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Functional Significance of the Alternative Transcript Processing of the Arabidopsis Floral Promoter *FCA*

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The Arabidopsis gene *FCA* encodes an RNA binding protein that functions to promote the floral transition. The *FCA* transcript is alternatively processed to yield four transcripts, the most abundant of which is polyadenylated within intron 3. We have analyzed the role of the alternative processing on the floral transition. The introduction of *FCA* intronless transgenes resulted in increased FCA protein levels and accelerated flowering, but no role in flowering was found for products of the shorter transcripts. The consequences of the alternative processing on the *FCA* expression pattern were determined using a series of translational *FCA*–β-glucuronidase fusions. The inclusion of *FCA* genomic sequence containing the alternatively processed intron 3 restricted the expression of the transgene predominantly to shoot and root apices and young flower buds. Expression of this fusion also was delayed developmentally. Therefore, the alternative processing of the *FCA* transcript limits, both spatially and temporally, the amount of functional FCA protein. Expression in roots prompted an analysis of root development, which indicated that FCA functions more generally than in the control of the floral transition.

INTRODUCTION

The transition to flowering in Arabidopsis is regulated by multiple environmental and developmental cues. Genetic pathways mediating the response to these cues have been defined. These pathways, composed of both floral promoters and repressors, redundantly activate sets of genes that are necessary to form a floral meristem (reviewed by Levy and Dean, 1998; Simpson et al., 1999; Colasanti and Sundaresan, 2000; Samach and Coupland, 2000). The major floral repressors *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) have been characterized through the analysis of naturally occurring late-flowering accessions. The genes promoting the floral transition, components of the autonomous, photoperiod, vernalization, and gibberellin floral pathways, were identified chiefly through the analysis of late-flowering mutants.

Three floral pathways determine whether a plant requires a long period of cold temperature for flowering (vernalization requirement) and how flowering is accelerated by cold temperature (vernalization response). *FRI*-mediated repression confers a dominant vernalization requirement by increasing RNA levels of the floral repressor *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999, 2000). Vernalization acts antagonistically to *FRI* by reducing *FLC* RNA levels (Michaels and Amasino, 1999; Sheldon et al., 1999, 2000). Recessive mutations in genes of the autonomous promotion pathway (e.g., in the *FCA* gene) cause an increase in *FLC* RNA levels and a late-flowering phenotype that can be rescued by vernalization. Therefore, the autonomous promotion pathway is considered to act in parallel with vernalization.

To further understand the molecular mechanisms involved in these floral pathways, we have been analyzing *FCA* as one component of the autonomous floral pathway (Macknight et al., 1997). *FCA* encodes a protein containing two RNA-recognition motifs and a WW protein interaction domain. The protein binds U- and G-ribohomopolymers in vitro, a property consistent with it functioning in vivo as an RNA binding protein. The *FCA* transcript is alternatively processed at two positions, resulting in four transcripts: α, β, γ, and δ (Figure 1). Differential processing of intron 3 yields three different transcripts: intron 3 remains in the transcript in transcript α; premature cleavage and polyadenylation within intron 3 yields transcript β; and excision of intron 3 yields transcript γ.

Alternative splicing occurs at intron 13, with a larger intron being excised to form transcript δ. The splice sites used for the alternative intron 13 splicing in transcript δ are not defined fully because they lie within a six-nucleotide direct

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repeat, but none of the possible combinations fits a consensus derived for either U2- or U12-dependent spliceosome-mediated intron excision. Transcript α accounts for <1%, transcript β accounts for 55%, transcript γ accounts for 35%, and transcript δ accounts for 10% of the *FCA* mRNA in seedlings (Macknight et al., 1997). Transcript γ is the only transcript that encodes the putative full-length FCA protein.

The relative abundance of transcripts α, β, γ, and δ, determined by RNase protection assays on total RNA samples, was found not to vary during development, within the plant, in differing environmental conditions, or in the early-flowering mutants *ap1-1* and *tfl1-2* (Macknight et al., 1997; Page et al., 1999). However, expression of the *FCA* gene from the strong 35S promoter of cauliflower mosaic virus resulted in a significant increase in transcript β but only a modest increase in transcripts γ and δ. This finding suggests that either the splicing of intron 3 requires limiting factor(s) or a feedback regulation exists that prevents too much transcript γ from being formed. Plants carrying the *35S-FCA* transgene flowered slightly but reproducibly earlier than controls, suggesting that FCA protein levels limited flowering (Macknight et al., 1997).

Here, we continue with the analysis of the regulation and role of the alternative processing of the *FCA* transcript. We have conducted a series of experiments to establish when and where the regulation of the processing occurs and whether this is functionally significant in flowering and other developmental processes.

RESULTS

Intron 3 Alternative Processing Is Conserved in *Brassica napus* and Pea

To determine if alternative processing of *FCA* intron 3 represents a general mechanism of *FCA* regulation, we analyzed the transcripts of *Brassica napus* and pea *FCA* homologs. Only two homologous copies of *FCA* are present in the *B. napus* genome, whereas the majority of Arabidopsis sequences are present at between four and six copies (Cavell et al., 1998). An *FCA* gene carried on a 12-kb *B. napus* λ clone was isolated and sequenced. The predicted structure of the *B. napus FCA* gene is very similar to that of the Arabidopsis gene. The *FCA* genes from both plants contain 21 introns (with 86.1% nucleotide sequence identity within the exons and 65.8% identity within the introns). The *B. napus* FCA γ protein shares 78% amino acid identity (87% similarity) with the Arabidopsis FCA protein and also contains two RNA binding domains and a WW protein interaction domain. We introduced the 12-kb genomic fragment containing the *B. napus FCA* gene into an Arabidopsis *fca-4* mutant. The progeny of the primary transformants segregated 3:1 (early-flowering:late-flowering plants), and all early-flowering plants carried the transgene. These plants flowered with a mean of 8.3 leaves compared with wild-type Landsberg *erecta* (L*er*) grown alongside, which flowered with 9.1 leaves, and *fca-4*, which flowered with 24.1 leaves. Thus, the *B. napus FCA* gene is a functional ortholog and must be processed correctly in Arabidopsis to produce a functional FCA protein.

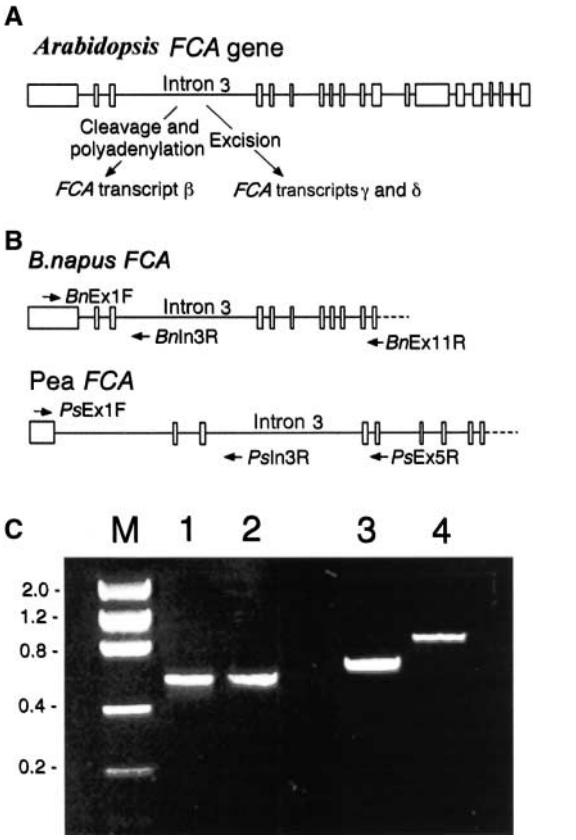

Figure 1. Intron 3 Processing in *B. napus* and Pea.

(A) Scheme of the alternative polyadenylation and splicing of the Arabidopsis *FCA* transcript. Exons are represented by open boxes. The low-abundance transcript α carries an unspliced intact intron 3.
(B) Schemes of the *B. napus* and pea *FCA* genes. Primer locations shown were used in RT-PCR experiments to amplify transcripts polyadenylated within intron 3 or with intron 3 spliced out.
(C) Ethidium bromide–stained gel showing products of the RT-PCR. Lane M, markers. Lanes 1 and 3 show products from pea RNA amplified with primers *Ps*Ex1F/*Ps*In3R and *Ps*Ex1F/*Ps*Ex5R, respectively. Lanes 2 and 4 show products from *B. napus* RNA amplified with primers *Bn*Ex1F/*Bn*In3R and *Bn*Ex1F/*Bn*Ex11R, respectively. All products were from 30 cycles of PCR. Units are in kb.

Reverse transcriptase–mediated polymerase chain reaction (RT-PCR) was used to identify *FCA* transcripts in young *B. napus* leaves. A product corresponding to either the *FCA* β or α transcript was amplified using primers to sequences within exon 1 and intron 3 and sequenced. Very low amounts of RT-PCR product were found when primers fur-

ther 3′ in intron 3 were used in combination with the exon 1 primer, suggesting that, as in Arabidopsis, transcript α has very low abundance (data not shown). To confirm that excision of intron 3 also could occur, RT-PCR was performed using primers to sequences within exon 1 and exon 11. Products were recovered and sequenced, and they were found to correspond to either the γ or δ transcript (Figure 1).

Transcript analysis also was undertaken for an *FCA* homolog isolated from pea. The pea gene shares an exon structure similar to that of the Arabidopsis and *B. napus* genes, and again, intron 3 was found to be the largest *FCA* intron (2074, 1877, and 2249 bp in Arabidopsis, *B. napus*, and pea, respectively). RT-PCR products were isolated from pea RNA, sequenced, and found to correspond to the *FCA* β/α and γ/δ transcripts (Figure 1). These experiments demonstrate that alternative processing of intron 3 is conserved in *B. napus* and pea, suggesting its functional significance. Comparison of the *FCA* intron 3 sequences from Arabidopsis, *B. napus*, and pea revealed only short (the longest being 8 bp) regions of identity. Functional analysis will be necessary to define the *cis*-acting sequences required for intron 3 polyadenylation.

Introduction of an Intronless *FCA* Transgene Results in Accelerated Flowering

To analyze the functional significance of the alternative processing, transgenic Arabidopsis plants carrying intronless *FCA* transgenes were produced. Two transgenes (called *35S-FCA-γ* and *35S-cab-FCA-γ*) were generated that contained an *FCA-γ* cDNA clone flanked by the 35S promoter and the 3′ untranslated region from the *FCA* gene with either an FCA 5′ region or the 5′ untranslated leader from the petunia chlorophyll *a/b* binding protein gene 22L (Dunsmuir, 1985). The flowering times of transformants carrying these two transgenes were very similar (data not shown), so detailed analysis was performed only for the *35S-FCA-γ* lines.

Because the only difference between *35S-FCA-γ* and *35S-FCA*-gene (described by Macknight et al., 1997) is the absence of introns, this allowed a direct analysis of the role of introns in *FCA* regulation. Flowering time was determined in long- and short-day photoperiods by counting leaf numbers of progeny homozygous for a single-locus transgene. The lines carrying *35S-FCA*-gene flowered slightly earlier than the nontransformed control when grown in short-day conditions (Table 1). In contrast, plants containing the intronless *35S-γ* transgene flowered significantly earlier in short-day conditions and slightly but consistently earlier in long-day conditions (Table 1).

To determine whether this earliness was attributable to high *FCA* expression from the use of the strong 35S promoter, we generated a number of transgenic lines containing the *FCA* promoter fused to the *FCA-γ* cDNA and the 3′ untranslated region from the *FCA* gene. The *FCA-γ* transformants flowered earlier than the wild type in long-day conditions (Table 1). In short-day conditions, flowering was accelerated significantly, with leaf number being reduced in

Table 1. Flowering Time, Measured as Leaf Number, of Lines Carrying *35S-γ*, *FCA-γ*, and *35S-FCA* Transgenes[a]

Construct	Background	Line	Leaf No. in Long Days	Leaf No. in Short Days (a)	Leaf No. in Short Days (b)
	L*er*		5.1 ± 0.1	46.3 ± 1.4	32.5 ± 0.7
	fca-1		20.9 ± 0.8[b]	>67[b]	
35S-γ	*fca-1*	*35S-γ*-1A	4.9 ± 0.1		19.8 ± 0.4[b]
35S-γ	*fca-1*	*35S-γ* 2A	4.5 ± 0.1[b]		
35S-γ	*fca-1*	*35S-γ* 4A	4.7 ± 0.1	33.8 ± 0.7[b]	19.5 ± 0.6[b]
	fca-4		16.7 ± 0.5[b]	>60[b]	
35S-FCA-gene	*fca-4*	*35S-FCA*-15	5.5 ± 0.1	41.2 ± 0.7[b]	
35S-FCA-gene	L*er*	*35S-FCA*-7	5.1 ± 0.1	45 ± 0.5	
	L*er*		6.8 ± 0.1		29.1 ± 0.7
	fca-4		17 ± 1.0[b]		
FCA-γ	L*er*	*FCA-γ*-20-2	5.3 ± 0.1[b]	37.3 ± 0.9[b]	17.1 ± 0.3[b]
	fca-4	*FCA-γ*-20-2	4.9 ± 0.1[b]		
	L*er*	*FCA-γ*-15	5.1 ± 0.1[b]		18.3 ± 0.5[b]
	fca-4	*FCA-γ*-15	5.2 ± 0.2[b]		
	L*er*	*FCA-γ*-9A	6.3 ± 0.1[b]		20.1 ± 0.9[b]
	L*er*	*FCA-γ*-17B	6.0 ± 0.1[b]		20.5 ± 0.9[b]
	L*er*	*FCA-γ*-13A	6.2 ± 0.1[b]		21.7 ± 0.5[b]
	L*er*	*FCA-γ*-20-1	6.3 ± 0.1[b]		26.2 ± 0.6[b]

[a] Two short-day conditions were used, a and b (see Methods). The late-flowering *fca* mutants grown in short-day condition a had not flowered at the end of the experiment, so a greater-than leaf number is given.

[b] Significantly different from the L*er* control at the 5% level.

Error bars indicate ±SE.

some cases to ~60% of the wild-type values (Table 1). This early flowering was a consequence of the transgene and not *FCA* gene dosage, because introgression of the *35S-γ* or *FCA-γ* transgene into an *fca-4* mutant background did not delay flowering time (Table 1).

RNA gel blot analysis was used to determine whether the γ transgenes were functioning in the same manner as wild-type *FCA*, namely, to reduce levels of the floral repressor *FLC*. *FLC* mRNA was analyzed in RNA isolated from long day–grown young seedlings and found to be low in wild-type L*er*, high in *fca-1*, and slightly lower than L*er* levels in the *FCA-γ fca-1* genotype (Figure 2).

Introns Limit FCA Protein Production

FCA protein levels in the transgenic lines were assayed using a polyclonal antibody that had been raised against an *Escherichia coli*–expressed, C-terminal FCA protein fragment extending from just after the second RNA-recognition motif to the end of the coding region. The polyclonal antibody (KL2) cross-reacts specifically with FCA, as judged by the absence of cross-hybridizing proteins in extracts from *fca-3*, an allele that produces a truncated protein that terminates before the beginning of the fragment used to produce the antibody (Figure 3B). The FCA protein produced from the *35S-FCA* gene and the *35S-cab-FCA-γ* and *35S-γ* transgenes is ~10 kD smaller than the major FCA isoform found in the L*er* and *FCA-γ* transformants. The fully complementing *35S-FCA* gene and the *35S-cab-FCA-γ* and *35S-γ* transgenes were constructed such that translation would initiate at the first Met codon of the predicted open reading frame.

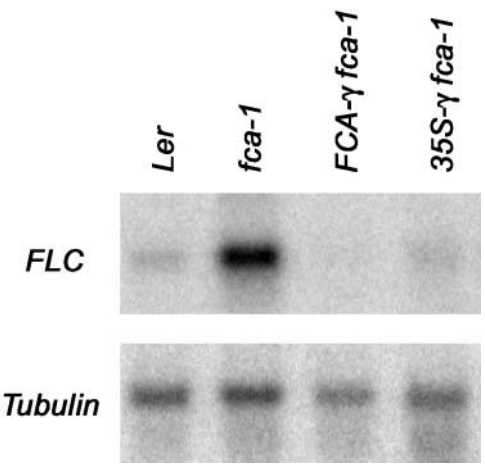

Figure 2. *FLC* RNA Levels in Plants Carrying Intronless *FCA* Transgenes.

RNA gel blot analysis of total RNA extracted from L*er*, *fca-1*, *FCA-γ fca-1*, and *35S-γ fca-1* seedlings. The blot was probed with a 403-bp *FLC*-specific probe (corresponding to nucleotides 297 to 700 of the *FLC* cDNA [Sheldon et al., 1999]). The ratio of *FLC* to *tubulin* is 1 (L*er*), 6 (*fca-1*), 0.74 (*FCA-γ fca-1*), and 0.98 (*35S-γ fca-1*).

The larger protein produced in the L*er* and *FCA-γ* transformants suggests that FCA translation in the wild-type context initiates at a non-Met codon upstream of the fusion point; we are investigating this possibility at present. Plants carrying the *35S-FCA* gene contained a small increase in FCA protein compared with the L*er* control (Figures 3A and 3C). However, a large increase was observed for the lines carrying the *35S-cab-FCA-γ* and *35S-γ* transgenes (Figures 3A and 3B). Plants expressing the *FCA-γ* transgene showed only a small increase in FCA protein (Figure 3C). *FCA-γ*-15, one of the earliest flowering *FCA-γ* lines, contained levels of FCA protein similar to those found in the *35S-FCA*-gene-15 line. Accelerated flowering therefore is associated with increased FCA protein, with small increases in FCA protein being sufficient to accelerate flowering significantly. This finding suggests that FCA levels limit flowering in a L*er* background.

Only Transcript γ Complements the *fca-1* Late-Flowering Phenotype

We further addressed the mechanism by which alternative processing limits FCA production and flowering time. It could exert its effect on flowering by limiting the production of transcript γ (with transcripts β and δ being nonfunctional by-products). Alternatively, because transcripts β and δ potentially encode protein isoforms containing partial or complete RNA binding domains, respectively, but that lack the WW protein-interaction domain, they might function antagonistically with the functional γ isoform by competing for binding of specific RNAs. We had shown previously that overexpression of transcript β did not complement the *fca-1* phenotype (Macknight et al., 1997). To assess more fully the role of the potential protein isoforms produced by the different transcripts, we tested the effects of overexpressing transcript δ in an *fca-1* background and the effects of overexpressing transcripts β and δ in a wild-type background.

35S-β and *35S-δ* transgenes were transformed into *fca-1*, and the progeny of the transformants were analyzed for flowering time. For each transgene, a single-locus line was selected for crossing into a wild-type L*er* background. Overexpression of β and δ transcripts was confirmed by RNase protection experiments, and overexpression of δ protein was confirmed by protein gel blot analysis (this could not be done for the β isoform because the antibody did not cross-react with this region of the FCA protein). Flowering time then was compared between individuals homozygous for the same transgene but in different genetic backgrounds (Figure 4). Like the *35S-β* transgene, the *35S-δ* transgene did not even partially complement the late-flowering phenotype of *fca-1*. Neither transgene delayed flowering in a L*er* background. These data show that high levels of transcripts β and δ do not interfere with wild-type *FCA* function.

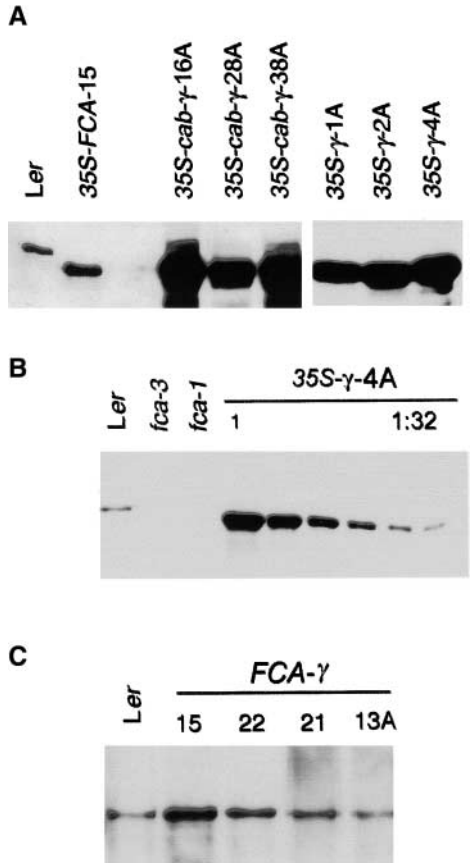

Figure 3. Immunodetection of the FCA Protein in Transgenic Lines.

(A) Protein gel blot analysis of total soluble protein extracts. From left to right are wild-type L*er* plants, a *35S-FCA* line (a 35S promoter fused to the entire *FCA* gene containing introns), independent transgenic lines carrying *35S-cab-γ* (a 35S fusion to a chlorophyll *a/b* binding gene 5′ untranslated leader and intronless *FCA* transgene), and *35S-γ* (a 35S fusion to an intronless *FCA* transgene).

(B) Relative quantitation of FCA protein levels. From left to right are wild-type L*er* plants, *fca-3* and *fca-1* seedlings, and a dilution series (twofold dilutions to 1:32) from transgenic line *35S-γ*-4A.

(C) FCA protein levels in plants carrying *FCA-γ* . Protein levels from independent transgenic lines carrying the *FCA-γ* transgene are shown. Lanes 15, 22, 21, and 13A correspond to transformants *FCA*-γ-15, *FCA*-γ-20-2, *FCA*-γ-20-1, and *FCA*-γ-13A.

The Presence of Introns 1 to 4 Influences the Expression Pattern of a β-Glucuronidase Translational Fusion

Our previous analysis of the relative abundance of the different *FCA* transcripts had used RNase protection assays on RNA extracted from seedlings of different ages, seedlings grown in different treatments, or from different parts of the plant. This kind of analysis does not provide information on the regulation of alternative processing at the cellular level. Therefore, three *FCA*–β-glucuronidase (*GUS*) translational gene fusions were constructed (Figure 5) to determine if intron processing affects the time and/or place at which the FCA protein is produced. The fusion point of the first construct, P_{FCA}–$FCA_{to\ ATG}$:*GUS*, was the third ATG codon of the *FCA* open reading frame. It carries the GUS coding sequences flanked by the same *FCA* promoter and 3′ sequences present in the complementing *FCA-γ* transgene. Thus, GUS activity would be detected in all tissues in which the *FCA* promoter is active.

The fusion point of the second construct, P_{FCA}–$FCA_{to\ exon5}$:*GUS*, was within *FCA* exon 5. For GUS activity to be detected, *FCA* introns 1 to 4 need to be spliced correctly. Given the fact that no alternative processing of introns 1, 2, and 4 has been detected in vivo, this construct was designed to monitor the alternative processing of intron 3. If the intron is excised as in transcript γ, then GUS activity would result. If cleavage and polyadenylation occur within intron 3, as in transcript β production, no GUS activity would be seen. The fusion point of the third construct was the TGA translation termination codon of the *FCA* cDNA expressed from the FCA promoter (P_{FCA}–$FCA_{to\ TGA}$:*GUS*). This construct tests the influence of *FCA* exon sequences and the removal of all intron sequences on the expression and pattern of GUS activity.

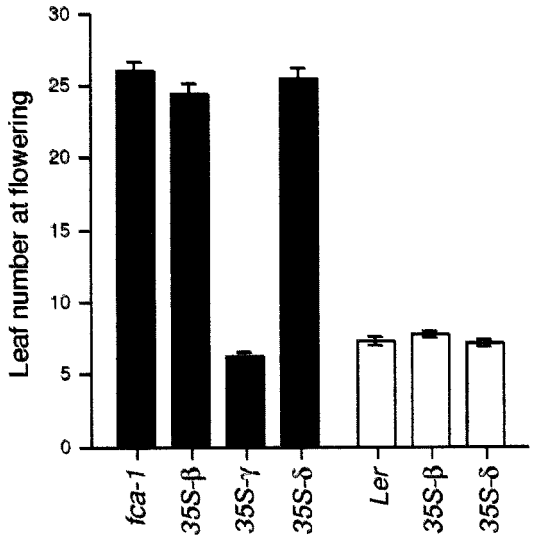

Figure 4. Flowering Time, Assayed as Leaf Number at Flowering, of Transgenic Lines Carrying the *35S-β*, *35S-γ*, and *35S-δ* Fusions.

For each transgene, single-locus lines showing a representative flowering response were introgressed from *fca-1* into a L*er* background. Populations represent pooled progeny from three independent transformants except for *35S-β* in the L*er* background, in which progeny from only two transformants were analyzed. *fca-1* genotypes are shown as black columns, and L*er* genotypes are shown as white columns.

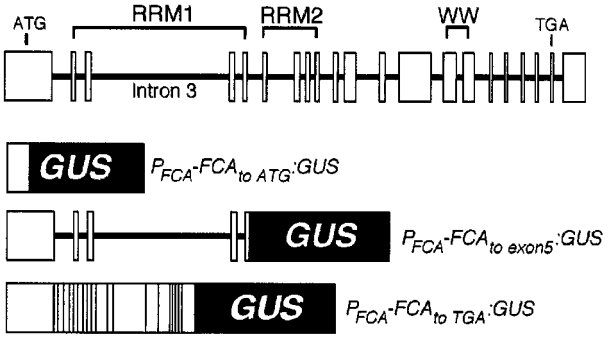

Figure 5. Scheme of the *FCA-GUS* Translational Fusions.

The 21 exons of the *FCA* gene are shown as white boxes, and the introns are shown as black lines. RRM1, RRM2, and WW represent the two RNA-recognition motifs and the WW protein interaction domain.

The pattern of GUS activity was constant between transformants carrying the same transgene, with the levels varying less than twofold. Two homozygous lines for each transgene were analyzed in detail. Representative photographs of the seedlings at 2, 4, 5, and 6 days after germination, together with close-ups of lateral roots, leaves, and flowers and cross-sections of the shoot meristem and young leaf primordia, are shown in Figure 6. The GUS activity from the P_{FCA}–$FCA_{to\ ATG}$:*GUS* transgene was high in newly emerged cotyledons, comparatively low in cotyledons 4 days after germination, and then increased progressively as the plant aged and more leaves formed. GUS activity was seen in the vasculature, main and lateral root tips, developing ovules, shoot meristem, and developing leaf primordia.

In contrast, GUS activity was not detected histochemically until several days after germination in the P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgenic lines in seed or germinating seedlings. The first detectable activity was seen 4 days after germination, when very low levels were detected in the shoot and root apical meristematic regions. By 6 days after germination, the shoot, root apices, and lateral root primordia showed high levels of GUS activity. In the shoot apex, GUS activity was confined to the meristematic region and to new leaf primordia and was below detection levels once the leaves reached >1 mm in length (Figures 6Q and 6V). No GUS activity was seen in the vasculature at any stage of development in the P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgenic lines.

The P_{FCA}–$FCA_{to\ TGA}$:*GUS* transgene showed the same pattern as P_{FCA}–$FCA_{to\ ATG}$:*GUS*, indicating that *FCA* exon sequences do not affect the GUS expression pattern (Figures 6W to 6Y). The different patterns of GUS activity from the P_{FCA}–$FCA_{to\ ATG}$:*GUS* and P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgenes indicate that although the *FCA* gene is transcribed in many parts of the plant, the distribution of *FCA* transcripts is limited by alternative transcript processing.

Next, we tested whether the sharp increase in GUS activity from the P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgene between 4 and 6 days after germination would be reflected in an increase in endogenous transcript γ at the same stage of development in both whole seedlings and seedlings from which leaves, cotyledons, and roots had been removed. Protein gel blot analysis detected full-length FCA protein in seed and seedlings assayed as early as 2 days after germination (data not shown). No significant increase was found using fluorometric quantitative analysis of GUS activity in whole seedlings or seedlings from which leaves, cotyledons, and roots had been removed. Therefore, the results from the different assays give a somewhat different picture of *FCA* regulation.

Our interpretation is that a low, basal level of intron 3 splicing occurs throughout the plant at a level too low to be detected in the GUS histochemical assay. Regulation of intron processing favoring the production of transcript γ then occurs in shoot and root meristems between 4 and 6 days after germination. The limited number of cells involved in this change in intron processing is not sufficient to cause a significant increase in the total levels of transcript γ or FCA protein throughout the plant, so it is not detected in RNA or total protein assays. In situ RNA analysis was attempted using probes specific for the γ transcript, but the level of *FCA* RNA was too low to detect (data not shown).

fca Mutations Cause Phenotypic Changes in Roots

The expression of the *FCA-GUS* fusions in the main and lateral root meristems was not expected for a gene whose function is associated with the control of flowering time. The roots of *fca* mutants were analyzed carefully to determine whether this expression was associated with a function in root development. The root length of the *fca-1* mutant was significantly shorter than that of the L*er* control after 12 days of growth in three different experiments (87 $\pm$ 1.5 mm compared with 98 $\pm$ 2.3 mm; $P < 0.001$). In addition, the number of lateral roots was lower in *fca-1*, and when expressed as lateral root number per unit (mm) of root length, to avoid the complications of shorter roots, *fca-1* was found to produce ~20% fewer lateral roots than L*er* (Table 2). The number of lateral roots per unit of root length was somewhat variable for the same genotype between experiments, but the relative differences were always observed. To ensure that this phenotype was caused by the loss of *FCA* function, the analysis was repeated with another strong mutant allele, *fca-6* (Koornneef et al., 1991).

Roots of an *fca-1* line carrying a complementing cosmid also were analyzed. The reduced lateral root phenotype was seen in both mutant *fca* alleles and was rescued by the complementing cosmid (Table 2). These data show that the phenotype is the result of the loss of *FCA* function, implicating the requirement for *FCA* in both root and shoot development. Because vernalization can rescue the late-flowering phenotype of *fca-1*, we also asked whether it could rescue

the lateral root phenotype. The roots of vernalized *fca-1* seedlings showed approximately the same root length and lateral root number per unit (mm) of root length as wild-type L*er* seedlings, significantly different from *fca-1*.

In flowering time control, *FRI* function acts antagonistically to *FCA* function and vernalization by increasing *FLC* levels. To determine if the autonomous, vernalization, and *FRI* repression pathways interact in a similar way in root development, lateral root number in *FRI*-containing plants also was analyzed. L*er* seedlings carrying an active *FRI* allele introgressed from ecotype San Feliu (Lee et al., 1994) showed reduced lateral root number per unit of root length

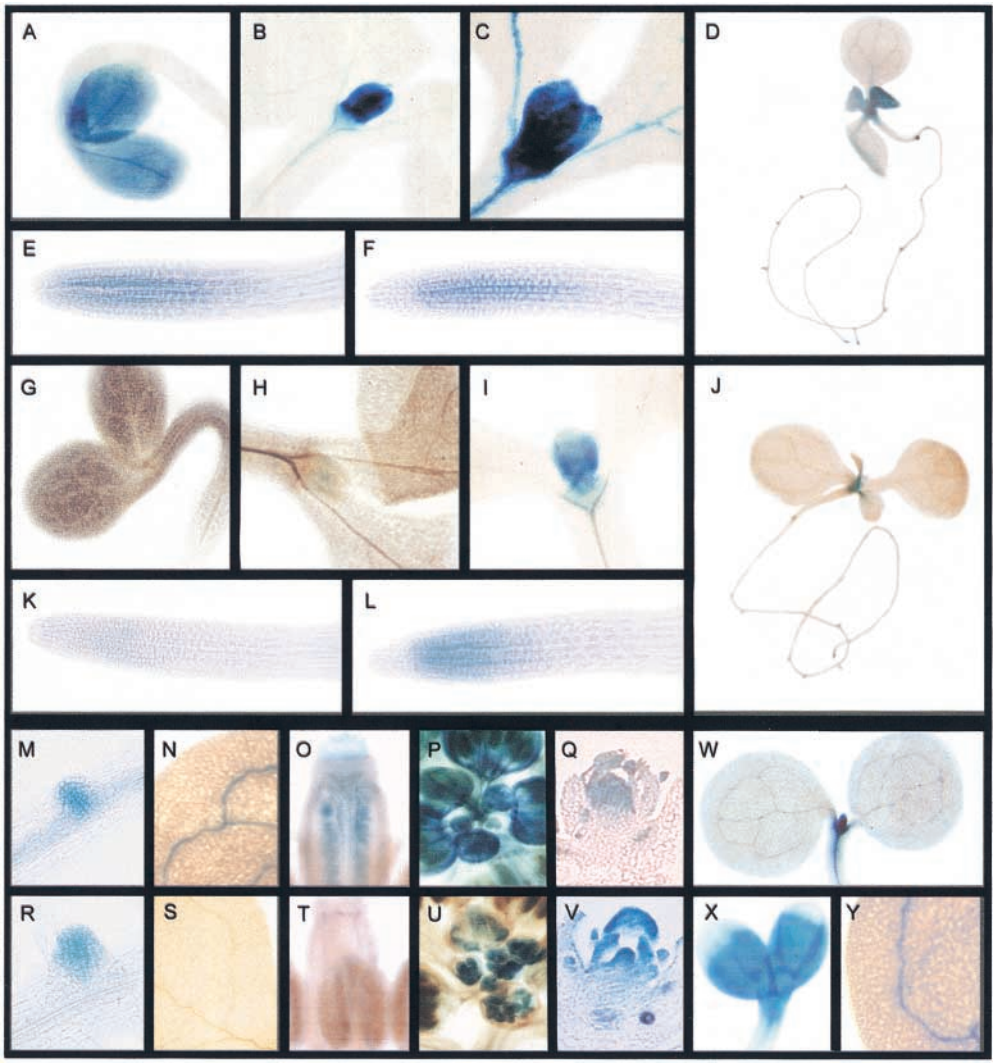

Figure 6. Histochemical Assay for GUS Activity in Seedlings Expressing P_{FCA}-$FCA_{to\ ATG}$:*GUS* and P_{FCA}-$FCA_{to\ exon5}$:*GUS*.

(A) to **(F)** P_{FCA}-$FCA_{to\ ATG}$:*GUS* at 2 **(A)**, 4 (**[B]** and **[D]**), 5 (**[C]** and **[E]**), and 6 **(F)** days after germination. **(A)** to **(C)** and **(F)** show seedlings incubated with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide for 16 hr. **(D)** and **(E)** show roots incubated for 10 hr.
(G) to **(L)** P_{FCA}-$FCA_{to\ exon5}$:*GUS* at 2 **(G)**, 4 (**[H]** and **[J]**), 5 (**[I]** and **[K]**), and 6 **(L)** days after germination. Incubation time was 16 hr.
(M) to **(Q)** P_{FCA}-$FCA_{to\ ATG}$:*GUS*. An emerging lateral root **(M)**, leaf vascular tissue **(N)**, pistil **(O)**, floral buds **(P)**, and an 8-μm section of the shoot apical meristem **(Q)** are shown. **(M)**, **(N)**, and **(Q)** show GUS activity in 10-day-old seedlings. Incubation times were 10 hr **(M)** and 16 hr (**[N]** to **[Q]**).
(R) to **(V)** P_{FCA}-$FCA_{to\ exon5}$:*GUS*. An emerging lateral root **(R)**, leaf vascular tissue **(S)**, pistil **(T)**, floral buds **(U)**, and a 15-μm section of the shoot apical meristem **(V)** are shown. **(R)**, **(S)**, and **(V)** show GUS activity in 10-day-old seedlings. Incubation time was 16 hr.
(W) to **(Y)** P_{FCA}-$FCA_{to\ TGA}$:*GUS* at 2 **(X)** and 4 (**[W]** and **[Y]**) days after germination.
All seedlings were incubated for 16 hr.

compared with wild-type L*er* seedlings (Table 2). This finding is in contrast to the results seen in *constans-2* (*co-2*), a mutant of the photoperiod promotion pathway that had a lateral root number per unit of root length 29% higher than *fca-1*, a value similar to that found in wild-type L*er*.

DISCUSSION

This study was designed to investigate the functional significance of the alternative processing of the *FCA* transcript on the control of the floral transition. Unlike the many examples in Drosophila and *Caenorhabditis elegans*, there are very few examples of developmental switches being controlled by post-transcriptional regulation in plants (Lorković et al., 2000). Our analyses suggest that *FCA* intron processing limits *FCA* expression both spatially and temporally and that this limits when the plants flower. Increased expression of the intronless transgenes and the limited expression of the P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgene also would have resulted if the intron sequences contained transcriptional silencers. This seems not to be the case, because plants containing the *35S-FCA*-plus-introns transgene showed very high levels of transcript β but not transcript γ, indicating that transcription of the transgene was high (Macknight et al., 1997). In addition, transcript β is made at wild-type levels from the P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgene, as judged by RT-PCR (data not shown). Thus, we believe that the multiple transcripts observed in wild-type plants are the result of alternative intron processing, with the ratio of transcript β and γ being determined through differential intron 3 splicing/intron 3 polyadenylation in the unprocessed transcript. Use of the intron 3 polyadenylation site would limit the production of transcript γ, which produces the isoform active in flowering time control.

A well-studied example of the regulation of gene expression through alternative pre-mRNA processing is the regulation of IgM heavy-chain synthesis during B cell differentiation (Proudfoot, 1996). In early development, a membrane-bound form of the protein is produced in pre-B and B cells through the use of a downstream polyadenylation site. Later in development, an upstream polyadenylation site is used, leading to a shorter, secreted form of the protein in plasma cells. The change in processing is complex in that the use of the downstream polyadenylation site is associated with an upstream splicing event that removes the upstream polyadenylation site. Levels of an essential polyadenylation fac-

Table 2. Lateral Root Number in Different Genotypes[a]

Genotype	Mean No. of Lateral Roots per Length of Primary Root	No. Counted	Difference Relative to *fca-1* from Each Experiment (%)
L*er*	0.444 ± 0.007	35	21
fca-1	0.367 ± 0.005[b]	63	
fca-6	0.360 ± 0.02[b]	7	−2
fca-1/*FCA* cosmid	0.451 ± 0.009[c]	37	23
fca-1/*35S-γ*	0.429 ± 0.009[c]	38	17
FRI (Sf2)	0.380 ± 0.009[b]	44	4
L*er*	0.469 ± 0.011	21	30
fca-1	0.362 ± 0.008[b]	30	
fca-1 vernalized	0.462 ± 0.014[c]	15	28
L*er*	0.484 ± 0.02	19	20
fca-1	0.405 ± 0.015[b]	24	
fca-1	0.31 ± 0.02	27	
fca-1 vernalized	0.36 ± 0.02	13	16
FRI (Sf2)	0.28 ± 0.02	13	−10
co-2	0.4 ± 0.015[c]	22	29

[a] Lateral root number per unit of root length was measured in four independent experiments on seedlings grown on square vertical agar plates 12 days after germination (just before the roots reached the bottom of the plate). The growth of the roots and the production of lateral roots were somewhat variable between experiments, but the relative differences were maintained and have been expressed as percentage difference relative to *fca-1* in each experiment.

[b] Significantly different from wild-type L*er* control ($P < 0.001$).

[c] Significantly different from *fca-1* ($P < 0.001$).

Error bars indicate ±SE.

tor (CstF-64) have been found to be lower in B cells, and this factor has been shown to play a key role in regulating which IgM form is produced (Takagaki et al., 1996).

Alternative polyadenylation site utilization has been found to control levels of the Drosophila protein Suppressor of Forked [Su(f)] in a situation that is very similar to that seen in *FCA*. Su(f) functions in the control of mRNA 3′ processing and the polyadenylation of cellular RNAs and is homologous with the CstF-77 protein of human CstF (cleavage stimulation factor). Polyadenylation within *su(f)* intron 4 leads to the production of a truncated and nonfunctional protein. A shift in polyadenylation site utilization to a site 3′ to the coding region results in the accumulation of the protein in mitotically active cells (Juge et al., 2000). In plants, the regulation of intron splicing/polyadenylation is less well understood. The regulation of *FCA* expression thus provides a good example to determine the molecular mechanisms that have evolved in plants to control pre-mRNA processing.

The alternative processing of the *FCA* transcript limits the overall level of *FCA* expression throughout the plant. Whether this regulation results in qualitative differences in expression or merely restricts expression quantitatively was analyzed carefully. Histochemical GUS staining suggested that the presence of introns 1 to 4 qualitatively regulated the pattern of expression. Despite prolonged staining, GUS expression was not detected histochemically until 4 to 5 days after germination and was never detected in the vasculature at any stage of development in lines carrying the P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgene. This compares with the fusions near the beginning of the open reading frame or over the TGA of the FCA open reading frame, both of which are expressed at much higher levels and more widely throughout development.

However, it is difficult to exclude completely the possibilities that the intron processing affects expression only quantitatively and that the observed apparent qualitative differences are the result of the threshold sensitivity of the histochemical assay. The early flowering of *35S-γ* and *FCA-γ* plants may be caused by quantitative and/or qualitative changes in *FCA* expression. The transgenes accelerated flowering and reduced *FLC* RNA levels to approximately the same extent, despite producing very different levels of FCA protein. However, the timing of the upregulation of FCA expression also was changed in the different lines. The ability to induce *FCA* function at a similar level but at different times of development may allow us to examine this issue further.

The increase in GUS expression from the P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgene, which reflects a shift of utilization from intron 3 to the 3′ untranslated region polyadenylation site and potentially generation of the functional γ transcript, occurs at a stage of development at which the floral transition would be initiated in wild-type seedlings. We now need to determine whether *FCA* intron processing is an actively regulated process aimed specifically at the downregulation of the floral repressor *FLC* at a time of development that coincides with the activities of other floral pathways. Simon et al. (1996) have shown that the induction of *CO* function by a single dexamethasone application to 8-day-old short day–grown *co* mutant seedlings carrying a *GLUCOCORTICOID RESPONSE ELEMENT* (*CO-GRE*) fusion fully activated the long day promotion floral pathway so that plants flowered at the same time as if they had been grown in long days. The similarity in the timing of the requirement of *CO* function and the upregulation of *FCA* intron 3 splicing in meristems may indicate that the activation of the different floral pathways is coordinated in vivo.

In addition to affecting expression temporally, the regulation of *FCA* intron processing results in P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgene expression being localized predominantly in regions of the plant where cells are undergoing division or have divided recently. One of the main functions of the autonomous pathway is to repress *FLC* function (Michaels and Amasino, 2001). *FLC* expression, as judged by GUS activity from an *FLC-GUS* transgene, is localized predominantly to the shoot meristematic region, developing leaf primordia, and root tips in young seedlings (Michaels and Amasino, 2000). Thus, the restricted expression of the P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgene results in a pattern of expression that overlaps that of *FLC*. Whether this indicates that *FCA* function is localized to meristematic regions is complicated by the finding that *FCA* function is not cell autonomous (Furner et al., 1996). The molecular mechanism by which FCA and FPA regulate *FLC* is undetermined, but the finding that another member of the autonomous promotion pathway, *FPA*, also encodes an RNA binding protein (Schomburg et al., 2001) suggests that downstream targets of FCA and FPA are likely to be regulated post-transcriptionally.

There are many questions regarding the molecular mechanisms that regulate *FCA* alternative transcript processing. Are there other genes that function to regulate polyadenylation site use in the *FCA* transcript, or does FCA regulate its own processing? It is possible that *FCA* intron processing is regulated via a floral-specific pathway or, alternatively, is tied into cell cycle regulation to ensure high levels of FCA as cells divide. Do environmental cues affect the regulation, or does the autonomous pathway act independently of environmental factors? Isolation of mutants altered in the ratio of intron 3 polyadenylation versus splicing should give an indication of the type of regulation that occurs. If intron 3 splicing is repressed actively, then mutations in the regulatory proteins would cause early flowering. If splicing requires a specific factor, with polyadenylation being the default pathway, mutations would be late flowering. Whatever the molecular basis of the regulation, the evolutionary conservation of intron 3 processing within members of two distantly related plant families (Fabaceae and Brassicaceae) suggests that it plays an important role in the regulation of *FCA* and flowering.

It is intriguing that all of the components of the autonomous promotion pathway analyzed to date are expressed in roots as well as shoots (Aukerman et al., 1999; Schomburg et al., 2001). The decision of the apex to undergo the transition to flowering is influenced by other tissues in a range of

plants. In maize, young leaf primordia influence the fate of the apex (Irish and Jegla, 1997), and roots are thought to produce a signal that maintains vegetative growth or prevents flowering in tobacco (McDaniel, 1996). Various aspects of root development were analyzed in *fca* mutants. Although the root phenotype of the *fca* mutants is quite subtle, a careful examination revealed significant differences, with *fca* mutations and active *FRI* alleles reducing lateral root number. This phenotype was reversed to wild-type levels by vernalization and was not observed in *co*, a mutation in the photoperiod promotion pathway.

This result shows that the changes in root development are not a secondary consequence of a delay in the floral transition. Whether the additional functions of *FCA*, *FRI*, and vernalization act via common targets such as *FLC* remains to be established. What is clear is that the autonomous promotion, *FRI*-mediated repression, and vernalization promotion pathways function more generally than at the shoot apex to control the timing of the floral transition. This may reflect the role of roots in controlling flowering or, alternatively, the fact that the autonomous promotion, *FRI*-mediated repression, and vernalization promotion pathways regulate an aspect of meristem function necessary for a range of developmental transitions that include the transition to flowering.

METHODS

Plant Material and Growth Conditions

The mutants *fca-1* and *fca-6* were provided by M. Koornneef (Wageningen University, The Netherlands) (Koornneef et al., 1991). The Landsberg *erecta* (L*er*) line containing the introgressed ecotype San Feliu 2 *FRI* gene was a gift from R. Amasino (University of Wisconsin, Madison) (Lee et al., 1994). The *35S-FCA* and *35S*-β transgenic lines were described by Macknight et al. (1997). *Arabidopsis thaliana* seed sown aseptically in Petri dishes containing GM medium (1 × Murashige and Skoog [1962] salts, 1% Glc, 0.5 mg/L pyridoxine, 0.5 mg/L nicotinic acid, 0.5 mg/L thymidine, 100 mg/L inositol, 0.5 g/L Mes, and 0.8% agar, pH 5.7) were stratified for 2 days at 4°C and planted in soil (mixture of Levingtons M3 compost [Scotts, Ipswich, UK] with grit) at the four-leaf stage. Plants were grown in controlled-environment rooms at 20°C under one of two short-day conditions: in a controlled environment room (Sanyo Gallenkamp, Loughborough, UK) with a 10-hr photoperiod from 400-W Wotan metal halide lamps supplemented with 100-W tungsten halide lamps (PAR, 114 μmol· m^{-2}·sec^{-1}; red:far red ratio, 2.4; Table 1, condition a) or in a room with a 10-hr photoperiod from a mixture of fluorescent and tungsten lights (PAR, 34.6 μmol·m^{-2}·sec^{-1}; red:far red ratio, 1.48; Table 1, condition b). Long-day conditions were the same as short-day conditions in the Sanyo Gallenkamp room except for the extension of the photoperiod by 6 hr using only the tungsten halide lamps (red:far red ratio, 0.66). Flowering time was measured by counting the number of rosette leaves at flowering. Plants were vernalized for 6 weeks immediately after sowing at 4°C with an 8-hr photoperiod (PAR, 9.5 μmol·m^{-2}·sec^{-1}; red:far red ratio, 3.9).

Construction of Chimeric Genes

FCA-γ

The intronless *FCA* gene construct was derived from the following DNA fragments: the *FCA* promoter was isolated from the cosmid CL58I16 (Macknight et al., 1997) as an EcoRI (present in cloning cassette)-SalI (bp 1470) fragment; the *FCA*-γ cDNA from SalI at bp 352 to SpeI at bp 2927; and the *FCA* 3′ untranslated region and terminator region from SpeI at bp 9168 to XhoI at 9763. The fragments were cloned together into pBluescript IISK+ vector (Stratagene) and cloned into the binary vector pSLJ1714 (Jones et al., 1992).

35S-γ and 35S-cab-γ

To produce *35S*-γ, the 35S promoter was cloned as an EcoRI-XhoI fragment into the EcoRI-SalI (bp 1470) sites of *FCA*-γ. The *35S-cab*-γ construct was made by introducing an HincII restriction site within the first ATG of *FCA*-γ using site-directed mutagenesis (primer 5′-GGAGGTTTCCCCCGGCTTAACGGTCCCCCAGAT-3′). The 35S promoter then was cloned into the EcoRI-HincII sites of this construct as an EcoRI-NcoI (Klenow-treated) fragment. The *35S-FCA* gene and *35S*-β constructs were described by Macknight et al. (1997). The 35S promoter used in these constructs was derived from the vector pJJ3431 (Jones et al., 1992).

P_{FCA}–$FCA_{to\ ATG}$:*GUS*, P_{FCA}–$FCA_{to\ exon\ 5}$:*GUS*, and P_{FCA}–$FCA_{to\ TGA}$:*GUS*

The β-glucuronidase (GUS) sequences were cloned from the plasmid pJJ3411 (Jones et al., 1992) as an NcoI (Klenow-treated)-XbaI fragment into the PstI (T4 DNA polymerase–treated)-XbaI sites of pUC18. A polymerase chain reaction (PCR) fragment containing the 3′ region of *FCA* was amplified from a pBluescript IISK− plasmid containing the 9763-bp *FCA* gene using the primer 5′-AAGAATAAATCTAGAGGTACATGAGACGAG-3′ (which contains an XbaI site after the stop codon of FCA) and the T7 primer (Stratagene). This was cloned into the pUC18-GUS vector as an XbaI-KpnI fragment to produce the construct pUC18-GUS-FCA-3′. To produce the three GUS constructs, SphI sites were introduced into the *FCA* gene and the *FCA*-γ constructs using site-directed mutagenesis. To make the P_{FCA}–$FCA_{to\ ATG}$:*GUS* construct, the SphI site was introduced at bp 1602 of the FCA gene (72 bp 3′ of the initiating Met, using the primer 5′-GTTTTCGGCGCATGCGGTTTGCC-3′); for P_{FCA}–$FCA_{to\ exon5}$:*GUS*, the SphI site was introduced within exon 5 at bp 4638 of the *FCA* gene (using the primer 5′-GACGGGGAGAGCATGCGCATAGG-3′); and for P_{FCA}–$FCA_{to\ TGA}$:*GUS*, the SphI site was introduced at bp 2658 of the *FCA*-γ construct. The GUS sequences then were cloned into the three *FCA* constructs as SphI-XhoI fragments, replacing the 3′ region of the *FCA* gene.

FCA C-Terminal Fragment for Expression in Escherichia coli

A BamHI-EcoRI fragment from *FCA*-γ was cloned into pRSETc (Invitrogen, Carlsbad, CA). This fragment expressed a polypeptide that extended from downstream of the second RNA-recognition motif to just after the translation stop codon.

Transformation of Arabidopsis

Constructs were mobilized into *Agrobacterium tumefaciens* strain C58C1, and the T-DNA was introduced into Arabidopsis ecotype L*er* or *fca-1* using either the root explant (Valvekens et al., 1988) or the vacuum infiltration (Bechtold et al., 1993) transformation protocol. Lines homozygous for the introduced T-DNA were selected using kanamycin resistance. Constructs were introduced into one genotype and then crossed into the other background. Three, five, and six independent transgenic lines were generated carrying *35S-γ*, *35S-cab-γ*, and *FCA-γ* transgenes, respectively. Nine, four, and four independent, single-locus transgenic lines were generated expressing the P_{FCA}–$FCA_{to\ ATG}$:*GUS*, P_{FCA}–$FCA_{to\ exon5}$:*GUS*, and P_{FCA}–$FCA_{to\ TGA}$:*GUS* transgenes, respectively.

Immunodetection of FCA Protein

Arabidopsis seedlings were ground in liquid nitrogen and homogenized in 3 volumes of SDS loading buffer (0.5 M Tris, pH 6.8, 10% SDS, 0.6 M DTT, and 0.012% bromphenol blue). The samples were boiled for 5 min, and the insoluble material was pelleted by centrifugation. The supernatant then was separated on a denaturing 8% polyacrylamide gel and blotted onto an Immobilon P nitrocellulose membrane (Millipore, Bedford, MA). FCA polyclonal antiserum was used at a dilution of 1:1000 (v/v). The immunoreactive proteins were visualized using the enhanced chemiluminescence protocol (Amersham), with the secondary antibody diluted 1:2000 (v/v), and by exposure to x-ray film (Kodak X-Omat AR) for 10 sec to 5 min.

Determination of GUS Activity in Transgenic Lines

Histochemical GUS staining of transgenic Arabidopsis plants was performed as described by Jefferson (1987). Plants grown in Petri dishes on GM medium were harvested from the plates and placed directly in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. Samples were placed in a vacuum desiccator for 10 min and then kept at 37°C overnight.

Root Analysis

Sterilized seed were placed individually in a line using a sterile syringe onto square plates containing GM medium. Plates were stratified for 2 days at 4°C and then placed vertically to allow the downward growth of roots. After 12 days, plants were harvested and placed in fixative (2.4% glutaraldehyde and 50 mM cacodylate buffer, pH 7.0). Measurements of primary root length and the number of lateral roots were made for each sample. All data was tested for normality using Kolmogrov Smirnov tests (Lillifors). One-way analysis of variance was performed using the statistical package Minitab (State College, PA).

Accession Number

GenBank accession number for the *Brassica napus FCA* gene is AF414188; GenBank accession number for the pea *FCA* intron 3 is AY072717. EMBL accession number for the Arabidopsis *FCA* gene is Z82992; EMBL accession number for *FCA-γ* cDNA is Z82989.

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