered polyadenylation site selection. Interestingly, however, both *fy-2* and *fy-5* showed a significant decrease in the proximally polyadenylated form of *FLC* (Fig. 7B). Thus FY acts to promote polyadenylation at the proximal site in *FLC* sense transcripts.

One of the striking findings of our work is the result that, depending on the genetic background, *fy* muta-

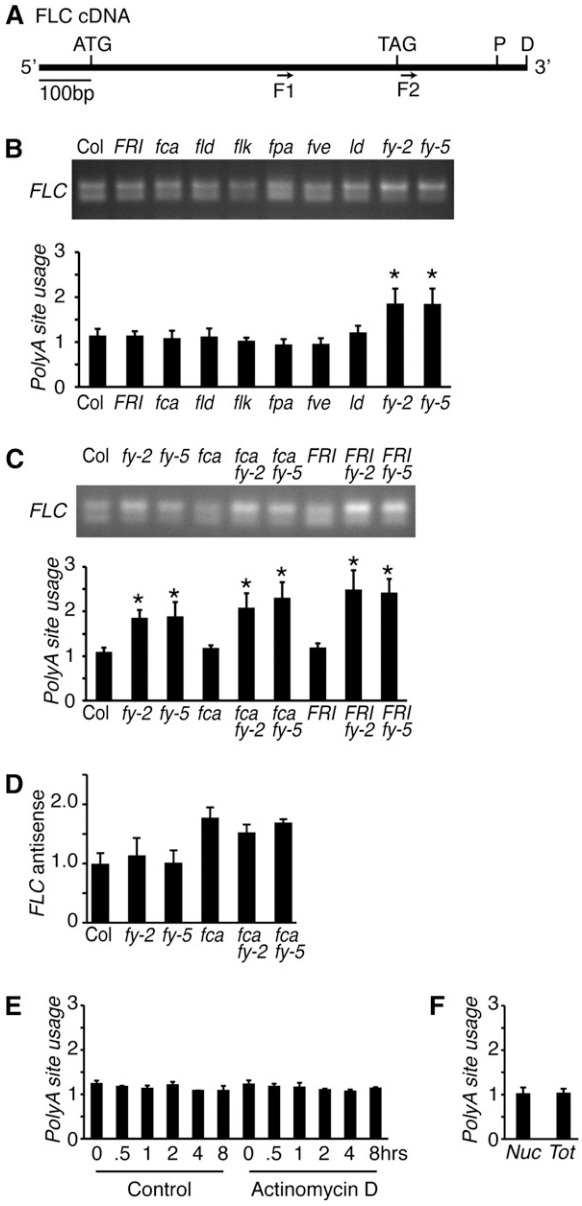

Figure 7. *FY* promotes proximal site polyadenylation in the *FLC* sense transcript. A, Schematic drawing of the *FLC* cDNA with the proximal (P) and distal (D) polyadenylation sites shown. F1 and F2 indicate the positions of primers used for 3′ RACE. B and C, 3′ RACE of *FLC* transcripts in the indicated genetic backgrounds and quantification of the ratio of distal/proximal site usage. Asterisks indicate differences that are significantly different ($P < 0.05$) from Col (B) or the *FY* wild-type reference genotype (C). D, Quantitative RT-PCR analysis of antisense *FLC* transcript levels. E, Effect of the transcription inhibitor actinomycin D on distal/proximal site usage. F, Quantification of the ratio of distal/proximal polyadenylated *FLC* transcripts in nuclear and total RNA.

tions can either increase or decrease *FLC* expression. We were therefore curious to determine if the effect of *fy* on polyadenylation site selection would be indicative of its effect on *FLC* expression. We determined the effect of *fy-2* and *fy-5* on distal/proximal ratios in a Col background (where *fy-5* mutations increase *FLC* and delay flowering) and in *fca-9* and *FRI* backgrounds (where *fy-5* mutations reduce *FLC* expression and accelerate flowering). Interestingly, *fy* mutations had similar effects in all three genetic backgrounds (Fig. 7C). In Col, *fca-9*, or *FRI*, both *fy-2* and *fy-5* mutations caused an increase in usage of the distal polyadenylation site. It has recently been shown that antisense transcripts play a role in the regulation of *FLC* (Swiezewski et al., 2009). This raises the possibility that *fy-2* and *fy-5* may differ in their effect on *FLC* antisense RNA; however, no significant differences in *FLC* antisense transcripts were observed between *fy-2* and *fy-5* lines (Fig. 7D). Thus, despite the fact that *fy-5* and *fy-2* have significantly different effects on flowering time (Figs. 1–3), their effects on *FLC* polyadenylation are indistinguishable. Thus differential polyadenylation of *FLC* transcripts cannot explain the differences in *FY* allele behavior. We also investigated whether the proximal and distal polyadenylation variants might differ in their stability or nuclear export. When treated with the transcription inhibitor actinomycin D, no change was observed in distal/proximal ratio (Fig. 7E), suggesting that both forms have similar levels of turn over. Similarly, the ratio of the two forms of *FLC* transcript was similar in total RNA or nuclear RNA (Fig. 7F).

Taken together with published findings, our work indicates that FY plays both *FCA*-dependent and *FCA*-independent roles in the regulation of *FLC* expression. Previous work has shown that an FY FCA complex influences the alternative splicing of the *FCA* transcript. Because only the gamma form of *FCA* gives rise to a functional repressor of *FLC*, the role of FY in *FCA* splicing is critical in setting basal *FLC* levels. Here we show that FY can also affect *FLC* expression and polyadenylation site selection in the absence of *FCA*. Although analyses using weak alleles must be interpreted with caution, the genetic interactions between *fy-5* and *FRI* or other autonomous pathway mutations are intriguing nonetheless. The observation that *fy-5* alleles cause increased *FLC* expression in some genetic backgrounds and decreased expression in others also supports the model that FY plays multiple roles in the regulation of *FLC*. The characterization of *fy-5* may also shed light on the evolution of *FY* function. Natural variation in the region of FY truncated in the *fy-5* allele has been implicated in the regulation of flowering time in response to light quality (Adams et al., 2009). In the future it will be interesting to determine the molecular interactions that underlie the various epistatic relationships between *FY*, other members of the autonomous pathway, and *FRI*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

FRI-Col (Lee et al., 1994b), *fca-9* (Bezerra et al., 2004), *fld-3* (He et al., 2003), *flk-4* (SALK_112850; Veley and Michaels, 2008), *fpa-7* (Michaels and Amasino, 2001), *fve-4* (Michaels and Amasino, 2001), *fy-2* (Simpson et al., 2003), *ld-1* (Rédei, 1962), and *flc-3* (Michaels and Amasino, 1999) are in the Col genetic

background and have been described previously. *fy-5* (SALK_005697) and *fca* (SALK_057540) were obtained from the Arabidopsis Biological Resource Center. Plants were grown at 22°C under cool-white fluorescent lights with a light intensity of 100 μmol m^{-2} s^{-1}. Long days consisted of 16 h of light followed by 8 h of darkness. For vernalization, seedlings were sown on agar-solidified medium (2.2 g L^{-1} Murashige and Skoog salts, 1% Suc, and 0.8% agar) and were kept at 4°C for the indicated periods of time.

RNA Expression Analysis

Total RNA isolation, reverse transcription (RT), semiquantitative RT-PCR analysis (Michaels et al., 2004), and quantitative RT-PCR analysis (Mockler et al., 2004) were performed as described previously. Detection of the FLC antisense transcript was performed as described (Swiezewski et al., 2009). Primer sequences are shown in Supplemental Table S2.

3′ RACE

M13-oligo(dT) primer (Supplemental Table S2) was used for RT. A 5-fold dilution of RT products was used as a template for 3′ RACE. Touchdown (TD) PCR was carried out using an M13 forward primer and an *FLC*-specific primer, 550-FLC-F1 (Supplemental Table S2). TD PCR conditions were: 94°C for 3 min; followed by 29 cycles of 94°C for 1 min, 65°C (with a per-cycle reduction of 0.5°C) for 2 min, 72°C (with elevation per cycle 0.2°C) for 1 min; then nine cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min; and concluded with a final extension of 72°C for 7 min. TD PCR products were then diluted 5-fold for a second round of nested PCR using the M13 forward primer and a FLC 3′ UTR-specific primer, 1121-FLC-3′UTR-F. Nested PCR conditions were: 95°C for 5 min; followed by 27 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 2 min; and then final extension at 72°C for 7 min. PCR products were resolved on 2.5% agarose gel and quantified using ImageJ (Abramoff et al., 2004).

RNA Stability Assay

RNA stability assay was performed as described previously (Mockler et al., 2004). Briefly, 10-d-old *FRI*-Col seedlings were placed in tubes and immersed with transcription inhibitor solution containing 1 mM PIPES buffer (pH 7.0), 1 mM KCl, 1 mM sodium citrate, 15 mM Suc, 100 mg mL^{-1} actinomycin D (Sigma). For the control group, *FRI*-Col seedlings were treated with the same buffer without actinomycin D. Samples were collected at the indicated time points up to 8 h.

Microscopy

Scanning electron microscopy was performed as described previously (Jacob et al., 2007).

Sequence data from this article can be found in EMBL/GenBank under the following accession numbers: FCA, At4g16280; FLD, At3g10390; FLK, At3g04610; FPA, At2g43410; FVE, At2g19520; FY, At5g13480; LD, At4g02560; FRI, At4g00650; CO, At5g15840; FT, At1g65480; SOC1, At2g45660; FLC, At5g10140; and UBQ, At4g05320.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. FLC mRNA quantification.

Supplemental Table S2. PCR and quantitative PCR primers used in this study.

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