Cell, Vol. 113, 777–787, June 13, 2003, Copyright ©2003 by Cell Press

FY Is an RNA 3′ End-Processing Factor that Interacts with FCA to Control the *Arabidopsis* Floral Transition

Gordon G. Simpson,[1,2] Paul P. Dijkwel,[1,3] Victor Quesada,[4] Ian Henderson, and Caroline Dean*
Department of Cell and Developmental Biology
John Innes Centre
Norwich
United Kingdom

Summary

The nuclear RNA binding protein, FCA, promotes *Arabidopsis* reproductive development. FCA contains a WW protein interaction domain that is essential for *FCA* function. We have identified FY as a protein partner for this domain. FY belongs to a highly conserved group of eukaryotic proteins represented in *Saccharomyces cerevisiae* by the RNA 3′ end-processing factor, Pfs2p. FY regulates RNA 3′ end processing in *Arabidopsis* as evidenced through its role in *FCA* regulation. *FCA* expression is autoregulated through the use of different polyadenylation sites within the *FCA* pre-mRNA, and the FCA/FY interaction is required for efficient selection of the promoter-proximal polyadenylation site. The FCA/FY interaction is also required for the downregulation of the floral repressor *FLC*. We propose that FCA controls 3′ end formation of specific transcripts and that in higher eukaryotes, proteins homologous to FY may have evolved as sites of association for regulators of RNA 3′ end processing.

Introduction

The floral transition is a major developmental switch in flowering plants. *Arabidopsis* flowering time is regulated by the quantitative integration of environmental signals with an endogenous program of development (Mouradov et al., 2002; Simpson and Dean, 2002). Genes required for the promotion of flowering have been identified through the characterization of late-flowering mutants that comprise genetically separable pathways (Mouradov et al., 2002; Simpson and Dean, 2002). Mutations that delay flowering in long- but not short-day photoperiods have been classified in the photoperiod promotion pathway. Mutations affecting biosynthesis of, or response to, the phytohormone gibberellin have a minor effect on flowering in long-day photoperiods but can drastically delay flowering in short-day photoperiods. A third pathway, the autonomous pathway, promotes flowering in long- and short-day photoperiods and controls the expression of a floral repressor, *FLOWERING LOCUS C (FLC)* (Michaels and Amasino, 1999; Sheldon et al., 1999), which modulates the activity of the other two pathways (Mouradov et al., 2002; Simpson and Dean, 2002).

The genetically defined autonomous pathway currently comprises six mutants, *fca, fy, fpa, fve, ld*, and *fld* (Simpson and Dean, 2002). *FCA* and *FPA* encode RNA binding proteins (Macknight et al., 1997; Schomburg et al., 2001), and *LD* encodes a homeodomain protein (Lee et al., 1994), while *FVE, FY*, and *FLD* have not yet been characterized. We have been studying FCA in order to understand the role and regulation of the autonomous pathway in flowering time control. FCA is a nuclear protein with two RNA recognition motif (RRM)-type RNA binding domains that can bind RNA in vitro (Macknight et al., 1997; Quesada et al., 2003). In addition, FCA contains a WW domain. This conserved protein interaction module is found in a range of eukaryotic proteins that function in diverse cellular processes (Sudol and Hunter, 2000). WW domains have recently been classified into four groups on the basis of their ligand specificity (Bedford et al., 2000; Sudol and Hunter, 2000). The resolution of cocrystal structures of WW domains complexed with their ligands has led to a clearer understanding of the mechanism by which specificity in these interactions is determined (Bedford et al., 2000; Huang et al., 2000; Verdecia et al., 2000; Zarrinpar and Lim, 2000). This classification therefore provides a predictive tool for identifying WW domain ligands.

The regulation of *FCA* expression is complex. *FCA* pre-mRNA is alternatively processed, resulting in the formation of four different transcripts, α, β, γ, and δ (Macknight et al., 1997). We have discovered that a principal level of control involves negative feedback of expression mediated by FCA itself (Quesada et al., 2003). Full-length FCA protein (FCA-γ) promotes premature cleavage and polyadenylation at a promoter-proximal site within intron 3 of its own pre-mRNA. This results in the production of a nonfunctional truncated transcript, known as *FCA*-β, at the expense of the fully spliced transcript, *FCA*-γ, which encodes the active protein. The autoregulation of *FCA* expression is developmentally regulated and has a functional consequence for flowering time control (Quesada et al., 2003). *FCA* autoregulation presets the level of active *FCA* expression (which is typically limiting; Macknight et al., 2002), which in turn controls the level of *FLC* mRNA. When the negative regulation of *FCA* expression is bypassed, the balance of the pathways controlling *FLC* is perturbed and flowering time is accelerated (Quesada et al., 2003).

In order to determine the mechanism by which FCA controls the floral transition, we searched for proteins that interacted with FCA through its WW domain. We have found that FCA interacts with FY, a previously uncharacterized component of the autonomous pathway. *FY* encodes a protein that is highly conserved in eukaryotes, and its homolog in *S. cerevisiae* functions in pre-mRNA 3′ end formation. We show that FY is required for the negative autoregulation of *FCA* expres-

*Correspondence: caroline.dean@bbsrc.ac.uk
[1]These authors contributed equally to this work.
[2]Present address: Gene Expression Programme, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland.
[3]Present address: Molecular Biology of Plants, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.
[4]Present address: Division de Genetica, Departamento de Biologia Aplicada, Universidad Miguel Hernandez, Campus de Elche, Edificio Vinalopo, 03202 Elche, Alicante, Spain.

sion, which involves alternative poly(A) site selection in the *FCA* pre-mRNA. Our results suggest that FCA and FY function together in a molecular complex to regulate RNA 3′ end formation and control the floral transition.

Results

The WW Domain Is Essential for the Function FCA Performs in Flowering Time Control

In order to determine whether the FCA WW protein interaction domain was required for the function FCA performs in flowering time control, we mutated the second signature tryptophan (W) to phenylalanine (F). The same mutation has previously been reported to maintain the folded state of the WW domain of p53 binding protein-2, while perturbing its ability to interact with its target ligand (Koepf et al., 1999). Wild-type FCA-WW and the mutant FCA-WF proteins were expressed from transgenes bearing the native FCA promoter and introduced into an *fca-1* loss-of-function background. The functionality of these proteins was assessed by measuring their capacity to complement the late-flowering phenotype of *fca-1* (Figure 1A). Leaf number is closely correlated with flowering time (Koornneef et al., 1991) and was used to quantify the time of the floral transition. The late flowering of *fca-1* was fully complemented by the expression of wild-type FCA-WW protein, but not by the FCA-WF mutant (Figures 1A and 1B). The expression level of the mutant protein in these transgenic plants matched that of the wild-type protein (Figure 1C). However, while the plants expressing FCA-WW protein exhibited reduced levels of *FLC* mRNA expression (consistent with them complementing the *fca-1* defect), the transgenic plants expressing FCA-WF did not (Figure 1D). We therefore conclude that an intact WW domain is essential for the function that FCA performs in repressing *FLC* mRNA expression in flowering time control.

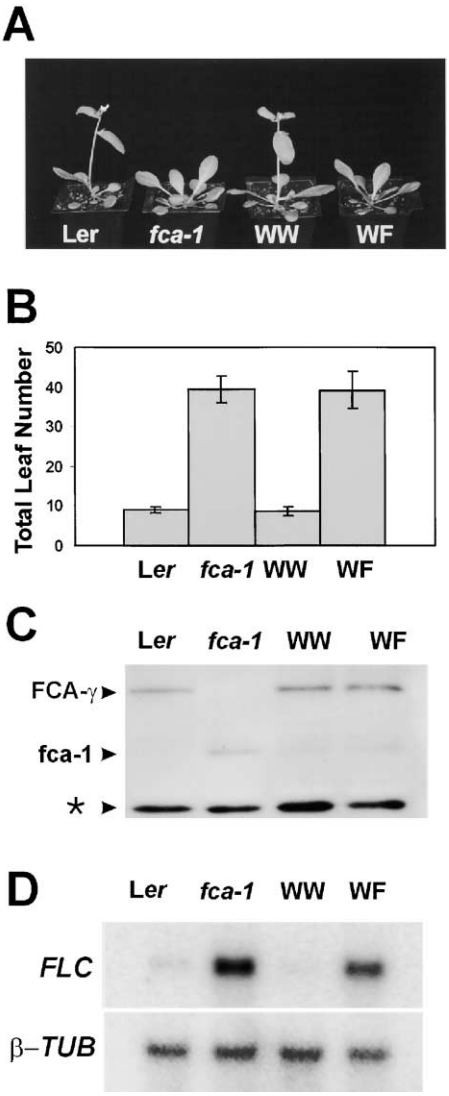

Figure 1. The WW Domain Is Essential for the Function FCA Performs in Flowering Time Control

(A) Contrasting phenotype of late- and early-flowering genotypes. All plants were sown at the same time and grown in identical conditions, but L*er* and *fca-1* plants expressing FCA-WW have already flowered while *fca-1* and *fca-1* plants expressing FCA-WF transgene continue to produce vegetative rosette leaves.

(B) Quantification of flowering time data. Total leaf number (rosette and cauline) was determined, since this is closely correlated with flowering time.

(C) Western analysis with anti-FCA (KL-4) antibodies of total protein extracts made from the same genotypes as in (A). The position of full-length FCA-γ protein and truncated mutant FCA protein produced in *fca-1* is indicated. Asterisk denotes a non-FCA protein that cross-reacts with KL-4 antibodies.

(D) Northern analysis of total RNA extracted from the same genotypes in (C) probed for *FLC* and β-*TUBULIN* as an internal control.

The FCA WW Domain Interacts with an *Arabidopsis* Protein in an *FY*-Dependent Manner

Since the WW domain is essential for FCA function, we searched for proteins that it might interact with. A two-step affinity purification, Far Western procedure was developed initially using shaved cauliflower curd, but we also investigated the interaction in *Arabidopsis* directly.

Using this approach, an interaction was detected between FCA and a protein from cauliflower extract that migrated with an apparent molecular weight of 84 kDa (Figure 2A). When the affinity precipitation step was performed with recombinant FCA protein carrying the WF mutation, this interaction could not be detected (Figure 2A). We tested whether this interaction was conserved in *Arabidopsis* and whether this protein corresponded to a factor involved in flowering time control by analyzing this interaction in mutant backgrounds defective in the floral transition. The interacting protein could be detected in extracts prepared from wild-type (*Arabidopsis thaliana* Landsberg *erecta* [L*er*]), *fpa-2, fve-1, fwa-1*, and *ap1* mutants, but not in extracts derived from *fy-1* (Figure 2B). Significantly, *FY* has been classified in the same genetic pathway as *FCA*. This raised two possibilities: first, the 84 kDa interacting protein could be FY, or second, *FY* normally functioned upstream of the interacting protein to affect its interaction with FCA.

Identification of FY

Based on the premise that the interacting protein might be FY, three pieces of information were available to

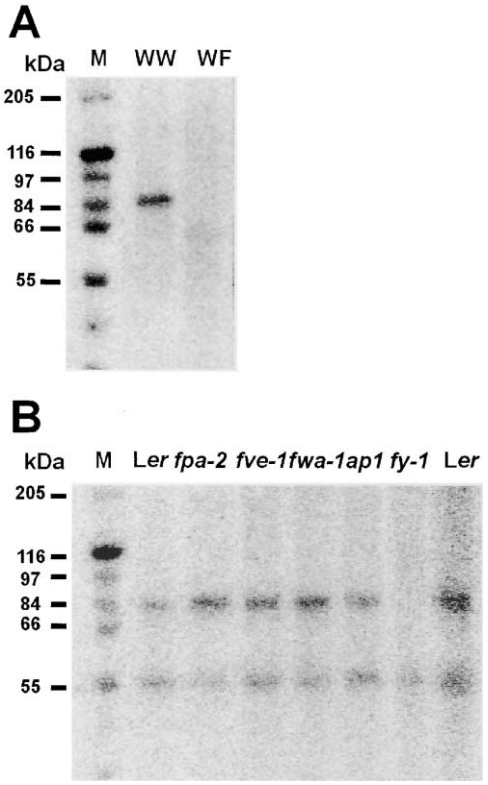

Figure 2. Affinity Precipitation and Far Western Analysis of Proteins Interacting with the FCA WW Domain

(A) Affinity precipitation with either FCA-WW- or FCA-WF-coupled agarose beads as indicated and cauliflower protein extract. The affinity-purified proteins were separated by SDS-PAGE, electroblotted, and probed with labeled recombinant FCA:HMK:WW protein as detailed in Experimental Procedures. When run alongside wide molecular weight markers (Sigma), this protein migrates with an apparent molecular weight of 84 kDa.

(B) Affinity precipitation with FCA WW-coupled agarose beads from extracts of *Arabidopsis* L*er*, *fpa-2*, *fve-1, fwa-1*, *ap1*, and *fy-1*, followed by Far Western analysis with FCA:HMK:WW reveals an interacting protein migrating with an apparent molecular weight of approximately 84 kDa in all backgrounds except *fy-1*.

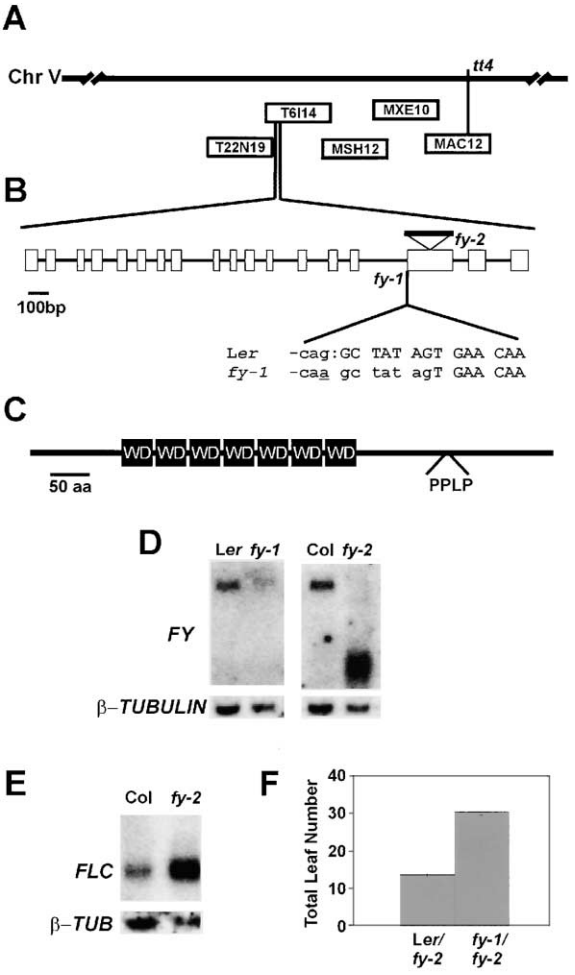

Figure 3. Identification of FY

(A) Physical map of Chromosome V in the region of *FY*. Bacterial artificial chromosomes (BACs) are shown as rectangles.

(B) Structure of the *FY* gene. Coding regions are shown as rectangles and introns as thick lines. The sequence distinction between L*er* and *fy-1* is shown below: lowercase denotes intron sequence, uppercase denotes exon sequence. The mutated G-A residue in *fy-1* is underlined. The next downstream AG is selected as a 3′ acceptor site in *fy-1*. Additional sequence excised as intron in *fy-1* is denoted by lowercase text. The T-DNA insertion site within *fy-2* is shown.

(C) Domain organization of FY protein.

(D) *FY* Northern. Northern blot analysis was performed with approximately 11 μg of total RNA isolated from 12-day-old L*er* and *fy-1*, Col and *fy-2* plants and probed with an *FY*-specific probe. The blot was stripped and reprobed with a β-*TUBULIN* probe as a loading control.

(E) *FLC* Northern. Northern blot analysis performed with approximately 11 μg of total RNA isolated from 12-day-old Col and *fy-2* plants, probed with an *FLC*-specific probe. The blot was stripped and reprobed with a β-*TUBULIN* probe as a loading control.

(F) Allelism test. Flowering time of *fy-1* × *fy-2* F1 plants compared to wild-type (L*er* × *fy-2* F1 plants).

facilitate its identification. First, the *fy-1* mutation had been genetically mapped to 0.6 cM north of *tt4* on *Arabidopsis* chromosome V (Koornneef et al., 1994). Second, the size of the predicted open reading frame (ORF) would be approximately 84 kDa. Third, WW domains typically interact with proline-rich sequences, so these would likely be present in the ligand. Since WW domains can be classified on the basis of their ligand specificity (Bedford et al., 2000; Sudol and Hunter, 2000) and the molecular basis of this specificity is increasingly well understood (Bedford et al., 2000; Huang et al., 2000; Verdecia et al., 2000; Zarrinpar and Lim, 2000), we examined the sequence of the FCA WW domain and found that it lacked elements important in the recognition of phosphorylated Ser-Pro or Thr-Pro (Zarrinpar and Lim, 2000), Pro-Pro-Tyr-Pro (Huang et al., 2000), or polyproline motifs flanked by Arg or Lys (Bedford et al., 2000). Instead, the primary sequence of the FCA WW domain most closely resembles that of FBP11 (Macknight et al., 1997), a group II WW domain containing protein that binds the consensus Pro-Pro-Leu-Pro (Sudol and Hunter, 2000).

An annotated but previously uncharacterized gene (At5g13480) north of *tt4* on Chromosome V (Figure 3A) predicted to encode a WD-repeat protein with Pro-Pro-Leu-Pro sequences in the C-terminal region was identi-

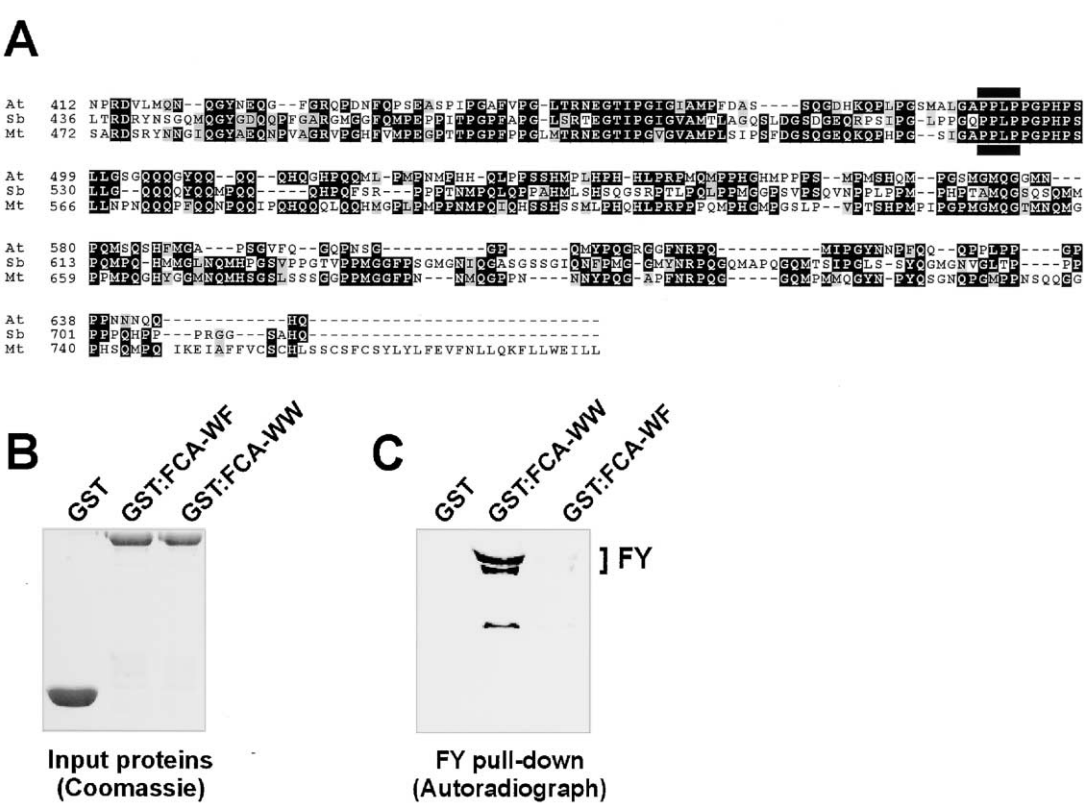

Figure 4. FCA/FY Interaction

(A) PPLP motif in FY-related sequence of other plants. Sequence alignment of C-terminal sequences of FY from *Arabidopsis thaliana* (At), *Medicago truncatulata* (BI308160) (Mt), and *Sorghum bicolor* (BG411156) (Sb) with numbers referring to amino acid number. Identical residues are shown as white type on a black background, while similar residues are shaded with gray. Dashes indicate gaps introduced to optimize the alignment. The conserved Pro-Pro-Leu-Pro motif is flanked by horizontal lines.

(B) The relative inputs of GST, GST:FCA-WW, and GST:FCA-WF proteins in the in vitro interaction study.

(C) [^{35}S]Met-labeled FY was produced by in vitro translation in rabbit reticulocyte extract. Lanes show protein retained in the following incubations: GST and FY, GST:FCA-WW and FY, and finally GST:FCA-WF and FY.

fied. A G-A mutation at a predicted 3′ splice acceptor site was detected in *fy-1* genomic DNA (Figure 3B). The existence of this mutation was confirmed through use of a derived cleaved amplified polymorphisms (dCAPs) marker. The sequence of *FY* cDNA was completed and found to differ from the predicted annotation. The gene comprises 18 exons and 17 introns and encodes a protein with 7 WD-repeats and Pro-Pro-Leu-Pro sequences in the C-terminal region (Figure 3C). The consequence of the mutation found in *fy-1* was assessed by sequencing cDNA amplified by RT-PCR from total *fy-1* RNA. We found a shift in splice acceptor site usage within exon 16, a change in reading frame, and the introduction ultimately of a premature termination codon (PTC) (Figure 3B). Consistent with the effect that a PTC can have on mRNA stability through nonsense-mediated decay, the levels of this transcript were reduced in RNA prepared from *fy-1* compared to wild-type (Figure 3D). The consequence of this mutation is that any protein made from this mRNA would be truncated, consisting only of the WD-repeats, and it would lack the Pro-Pro-Leu-Pro sequences we predicted would interact with the FCA WW domain. This is consistent with our inability to detect an interaction between FCA and the 84 kDa protein in extracts of *fy-1* (see Figure 2B). We subsequently identified a second allele, *fy-2*, in the Syngenta *Arabidopsis* T-DNA insert population, which results from a T-DNA insertion within *FY* exon 16 (Figure 3B). *FY* transcripts of abnormal size are produced in *fy-2* (Figure 3D), and the consequence of this mutation, as with *fy-1*, is that any FY protein produced would encode the WD-repeat region but lack the Pro-Pro-Leu-Pro sequences we expect FCA to interact with. Like *fy-1, fy-2* plants flower late, respond to vernalization, and exhibit elevated levels of *FLC* mRNA (Figure 3E). The allelism of *fy-1* and *fy-2* was confirmed by the fact that F1 progeny resulting from crossing these parents flowered late (Figure 3F). The late flowering of *fy-1* was complemented by introduction of an *FY* transgene fused to the cauliflower mosaic virus (CaMV) 35S promoter (see Experimental Procedures).

FY is a highly conserved plant protein. The full-length sequence of related expressed sequence tags (ESTs) from *Sorghum bicolor* and *Medicago truncatulata* was determined. The alignment of these sequences with *Arabidopsis FY* reveals that the WD repeat region is particularly highly conserved, exhibiting 84% and 85% identity with the *Sorghum* and *Medicago* sequences, respectively. The C-terminal region of *Arabidopsis FY* is less well conserved, exhibiting only 42% and 43%

identity with the *Sorghum* and *Medicago* sequences, respectively. Notably, however, as shown in Figure 4A, the first of the Pro-Pro-Leu-Pro motifs in this otherwise less well-conserved region is invariant among the FY sequences from different plant species.

Recombinant FCA and FY Interact In Vitro

To confirm that the interacting protein identified through the Far Western analysis was FY, the interaction of FCA and FY was tested in vitro. GST:FCA-WW pulled down FY and this interaction was impaired when GST:FCA-WF was tested (Figures 4B and 4C). Therefore, recombinant FCA and FY proteins do interact in vitro in a manner that is dependent on an intact FCA WW domain.

FY Is Necessary for the Function FCA Performs in Flowering Time Control

We next analyzed the significance of the FCA-FY interaction in flowering time control genetically. *FCA* can be overexpressed in *Arabidopsis* by driving expression with the CaMV 35S promoter (35S) and removing regulatory sequences such as the introns and 5′ region. FCA protein produced from this transgene is shorter than wild-type, but nevertheless plants expressing *35S::FCA*-γ flower early (Macknight et al., 2002) and can accelerate the floral transition of certain late-flowering mutants (our unpublished results). However, when this transgene was introduced from *fca-1* into an *fy-1* mutant background (by crossing), *fy-1* was found to be epistatic to the early flowering of *35S::FCA*-γ (Figure 5A). FCA protein derived from the *35S::FCA*-γ transgene was overexpressed in *fy-1* (Figure 5B). Therefore, this epistasis does not result from a requirement for *FY* in the proper expression of active *FCA*. Instead, the level of *FLC* mRNA in *fy-1* plants expressing *35S::FCA*-γ was higher than *fca-1* plants expressing *35S::FCA*-γ (Figure 5C). This reveals that loss of *FY* function compromises the ability of FCA to prevent the accumulation of *FLC* mRNA. We therefore conclude that *FY* is necessary for the function FCA performs in flowering time control.

FY Is Related to the *S. cerevisiae* 3′ End-Processing Factor Pfs2p and Affects 3′ End Processing in *Arabidopsis*

The FY sequence was found to be closely related to several nonplant proteins, including deduced proteins from humans (Ito et al., 2001), *Tetraodon nigroviridis, Caenorhabditis elegans*, *Drosophila melanogaster*, and *S. cerevisiae* (Figure 6A). Each of these proteins is more related to each other than to any other protein originating from the same organism. The relatedness is confined to the WD-repeat region (for example, there is 61% identity between the WD repeat regions of FY and the human protein WDC146). Genetic and molecular analysis has shown that the related protein from *S. cerevisiae*, Pfs2p, functions in pre-mRNA 3′ end processing (Ohnacker et al., 2000).

Viable mutations in RNA 3′ end-processing factors are rare in higher eukaryotes. However, viable alleles of the *D. melanogaster* polyadenylation factor *suppressor of forked* (*su(f)*) exist (Mitchelson et al., 1993). These mutants show inefficient selection of weak proximal poly(A) sites (Brogna and Ashburner, 1997; Mitchelson et al., 1993). The selection of distinct poly(A) sites within *Arabidopsis* pre-mRNAs (aside from the heterogeneity in site selection within the 3′ UTR) is rarely reported. However, *FCA* pre-mRNA maturation is regulated by alternative 3′ end formation, involving the selection of a promoter proximal site within intron 3 to produce *FCA* transcript β and the selection of a distal site in the conventional 3′ UTR to produce the other *FCA* transcripts, α, δ, and γ (Macknight et al., 1997). We therefore asked whether the selection of the proximal site in *FCA* pre-mRNA was affected in an *fy-1* mutant background. The

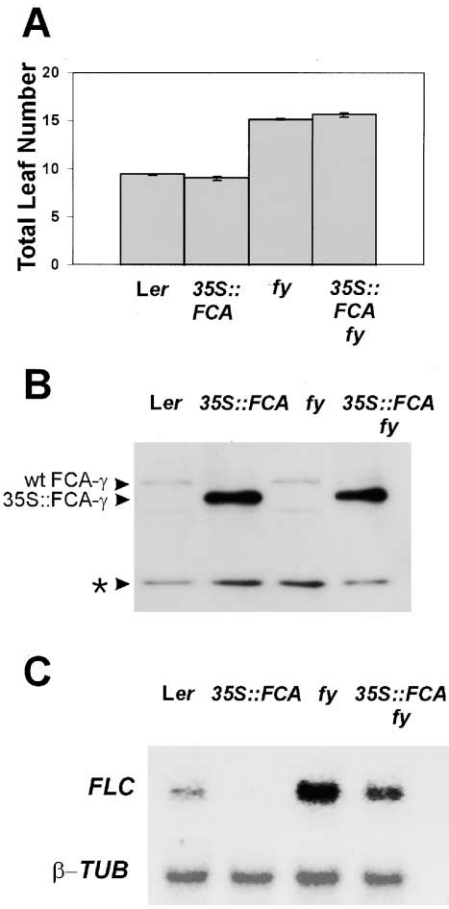

Figure 5. *fy-1* Is Epistatic to *35S::FCA*-γ

(A) The flowering time of L*er, 35S::FCA*-γ *fca-1*, *fy-1*, and *35S::FCA*-γ introduced into *fy-1* from *fca-1* by crossing (*F3* homozygous *35S::FCA*-γ and *fy-1,* but segregating for *fca-1*) was determined in controlled environment conditions.

(B) Western analysis of FCA expression with anti-FCA KL-4 antibodies. Total protein was extracted from the same genotype as (A), harvested 12 days after germination. Protein expressed from the *35S::FCA*-γ transgene is some 10 kDa shorter than the native wild-type protein but complements the late-flowering phenotype of *fca-1* by preventing the accumulation of *FLC* mRNA (Macknight et al., 2002). The position of the endogenous full-length protein FCA-γ is indicated as is the truncated protein overexpressed from the transgene, *35S::FCA*-γ. Asterisk denotes a non-FCA protein that crossreacts with KL-4 antibodies.

(C) Northern analysis of *FLC* expression using the same material as above. *FLC* mRNA expression is elevated in *fy-1* mutant backgrounds. After exposure, the blot was stripped and reprobed with β-*TUBULIN*.

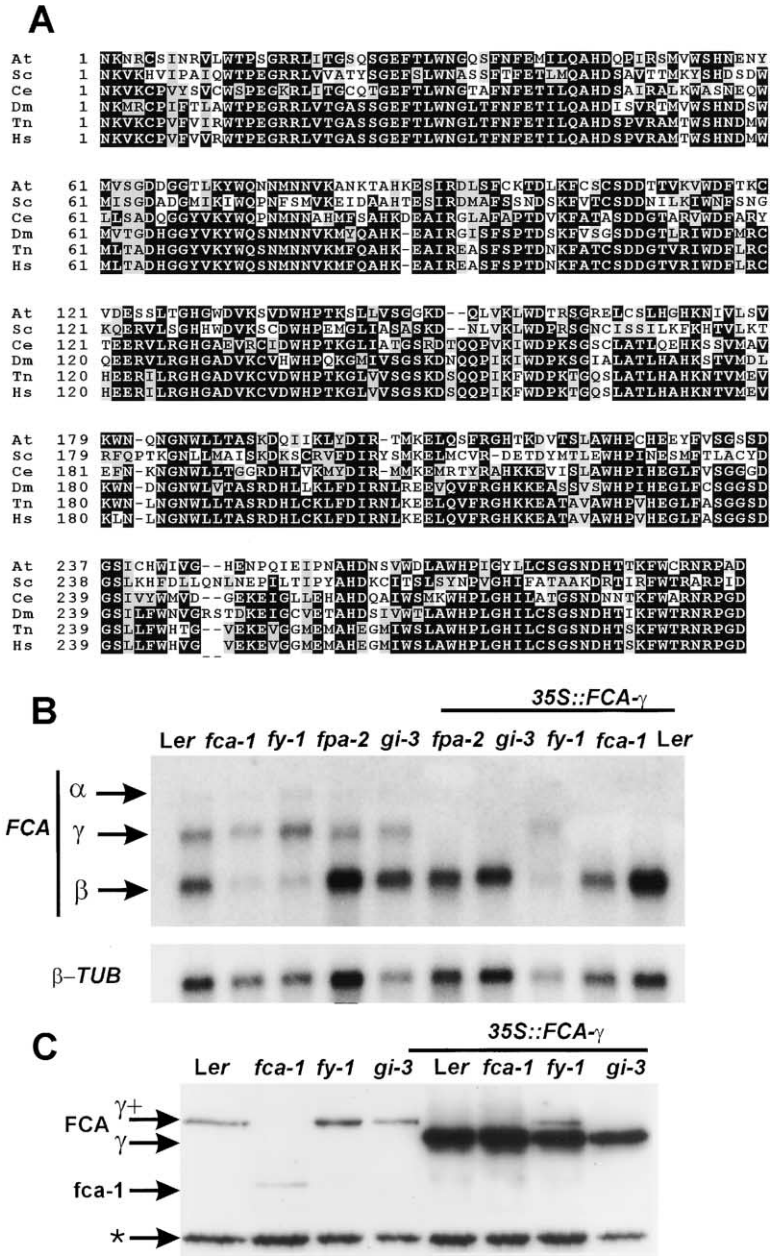

Figure 6. FY Is Related to the 3′ End-Processing Factor Pfs2p, and *fy-1* Loss-of-Function Mutants Exhibit Defects in 3′ End Formation

(A) Alignment of the 7 × WD repeat region of (At) *Arabidopsis thaliana* FY with (Sc) *Saccharomyces cerevisiae* Pfs2p [NP_014082], (Ce) *Caenorhabditis elegans* [NP496985], (Dm) *Drosophila melanogaster* [AAK93119], (Tn) *Tetraodon nigroviridis* [CAD27805], and (Hs) *Homo sapiens* [BAB 32435].

(B) Northern blot analysis of *FCA* transcripts processed at proximal (β) and distal (α, γ) poly(A) sites. Approximately 2 μg poly(A)$^+$ RNA was hybridized with a probe to the 5′ of *FCA*. The blot was then stripped and rehybridized with a β-*TUBULIN* probe as a loading control. The level of *FCA*-β (polyadenylated at the proximal site) is reduced in *fy-1* and *fca-1* backgrounds. The ability of overexpressed FCA (*35S::FCA*-γ) to promote proximal poly(A) site usage of native *FCA* gene expression is compromised in an *fy-1* background—this is the only background where *FCA*-γ, (polyadenylated at the distal site) can still be detected. *FCA* transcript abundance was quantified with respect to β-*TUBULIN*. The ratio of α,γ/β in the characterized genotypes was determined: L*er* (0.5), *fca-1* (1.4), *fy-1* (2.0), *fpa-2* (0.1), *gi-3* (0.2), *35S::FCA fpa-2* (0), *35S::FCA gi* (0), *35S::FCA fy-1* (1.1), *35S::FCA fca-1* (0), *35S::FCA* L*er* (0). This distinctive pattern in *fy-1* was reproduced in at least four independent analyses.

(C) Western blot of FCA protein with anti-FCA (KL-4) antibodies. The expression of full-length FCAγ+ protein is shown in different genetic backgrounds affecting flowering time. When the truncated FCA-γ protein is overexpressed (*35S::FCA*-γ), full-length endogenous FCAγ+ protein disappears in all backgrounds except *fy-1*. This is consistent with the effect on endogenous *FCA* transcript accumulation depicted in (B). Asterisk denotes a non-FCA protein that crossreacts with KL-4 antibodies.

relative abundance of *FCA* transcripts formed at either the distal or proximal site was determined by Northern analysis of poly(A)$^+$ RNA hybridized with a probe to the 5′ leader of *FCA*. As shown in Figure 6B, the level of *FCA*-β, which is polyadenylated at the proximal site within *FCA* pre-mRNA, is specifically reduced in *fy-1* but not other late-flowering mutants (with the exception of *fca-1*). There is a corresponding shift in the amount of transcripts polyadenylated at the distal site, since the level of *FCA*-γ and *FCA*-α (in which intron 3 is retained) increases in *fy-1*.

We have recently discovered that FCA negatively autoregulates its expression by modulating the site of 3′ end formation in its own pre-mRNA (Quesada et al., 2003). FCA overexpressed from an intronless transgene actively promotes the selection of the proximal poly(A) site within intron 3 of pre-mRNA produced from the native *FCA* gene, resulting in the formation of the truncated *FCA*-β transcript. This posttranscriptional control has the consequence of limiting the expression of the only active isoform, *FCA*-γ (Quesada et al., 2003). An intact WW domain is necessary for *FCA* autoregulation (Quesada et al., 2003). Since FY is a partner for this domain and can function in 3′ end processing, we asked whether *FY* was necessary for FCA to autoregulate its expression in this way. A transgene designed to overexpress *FCA* (consisting of an intronless *FCA* sequence lacking the 5′ 349 nt driven by the CaMV 35S promoter) was introduced into *fy-1* and other late-flowering mutants, including *gigantea-3* and *fpa-2*, by crossing from an *fca-1* background. In each case, a dCAPs test was used to verify that the F3 individuals analyzed were wild-

type at the *FCA* locus. The expression of different *FCA* transcripts derived from the native gene was analyzed by hybridizing a Northern blot of poly(A)$^+$ RNA with a probe to the 5′ leader sequence of *FCA*. This probe detects all the native *FCA* transcripts, but not transcripts derived from the *35S::FCA*-γ transgene, since this construct lacks this sequence (Macknight et al., 2002; Quesada et al., 2003). When FCA is overexpressed from a transgene, it promotes the selection of the proximal poly(A) site in native *FCA* pre-mRNA, resulting in the exclusive formation of *FCA*-β mRNA at the expense of the active *FCA*-γ isoform. FCA can function in this negative regulation in all the late-flowering mutant backgrounds investigated, with the exception of *fy-1* (Figure 6B), since in this case, less *FCA*-β mRNA accumulates and *FCA*-γ can still be detected. A Western of FCA protein produced from these lines is shown in Figure 6C. Here, the negative regulatory effect of overexpressing transgenic FCA results in loss of the endogenous FCA except when *35S::FCA* is expressed in *fy-1*. Therefore, FCA requires *FY*, not only for the function it performs in flowering time control, but also to autoregulate its expression by modulating the site of 3′ end formation in its own pre-mRNA.

On the basis of conserved sequence and proximal poly(A) site usage phenotypes, we therefore propose that FY functions as an RNA 3′ end-processing factor.

Discussion

We have demonstrated that the WW protein interaction domain is essential for the function FCA performs in flowering time control and identified FY as a molecular partner for this domain. In doing so, we have characterized one of the original late-flowering mutants (Koornneef et al., 1991). Loss-of-function *fy* mutants exhibit the same flowering time phenotypes as *fca*, and as such they have been classified together in the same genetically defined autonomous pathway. *fca-1* is epistatic to *fy-1* (Koornneef et al., 1998) and *fy-1* is epistatic to *35S::FCA*-γ. We have demonstrated that *FCA* and *FY* do not function in a linear pathway to control each other's expression. Instead, the molecular basis of this genetic epistasis is the interaction of FCA and FY proteins. FY encodes a protein that is highly related to an RNA 3′ end-processing factor, and we have demonstrated phenotypic defects in *FCA* RNA 3′ end formation in *fy* loss-of-function mutants. We therefore propose that FCA and FY interact to regulate RNA 3′ end formation.

In theory, FCA could interact with FY either to inhibit its activity and prevent 3′ end formation or to tether the 3′ end machinery to a regulated site and thus promote 3′ end formation. We have been able to distinguish between these two possibilities and understand the mechanistic consequence of the FCA-FY interaction by analyzing the molecular basis of *FCA* autoregulation. An intact WW domain is required for *FCA* autoregulation (Quesada et al., 2003) and, consistent with the interaction of FY with this domain, we have now demonstrated a genetic requirement for *FY* in this process. Two alternative 3′ ends are selected in *FCA* pre-mRNA: a promoter-proximal site within intron 3 and a promoter-distal site in the conventional 3′ UTR. FCA negatively regulates its expression by promoting selection of the proximal site (Quesada et al., 2003). FCA could interact with FY at the distal site to block its usage, with 3′ end formation then occurring at the proximal site by default. However, we have made use of the γ-ray induced *fca-4* allele, in which the two poly(A) sites are expressed on separate transcripts (as a result of a chromosomal breakpoint and rearrangement within this allele of *FCA*) to demonstrate that FCA actively promotes selection of the proximal 3′ end (Quesada et al., 2003). Our data therefore supports a model in which FCA would promote proximal poly(A) site selection by binding RNA close to this site (with its N-terminal RNA binding domains), while simultaneously interacting with FY and the 3′ end-processing machinery through its WW domain. In this way, FCA would act as a regulator of 3′ end formation by tethering or stabilizing the 3′ end-processing machinery to an otherwise weak poly(A) site and thereby actively promoting its selection (Figure 7). No precedent exists for a regulatory factor interacting directly with the 3′ end-processing machinery in order to modulate the site of 3′ end formation (Zhao et al., 1999).

We propose that FCA is a regulator of 3′ end formation, while FY (by sequence similarity) is a conserved component of the 3′ end-processing machinery. It seems unlikely that FCA is involved in constitutive 3′ end formation. First, since the early flowering *flc-3* null allele is epistatic to *fca* loss-of-function mutants, the late-flowering phenotype of *fca* can be explained exclusively by its role in regulating one gene, *FLC*, as opposed to the accumulated misregulation of multiple transcripts (Michaels and Amasino, 2001). Second, consistent with a regulatory role, the level of *FCA* expression is normally limiting for flowering, since elevated levels of FCA protein promote precocious flowering (Macknight et al., 2002; Quesada et al., 2003). Third, the role of FCA and FY can be genetically separated: *fy-1fpa-1* double mutants are lethal, but *fca-1fy-1* or *fca-1fpa-1* double mutants are not (Koornneef et al., 1998). Therefore, FY must perform an additional essential function separate from its interaction with FCA. Although the hypomorphic nature of *fy-1* and *fy-2* means that we cannot as yet distinguish whether FY acts in constitutive or regulated 3′ end formation, these genetic data reveal that FCA plays a more limited regulatory role in plant development than FY. The further examination of this model will require the characterization of null *fca* and *fy* alleles and the identification of FY protein partners.

The known downstream target for FCA and FY in flowering time control is *FLC* (Michaels and Amasino, 1999, 2001; Sheldon et al., 1999). Through genetic analysis, we have shown here that FY and an intact WW domain are necessary both for FCA to autoregulate its expression and for the function FCA performs in regulating *FLC* mRNA accumulation. This indicates that the molecular mechanisms involved in both these processes may be similar (Figure 7). FCA and FY could therefore regulate 3′ end formation of either *FLC* pre-mRNA directly or of an unidentified intermediate. Molecular and genetic approaches are now being undertaken to distinguish between these possibilities. So far, we have not detected alternatively polyadenylated transcripts of *FLC* through Northern analysis of wild-type, *fca-1*, or *fy-1* mutant backgrounds. This may be because such tran-

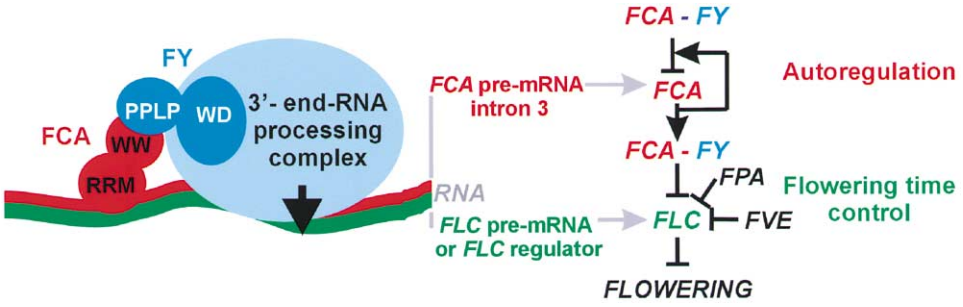

Figure 7. Model for FCA and FY Function

We propose that FCA (red) binds target RNA through its two N-terminal RRMs and tethers the 3′ end-processing machinery (pale blue) to this RNA via an interaction between the FCA WW domain and the PPLP domain of the 3′ end-processing factor, FY (blue). One target of this interaction is *FCA* pre-mRNA (red). Autoregulation of *FCA* expression presets the level of active FCA protein available to regulate the floral repressor, *FLC* (green), which is executed by FCA again interacting with FY. It is not yet known whether the regulation of *FLC* pre-mRNA is direct or if an intermediate RNA is involved. The activity of other regulators of *FLC* (such as *FPA* and *FVE*) is not controlled by FCA (at the RNA level, at least) and conversely, they do not regulate *FLC* by controlling *FCA* expression.

scripts are not stable: transcripts that are cleaved and polyadenylated at sites that do not include in-frame upstream stop codons are subject to nonstop decay (Frischmeyer et al., 2002). It is conceivable that this RNA decay pathway could be deliberately exploited through regulated 3′ end formation as a means to control gene expression.

The identification of FY and its interaction with FCA provides insight into the molecular nature of the autonomous pathway in flowering time control. The interaction of FCA and FY provides the only clear example of genetic epistasis between autonomous pathway mutants as classified by Koornneef (Koornneef et al., 1998). The molecular interaction of FCA and FY explains this genetic epistasis and the identification of FY suggests the molecular mechanism by which they act. Other autonomous pathway components appear to function in a genetically parallel manner to FCA and FY (Koornneef et al., 1998; our unpublished results). None of the other autonomous pathway components appear to be involved in 3′ end formation: *LD* encodes a homeodomain protein (Lee et al., 1994) and although *FPA* encodes an RNA binding protein (Schomburg et al., 2001), it is not required for the function *FCA* performs in flowering time control (our unpublished results). Indeed, none of the other components of the autonomous pathway (except FY) are required for the role FCA plays in flowering time control, nor are any required for FCA expression (our unpublished results). Therefore, the genetically defined autonomous pathway does not appear to function in a conventional linear manner controlled by an upstream input signal. Instead, it may represent the evolution of multiple, divergent mechanisms that function in parallel to control the level of the critical regulator of *Arabidopsis* flowering time, *FLC* (Figure 7).

Our work raises general questions about the composition of complexes regulating RNA 3′ end formation in all eukaryotes. Pfs2p/FY are highly conserved eukaryotic proteins (Figure 7A). The function of Pfs2p in *S. cerevisiae* 3′ end formation has been clearly demonstrated (Ohnacker et al., 2000), and we have shown here that FY is required for the regulated 3′ end formation of *FCA* pre-mRNA. It therefore seems likely that all these Pfs2p/FY-like proteins will turn out to function in 3′ end formation. However, the human homolog, WDC146, has not been identified in complexes that carry out cleavage and polyadenylation in vitro (Zhao et al., 1999). This may be explained by the fact that the expression of this gene is under developmental control (WDC146 exhibits its highest level of expression in spermatocytes), and so WDC146 may not function in constitutive 3′ end formation (Ito et al., 2001). Instead, another mammalian protein, cleavage stimulatory factor 50 kDa (CstF50), has been suggested to be the functional equivalent of Pfs2p (Ohnacker et al., 2000). CstF50 is an established component of mammalian complexes that carry out cleavage and polyadenylation in vitro and it resembles Pfs2p in domain organization (7 × WD repeats) and conservation of protein-protein interactions within 3′ end-forming complexes (Ohnacker et al., 2000; Takagaki and Manley, 1992, 2000). Our searches reveal that Pfs2p/FY and CstF50 homologs are highly conserved in model eukaryotic genomes. However, while *Arabidopsis*, *D. melanogaster*, *C. elegans*, and human genomes encode both Pfs2p/FY and CstF50 proteins, *S. cerevisiae* encodes only Pfs2p. This raises the possibility that in higher eukaryotes, the functional relatedness of these proteins has enabled the evolution of compositionally distinct 3′ end-processing complexes that may distinguish constitutive 3′ end formation from regulated 3′ end formation. The comparative characterization of *Arabidopsis* FY and CstF50-containing complexes will enable this question to be addressed. This is important because although alternative polyadenylation is increasingly well documented and can have profound effects on gene expression (Beaudoing and Gautheret, 2001; Edwalds-Gilbert et al., 1997), little is known about *trans*-acting factors that regulate it and no tissue-specific or transcript-specific factors have been discovered (Barabino and Keller, 1999). Instead, two well-characterized cases of alternative polyadenylation within pre-mRNA introns can be explained by changes in general polyadenylation factor abundance; autoregulation of expression of the *Drosophila* polyadenylation factor suppressor of forked, Su(f) (Juge et al., 2000), and processing of mammalian immunoglobulin heavy chain transcripts (IgM) in B cell

maturation (reviewed by Zhao et al., 1999). In both cases, there are competing, suboptimal splicing and polyadenylation processing sites and it has been proposed that small changes in the efficiency of these reactions can tip the balance in favor of one of these processing reactions. The specificity of the FCA-FY interaction, described here, may therefore establish a regulatory paradigm and suggests that the search for regulators of 3′ end formation in humans could begin with the characterization of WDC146 and its protein partners.

The *Arabidopsis* genome encodes many more RRM-type RNA binding proteins than that of *D. melanogaster* and *C. elegans* (Lorkovic and Barta, 2002). More than half of these genes have no obvious metazoan homolog (Lorkovic and Barta, 2002). This indicates that these RNA binding proteins might regulate plant-specific processes (like FCA in controlling flowering time), but how do they function? They must act either in as yet undiscovered mechanisms of RNA processing or as novel regulators of established RNA processing events. Here, we have shown that FCA interacts with a plant-specific Pro-Pro-Leu-Pro-containing domain of an otherwise highly conserved component of the 3′ end-processing machinery. We speculate that the evolution of this domain has facilitated the interaction of this conserved RNA processing complex with a regulatory RNA binding protein, and together, this has enabled regulated gene expression to control the timing of higher plant reproductive development. Likewise, the variable C-terminal sequence of Pfs2p/FY related proteins in other higher eukaryotes may have evolved as a platform to enable the association of the 3′ end-processing machinery with additional regulatory proteins.

Experimental Procedures

Plant Material, Growth Conditions, and Flowering Time Analyses

The L*er*, *fca-1*, *fpa -2, fwa-1, fve-1*, and *fy-1* lines were originally obtained from M. Koornneef (Wageningen University), *fy-2* was obtained from Syngenta, and *gi-3* was from George Coupland (Max-Planck Institute, Cologne). Flowering time measurements were performed as previously described (Macknight et al., 2002).

Plasmid Construction

The WF mutation was introduced into pFCAcDNA (Macknight et al., 2002) with oligo W23F (5′-cctcaggtttttcgaacttgctttcacc) by using the U.S.E. Mutagenesis kit (Pharmacia Biotech) to create pFCAcDNAWF. An EcoRI, XhoI fragment of pFCAcDNAWF was subcloned into pSLJ755I5 (Jones et al., 1992). GST-HMK-FCA was prepared by amplifying pFCAcDNA (Macknight et al., 2002) by PCR with FCA_E2 (5′-ctgccaccacaggaattcatcagccgtgc) and FCA3′a (5′-aggc cattgtttggcagctc). Amplified DNA was cut with EcoRI and EcoRV and cloned into pGTK (Stone et al., 1994) to make pGTK-A1. A BamHI, XhoI fragment was subcloned from this vector into pGex-6P-1 (Pharmacia Biotech) to create pGex6P-A1. The W-F mutation was introduced into pGex6p-A1 using oligo W23F as described above. GST:FCA-WW was prepared by mutating pFCAcDNA (Macknight et al., 2002) with the Kunkel procedure (Kunkel, 1985) to introduce an EcoRI site with GSO213 (5′-gagccaaagaggcctaaatctaga gaattccctggcgagtcaagggac) and a SalI site with GSO210 (5′-atgtg gaagaataaaacttgacgtcgacctggtacatgagacgaggag) to create pGGS214. An EcoRI, SalI fragment was subcloned from pGGS214 into pGEX-6p-1 to make GST:FCA-WW. GST:FCA-WF was prepared by subcloning an EcoNI fragment from pFCAcDNA-WF into pGGS214 to create pGGS214WF and an EcoRI, SalI fragment was then subcloned from here into pGEX-6p-1. A 5′ fragment of *FY* was obtained by performing RT-PCR on *Arabidopsis* (Col) RNA with GSO365 (5′-gtaatctgcagctgtatgatataagg) and GSO395 (5′-cattg gaagcatttgctgggg). The amplified product was cloned into pGEM-Teasy (Promega), creating pGGS322. *FY* coding sequence was amplified by PCR with GSO445 (5′-cggaattcatgtacgccggcggcg) and GSO385 (5′-cctccatcatcaccagaaacc). Amplified product was cut with EcoRI and HindIII and subcloned into *FY* EST (BG459222) to create full-length FY, pGGS338. *35S::FY* was prepared by amplifying pGGS338 by PCR with GSO446b (5′-ggaattcaattcaataaaccatg tacgccggcggcg) and 35SR (5′-cgggatccctactgatgttgctgattgtt). Amplified product was cleaved with EcoRI and BamHI and cloned into pSOV (Mylne and Botella, 1998).

Expression Analysis

Protein and total RNA extractions were made with TRIZOL (Invitrogen), while poly(A)$^+$ RNA was purified as described (Quesada et al., 2003). Western and Northern analyses were performed as previously described (Quesada et al., 2003). Northern analysis of *FY* expression was performed with a probe prepared by amplifying the FY cDNA with FY1F (5′-aatcccgaattcgttcttatgcagaacc) and FY1R (5′-cgcggatccctactgatgttgctgattg).

Plant Transformation

Transgenes were introduced into *Agrobacterium* strain C58C1 pGV2260 by triparental mating and transformed into either *Arabidopsis thaliana* L*er* or *fca-1* by floral dip (Clough and Bent, 1998).

Affinity Precipitation and Far Western

FCA#6 protein (Macknight et al., 2002) was coupled to N-hydroxysuccimidyl chloroformate activated agarose (Sigma) as described (Harlow and Lane, 1988). The coupling reaction was buffered in 0.1 M $NaPO_4$ (pH 7.6), and the agarose beads were blocked with 0.1 M ethanolamine throughout the coupling reaction in order to reach approximately 80% coupling efficiency. Beads with FCA protein bound in the range of 0.6 to 1.0 μg protein per μl beads were used in affinity precipitation experiments.

400 mg fresh-weight of *Arabidopsis* seedlings (harvested 12 days after germination) were crushed in liquid N_2 and resuspended in 12.5 mM Tris (pH 7.5), 150 mM NaCl, 1% SDS. Following incubation at 100°C for 5 min, cell debris was removed by centrifugation at 13,000 × g for 3 min. SDS was precipitated from the supernatant by incubation in an ice/water bath. 1 ml of the supernatant was then transferred to a fresh microfuge tube containing 20 μl FCA#6 protein-coupled beads. The beads and extract were mixed by end-over rotation for 2 hr at 4°C and then washed three times with 500 μl TBST (25 mM Tris [pH 8.0], 137 mM NaCl, 2.7 mM KCl, 0.1% v/v Tween-20). After the last wash, 20 μl SDS-PAGE loading buffer was added and the sample was incubated at 100°C for 3 min and centrifuged, and the supernatant was separated by SDS-PAGE. Proteins were electroblotted to PVDF membrane (Millipore). The membrane was blocked in TBST plus 1.1% w/v dried milk powder for 90 min. Fresh blocking buffer was added with 0.5×10^6 cpm of labeled WW domain protein (1 μg purified HMK target sequence tagged-FCA protein was phosphorylated as described [Braun et al., 1997]) in 15 μl using 25 μCi [γ-^{32}P]ATP and incubated with gentle agitation for 6 hr at 4°C. The membrane was washed four times with cold TBST at 4°C and the filter was exposed to a phosphoimaging screen.

GST In Vitro Pull-Down Assay

FY was translated in vitro with TnT T3/T7 coupled reticulocyte lysate (Promega). 10 μl bed volume of washed GST, GST-WW, and GST-WF bound Sepharose was blocked for 30 min at 4°C in 1.5 mg·ml^{-1} BSA, 0.1 mg·ml^{-1} glycogen. After blocking, the GST-coupled Sepharose was washed three times with IP buffer (12.5 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.01% v/v NP-40). TnT translation products (15 μl) were added to the blocked and washed GST fusion Sepharose, mixed by end-over rotation at 4°C for 2 hr, and washed three times with IP buffer. The Sepharose beads were then resuspended in 20 μl SDS-PAGE loading buffer and incubated for 5 min at 100°C, and released proteins were separated by SDS-PAGE. Following electrophoresis, gels were fixed, dried, and exposed to a phosphoimaging screen for detection of labeled proteins.

Identification of *fy-1* and *fy-2* Mutations

DNA was isolated from *fy-1* and amplified by PCR and sequenced. The *fy-1* mutation was confirmed by dCAPs: DNA was amplified by PCR with GSO420 (5′-gcgaccaaaaccttgttcactaaagc) and GSO421 (5′-aactcagacccaagtactcgg). PCR product was cleaved with HindIII. DNA amplified from *fy-1* is cut in this reaction, while DNA amplified from wild-type plants is not.

fy-2 was identified by searching the Syngenta T-DNA insert collection for insertions in *FY*. Line GARLIC 657D4 has an insertion within *FY* exon 16. The insertion site was confirmed by sequencing and monitored by PCR amplification with FY4 (5′-ctgttggaaagggttgttg tagcctggaatc) and LB3 (5′-tagcatctgaatttcataaccaatctcgatacac).

Complementation of *fy*

Despite exhaustive attempts, we were unable to transform *fy-1* directly by conventional procedures (Clough and Bent, 1998). Therefore, a transgene designed to express *FY* from the CaMV 35S promoter was introduced into wild-type L*er*. Transgenic plants carrying single locus insertions were identified and a *35S::FY* line was crossed to *fy-1*. The resulting segregating F2 populations were analyzed for flowering time. All homozygous *fy-1* plants that expressed *35S::FY* flowered as early as wild-type.

Acknowledgments

We thank J. Walker for pGTK; the *Arabidopsis* stock center (AIMS), M. Pratt, and M. Grusak for *FY* ESTs; and Syngenta for *fy-2*. We thank L. Clissold, C. Nicholls, and M. Smith for technical assistance; A.R. Gendall for helpful discussions; and D. Bradley and D. Baulcombe for comments on the manuscript. This work was supported by Marie Curie fellowships to V.Q. and P.P.D., Ministerio de Educacion y Cultura of Spain (V.Q.), Gatsby Charitable Trust (I.H.), and a Core Strategic Grant from the Biotechnology and Biological Sciences Research Council.

Received: February 5, 2003
Revised: May 13, 2003
Accepted: May 20, 2003
Published: June 12, 2003

References

Barabino, S.M., and Keller, W. (1999). Last but not least: regulated poly(A) tail formation. Cell *99*, 9–11.

Beaudoing, E., and Gautheret, D. (2001). Identification of alternate polyadenylation sites and analysis of their tissue distribution using EST data. Genome Res. *11*, 1520–1526.

Bedford, M.T., Sarbassova, D., Xu, J., Leder, P., and Yaffe, M.B. (2000). A novel Pro-Arg motif recognized by WW domains. J. Biol. Chem. *275*, 10359–10369.

Braun, D.M., Stone, J.M., and Walker, J.C. (1997). Interaction of the maize and *Arabidopsis* kinase interaction domains with a subset of receptor-like protein kinases: implications for transmembrane signaling in plants. Plant J. *12*, 83–95.

Brogna, S., and Ashburner, M. (1997). The Adh-related gene of *Drosophila melanogaster* is expressed as a functional dicistronic messenger RNA: multigenic transcription in higher organisms. EMBO J. *16*, 2023–2031.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. *16*, 735–743.

Edwalds-Gilbert, G., Veraldi, K.L., and Milcarek, C. (1997). Alternative poly(A) site selection in complex transcription units: means to an end? Nucleic Acids Res. *25*, 2547–2561.

Frischmeyer, P.A., van Hoof, A., O'Donnell, K., Guerrerio, A.L., Parker, R., and Dietz, H.C. (2002). An mRNA surveillance mechanism that eliminates transcripts lacking termination codons. Science *295*, 2258–2261.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual. (Cold Spring Harbor, Neew York: Cold Spring Harbor Laboratory).

Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M., and Eck, M.J. (2000). Structure of a WW domain containing fragment of dystrophin in complex with beta-dystroglycan. Nat. Struct. Biol. *7*, 634–638.

Ito, S., Sakai, A., Nomura, T., Miki, Y., Ouchida, M., Sasaki, J., and Shimizu, K. (2001). A novel WD40 repeat protein, WDC146, highly expressed during spermatogenesis in a stage-specific manner. Biochem. Biophys. Res. Commun. *280*, 656–663.

Jones, J.D.G., Shlumukov, L., Carland, F., English, J., Scofield, S.R., Bishop, G.J., and Harrison, K. (1992). Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. Transgenic Res. *1*, 285–297.

Juge, F., Audibert, A., Benoit, B., and Simonelig, M. (2000). Tissue-specific autoregulation of *Drosophila* suppressor of forked by alternative poly(A) site utilization leads to accumulation of the suppressor of forked protein in mitotically active cells. RNA *6*, 1529–1538.

Koepf, E.K., Petrassi, H.M., Ratnaswamy, G., Huff, M.E., Sudol, M., and Kelly, J.W. (1999). Characterization of the structure and function of W → F WW domain variants: identification of a natively unfolded protein that folds upon ligand binding. Biochemistry *38*, 14338–14351.

Koornneef, M., Hanhart, C.J., and Van der Veen, J.H. (1991). A genetic and physiological, analysis of late flowering mutants in *Arabidopsis thaliana*. Mol. Gen. Genet. *229*, 57–66.

Koornneef, M., Blankestijn-de Vries, H., Hanhart, C., Soppe, W., and Peeters, T. (1994). The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. Plant J. *6*, 911–919.

Koornneef, M., Alonso-Blanco, C., Blankestijn-de Vries, H., Hanhart, C.J., and Peeters, A.J.M. (1998). Genetic interactions among late flowering mutants of *Arabidopsis*. Genetics *148*, 885–892.

Kunkel, T. (1985). Rapid and efficient site-directed mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA *82*, 488–492.

Lee, I., Aukerman, M.J., Gore, S.L., Lohman, K.N., Michaels, S.D., Weaver, L.M., John, M.C., Feldmann, K.A., and Amasino, R.M. (1994). Isolation of *LUMINIDEPENDENS*: a gene involved in the control of flowering time in *Arabidopsis*. Plant Cell *6*, 75–83.

Lorkovic, Z.J., and Barta, A. (2002). Genome analysis: RNA recognition motif (RRM) and K homology (KH) domain RNA-binding proteins from the flowering plant *Arabidopsis thaliana*. Nucleic Acids Res. *30*, 623–635.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997). *FCA*, a gene controlling flowering time in *Arabidopsis*, encodes a protein containing RNA-binding domains. Cell *89*, 737–745.

Macknight, R., Duroux, M., Laurie, R., Dijkwel, P., Simpson, G., and Dean, C. (2002). Functional significance of the alternative transcript processing of the *Arabidopsis* floral promoter FCA. Plant Cell *14*, 877–888.

Michaels, S.D., and Amasino, R.M. (1999). *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell *11*, 949–956.

Michaels, S.D., and Amasino, R.M. (2001). Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. Plant Cell *13*, 935–941.

Mitchelson, A., Simonelig, M., Williams, C., and O'Hare, K. (1993). Homology with *Saccharomyces cerevisiae* RNA14 suggests that phenotypic suppression in *Drosophila melanogaster* by suppressor of forked occurs at the level of RNA stability. Genes Dev. *7*, 241–249.

Mouradov, A., Cremer, F., and Coupland, G. (2002). Control of flowering time: interacting pathways as a basis for diversity. Plant Cell *14*, S111–S130.

Mylne, J.S., and Botella, J.R. (1998). Binary vectors for sense and antisense expression of *Arabidopsis* ESTs. Plant Mol. Biol. Rep. *16*, 257–262.

Ohnacker, M., Barabino, S.M., Preker, P.J., and Keller, W. (2000). The WD-repeat protein pfs2p bridges two essential factors within

the yeast pre-mRNA 3′-end-processing complex. EMBO J. *19*, 37–47.

Quesada, V., Macknight, R., Dean, C., and Simpson, G.G. (2003). Autoregulation of the site of 3′ end formation in *FCA* pre-mRNA prevents precocious flowering. EMBO J. *22*, 3142–3152.

Schomburg, F.M., Patton, D.A., Meinke, D.W., and Amasino, R.M. (2001). *FPA*, a gene involved in floral induction in *Arabidopsis*, encodes a protein containing RNA-recognition motifs. Plant Cell *13*, 1427–1436.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999). The *FLF* MADS box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. Plant Cell *11*, 445–458.

Simpson, G.G., and Dean, C. (2002). *Arabidopsis*, the Rosetta stone of flowering time? Science *296*, 285–289.

Stone, J.M., Collinge, M.A., Smith, R.D., Horn, M.A., and Walker, J.C. (1994). Interaction of a protein phosphatase with an *Arabidopsis* serine-threonine receptor kinase. Science *266*, 793–795.

Sudol, M., and Hunter, T. (2000). NeW wrinkles for an old domain. Cell *103*, 1001–1004.

Takagaki, Y., and Manley, J.L. (1992). A human polyadenylation factor is a G protein beta-subunit homologue. J. Biol. Chem. *267*, 23471–23474.

Takagaki, Y., and Manley, J.L. (2000). Complex protein interactions within the human polyadenylation machinery identify a novel component. Mol. Cell. Biol. *20*, 1515–1525.

Verdecia, M.A., Bowman, M.E., Lu, K.P., Hunter, T., and Noel, J.P. (2000). Structural basis for phosphoserine-proline recognition by group IV WW domains. Nat. Struct. Biol. *7*, 639–643.

Zarrinpar, A., and Lim, W.A. (2000). Converging on proline: the mechanism of WW domain peptide recognition. Nat. Struct. Biol. *7*, 611–613.

Zhao, J., Hyman, L., and Moore, C. (1999). Formation of mRNA 3′ ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. Microbiol. Mol. Biol. Rev. *63*, 405–445.