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Autoregulation of *FCA* pre-mRNA processing controls *Arabidopsis* flowering time

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The timing of the transition to flowering is critical for reproductive success in plants. *Arabidopsis FCA* encodes an RNA-binding protein that promotes flowering. *FCA* expression is regulated through alternative processing of its pre-mRNA. We demonstrate here that FCA negatively regulates its own expression by ultimately promoting cleavage and polyadenylation within intron 3. This causes the production of a truncated, inactive transcript at the expense of the full-length *FCA* mRNA, thus limiting the expression of active FCA protein. We show that this negative autoregulation is under developmental control and requires the FCA WW protein interaction domain. Removal of introns from *FCA* bypasses the autoregulation, and the resulting increased levels of FCA protein overcomes the repression of flowering normally conferred through the up-regulation of *FLC* by active *FRI* alleles. The negative autoregulation of *FCA* may therefore have evolved to limit *FCA* activity and hence control flowering time.
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Introduction

The switch from vegetative to reproductive development is the major developmental transition in flowering plants (Mouradov *et al*., 2002; Simpson and Dean, 2002). The integration of environmental signals and the endogenous developmental competence of the plant determine the timing of this transition. Flowering time is closely allied to seasonal progression and is an important trait governing adaptation to environment. Naturally occurring *Arabidopsis* ecotypes have evolved two broadly different flowering strategies: rapid cycling and a winter annual habit (Simpson and Dean, 2002). Rapid cycling accessions complete their life-cycle in as little as 6 weeks and may complete more than one life cycle in a growing season. This is one property that has led to the widespread use of *Arabidopsis* as a model for plant biology, and the most commonly studied laboratory accessions, Landsberg *erecta* (L*er*) and Columbia (Col), are in this class. However, most naturally occurring *Arabidopsis* ecotypes are winter annuals. That is, they germinate before winter and flower in the favourable conditions of the following spring. Winter annuals therefore complete only one life-cycle in a growing season. Unlike rapid cycling accessions, winter annuals require the long cold treatment of winter to accelerate flowering, in a process known as vernalization.

In *Arabidopsis*, genes that promote flowering have been identified through the characterization of late flowering mutants (Koornneef *et al*., 1991). These genes have been placed into genetically separable pathways on account of the shared phenotypic and epistatic interactions of the corresponding mutants (Mouradov *et al*., 2002; Simpson and Dean, 2002). A simplified representation of these pathways is shown in Figure 1A and B. Flowering in *Arabidopsis* is promoted by long days and the photoperiod pathway mediates this response. In addition, a genetically separable gibberellin signal transduction pathway promotes flowering in both long and short day conditions (Mouradov *et al*., 2002; Simpson and Dean, 2002). These pathways ultimately activate genes that switch meristem identity from vegetative to floral and thus execute the floral transition. The capacity of these pathways to promote flowering is antagonized by a key repressor of the *Arabidopsis* floral transition, the MADS-domain transcription factor FLOWERING LOCUS C (FLC) (see Hepworth *et al*., 2002; Simpson and Dean, 2002). The expression of *FLC* correlates with flowering time, with high levels of *FLC* mRNA being associated with late flowering (Michaels and Amasino, 1999; Sheldon *et al*., 1999). *FLC* expression is regulated by pathways that act antagonistically to either promote or down-regulate its expression (Michaels and Amasino, 1999; Sheldon *et al*., 1999). *FLC* expression is down-regulated by the autonomous pathway (Michaels and Amasino, 1999; Sheldon *et al*., 1999). However, in winter annual accessions like San Feliu-2 (SF-2), active *FRIGIDA* (*FRI*) alleles override the activity of the autonomous pathway and promote elevated levels of *FLC* mRNA expression (Michaels and Amasino, 1999; Sheldon *et al*., 1999) (Figure 1A). This function of *FRI* is antagonized by the vernalization pathway, which acts to down-regulate *FLC* in response to the long cold temperature treatment of winter (Sheldon *et al*., 1999, 2000; Gendall *et al*., 2001) (Figure 1A). In rapid cycling accessions, like L*er*, that carry loss-of-function *fri* alleles (Johanson *et al*., 2000), the autonomous pathway is the major pathway controlling the expression of *FLC* (Figure 1B).

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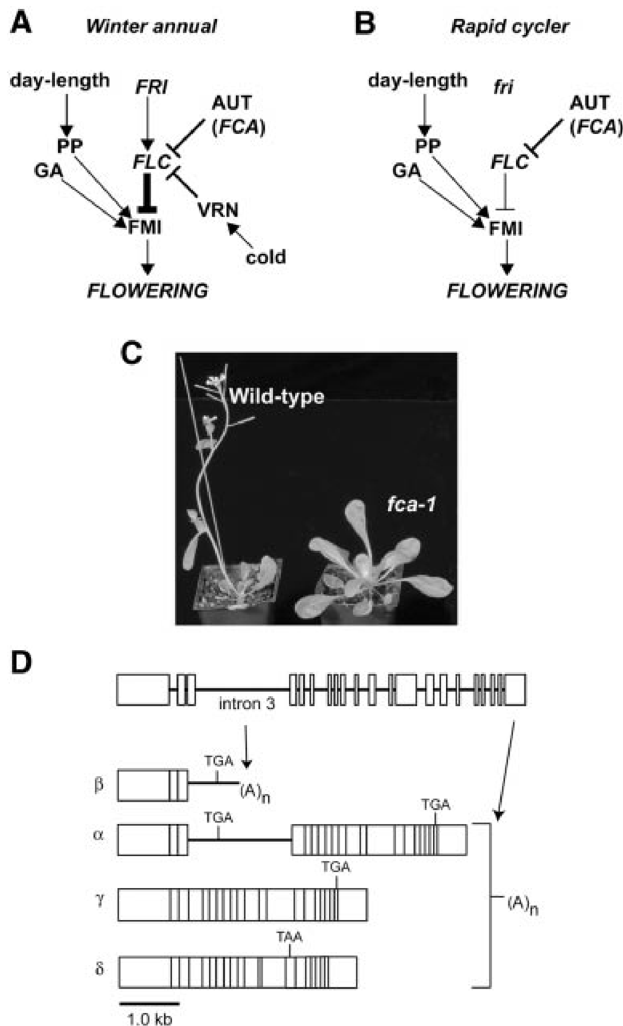

Fig. 1. FCA and the control of *Arabidopsis* flowering time. (**A** and **B**) Schematic representation of the principal genetic pathways controlling flowering time in winter annual and rapid cycling accessions of *Arabidopsis.* Promotive activities are denoted by arrowheads, repressive activities are denoted by T-bars. The photoperiod (PP) and gibberellin signal transduction (GA) pathways are shown activating genes with a floral meristem identity function (FMI), while FLC is shown to repress this. FRI, the FCA-containing autonomous pathway (AUT) and vernalization pathway (VRN) regulate *FLC* in an antagonistic manner. The most frequently found difference between winter annual (A) and rapid cycling (B) *Arabidopsis* accessions is allelic variation at *FRI*, with most rapid cycling accessions carrying inactive, loss-of-function *fri* alleles. (**C**) A comparison of the phenotype of wild-type L*er* plants and late flowering *fca-1* mutant plants. Although grown for the same time and under identical conditions, wild-type plants have already flowered while the *fca-1* plants have remained in the vegetative state and continued to produce more leaves as opposed to floral organs. (**D**) Schematic representation of the alternative processing of *Arabidopsis FCA* pre-mRNA. Exons are represented as filled boxes and intron by lines.

The autonomous pathway is currently defined by six recessive late-flowering mutants, *fca*, *fy*, *fve*, *fld*, *ld* and *fpa*, all of which flower later than wild type in both long and short day conditions (Koornneef *et al*., 1998). Each appears to negatively regulate *FLC*, since autonomous pathway mutant backgrounds show elevated levels of *FLC* mRNA (Michaels and Amasino, 1999; Sheldon *et al*., 1999). In addition, the early flowering *flc-3* null mutation is epistatic to autonomous pathway mutations, indicating that the regulation of *FLC* expression can account for their late flowering phenotype (Michaels and Amasino, 2001). In contrast to the environmental inputs that control the photoperiod and vernalization pathways, it is not known how the activity of the autonomous pathway is regulated.

In order to understand how the autonomous pathway is controlled, we are analysing the pathway component, *FCA*, which encodes a protein with two RRM-type RNA binding domains and a WW protein interaction domain (Macknight *et al*., 1997). The late flowering allele, *fca-1* (Figure 1C), results from a point mutation in exon 13 that introduces a premature stop codon. A truncated FCA protein expressed from this mutated gene lacks the WW domain (Macknight *et al*., 1997). *FCA* expression is regulated post-transcriptionally by alternative processing of the pre-mRNA. Four different transcripts are processed from *FCA* pre-mRNA: α, β, δ and γ (Figure 1D). *FCA*-γ and -δ transcripts result from excision of all introns, but they are distinguished by an alternative splicing event around intron 13 that produces transcript δ. While γ encodes the full-length active FCA protein, the alternative splicing that produces transcript δ results in a shift in reading frame that leads to the introduction of a premature termination codon (PTC). As a result, δ mRNA encodes a truncated isoform of FCA that lacks the WW domain. Transcript α retains intron 3, but all other introns are excised. The fourth transcript, β, arises from cleavage and polyadenylation within intron 3. Because of the presence of in-frame stop codons within intron 3 (Figure 1D), both α and β encode truncated isoforms of FCA that lack intact RNA binding domains. While *FCA*-β is cleaved and polyadenylated at a promoter-proximal site within intron 3, the other transcripts are cleaved and polyadenylated at a distal site in the 3′ untranslated region (UTR) (Figure 1D). Therefore, the processing of *FCA* pre-mRNA involves alternative splicing and alternative polyadenylation, with three different transcripts resulting from the alternative processing of intron 3. Only the fully spliced *FCA*-γ transcript appears to be functional in flowering time control, since the overexpression of the other transcripts neither promotes flowering in an *fca-1* background nor delays it in wild type (Macknight *et al*., 1997, 2002). The conservation of the alternative processing of *FCA* pre-mRNA in other plants and its temporal and spatial regulation (Macknight *et al*., 2002) suggests that it plays a key role in regulating *FCA* expression levels.

In this report we show that FCA negatively regulates its expression by ultimately promoting proximal poly(A) site usage within intron 3 of its own pre-mRNA. We show that FCA is a nuclear RNA-binding protein and that its WW domain is required for this autoregulation. This post-transcriptional regulation has a functional consequence for growth, development and flowering time. The removal of this control through the use of intronless transgenes changed the balance of the activities of pathways regulating *FLC* mRNA levels, resulting in precocious flowering.

Results

Endogenous FCA protein expression is undetectable in Arabidopsis plants overexpressing FCA from a transgene

FCA can be overexpressed in stable transgenic lines from a transgene consisting of a cauliflower mosaic virus (CaMV) 35S promoter driving the expression of an intronless *FCA* gene (Macknight *et al*., 2002). In constructing this transgene, the CaMV 35S promoter was inserted at position +349 (relative to the *FCA* transcription initiation site) and upstream of the first in-frame AUG codon (Macknight *et al*., 2002; see Figure 2A). As a result, sequences encoding the first 349 nucleotides of the 5′ end of the *FCA* transcript are deleted from this construct. We have subsequently discovered that translation of endogenous FCA actually initiates at a non-AUG codon upstream of the first in-frame AUG (Figure 2A; unpublished data) and as a result, the protein expressed from this transgene is 10 kDa shorter than the native or endogenous FCA protein (Macknight *et al*., 2002). However, the truncated transgene-derived protein is functional, since it complements the late-flowering phenotype of *fca-1* by reducing the levels of *FLC* mRNA (Macknight *et al*., 2002). The difference in size between the endogenous and transgene-derived proteins should have enabled their simultaneous detection in Western analysis with anti-FCA antibodies. When *35S::FCA-*γ was introduced into a wild-type L*er* background from an *fca-1* mutant background by crossing (hereafter referred to as introgression), we were able to confirm that the truncated FCA protein derived from the transgene was overexpressed in both backgrounds. However, we were unable to detect the simultaneous expression of the transgene-derived protein with either full-length endogenous FCA protein or the truncated mutant protein derived from *fca-1* (Figure 2B). This suggested that the overexpression of FCA from a transgene was associated with reduced expression of FCA protein from the endogenous gene.

Negative regulation of FCA expression occurs at the level of intron 3 processing

In order to determine the molecular basis for reduced expression of endogenous FCA protein in plants overexpressing transgenic FCA, we analysed endogenous *FCA* gene expression at the RNA level. Northern analysis was performed using poly(A)$^+$ RNA purified from wild-type (L*er*), loss-of-function *fca-1* and *fca-4* alleles, and from transgenic plants overexpressing *FCA* in either a L*er* or a *fca-1* mutant background (Figure 2C). To detect only the expression of the endogenous *FCA* gene, a probe to the 5′ end of *FCA* was used (Figure 2A). This region is present in all the isoforms generated by alternative processing of endogenous *FCA* pre-mRNA, but is absent from the *FCA* transgene (Macknight *et al*., 2002). To confirm the identity of the different *FCA* transcripts we included *fca-4* as a positive control. *fca-4* is a γ-ray-induced allele containing a breakpoint and chromosomal rearrangement within intron 4 of *FCA* (Page *et al*., 1999). This results in *FCA* intron 4 being fused to the predicted gene, *At4g1550* (DDBJ/EMBL/GenBank accession No. Z97335). Since *FCA*-β results from cleavage and polyadenylation within intron 3, it should be the same size in *fca-4* as in wild-type

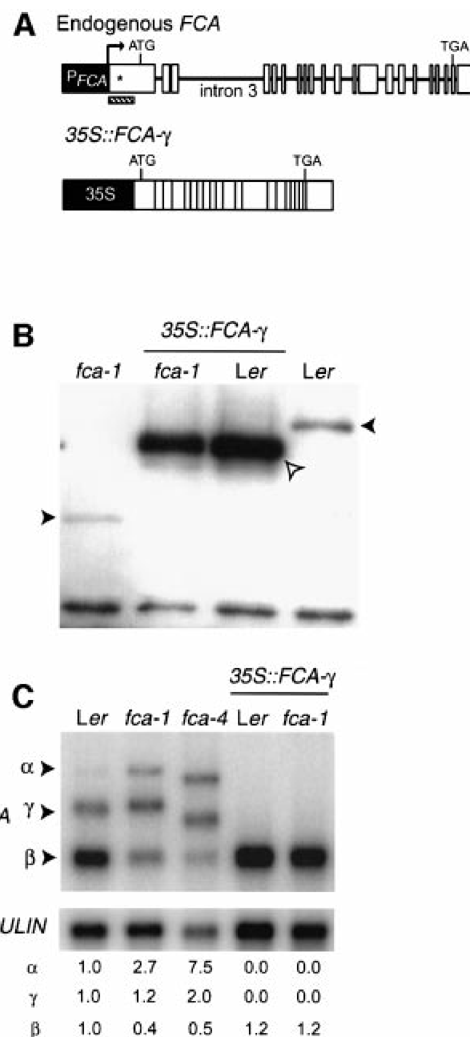

Fig. 2. *FCA* negatively autoregulates its expression at the level of poly(A) site choice. (**A**) Schematic representation illustrating the differences between the endogenous *FCA* gene and *35S::FCA-*γ transgene. The CaMV 35S promoter is inserted at a site upstream of the first in-frame AUG codon, resulting in the deletion of 349 bp of *FCA* exon 1 from *35S::FCA* transgenes. The position of the non-canonical initiation codon from which FCA is translated is denoted with an asterisk. The region used as a probe in northern analysis is denoted by a hatched bar. (**B**) Immunodetection of FCA protein. Western blot analysis of total soluble protein extracts from seedlings of L*er*, *fca-1*, and the transgenic lines *35S::FCA-*γ in L*er* and *35S::FCA*γ in *fca-1* (a *35S* fusion to an intronless *FCA* transgene). Filled and unfilled arrowheads denote endogenous and transgenic FCA proteins respectively. Lower bands are unspecific binding products used as loading controls. (**C**) Northern blot analysis of poly(A)$^+$ RNA isolated from L*er*, *fca-1*, *fca-4*, and the transgenic lines *35S::FCA-*γ in L*er* and *35S::FCA-*γ in *fca-1* [the transgene described in (A)]. The blot was probed first with an *FCA* 5′ leader probe and later stripped and re-probed with a β-*TUBULIN* probe as a loading control. Numbers below compare the expression level of each *FCA* transcript between the different samples analysed. They were calculated as a ratio between a normalized *FCA* transcript signal (against that for β-*TUBULIN*) of a given sample and the same *FCA* transcript signal normalized in the wild type (L*er*).

plants. However, since transcripts α, δ and γ are processed to include both the 5′ part of *FCA* and the fusion to the rearranged gene downstream, they should differ in size between *fca-4* and wild-type.

Transcripts corresponding to *FCA*-β, -α and -γ were detected by northern analysis in wild-type plants (Figure 2C). *FCA*-β was the same size in *fca-4* as in

wild type, whereas transcripts α and γ were smaller in *fca-4* compared with wild type (Figure 2C), confirming identification of the correct transcripts (*FCA*-δ was not detected using this approach because of its low abundance and similarity in size to γ; Macknight *et al.*, 1997). *FCA*-β was detected in both *35S::FCA*-γ lines overexpressing *FCA*; however, transcripts α and γ were not (Figure 2C). The presence of increased levels of transcript β (which arises from cleavage and polyadenylation at the proximal site within intron 3) and the corresponding absence of transcripts cleaved and polyadenylated at the distal site (α and γ) indicates that the negative regulation of endogenous *FCA* expression occurs at the post-transcriptional level, ultimately through a shift in poly(A) site choice. The absence of transcript γ explains our inability to detect full-length FCA protein in these lines. The comparison of the ratio of the *FCA* transcripts in wild-type and the loss-of-function *fca-1* and *fca-4* mutants showed that less β and more α transcript accumulates in *fca* mutants (Figure 2C). This indicates that regulation of intron 3 processing may be a normal feature of FCA function and regulation, and not simply a gain-of-function phenotype from the ectopic overexpression of an *FCA* transgene.

We conclude that the negative regulation of *FCA* expression occurs post-transcriptionally, at the level of splicing versus poly(A) site selection at intron 3, and that this is a normal feature of the regulation and activity of *FCA*.

FCA negatively regulates its expression ultimately by promoting proximal poly(A) site usage of its own pre-mRNA

The regulation of *FCA* mRNA 3′ end formation could result from two alternative mechanisms. The overexpression of FCA may result in the inhibition of cleavage and polyadenylation at the distal site in the conventional 3′UTR, with the proximal site within intron 3 then being used by default. Alternatively, the overexpression of FCA may actively promote proximal poly(A) site selection (either directly, or as a consequence of affecting splicing of intron 3), so that all transcripts are cleaved and polyadenylated within intron 3. To distinguish between these possibilities, we made use of the *fca-4* allele. In this background, the proximal and distal poly(A) sites are physically separated by the chromosomal breakpoint and rearrangement and are therefore expressed on separate transcripts. We reasoned that if the responsive *cis*-elements to this control lay at the proximal site, then overexpression of *FCA* in an *fca-4* background should result in increased accumulation of the β transcript. However, if the responsive *cis*-elements lay at the *FCA* distal site, then the accumulation of *FCA*-β should be unaffected by *FCA* overexpression in *fca-4*. To test this, we introduced a transgene (P_{FCA}*::FCA*-γ) expressing a full-length, intronless *FCA* gene driven by the endogenous promoter into *fca-4*. Significantly, we detected increased accumulation of endogenous *FCA*-β transcripts and a corresponding disappearance of the size-shifted *fca-4* α and γ transcripts (Figure 3). These data are all consistent with FCA ultimately promoting selection of the proximal poly(A) site.

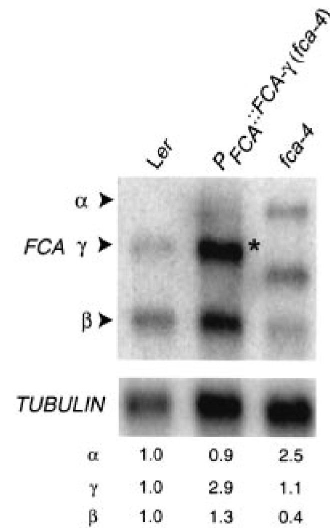

Fig. 3. FCA actively promotes the use of the proximal poly(A) site in its pre-mRNA. Northern blot analysis of poly(A)+ RNA isolated from L*er*, *fca-4* and the transgenic line P_{FCA}*::FCA*-γ in *fca-4* (an intronless *FCA* transgene fused to the native *FCA* promoter). Blot was probed as indicated in Figure 2C. Numbers denote relative expression and were calculated as in Figure 2C. Asterisk denotes the *FCA*-γ transcript belonging to the P_{FCA}*::FCA*-γ transgene.

Autoregulation of FCA expression requires an intact FCA WW domain

To determine whether the RNA-binding domains of FCA were sufficient for the negative feedback control, we examined the effects of overexpressing *FCA*-δ from an intronless transgene driven by the CaMV 35S promoter (*35S::FCA*-δ) in a L*er* background. This isoform encodes the RNA binding domains of FCA but lacks an intact WW protein interaction domain. Overexpression of FCA-δ protein had no effect on either the expression of endogenous FCA protein (Figure 4A) or the pattern of endogenous *FCA* mRNA expression (Figure 4B). This is consistent with the observed negative regulation not simply being the result of ectopically overexpressing an RNA binding protein in these plants. Since FCA-δ lacks the WW protein interaction domain as well as other C-terminal sequences of FCA, we tested whether the WW domain was necessary for the negative regulation of *FCA* expression. The second signature tryptophan of the WW domain was mutated to phenylalanine, and plants expressing this WF mutant protein in an *fca-1* mutant background accumulated wild-type levels of this protein (Simpson *et al.*, 2003). While the increased accumulation of β transcript could be detected in *fca-1* plants expressing intact FCA from an intronless transgene, no increase in the accumulation of β could be detected in *fca-1* plants expressing the WF mutation (Figure 4C). We therefore conclude that the autoregulation of *FCA* expression requires an intact WW domain.

FCA is a nuclear protein

As FCA is an RNA-binding protein (Macknight *et al.*, 1997) it is possible that it carries out the negative regulation of its expression directly. In order to establish whether this was a possibility, we determined whether FCA was nuclear localized. A chimeric FCA:GUS fusion protein was transiently expressed in onion epidermal cells

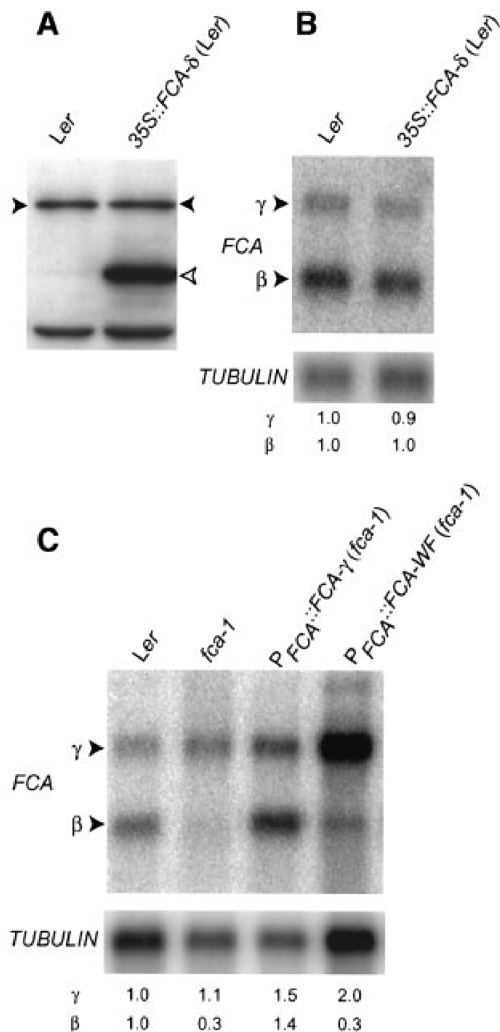

Fig. 4. *FCA* autoregulation requires an intact WW domain. (**A**) Immunodetection of FCA protein. Western blot analysis of total soluble protein extracts from seedlings of L*er* and the transgenic line *35S::FCA*-δ in L*er* (a *35S* fusion to an intronless *FCA*-δ transgene). Lower bands are unspecific binding products used as loading controls. Filled and unfilled arrowheads denote endogenous and transgenic FCA proteins, respectively. (**B**) Northern blot analysis of poly(A)$^+$ RNA isolated from L*er* and the transgenic line *35S::FCA*-δ in L*er* [the same transgenic line as reported in (A)]. The blot was probed as indicated in Figure 2C. Numbers denote relative expression and were calculated as in Figure 2C. (**C**) Northern blot analysis of poly(A)$^+$ RNA isolated from L*er*, *fca-1*, and the transgenic lines P_{FCA}*::FCA*-γ in *fca-1* (the same transgene reported in Figure 3) and P_{FCA}*::FCA-WF* in *fca-1* (an intronless *FCA* transgene fused to the endogenous promoter carrying a mutation resulting in a tryptophan to phenylalanine substitution in the WW domain). The blot was probed as indicated in Figure 2C. Numbers denote relative expression and were calculated as in Figure 2C.

(Varagona *et al*., 1992). When transfected with a plasmid expressing only the GUS protein, cells showed a uniform distribution of GUS activity (not shown). In contrast, cells transfected with *FCA:GUS* showed GUS activity predominantly in the nucleus indicating that FCA is a nuclear-localized protein (Figure 5A and B).

Autoregulation of FCA expression normally controls the levels of active FCA transcript γ

The presence of introns in the *35S::FCA-gene* limited the capacity of *FCA*-γ RNA to be overexpressed (Macknight

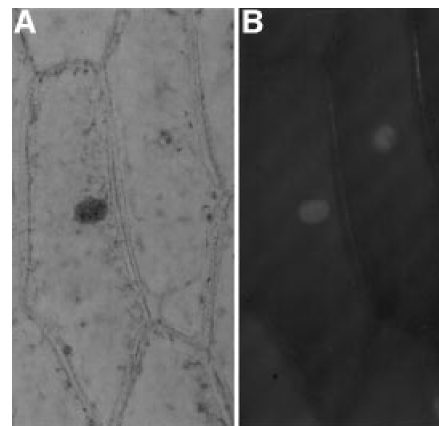

Fig. 5. Nuclear localization of FCA protein. (**A**) Histochemical staining for GUS activity (dark staining) of transiently transfected onion epidermal cells. (**B**) DAPI counter staining of same cells as in (A), revealing location of nuclei.

et al., 1997, 2002). As shown in Figure 2B, when the truncated transgenic FCA protein was expressed from an intronless construct with the 35S promoter (*35S::FCA*-γ), full-length endogenous FCA protein could not be detected. However, when this same truncated FCA protein was expressed from a transgene that contained all the endogenous *FCA* introns (*35S::FCA-gene*), we detected low levels of the transgenic protein and were still able to detect the endogenous wild-type protein (Figure 6A). In addition, RNA expression from the endogenous *FCA* gene was unaffected (Figure 6B). These findings support the idea that autoregulation of *FCA* expression prevents overexpression of the active FCA-γ isoform.

Quantitation of transcript levels in *fca-1* revealed that while the level of *FCA*-β was reduced compared with wild type, there was no corresponding difference in the steady state level of *FCA*-γ between these plants (Figure 6C). If the negative feedback regulation had a functional consequence for the level of active *FCA* expression, one would have expected the levels of transcripts β and γ to change reciprocally, i.e. if less pre-mRNA was cleaved and polyadenylated at the promoter proximal site, more pre-mRNA should be available for processing at the distal site. One explanation for this apparent discrepancy is that *FCA* transcripts in *fca-1* mutant plants might be subject to nonsense-mediated decay (Hentze and Kulozik, 1999) due to the introduction of a PTC within exon 13 in *fca-1* (Macknight *et al*., 1997). The transcripts α, γ and δ would all carry this PTC, but *FCA*-β would not. Equal amounts of β can be detected in *fca-1* mutant and wild-type plants overexpressing *FCA* from the *35S::FCA*-γ transgene (see Figure 2C). However, when all the *FCA* transcripts are quantified, ~35% less *FCA* RNA can be detected in *fca-1* compared with wild type (Figure 6C). This supports the idea that the steady-state levels of the γ transcript in *fca-1* mutant plants are lower than might be expected because of the susceptibility of this transcript to nonsense-mediated decay. Therefore, in *fca-1* the expected reciprocal increase in γ levels may be effectively masked by the effect that the PTC has on RNA accumulation.

To test whether the removal of *FCA* autoregulation would result in elevated levels of the γ transcript in another way, we examined the processing of *FCA* intron 3 in

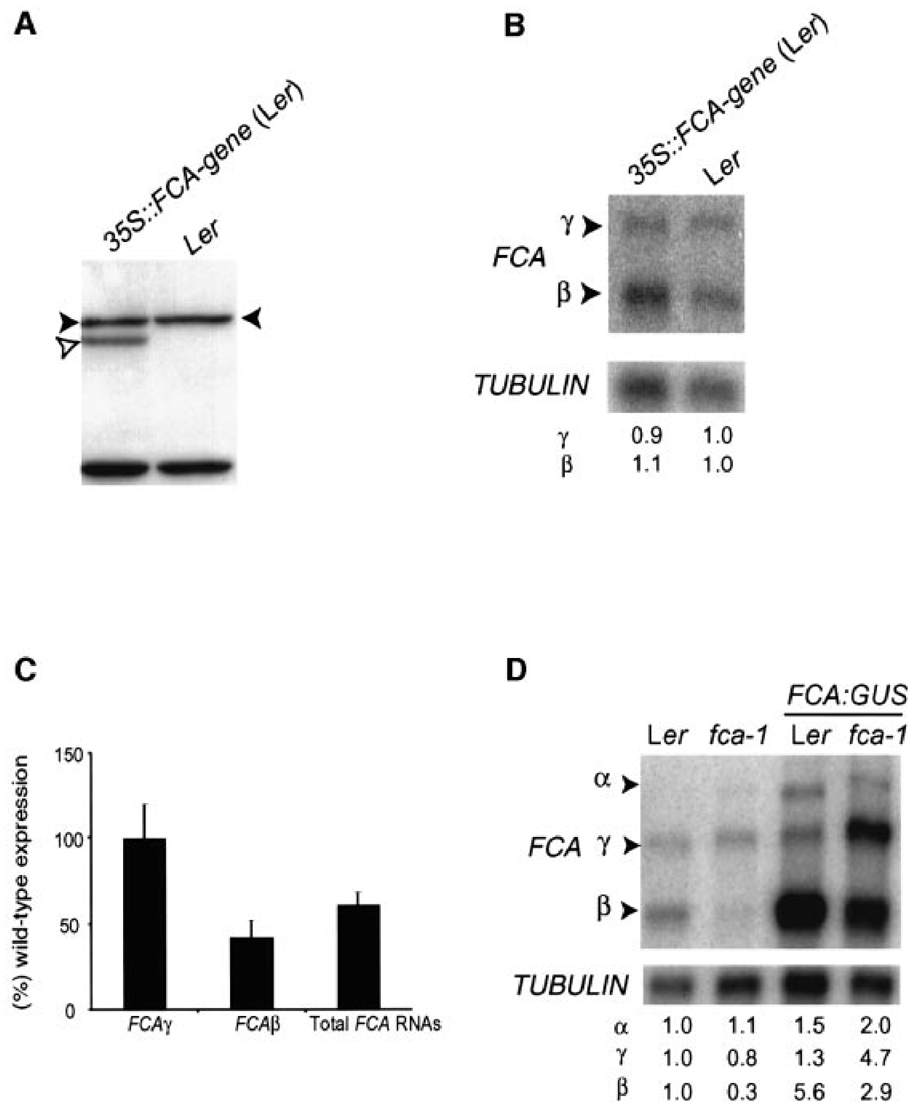

Fig. 6. *FCA* autoregulation controls the level of functional transcript γ. (**A**) Immunodetection of FCA protein. Western blot analysis of total soluble protein extracts from seedlings of L*er* and the transgenic line *35S::FCA*-gene (a *35S* fusion to the *FCA* gene) in L*er*. Filled and unfilled arrowheads denote endogenous and transgenic FCA proteins, respectively. Lower bands are unspecific binding products used as loading controls. (**B**) Northern blot analysis of poly(A)$^+$ RNA isolated from L*er* and the transgenic line *35S::FCA*-gene in L*er* [the same transgene as in (A)]. The blot was probed as indicated in Figure 2C. Numbers denote relative expression and were calculated as in Figure 2C. (**C**) Quantification of the *FCA* transcripts levels in the *fca-1* mutant. Data are the average of six different experiments and the *FCA* transcript signals obtained by northern analysis were normalized against that for β-*TUBULIN*. (**D**) Northern blot analysis of poly(A)$^+$ RNA isolated from L*er*, *fca-1*, and the transgenic lines $P_{FCA}::FCA_{to\ exon\ 5}:GUS$ in L*er* and $P_{FCA}::FCA_{to\ exon\ 5}:GUS$ in *fca-1*. The blot was probed as indicated in Figure 2C. Numbers denote relative expression and were calculated as in Figure 2C.

transgenic plants stably expressing an *FCA:GUS* reporter gene fusion ($P_{FCA}::FCA_{to\ exon\ 5}:GUS$) (Macknight *et al.*, 2002). We monitored the effects of loss of *FCA* function on the processing of *FCA* intron 3 *in vivo* by comparing the profile of transcripts expressed from this transgene in wild-type and *fca-1* backgrounds. As shown in Figure 6D, there is indeed a corresponding shift in the accumulation of β and γ transcripts between wild type and *fca-1*: less *FCA*-β, but more *FCA:GUS*-γ accumulates in *fca-1* compared with wild type.

We conclude that the negative regulation in wild-type plants will cause changes in abundance of the active *FCA*-γ transcript, and therefore is likely to have a functional consequence for the control of flowering time.

Autoregulation of FCA expression controls the temporal and spatial distribution of active FCA

The processing of *FCA* intron 3 limits *FCA* expression in a temporal and spatial dependent manner, restricting elevated levels of γ expression to proliferating cells and to a level that was undetectable (by histochemistry) until 4–6 days after germination (Macknight *et al.*, 2002). To assess the *in vivo* relevance of negative feedback regulation on this pattern, the expression of the $P_{FCA}::FCA_{to\ exon\ 5}:GUS$ transgene was compared histochemically in *fca-1* and wild-type backgrounds. As we had reported previously (Figure 7A–D; Macknight *et al.*, 2002), GUS activity (and by extension, intron 3 excision) cannot be detected until 4–6 days after germination in a

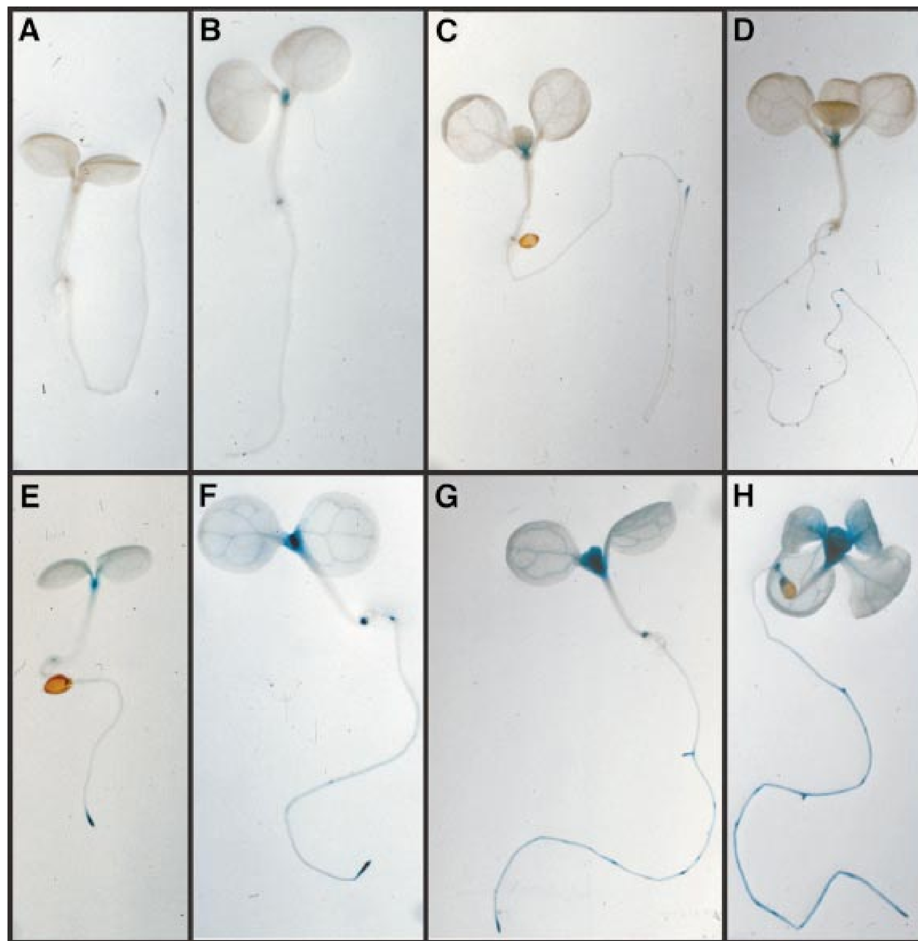

Fig. 7. Histochemical assay for GUS activity in seedlings expressing P_{FCA}::$FCA_{to\ exon\ 5}$:*GUS* in a L*er* or *fca-1* background. (**A–D**) P_{FCA}::$FCA_{to\ exon\ 5}$:*GUS* (L*er*) at 2 (A), 4 (B), 6 (C) and 10 (D) days after germination. (**E–H**) P_{FCA}::$FCA_{to\ exon\ 5}$:*GUS* (*fca-1*) at 2 (E), 4 (F), 6 (G) and 10 (H) days after germination. All panels show seedlings incubated with X-Gluc for 16 h.

wild-type background. When GUS activity is detected, it is limited to regions of active cell proliferation in the shoot and root apices (Figure 7B–D). However, in *fca-1*, GUS activity is detected earlier (2 days after germination) and in a much broader distribution (Figure 7E–H); i.e. vasculature of cotyledons, shoot apex, emerging leaf primordia, main and lateral roots and in developed leaves. This pattern more closely resembles the expression of *FCA* promoter:*GUS* fusions (Macknight *et al*., 2002). We interpret these data to show that *FCA* is normally constitutively transcribed, but the negative feedback maintains γ at a low level throughout much of the plant. This autoregulation, however, is either less efficient or does not function in meristematic cells at a specific time in development.

We therefore conclude that the autoregulation of *FCA* expression at the level of intron 3 processing governs the level of active *FCA* expression in a temporally and spatially dependent manner.

High overexpression of FCA perturbs Arabidopsis development

The *35S::FCA-*γ transgenic line described above carried a fusion between the CaMV 35S promoter and the *FCA* cDNA (Macknight *et al*., 2002). Except for flowering earlier, these plants had a similar phenotype to the wild-type L*er*. We had previously also generated lines carrying a fusion of the CaMV 35S promoter, chlorophyll a/b binding gene 22L 5′ untranslated leader and the *FCA* cDNA (*35S*::*cab-FCA-*γ), and these lines showed much higher levels of FCA protein than *35S::FCA-*γ (Macknight *et al*., 2002). The highest expressing *35S::cab-FCA-*γ lines showed additional phenotypes apart from early flowering (Figure 8) that ranged from small stature (Figure 8C) and premature senescence of rosette leaves (Figure 8D) to arrested development, epinasty and yellowing of the leaves, premature senescence and death (Figure 8F and G). The occurrence of these phenotypes correlated with the level of FCA protein (data not shown). These deleterious effects of *FCA* overexpression might therefore account for the fine control of *FCA* expression levels and the evolution of an autoregulatory mechanism.

FCA autoregulation prevents precocious flowering

A second rationale for establishment and maintenance of *FCA* negative autoregulation might be to avoid precocious flowering by controlling *FLC* expression. *FCA* functions in parallel to the vernalization pathway to down-regulate RNA levels of the floral repressor *FLC*. *FRI* acts antagonistically to these pathways to up-regulate *FLC* expression levels (Simpson and Dean, 2002). To investigate the relevance of the *FCA* negative feedback control

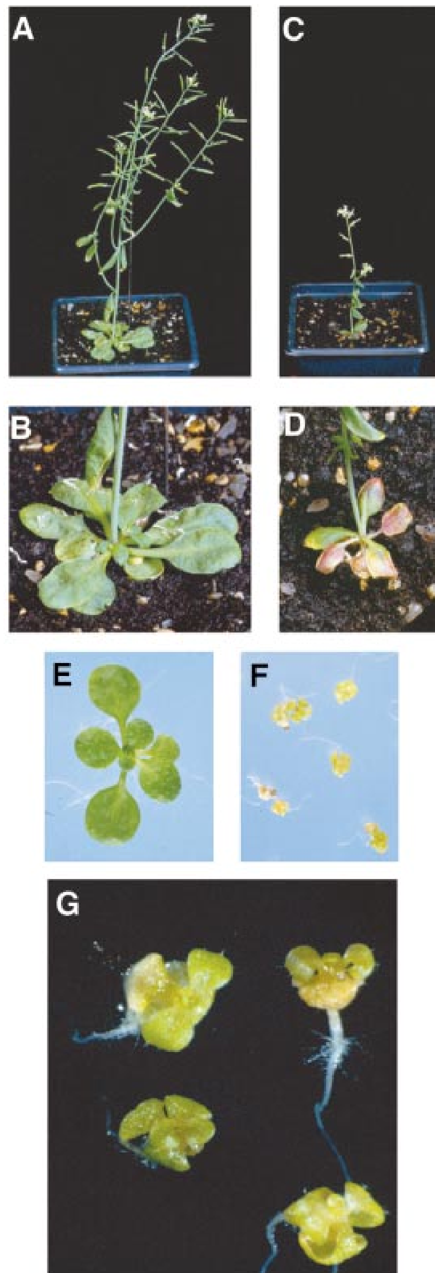

Fig. 8. High overexpression of FCA is detrimental for *Arabidopsis* development. (**A**) Wild-type L*er* plants grown in long day photoperiod (16 h light) and (**B**) close-up of rosette leaves. (**C**) A transgenic plant expressing the *35S::cab-FCA-γ* transgene, grown under the same conditions as the wild-type plant shown in (A). (**D**) A close-up of the rosette leaves of the plants shown in (C). (**E**) Wild-type L*er* seedlings. (**F**) *35S::cab-FCA-γ* plants that gave a more severe phenotype, shown at the same magnification as (E). (**G**) The *35S::cab-FCA-γ* plants that gave a more severe phenotype, shown at a higher magnification.

on flowering time, we introgressed the intronless P_{FCA}*::FCA-γ* transgene with expression driven by the native *FCA* promoter, as described above, into L*er* plants carrying an active *FRI* allele (introgressed from the SF-2 accession). L*er* plants with a functional *FRI* allele flower later than L*er* plants without active *FRI* (Table I). However, the presence of the *FCA-γ* transgene overcomes *FRI* epistasis and plants flower earlier (Table I). The expression of *FCA* is unaffected in an active *FRI*

Table I. Flowering time, measured as leaf number, of lines carrying a *FCA-γ* transgene

Construct	Background	Line	Leaf no. in long days[a]
	L*er*		8.6 ± 0.1
FCA-γ	L*er*	*FCA-γ-20-2*	8.1 ± 0.2
	FRI (SF-2)		21.8 ± 0.3
FCA-γ	*FRI* (SF-2)	*FCA-γ-20-2*	13.5 ± 0.3

[a]Errors indicate ±SE.

background (G.G.Simpson, R.Macknight and C.Dean, in preparation) and thus this distinction is not related to *FRI* controlling the processing of *FCA* pre-mRNA. The acceleration of flowering by the P_{FCA}*::FCA-γ* transgene in a L*er* background is relatively modest, since *FLC* mRNA levels are already low and its role in repressing flowering is minor. However, in winter annuals with active *FRI* alleles, the dramatic up-regulation of *FLC* mRNA constitutes the major activity repressing flowering. Therefore, we conclude that removal of the capacity for *FCA* autoregulation results in an increase in FCA activity that can perturb the balance of the pathways controlling *FLC* expression, and hence flowering time.

Discussion

In this report we show that *FCA* negative autoregulation results from FCA ultimately promoting premature cleavage and polyadenylation of its own pre-mRNA. This negative feedback functions to limit the level of active *FCA-γ* expression in a temporally and spatially dependent manner. We demonstrate that *FCA* autoregulation has functional consequences for *Arabidopsis* development, since it limits active *FCA-γ* expression, which is limiting for the floral transition, and it prevents increased expression of *FCA-γ*, which can be deleterious to *Arabidopsis* viability. Increased levels of *FCA-γ* alters the balance of pathways controlling flowering time leading to precocious flowering. Our analysis provides insight into a novel example of post-transcriptional autoregulation, which has important consequences for the life-cycle of *Arabidopsis*.

This negative regulation provides an explanation for our previous failure to overexpress FCA-γ protein from intron-containing transgenes (Macknight *et al*., 1997, 2002): When FCA-γ protein reaches a threshold level it will ultimately promote proximal poly(A) site selection within intron 3 of its own pre-mRNA, and therefore prevent the further accumulation of γ transcript. Consistent with this, we had previously found a dramatic increase in the accumulation of *FCA-β*, but only a modest increase in γ levels, when the *FCA* gene was overexpressed with the CaMV 35S promoter (Macknight *et al*., 1997). Consequently, it is only when the *cis* element within intron 3 required for this negative feedback is removed that active *FCA-γ* transcript can be overexpressed. FCA is a highly conserved plant protein and the alternative processing of its pre-mRNA is also conserved (Macknight *et al*., 2002). The relatively large size of intron 3 is conserved in *FCA* from other plants and transcripts equivalent to *FCA-β* and -γ can be detected in all plant species that we have examined (Macknight *et al*., 2002; unpublished data). This suggests that attempts to

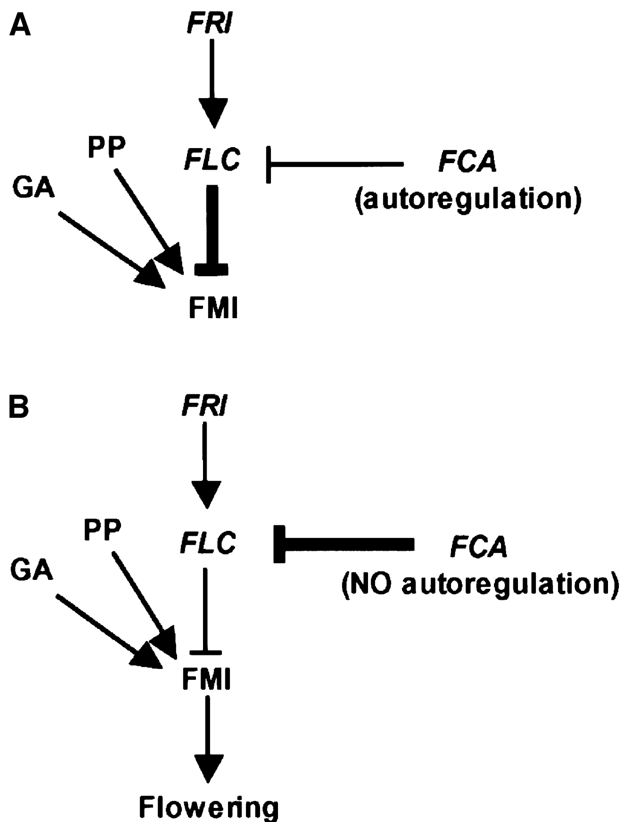

Fig. 9. Model of the interplay of flowering time control pathways. (**A**) *FRI* represses flowering in the absence of vernalization by up-regulating the floral repressor, *FLC*, which antagonizes the promotive effects of the photoperiod (PP) and gibberellin (GA) signal transduction pathways on the activation of floral meristem identity genes (*FMI*). (**B**) When *FCA* autoregulation is removed, increased *FCA* activity perturbs the quantitative interaction of these pathways. Increased FCA activity down-regulates *FLC*, enabling the *FMI* genes to respond to the promotive pathways and accelerate flowering. Promotive activities are denoted by arrowheads, repressive activities are denoted by T-bars.

manipulate flowering time in other plants by the over-expression of *FCA* may also require the removal of intron 3 from expression constructs.

The processing of *FCA* intron 3 limits *FCA*-γ expression in a temporal and spatial dependent manner (Macknight *et al.*, 2002). The production of elevated levels of transcript γ is restricted to regions of active cell proliferation such as the shoot and root apices, and this up-regulation cannot be detected until 4–6 days after germination. Our analysis here indicates that *FCA* is present and functional in regulating its own expression before then. This has implications for understanding how FCA regulates *FLC* levels, as it suggests that *FCA* might also regulate *FLC* expression throughout the plant and not just within the meristematic regions. Alternatively, the localized elevated level of *FCA* expression seen in proliferating apical cells at 4–6 days after germination may be associated with the timed down-regulation of *FLC* expression. Since elevated levels of *FLC* expression can be detected by northern analysis of *fca-1* seedlings at 3 days after germination (Gendall *et al.*, 2001), the localized up-regulation of *FCA* expression does not seem to be a prerequisite for its ability to regulate *FLC*. In order to distinguish between these possibilities we are now examining the expression of *FLC:GUS* reporters in these backgrounds in detail.

That transcript γ accumulates in regions of active cell proliferation at a specific time in development indicates that *FCA* negative feedback is less effective in these cells at this time. Since these regions are actively dividing prior to this localized upregulation of *FCA* expression, this distinction cannot be correlated with cell proliferation alone. Notably, we have found that the expression pattern of the P_{FCA}*::FCA*$_{to\ exon\ 5}$*:GUS* transgene is unaffected when combined with *35S::FCA*-γ (data not shown). This indicates that these regions are resistant to negative feedback of *FCA* expression. This may be due to an increased activity of splicing factor(s) in these cells promoting intron 3 excision or a reduction in polyadenylation factor(s) required to carry out negative feedback. It has previously been shown that cell-specific changes in the expression level of constitutive RNA processing factors such as the essential polyadenylation factor, CstF64, can affect poly(A) site selection and alternative splicing of pre-mRNAs (Takagaki *et al.*, 1996). *FCA*-β and -α appear to be non-functional by-products of the negative regulation of *FCA* expression by proximal poly(A) site selection. Since negative regulation by proximal poly(A) site usage is not effective in apical meristems, then the alternative splicing that produces *FCA*-δ (which is also non-functional in flowering time control; Macknight *et al.*, 2002) may have evolved as another means to negatively regulate *FCA* expression in these cells.

To begin to address the molecular basis of FCA mediated negative feedback we showed that cleavage and polyadenylation of intron 3 is ultimately promoted by FCA and is not an indirect effect resulting from FCA blocking the use of the distal poly(A) site. Given that FCA can bind RNA *in vitro* (Macknight *et al.*, 1997), it is possible that FCA protein binds to its own pre-mRNA to control this processing directly. Consistent with this possibility and the fact that pre-mRNA processing is a nuclear event, we found that FCA is localized to the nucleus. Since an intact FCA WW protein interaction domain is required for this regulation, it is possible that FCA interacts with factors that either inhibit splicing of intron 3 or promote proximal poly(A) site usage more directly. Alternative polyadenylation can be intimately connected to alternative splicing as the analysis of mammalian calcitonin/CGRP and immunoglobulin transcripts has revealed (for a review, see Zhao *et al.*, 1999). The processing choice in *FCA* most closely resembles that found in IgM heavy chain transcripts, where two poly(A) sites, each forming part of a different 3′ terminal exon, are present on the primary transcript. The competing processing sites that define the splicing and polyadenylation signals are suboptimal, and it has been proposed that small changes in the efficiency of either the splicing or polyadenylation reactions could tip the balance in favour of one of the processing reactions. Consistent with this, changes in the abundance of either splicing or polyadenylation factors can alter the efficiency of these respective processing events (Zhao *et al.*, 1999). It is possible that a different mechanism is involved in the regulation of *FCA*. Since we found no increase in the transcript α (from which intron 3 is not processed) when *FCA* was overexpressed, and indeed found elevated levels

of unspliced intron 3 in loss-of-function *fca* backgrounds, it seems more likely that FCA promotes poly(A) site selection rather than inhibits splicing. Consistent with this, we have recently found that FCA interacts with a conserved 3′ end processing factor, Pfs2p/FY, through its WW domain and that this interaction is genetically required for *FCA* autoregulation (Simpson *et al*., 2003). Clearly, it will be interesting to dissect this process further at the molecular level.

In other cases of post-transcriptional autoregulation of gene expression by RNA binding proteins the mechanism involved is closely related to the normal function these proteins perform. For example the splicing factors *Drosophila tra-2* (McGuffin *et al*., 1998) and *Arabidopsis* atSRp30 (Lopato *et al*., 1999) autoregulate their expression by controlling splice-site selection of their own pre-mRNAs. However, not all autoregulation of RNA-binding protein expression occurs by a mechanism related to their normal function. For example, the mammalian splicing factor, U1A, autoregulates its expression by inhibiting polyadenylation of its own pre-mRNA (Boelens *et al*., 1993; Gunderson *et al*., 1994). Nevertheless, these findings at least raise the possibility that *FCA* functions in the control of flowering time through the control of 3′ end formation. Northern analysis has not yet revealed a significant reciprocal accumulation of full-length *FLC* and truncated *FLC* transcripts in wild-type and mutant *fca* plants, but we do not yet know whether the regulation of *FLC* by FCA is direct. In addition, since transcripts that are polyadenylated upstream of an in-frame stop codon are targeted for degradation (Frischmeyer *et al*., 2002), regulated *FLC* transcripts may simply not accumulate to detectable levels.

Removal of *FCA* negative autoregulation changed the interaction of the autonomous and *FRI* pathways. The antagonistic functions of *FCA* and *FRI* determine the levels of *FLC* mRNA in non-vernalized plants (Figure 9). Since flowering time is directly correlated with *FLC* levels, the balance of *FRI*/*FCA* activities is key to ensuring the correct timing of flowering. In wild-type winter annuals, *FRI* is epistatic to *FCA* function so *FRI* activity maintains high *FLC* mRNA levels, thus preventing flowering and causing plants to over-winter vegetatively (Figure 9A). Vernalization overcomes the *FRI* up-regulation of *FLC* resulting in flowering in spring. A change in epistasis between *FRI* and *FCA*, through removal of the negative autoregulation of *FCA*, changes the reproductive strategy of the plant, causing a shift from winter annual to rapid-cycling habit, namely flowering without the need for vernalization (Figure 9B). A winter annual habit is considered to be an adaptation to growth in different climates, for example being advantageous in localities with moderate winters and/or hot, dry summers. A requirement for this habit would therefore act as a strong selection for maintenance of *FCA* negative autoregulation. The main control of *FLC* expression in rapid-cycling accessions carrying loss-of-function *fri* alleles is the autonomous pathway. These accessions are adapted to environments where rapid flowering is beneficial. It is clear that mutations that affect the threshold at which FCA will autoregulate its expression will affect the available level of active FCA and hence the level of *FLC* mRNA. Natural variation in this autoregulation could therefore provide a mechanism by which *FLC* levels are controlled to facilitate adaptation to environment through regulated flowering time. The analysis of *FCA* autoregulation in natural *Arabidopsis* accessions varying in flowering time should be informative with respect to these selective forces. One rationale for characterizing the regulation of the autonomous pathway was to understand the molecular basis of cues that regulate flowering. This analysis reveals that the level of FCA limits flowering time in a manner effectively pre-set by FCA itself. This may constitute the molecular basis of an endogenous cue controlling flowering time. However, it will be interesting to investigate the influence of environmental cues on this regulation.

We have discovered that the major mechanism controlling *FCA* expression is post-transcriptional autoregulation at the level of pre-mRNA processing, possibly through alternative polyadenylation. Although the consequences of alternative splicing and alternative polyadenylation on gene expression can be similar, we know relatively little about the mechanisms that underpin alternative polyadenylation. We have recently identified a mutant defective in *FCA* autoregulation at the level of poly(A) site choice. Therefore, in addition to unravelling the complexities of flowering time control, our characterization of the regulation of *FCA* expression may also provide a new genetically tractable means to study the molecular basis of alternative polyadenylation.

Materials and methods

Plant material and growth conditions

The mutants *fca-1* and *fca-4* were provided by M.Koornneef (Koornneef *et al*., 1991). The *35S::FCA-gene* and the *35S::FCA-γ, 35S::FCA-δ, 35::cab-FCA-γ*, P_{FCA}*::FCA-γ*, P_{FCA}*::*$FCA_{to\ exon\ 5}$*:GUS* transgenic lines were as described in Macknight *et al*. (1997) and Macknight *et al*. (2002), respectively. *Arabidopsis* seeds were sown aseptically in Petri dishes containing GM medium as described in Macknight *et al*. (2002), stratified for 2 days at 4°C and planted in soil (mixture of Levingtons M3 compost with grit) at the four-leaf stage. Plants were grown in controlled environment rooms at 20°C under the conditions described in Macknight *et al*. (2002). Flowering time was measured by counting the number of rosette leaves at flowering.

RNA analysis

Three to 4 g of plant tissue from 2-week-old seedlings were ground in liquid nitrogen, and homogenized in NTES buffer (100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, 1% SDS). Total RNA was phenol:chloroform:isoamyl alcohol (25:24:1) (7.5:5 v/v) extracted, LiAcO and ethanol precipitated and resuspended in 200 µl of DEPC-treated water. Poly(A)$^+$ RNA was isolated from ~400 µg of total RNA using oligo(dT) and streptavidin paramagnetic particles (Promega). Poly(A)$^+$ RNA was ethanol precipitated, resuspended in 20 µl of DEPC-treated water and fractionated in formaldehyde–agarose containing gels. RNA was transferred to Hybond N$^+$ nylon filters (Amersham) by conventional northern blotting.

The 5′ leader *FCA* probe was a 284 bp PCR-amplified DNA fragment (primers: forward 5′-AATTCATCATCTTCGATACTCG-3′ and reverse 5′-TTGCTAGGGCTGCTTCCACG-3′), corresponding to nucleotides 22–306 of *FCA* DNA. To normalize loading, membranes were stripped in boiling 0.5% SDS and rehybridized with a β-*TUBULIN* coding region probe (Snustad *et al*., 1992). Blots were exposed to PhosphorImager screens (Molecular Dynamics).

Immunodetection of FCA protein

To isolate proteins, 2-week-old *Arabidopsis* seedlings were frozen in liquid nitrogen and homogenized in 1 ml Trizol (Gibco-BRL). Protein pellets were washed in guanidine hydrochloride (95% EtOH), EtOH 100% and resuspended in 100 µl of (8 M urea, 40 mM Tris, pH 6.8,

0.1 mM EDTA, 1% SDS) buffer. Approximately 20 µg of protein from each sample were resuspended in 2 vol. of SDS loading buffer (62.5 mM Tris, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 5% v/v 2-β-mercaptoethanol, 0.05% w/v bromophenol blue). The samples were boiled for 5 min and the insoluble material was pelleted by centrifugation. The supernatant was then separated on a denaturing 8% polyacrylamide gel and blotted onto Immobilon-PVDF membrane (Millipore). FCA polyclonal antibody (KL4) serum was used at a dilution of 1:1500 (v/v). The immunoreactive proteins were visualized using Pierce picosignal reagents, with the secondary antibody diluted 1:2000 (v/v), and by exposure to X-ray film (Amersham Hyperfilm) for 10 s to 5 min.

Transient subcellular localization

FCA:GUS fusion from $P_{FCA}::FCA_{to\ TGA}:GUS$ (Macknight *et al*., 2002) was transferred to pMF6 by digestion with *Sal*I and *Kpn*I and cloning into pMF6 cut with *Xho*I and *Kpn*I. Transient transfections of onion epidermal cells were performed as described previously (Varagona *et al*., 1992), except 10 µg of DNA was used to coat 2 mg of 1.6 µm gold particles. Nuclei were counter-stained for 10 min with 10 mg/ml DAPI.

Determination of GUS activity in transgenic lines

Histochemical GUS staining of transgenic *Arabidopsis* plants was performed as described in Jefferson *et al*. (1987). Seedlings grown in Petri dishes on GM medium were harvested from the plates at different time points and placed directly in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). Samples were incubated at 37°C overnight.

Molecular determination of the presence of fca-1 and fca-4 alleles and $P_{FCA}::FCA_{to\ exon\ 5}:GUS$ transgene

The *fca-1* allele was detected by PCR using the primers GSO344 (5′-AACCTCTTCACAGTCCACAGGG) and GSO345 (5′-TTGGCCGTA-GATTAATGTTCAAAGG). After amplification, PCR products were digested with *Mse*I (NEBL), which only cuts in the mutant allele producing 158 and 30 bp fragments.

To detect the *fca-4* allele we used primers flanking the breakpoint in this allele. We designed a primer specific for the AT4g1550 gene (GS0505, 5′-GGTAGCAGCTTTATACAGTATGC-3′) and combined it with an *FCA*-specific primer (Fw4, 5′-ATGAGTTATCTTGCCC-ATAAC-3′). A PCR product of the expected size (210 bp) was obtained only from *fca-4* DNA.

To verify the presence of the $P_{FCA}::FCA_{to\ exon\ 5}:GUS$ transgene, PCR amplifications were performed using *FCA* intron 3- (Fw3, 5′-GTT-GAGTAGCTCTTATGTCTG-3′ and Fw4, 5′-ATGAGTTATCTTG-CCCATAAC-3′) and GUS- (318, 5′-CACCAACGCTGATCAATTCC-3′)] specific primers. PCR fragments of the expected size, 1424 (Fw3 and 318) and 731 bp (Fw4 and 318) were obtained.

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