An allelic series reveals essential roles for FY in plant development in addition to flowering-time control

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Summary

The autonomous pathway functions to promote flowering in *Arabidopsis* by limiting the accumulation of the floral repressor *FLOWERING LOCUS C* (*FLC*). Within this pathway *FCA* is a plant-specific, nuclear RNA-binding protein, which interacts with FY, a highly conserved eukaryotic polyadenylation factor. FCA and FY function to control polyadenylation site choice during processing of the *FCA* transcript. Null mutations in the yeast FY homologue Pfs2p are lethal. This raises the question as to whether these essential RNA processing functions are conserved in plants. Characterisation of an allelic series of *fy* mutations reveals that null alleles are embryo lethal. Furthermore, silencing of *FY*, but not *FCA*, is deleterious to growth in *Nicotiana*. The late-flowering *fy* alleles are hypomorphic and indicate a requirement for both intact FY WD repeats and the C-terminal domain in repression of *FLC*. The FY C-terminal domain binds FCA and in vitro assays demonstrate a requirement for both C-terminal FY-PPLPP repeats during this interaction. The expression domain of *FY* supports its roles in essential and flowering-time functions. Hence, FY may mediate both regulated and constitutive RNA 3′-end processing.

Key words: *Arabidopsis thaliana*, flowering, polyadenylation, FCA, FY

Introduction

The switch to flowering is a key developmental transition in the plant life cycle. Entry into reproductive development is modulated with respect to both environmental and endogenous cues (Simpson and Dean, 2002). Genetic analysis in the model plant *Arabidopsis* has identified several pathways that regulate flowering time (Simpson and Dean, 2002). These pathways quantitatively regulate a key set of floral-integrator genes, the activity of which are important for flowering (Simpson and Dean, 2002). Wild accessions of *Arabidopsis* either overwinter vegetatively (winter annuals) or show a rapid-cycling habit, with flowering of winter annuals being promoted by the extended cold of winter, a process known as vernalization. Vernalization requirement is conferred by two genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Johanson et al., 2000; Michaels and Amasino, 1999; Sheldon et al., 1999). Active *FRI* alleles increase the accumulation of *FLC* mRNA, which encodes a MADS-box transcription factor that is a potent repressor of flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). FLC functions to repress flowering by antagonising the activation of floral-integrator genes such as *AGAMOUS-LIKE 20/SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*(*AGL20*/*SOC1*) and *FT* (Hepworth et al., 2002; Michaels and Amasino, 2001; Moon et al., 2003; Samach et al., 2000). The vernalization pathway acts in opposition to *FRI* by repressing *FLC* expression in response to the cold (Michaels and Amasino, 1999; Sheldon et al., 1999).

In parallel to vernalization, the autonomous pathway also acts to promote flowering by repressing expression of *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999). Loss of autonomous function confers a recessive vernalization requirement (Koornneef et al., 1991). The autonomous pathway currently comprises seven genes; *FCA*, *FY*, *FPA*, *FVE*, *FLD*, *FLK* and *LUMINIDEPENDENS* (*LD*), which encode putative transcriptional (*FLD*, *LD*, *FVE*) and post-transcriptional (*FCA*, *FY*, *FPA*, *FLK*) regulators of gene expression (Ausin et al., 2004; He et al., 2003; Lee et al., 1994; Lim et al., 2004; Macknight et al., 1997; Mockler et al., 2004; Schomburg et al., 2001; Simpson et al., 2003). Epistasis analysis indicates that these genes repress *FLC* in a partially non-redundant manner (Koornneef et al., 1998). *FCA* and *FY* make up one epistasis group and this reflects functional interaction of their gene products (Simpson et al., 2003). FCA is a plant-specific, nuclear RNA binding protein with a C-terminal WW protein interaction domain (Macknight et al., 1997). FCA interacts with FY, which is homologous to a highly conserved polyadenylation factor, Pfs2p (Ohnacker et al., 2000; Simpson et al., 2003).

In eukaryotes, the 3′ ends of RNA polymerase-II-generated transcripts are cleaved and polyadenylated and this is an essential step for transcript stability (Zhao et al., 1999). Genetic and biochemical approaches in *Saccharomyces cerevisiae* have defined a large number of conserved proteins required for RNA 3′-end processing, including the polyadenylation factor Pfs2p (Zhao et al., 1999). Pfs2p contains seven WD repeats and acts as an interaction surface

within the cleavage and polyadenylation factor (CPF) 3′-end processing complex (Ohnacker et al., 2000). CPF acts with the cleavage factor I (CFI) complex to direct 3′-end processing of pre-mRNA transcripts (Zhao et al., 1999). The *Arabidopsis* homologue of Pfs2p is FY and this was revealed to play a role in RNA 3′-end processing through analysis of *FCA* gene regulation (Quesada et al., 2003; Simpson et al., 2003). FCA negatively autoregulates its expression by promoting premature cleavage and polyadenylation within intron 3, to generate the non-functional *FCA-β* transcript (Macknight et al., 2002; Quesada et al., 2003). This feedback mechanism requires an interaction between FCA and FY (Quesada et al., 2003; Simpson et al., 2003). The mechanism by which FCA and FY regulate *FLC* is unknown, but this may also involve regulated 3′-end processing. In plants, FY possesses an extended C-terminal domain in addition to seven conserved WD repeats (Fig. 1). This C terminus carries sequences predicted to interact with the FCA WW domain (Sudol and Hunter, 2000). Hence, the novel RNA-binding protein FCA is recruited to FY, a conserved polyadenylation factor, to mediate regulated 3′-processing.

Mutations in the 3′-end processing machinery are generally lethal and this is true for null *pfs2* mutations in yeast (Ohnacker et al., 2000; Wang et al., 2005). However, hypomorphic mutations that are viable can also be recovered (Ohnacker et al., 2000; Zhao et al., 1999). Viable mutations in the *Drosophila* polyadenylation factor, *suppressor of forked* (*su(f)*) were identified as modifiers of retrotransposon insertions in the *Forked* gene (Parkhurst and Corces, 1985; Parkhurst and Corces, 1986). The *su(f)* mutation modified usage of premature polyadenylation sites within the retrotransposon long terminal repeats, restoring *forked* gene expression (Parkhurst and Corces, 1985; Parkhurst and Corces, 1986). However, strong or null *su(f)* alleles are cell-autonomous, lethal mutations that prevent mitotic proliferation (Audibert et al., 1998; Audibert and Simonelig, 1999). To investigate whether *FY* is also an essential gene in *Arabidopsis*, an allelic series of *fy* mutations was characterised. A null *fy* mutation combined with conditional silencing of *FY* in *Nicotiana* demonstrate that FY is required for growth and development in plants. In addition, the different *fy* alleles indicate a requirement for both the conserved FY WD repeats and the C terminus in repression of *FLC*.

Materials and methods

Plant strains, genotyping and cloning

Growth conditions used for flowering-time analyses were as described previously (Macknight et al., 2002). The *fy-1* and *fca-1* mutants were provided by M. Koornneef (Wageningen University, The Netherlands) (Koornneef et al., 1991). The *fca-9* mutant was provided by Chang-Hsien Yang (National Chung Hsing University, Taiwan). The *fy-2* mutant was provided by Syngenta (Sessions et al., 2002). The *fy-3* and *fy-4* mutants were provided by the *Arabidopsis* TILLING project (Targeted Induced Local Lesions IN Genomes; ATP) (McCallum et al., 2000).

Genotyping the *fy* alleles

Plants carrying *fy* mutations were genotyped using PCR markers. The *fy-3* mutation was genotyped by amplifying with the *FY3F* (5′-ACCACACCTTCAGGAAGACGTCTTATCTAG-3′) and *FY3XBA* (5′-TCACCAGAAACCATATAATTTTCATTGTGG-3′) primers, the PCR product was cut with *Xba*I in *fy-3* mutants. The *fy-4* mutation was genotyped by amplifying with the *FY4F* (5′-CCCAAAGTGGGGAGTTTACTCTCT-3′) and *FY4XBA* (5′-CCTCCATCATCACCAGAAACC-3′) primers, the PCR product was cut by *Xba*I in *fy-4* mutants. The dCAPs marker used to identify *fy-1* was as described previously (Simpson et al., 2003). Plants carrying *fy-2* were identified by herbicide (BASTA) resistance conferred by the T-DNA insertion or by PCR. Amplifying with a T-DNA left border primer *LB* (5′-TGGTTCACGTAGTGGGCCATCG-3′) and *FY4* (5′-CTGTTGGAAAGGGTTGTTGTAGCCTGGAATC-3′) produces a product in the presence of the *fy-2* T-DNA insertion.

Construction of the *FY-GUS* transgene

The *FY::GUS* transgene was constructed by PCR, amplifying the *FY* promoter from *Arabidopsis* genomic DNA, made from the Columbia accession, using the primers *PFYF* (5′-CGAGCTCGGTGT-GTTTTTGGG-3′) and *PFYR* (5′-CATGCCATGGTTGCCCACGGA-GAAC-3′). The 1.3 kb PCR product was cloned and sequenced. The P_{FY} sequence was then introduced upstream of β-glucuronidase (GUS) as a *Nco*I-*Sac*I fragment in the pGreen0029 binary vector (Hellens et al., 2000). The *FY::GUS* transgene was introduced into *Arabidopsis* by *Agrobacterium*-mediated floral dip transformation (Bechtold et al., 1993). Transformed lines were selected using kanamycin resistance.

Construction of the *pFY::FY* complementation transgene

The complementation transgene was constructed using the *FY* open-reading frame amplified by RT-PCR using RNA extracted from Col seedlings with the primers *FYBstEIIF* (GGGTCTAGAGGTAACC-TAAATTCGAACACTTTCGCAG) and *F20SalIR* (CCCGTCGACC-TACTGATGTTGCTGATTGTT). The *FY* cDNA was cloned into pCAMBIA1300 as a *Xba*I/*Sal*I fragment. The *FY* promoter was amplified using Pfu (Stratagene) from Columbia genomic DNA with the primers *Pfy5* (GAGGGATCCACTATAGGTGTGGCAAAGCT-CAT) and *Pfy3* (GTTGCCCACGGAGAACAGT) and cloned as a *Bam*HI/*Bst*EII fragment upstream of the *FY* cDNA in pCAMBIA1300. The *FY* 3′-UTR was amplified from Col genomic DNA using the primers *3UTR-1* (GTAGGTCGACGTTGTATTAGTA-CATTAGTTT) and *3UTR-2* (CTCCGTCGACGTCTGCTGTGGTG-GCTTGGGTCTT) and cloned as a *Sal*I fragment downstream of the *FY* cDNA. The pCAMBIA-*pFY::FY* plasmid was transformed into *Agrobacterium* strain GV3101 and used to transform *fy-4/FY* heterozygote plants. Inheritance of *fy-4* in T_1 progeny was analysed by genotyping with a dCAPs marker amplified with *FY4F* and *FY4R* (TT-TAAACAGTCAATACCAGGAGCAG) and digested with *Xba*I.

In situ hybridization, light microscopy and GUS histochemical staining

To analyse seed development whole siliques were fixed for 1 hour at room temperature in Cornoy's solution (acetic acid:ethanol, 1:9) and then washed for 1 hour in 80% ethanol followed by 70% ethanol. The ethanol was then replaced with fresh clearing solution (chloral hydrate:H_2O:glycerol, 8:2:1) and left overnight at room temperature. Cleared seeds were dissected from siliques using 0.2 μm needles and mounted on a slide. Seeds were viewed using differential interference contrast (DIC) microscopy with a Nikon Microphot microscope (×20 or ×40 objectives). Pictures were taken using a Nikon digital camera.

Plants were stained for GUS expression as described previously (Jefferson, 1987). To analyse GUS expression during seed development, material was first fixed in Cornoy's solution for 1 hour at room temperature. After fixation the seed was extensively washed in GUS staining buffer and then GUS stained as described previously (Jefferson, 1987). After staining, seed was cleared as described above and analysed with DIC microscopy.

mRNA in situ hybridization was performed using a published protocol (Coen et al., 1990). 8 μm cross sections of L*er* 10-day shoot meristems and longitudinal sections through siliques at several stages

of development were used. Antisense and sense probes were constructed using the *FY-CT* construct as a template. The insert was amplified using M13 forward and reverse primers and transcribed with T3 RNA polymerase (antisense) and T7 RNA polymerase (sense).

Virus-induced gene silencing

LeFCA sequence from tomato (*Lycopersicon esculentum*) was provided by Dr R. Macknight (University of Otago, New Zealand). *LeFCA* sequence was PCR amplified using *FCAVBAM* (5′-CGGGATCCTTGTTGGATCTGTTCCTAGAAC-3′) and *FCAVHIND* (5′-TTCATCGATTCAGCAAATCTAACAATCAGAGG-3′) primers and cloned into the TRV-00 vector as a *Bam*HI-*Hind*III fragment (Ratcliff et al., 2001). The potato (*Solanum tuberosum*) EST BI176637 provided *StFY* Solanaceous sequence. *StFY* sequence was PCR amplified using *FYVBAM* (5′-CGGGATCCAGGACAGTGTTACAACCTAGC-3′) and *FYVHIND* (5′-TTCATCGATTCTCGTATTGATTCTTTATGTGC-3′) primers and cloned into the TRV-00 vector as a *Bam*HI-*Hind*III fragment. The TRV vectors were transformed into *Agrobacterium* and used to inoculate young tobacco plants as described previously (Ratcliff et al., 2001).

To analyse gene expression, RNA was extracted from leaves systemically infected with either TRV-00 (empty vector), TRV-FY or TRV-FCA and used to generate cDNA. The expression of *FY*, *FCA* and *ACTIN* was analysed by PCR amplification using the following primers: *FYF* (5′-ATGATGCGGCAGCCATCTGCATCC-3′), *FYR* (5′-ACCAGTGACCATCCAGTTATC-3′), *FCAF* (5′-ATTTGTTGGATCTGTTCCTAG-3′), *FCAR* (5′-TCTCGTATTGATTCTTTATGTGC-3′), *ACTINF* (5′-ATGGCAGACGGTGAGGATATTCA-3′), *ACTINR* (5′-GCCTTTGCAATCCACATCTGTTG-3′). PCR reactions were amplified for 27, 30, 32, 35 and 40 cycles and analysed using agarose gel electrophoresis with ethidium bromide staining. Gels were visualised using a fluorescence scanner (Amersham-Pharmacia).

Generation of an FY antibody

FY (residues 416-646) sequence was amplified by PCR and cloned into the pET19b vector (Novagen) using the primers *FYNDEI* (5′-AATCCCAATGTTCTTATGCAGAACC-3′) and *FYR* (5′-CCGGTATACCTACTGCTGTTGCTGATTGTT-3′). This allowed inducible expression and purification of the FY-CT protein with a 6× histidine tag. After elution from the nickel affinity resin, the FY-CT protein was further purified using SDS-PAGE (8% acrylamide) and electroelution (BioRad). The FY-CT antigen was then concentrated using Centricon spin columns (Amicon) and dialysed against PBS. FY-CT was used to immunise two female New Zealand White rabbits according to standard procedures (Harlow and Lane, 1988). Specific FY antibodies were purified from FY cross-reactive serum using affinity purification techniques (AminoLink, Pierce). Purified antibodies were concentrated, dialysed against PBS, supplemented with 10 mg/ml BSA and 0.1% sodium azide as a preservative and stored at –80°C. The FY antibody was used at a concentration of 1:1000 according to standard procedures.

In vitro protein interaction assays

Interaction of FCA and FY proteins was tested using the in vitro GST pull-down assay previously described (Simpson et al., 2003). FY was subcloned as either WD (residues 1-415) or CT domains (residues 416-646). The *FY-WD* subclone was generated by PCR amplification using the *WDF* (5′-GGAATTCAATAAACCATGTACGCCGGCGGCGATATG-3′) and *WDR* (5′-CGGGATCCCTAATCTCGGGGATTATCTGC-3′) primers and cloning the PCR product under the T7 promoter in pBLUESCRIPT IISK–. The *FY-CT* subclone was generated by PCR amplification using the *CTF* (5′-GGAATTCAATAAACCATGGTTCTTATGCAGAACCAAGGC-3′) and *CTR* (5′-CGGGATCCCTACTGATGTTGCTGATTGTTG-3′) primers and cloning the PCR product under the T7 promoter in pBLUESCRIPTIISK-. The FY-CT subclone was then mutagenised to generate the PPLPP→AAAAA mutants using the Quikchange method (Stratagene). PPLPP-1 was mutagenised in two steps using the *PA1A1* (5′-CCATGGCACTGGGGGCTGCTGCTGCGGCACCTGGTCCCCACCCATCG-3′) and *PA1A2* (5′-CGATGGGTGGGGACCAGGTGCCGCAGCAGCAGCCCCCAGTGCCATGG-3′) oligos first, followed by the *PA1B1* (5′-GGGGCTGCTGCTGCGGCAGCTGCTGCCCACGCATCGCTTCTTGGAAGTGGC-3′) and *PA1B2* (5′-GCCACTTCCAAGAAGCGATGCGTGGGCAGCAGCTGCCGCAGCAGCAGCCCC-3′) oligos. PPLPP-2 was mutagenised using the *PA21* (5′-AACAACCCTTTCCAACAGCAGGCAGCTGCAGCTGCTGGCGCTGCACCAAACAACAATCAGCAAC-3′) and *PA22* (5′-GTTGCTGATTGTTGTTTGGTGCAGCGCCAGCAGCTGCAGCTGCCTGCTGTTGGAAAGGGTTGTT-3′) oligos.

Results

The PPLPP repeats within the carboxyl terminus of FY mediate FCA binding

Proteins with strong similarity to FY can be identified in many eukaryotes (Fig. 1). However, the similarity with FY is restricted to the seven N-terminal WD repeats (Fig. 1). In addition to WD repeats many FY-like proteins have extended C-terminal domains that are not highly conserved in sequence between different eukaryotes (Fig. 1). These regions typically consist of low complexity sequence but in some cases carry defined motifs. For example, the human and mouse WDC146 (HsWDC146 and MmWDC146) C termini display similarity

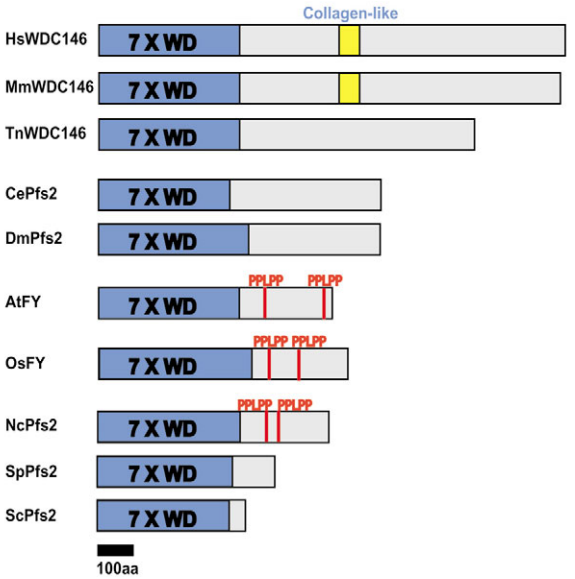

Fig. 1. Domain structure of proteins showing similarity to FY. BLAST searches identified eukaryotic proteins with amino acid similarity to FY (Altschul et al., 1990). The conserved WD repeats are shared between each of the proteins and are shown in blue. The divergent C-terminal domains are shown in grey with the presence of PPLPP motifs or collagen-like domains indicated. These C-terminal domains show little similarity between proteins, apart from the highlighted motifs. The sequences represented are: *Saccharomyces cerevisae* ScPfs2 (NP_014082), *Schizosaccharomyces pombe* SpPfs2 (S62544), *Neurospora crassa* NcPfs2 (XP_322123), *Arabidopsis thaliana* AtFY (NP_196852), *Oryza sativa* OsFY (BAB86205), *Drosophila melanogaster* DmPfs2 (NP_730982), *Caenorhabditis elegans* CePfs2 (NP_496985), *Homo sapiens* HsWDC146 (NP_060853.2), *Mus musculus* (BAC00776) and *Tetraodon nigroviridis* TnWDC146 (CAD27805).

to collagen (Ito et al., 2001) (Fig. 1). The FY C terminus carries two PPLPP motifs, the proline-rich consensus ligand for group-II WW domains such as that present in FCA (Sudol and Hunter, 2000) (Fig. 1). Binding assays were utilised in order to test whether this domain mediates recruitment of FCA to FY polyadenylation complexes directly in vitro.

The C terminus of FCA containing the WW domain was fused to glutathione S-transferase (GST) and purified from *E. coli* as a recombinant protein (Simpson et al., 2003). FY translated in vitro in the presence of [^{35}S]methionine was then tested in a pull-down assay for interaction with GST-FCA. Both the seven N-terminal WD repeats (FY-WD) that mediate protein-protein interactions (Smith et al., 1999) (Fig. 2B) and the C terminus of FY (FY-CT) were tested. FY-CT, but not FY-WD, was found to have strong interaction with GST-FCA (Fig. 2A). We previously demonstrated that FY fails to interact in vitro with mutated GST-FCA-WF protein, and this mutation was also found to block interaction with FY-CT (Fig. 2A) (Simpson et al., 2003). The WF mutation results in the substitution of the second conserved tryptophan of the FCA-WW domain by a phenylalanine and abolishes FCA function in vivo (Simpson et al., 2003). Hence, the FY-CT domain interacts with FCA in a WW domain-dependent manner. To investigate a binding requirement for the FY-PPLPP repeats, site-directed mutagenesis was performed. Structural work indicates that a proline-rich backbone is important for group-II WW domain binding and both FY-PPLPP repeats occur embedded in polyproline tracts (Macias et al., 2002). The PPLPP repeats and flanking prolines were mutated to alanines independently and together to generate single and double PPLPP→AAAAA mutants (Fig. 2D). Interaction assays revealed that mutation of either PPLPP repeat alone did not prevent FY-CT binding to GST-FCA in vitro (Fig. 2C). However, the double PPLPP→AAAAA mutant FY-CT showed no interaction (Fig. 2C). This demonstrates that both PPLPP within the FY-CT are capable of interaction with the FCA WW domain in vitro. Hence, the FY C-terminal domain mediates recruitment of the RNA-binding protein FCA.

FY regulates FLC mRNA accumulation to control flowering time

The FY WD repeats and C terminus are distinct in their degree of conservation throughout eukaryotes (Fig. 1). To investigate the requirement of these domains for FY function in vivo, an allelic series of *fy* mutations was characterised. Forward and reverse genetics provided four *fy* alleles. The *fy-1* mutation was isolated from an EMS screen for late-flowering mutants (Koornneef et al., 1991). Sequencing revealed a splice-acceptor mutation at exon 16 in *fy-1* (Simpson et al., 2003) (Fig. 3A). Mutations caused by insertion of T-DNA can be isolated from the Syngenta SAIL (Syngenta *Arabidopsis* Insertion Library) collection (Sessions et al., 2002). This collection provided the *fy-2* allele, which carries a T-DNA insertion within exon 16 (Fig. 3A). The *fy-3* and *fy-4* mutations were isolated by the *Arabidopsis* TILLING project (McCallum et al., 2000). TILLING allows reverse genetic isolation of EMS-induced mutations in a gene of interest. These *fy* alleles affect the first FY-WD repeat and introduce glycine to serine (G141S) and tryptophan to stop-codon (W150*) substitutions, respectively (Fig. 3A). Together these alleles provide mutations in both the conserved FY WD repeats and the C-terminal domain.

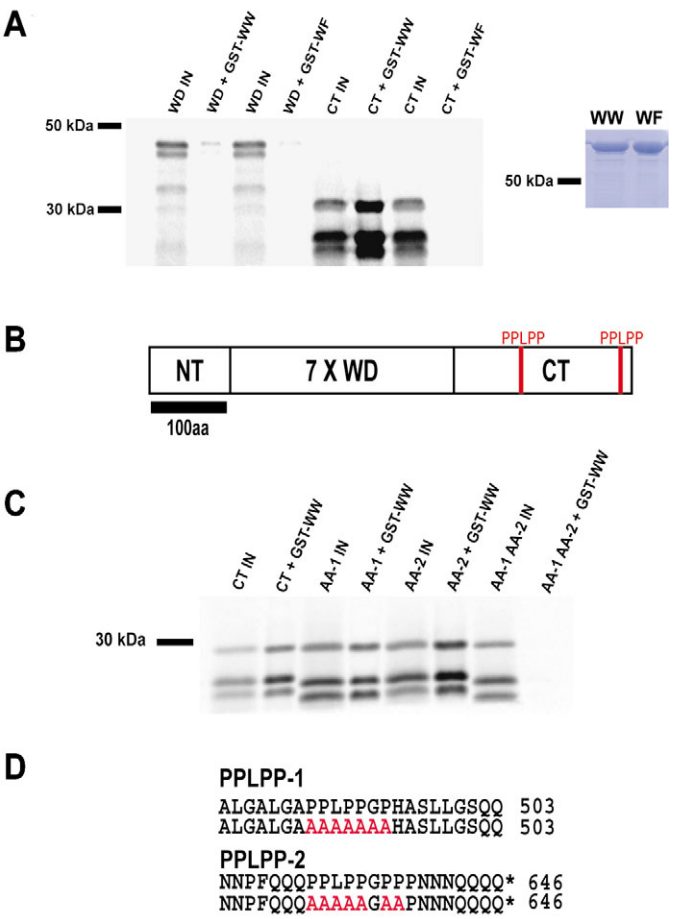

Fig. 2. In vitro interactions between FCA and FY. (A-D) The FY C-terminal PPLPP motifs interact with the FCA WW domain. (A) Autoradiograph of an acrylamide gel loaded with in-vitro translated, ^{35}S labelled FY proteins present before and after binding to GST-FCA proteins. 10% input (IN) lanes were loaded to represent FCA pre-binding. The lanes adjacent to each input lane show FY expressed as either 7×WD repeats (WD) or the C-terminal domain (CT) that was retained after binding to either GST-FCA-WW (GST-WW) or GST-FCA-WF (GST-WF). (Right) A Coomassie Blue-stained acrylamide gel showing that GST-WW and GST-WF proteins were present in equivalent amounts. (B,D) The CT domain carries two PPLPP motifs (B), which were mutated to stretches of alanines (D). (C) CT domains with either PPLPP-1 (AA-1) or PPLPP-2 (AA-2) or both (AA-1,AA-2) mutated to alanines were tested for binding to GST-WW.

The *fy-1*, *fy-2* and *fy-3* mutations are viable and confer late flowering (Fig. 3B). Northern blotting and hybridization reveal that these alleles misregulate *FLC*, the degree of which correlates well with the severity of the late-flowering phenotype (Fig. 3C and Table 1). The *fy-2* mutation has the strongest delay in flowering and the highest level of *FLC* expression (Fig. 3C and Table 1). *FLC* expression levels in *fy-3* and *fy-1* are both lower (Fig. 3C and Table 1). Although *fy-2* and *fy-3* flower later than *fy-1*, a direct comparison of their flowering phenotypes is complicated by differences in genetic backgrounds. The L*er* (*fy-1, fca-1*) accession has a much weaker *FLC* allele relative to Col (*fy-2*, *fy-3*, *fca-9*), caused by the insertion of a Mutator-like element within *FLC* intron 1

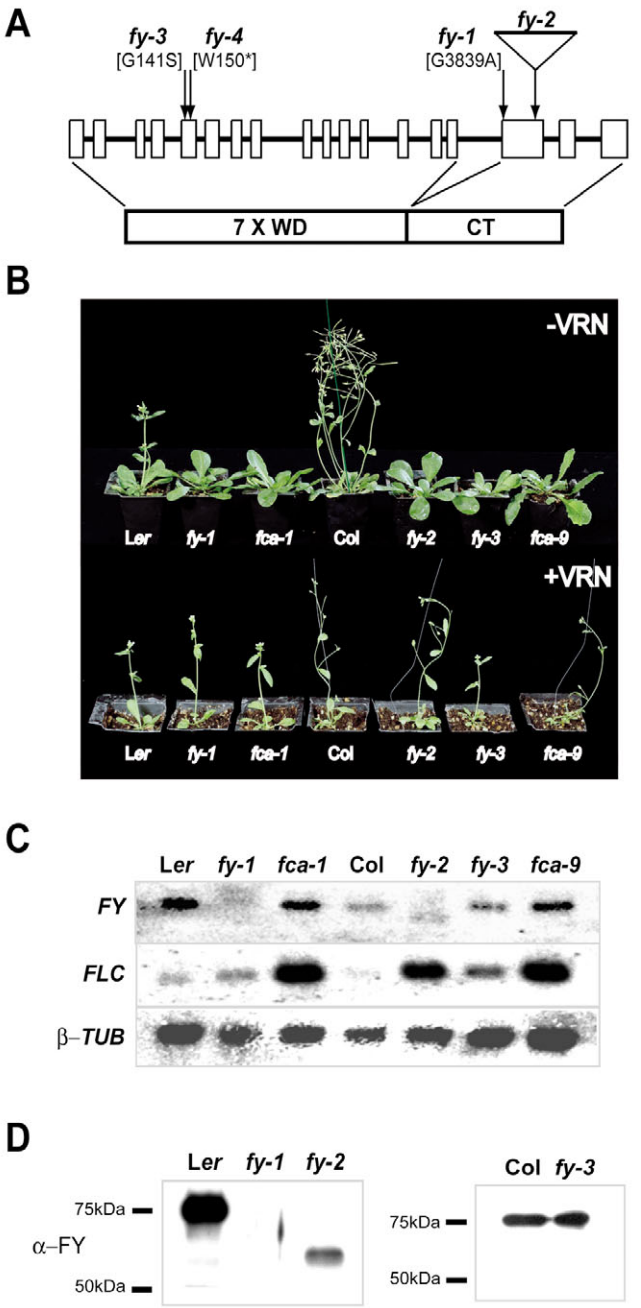

Fig. 3. Analysis of the late-flowering *fy* and *fca* mutants. (A) Schematic diagram of *FY* gene and protein and position of *fy* mutations. The amino acid changes in *fy-3* and *fy-4* and nucleotide change in *fy-1* are indicated in parentheses. Exons are represented as boxes and introns as lines. FY protein is represented as two protein domains, the WD repeats (7×WD) and C-terminal (CT) domain. (B) Phenotypes of late-flowering *fy* and *fca* mutations with (+VRN) and without (–VRN) 6-week vernalization treatments at 4°C. (B) The late-flowering mutants are grown alongside their wild-type, parental accession, either L*er* (*fy-1*, *fca-1*) or Col (*fy-2*, *fy-3*, *fca-9*). (C) Northern blotting and hybridization analysis of *FY*, *FLC* and *β-TUB* mRNA expression in wild-type, *fy* and *fca* mutant backgrounds. (D) Western blot analysis of FY accumulation in wild-type and *fy* mutant backgrounds. For northern and western blot analysis RNA and protein were extracted from non-vernalized, 10-day-old *Arabidopsis* seedlings.

Table 1. Flowering-time of *fy* and *fca* mutants

Genotype	LD–VRN	LD+VRN	SD–VRN
L*er*	9.8±0.21	8.6±0.12	24.9±1.85
fy-1	15.4±0.34	9.3±0.13	49.4±1.118
fca-1	42.1±1.12	10.7±0.19	62.4±1.14
Col	13.6±0.27	10.1±0.25	51.2±3.21
fy-2	55.1±1.31	10.9±0.26	88.3±2.46
fy-3	29.5±0.69	10.8±0.27	66.9±1.14
fca-9	43.0±0.96	10.3±0.20	83.4±2.28
flc-3	10.2±0.44	n.d.	n.d.
fy-2 flc-3	13.6±0.42	n.d.	n.d.
FY / fy-4	10.8±1/1	n.d.	n.d.
fy-2/fy-4	77.3±7.7	n.d.	n.d.

Total leaf-count data for *fy* and *fca* mutant backgrounds. Plants were grown in a controlled environment under long-day (LD) or short-day (SD) photoperiods, with (+VRN) or without (–VRN) a 6-week vernalization treatment at 4°C. Values are±standard error.

(Gazzani et al., 2003; Michaels et al., 2003). The L*er* and Col genotypes are rapid-cycling accessions in which the *fy* and *fca* mutations were isolated. Within the Col background *fy-2* and *fca-9* showed a similar delay in flowering time and an increase in *FLC* expression (Fig. 3C and Table 1). In contrast, *fy-1* shows a much weaker delay in flowering than *fca-1*, which is also in the L*er* background (Fig. 3C and Table 1). Hence, strong *fy* flowering-time alleles are comparable with *fca* in their effects on *FLC* expression, and *fy-1* is likely to be a weak allele.

In order to determine whether the late-flowering phenotype of *fy* occurs through a specific effect on *FLC* regulation or misregulation of multiple transcripts, an *fy-2 flc-3* double mutant was generated. The *flc-3* mutation is a null allele and can suppress the late flowering of mutants in the autonomous pathway and *FRI*-containing lines (Michaels and Amasino, 2001). The *flc-3* mutation was found to suppress *fy-2* late flowering (Fig. 4 and Table 1). A one-way ANOVA shows these differences to be significant ($P<0.001$). This demonstrates that the effects of *fy-2* on flowering time are a specific effect on *FLC* regulation and not a pleiotropic consequence of defects in RNA metabolism. Furthermore, after a 6-week vernalization treatment to repress *FLC* expression, the *fy* mutants flowered early and closely resembled wild type (Fig. 3B and Table 1).

To investigate whether the late-flowering *fy* alleles were null mutations, the expression of *FY* mRNA and FY protein was analysed. Northern blot analysis showed reduced abundance of *FY* mRNA in *fy-1* and the presence of transcripts varying in size (Fig. 3C). This size variation is likely to be due to the utilization of multiple, cryptic splice-acceptor sites and potentially exon skipping (Simpson et al., 1998). Sequencing revealed the presence of premature stop codons in mutant *fy-1* mRNAs, which would disrupt expression of the C-terminal domain (Simpson et al., 2003). No FY protein was found to accumulate in *fy-1* (Fig. 3D), however, the FY antibody was raised using the C-terminal domain as an antigen. A translation product of mutant *fy-1* mRNA would only overlap by approximately seven amino acids with the peptide used to raise antibodies. Hence, although no FY protein is detectable in *fy-1*, this may be due to a lack of expressed epitopes rather than it being a null allele. The possibility of *fy-1* being a hypomorphic allele is also supported by its weak effect on *FLC* expression and flowering time (Fig. 3C and Table 1). The T-

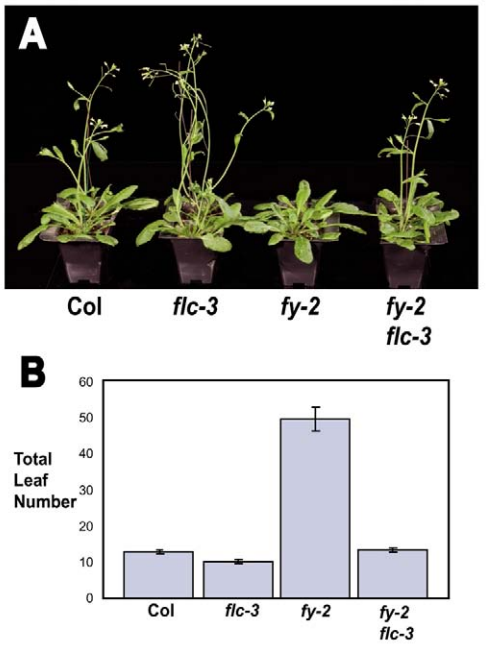

Fig. 4. Flowering time of *fy flc* double mutants. (A) Phenotypes of Col, *flc-3*, *fy-2* and *fy-2 flc-3* plants grown under long day (LD) conditions. (B) Average total number of leaves from 20 plants of each genotype, grown simultaneously under LD conditions (see Table1). Bars indicate the standard error of the mean.

DNA in *fy-2* is inserted within exon 16, 320 nucleotides downstream of the *fy-1* mutation. Although *FY* mRNA is reduced in *fy-2* (Fig. 3C), the 3′ T-DNA insertion site means a truncated protein of 62.4 kDa could be produced, encoding the conserved WD repeats but lacking an intact C-terminal domain. Western blot analysis revealed the presence of this truncated FY protein in *fy-2* (Fig. 3D). Hence, it appears that in both *fy-1* and *fy-2* the FY C-terminal domain is disrupted and this results in *FLC* misexpression. Disruption of this domain is likely to impair recruitment of FCA to FY complexes in vivo because of loss of the PPLPP repeats.

Genetic evidence supporting the function of the FY WD repeats in flowering time is provided by the *fy-3* (G141S) substitution allele. Western blotting reveals that mutant *fy-3* protein accumulates normally (Fig. 3D). Indeed, serine is a relatively common alternative residue at this position within WD repeats and would not be expected to cause severe misfolding (Smith et al., 1999). Although the C-terminal domain is severely disrupted in *fy-1* and *fy-2*, the *fy-3* allele introduces only a minor change to the WD repeats.

FY is required for embryogenesis in *Arabidopsis*

Screening performed by the *Arabidopsis* TILLING project isolated the null *fy-4* mutation (McCallum et al., 2000). This allele carries a premature stop codon at the end of the first FY-WD repeat (Fig. 3A). The tryptophan affected is the signature W residue at the end of the first WD repeat and would lead to

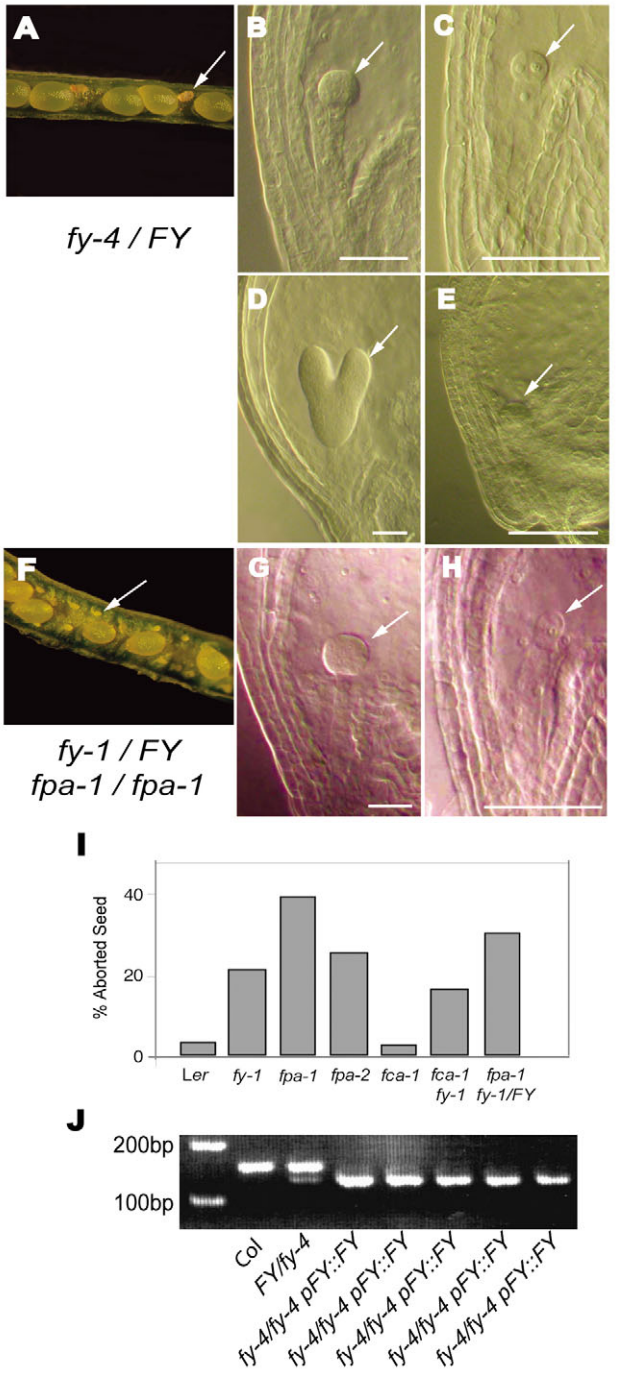

Fig. 5. *FY* is an essential gene in *Arabidopsis.* (A-E) Phenotypic analysis of reproductive development in *fy-4/FY* plants. (A) Gross phenotype of a *fy-4/FY* silique showing healthy, green seeds alongside brown, aborted siblings (arrow). (B-E) DIC images of healthy and aborted seeds in *fy-4/FY* siliques; embryos are indicated with arrows. Healthy embryos are shown at globular (B) and heart (D) stage alongside aborted sibling embryos (C and E, respectively). Scale bars: 25 μm. (F-H) Phenotypic analysis of reproductive development in *fy-1/FY, fpa-1/fpa-1* plants. (F) Gross phenotype of an *fy-1/FY, fpa-1/fpa-1* silique. (G,H) DIC image of wild-type seed at globular stage of embryogenesis (G) and a sibling aborted seed (H) in an *fy-1/FY, fpa-1/fpa-1* silique. White arrows indicate the embryo. Scale bars: 25 μm. (I) Quantification of seed abortion in *fy, fpa* and *fca* mutant backgrounds. (J) Complementation of *fy-4* by the *pFY::FY* transgene is shown by dCAPs analysis. The lower DNA band on the ethidium bromide-stained gel is the *Xba*I-digested product from the *fy-4* allele. Homozygote *fy-4/fy-4* plants are only obtained after transformation with *pFY::FY*.

Table 2. Gametophytic transmission of fy mutations

Cross (paternal × maternal)	F1 genotypes and frequencies		Total
	FY/FY FPA/fpa-1	*FY/fy-1 FPA/fpa-1*	
L*er* × *FY/fy-1 fpa-1 fpa-1*	60% (72)	40% (49)	(121)
FY/fy-1 fpa-1 fpa-1 × L*er*	79% (31)	31% (16)	(51)
	FY/FY	*FY/fy-4*	
Col × *FY/fy-4*	45% (40)	55% (48)	(88)
FY/fy-4 × Col	50% (59)	50% (58)	(117)

Figures in parentheses are numbers of plants analysed.

expression of a severely truncated protein. A dCAPs (derived Cleaved Amplified Polymorphic Sequence) marker designed against this mutation identified *fy-4* heterozygote individuals (Fig. 5J) (Michaels and Amasino, 1998; Neff et al., 1998). However, self-fertilization of *fy-4* heterozygotes yielded neither late-flowering plants nor *fy-4* homozygotes, raising the possibility that this allele is lethal. Indeed, the siliques of *fy-4/FY* plants displayed high levels of seed abortion (Fig. 5A). To analyse the contribution of gametophytic defects to this abortion, *fy-4/FY* plants were crossed reciprocally to Col and the F_1 progeny genotyped for the presence of *fy-4* heterozygotes. In the absence of any gametophytic defect, 50% of the F_1 should be *fy-4* heterozygous. If either *fy-4* gametophyte is lethal, then no transmission will be observed. Crosses using *fy-4/FY* as either pollen donor or acceptor demonstrated normal transmission of *fy-4* through both gametophytes (Table 2). We then analysed seed development in *fy-4/FY* siliques. Plants were fixed, cleared and analysed by differential interference contrast (DIC) microscopy. Siliques were harvested when wild-type seed contained embryos at globular or heart stage (Fig. 5B,D). Alongside their healthy siblings, mutant individuals had aborted development at a very early stage post-fertilization, with the embryo only undergoing a few mitotic divisions (Fig. 5C,E). Hence, disruption of the conserved FY-WD repeats causes a failure in embryogenesis and reveals that *FY* is an essential gene in *Arabidopsis*, consistent with a conserved function in constitutive RNA 3′-end processing.

An *fy-4* heterozygote was also crossed to *fy-2* mutant plants. The F_1 from this cross was genotyped to find individuals carrying both the *fy-2* and *fy-4* alleles and their flowering time analysed. Consistent with *fy-4* being a null allele and *fy-2* providing a partially functional, truncated gene product these *fy-4/fy-2* plants were viable yet late flowering (Table 1). To demonstrate that the lethality observed in *fy-4* was due to disruption of *FY* we performed a complementation experiment. A transgene was constructed to express the *FY* open reading frame (cDNA) under control of the endogenous *FY* promoter and 3′-UTR (*pFY::FY*). This transgene was transformed into *fy-4/FY* heterozygotes and the T_1 progeny were genotyped for the inheritance of *fy-4*. Among 133 T_1 transformants 44 were *FY/FY*, 78 were *FY/fy-4* and 11 were *fy-4/fy-4* homozygotes (Fig. 5J). As self-fertilization of *fy-4/FY* heterozygotes has never produced *fy-4/fy-4* homozygotes we conclude that complementation was observed.

Pleiotropic functions for FY in development were suggested previously by genetic analysis within the autonomous pathway (Koornneef et al., 1998). An *fy fpa* double mutant was never recovered, suggestive of lethality. In contrast, both *fca fy* and *fca fpa* double mutants are viable and late flowering (Koornneef et al., 1998). To provide further insight into these interactions, reproductive defects in plants heterozygous for *fy-1* and homozygous for *fpa-1* mutations were analysed. The siliques of *fy-1/FY, fpa-1/fpa-1* plants displayed a high incidence of aborted seed (Fig. 5F). To analyse possible gametophytic defects mutant plants were crossed reciprocally to L*er* plants. Significant transmission of *fpa-1 fy-1* gametes was observed through both crosses, indicating that gametophytic defects are unlikely to be the major cause of *fy fpa* lethality (Table 2). However, less than 50% of the F_1 progeny were *fy-1* heterozygous when plants were used as either pollen-donor or acceptor (Table 2), meaning that although *fy-1 fpa-1* gametes participate in fertilization, they have reduced vigour or viability. Analysis of seed development in *fy-1/FY, fpa-1/fpa-1* plants revealed that mutant seed had ceased dividing and aborted very early after fertilization, with a similar phenotype to that observed in *fy-4* (Fig. 5H). Furthermore, examination of *fy-1*, *fpa-1* and *fpa-2* single mutants revealed elevated levels of seed abortion relative to L*er* (Fig. 5I). In contrast, *fca-1* siliques were indistinguishable from wild type and *fca-1* did not enhance the defect observed in *fy-1* in the *fca-1 fy-1* double mutant (Fig. 5I). A one-way ANOVA shows these differences to be significant ($P<0.001$). Hence, *fy-1* and *fpa-1* have weakly penetrant defects in seed development, which combine to cause synergistic lethality. These interactions are likely to reflect essential FY functions. The fact that *fca-1* lacks these phenotypes indicates that *FCA* acts more specifically in development than *FY* and *FPA*.

Conditional silencing of FY, but not FCA, is deleterious

The embryo lethality of null *fy-4* mutations precludes analysis of loss of *FY* function later in development. Virus-induced gene silencing (VIGS) allows conditional silencing of target genes in *Nicotiana benthamiana* (Lu et al., 2003). Infection of *N. benthamiana* with a tobacco rattle virus (TRV) vector carrying target gene sequences leads to gene silencing of the endogenous target mRNA (Ratcliff et al., 2001). This system was utilised to assay the effect of silencing *FCA* and *FY* expression during post-embryonic development. *FCA* and *FY* are well conserved throughout higher plants and BLAST searches identified several potato and tomato EST sequences with strong nucleotide similarity to *AtFCA* and *AtFY* (Macknight et al., 1997; Simpson et al., 2003). Within the Solanaceae the sequences homologous to *FCA* and *FY* were >90% identical, making them suitable for use in TRV VIGS in *N. benthamiana* (Lu et al., 2003). Potato (*StFCA*) and tomato (*LeFCA*) *FCA* sequences were cloned into a TRV vector and

called TRV-FCA and TRV-FY respectively. The *LeFCA* sequence was very similar to *AtFCA* sequence from exons 3 and 4, which spans the alternatively processed *FCA* intron 3. The four alternative *FCA* mRNAs contain exon 3 sequence and hence VIGS against this sequence should target all *FCA* transcripts (Macknight et al., 1997; Macknight et al., 2002).

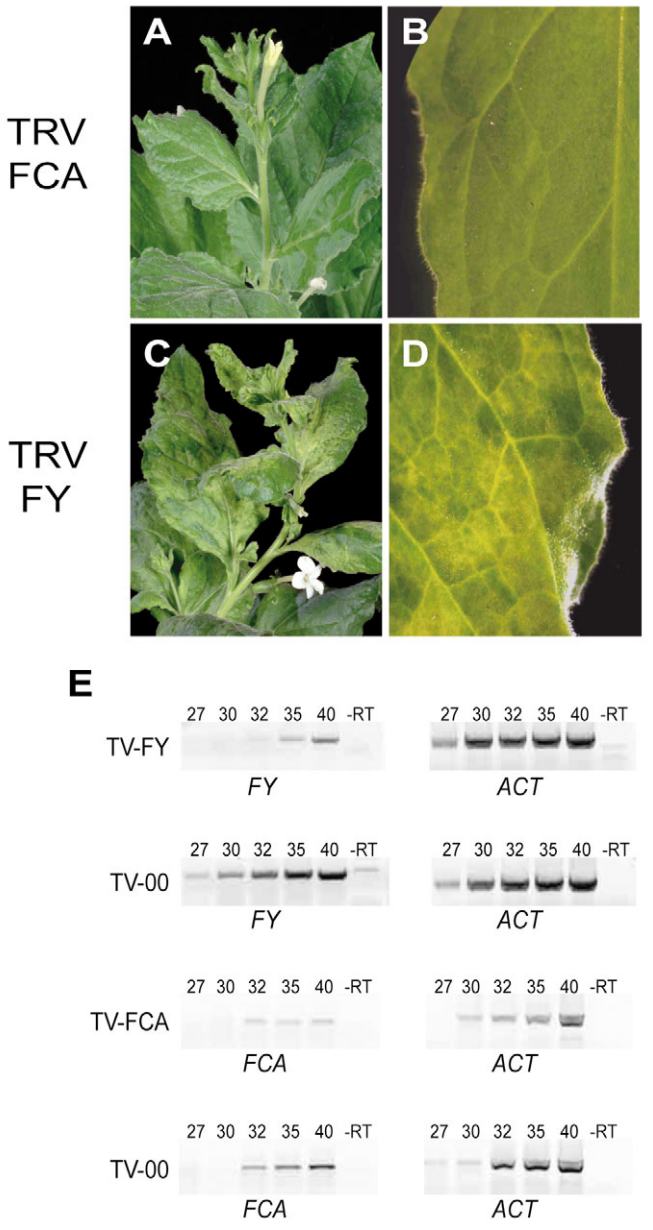

Fig. 6. Virus-induced gene silencing (VIGS) of *FCA* and *FY* in *Nicotiana benthamiana*. (A,B) TRV-FCA (tobacco rattle virus-FCA) infection leads to asymptomatic silencing of *FCA*. Whole shoot (A) and leaf (B) phenotypes of *Nicotiana* plants infected with TRV-FCA. (C,D) TRV-FY infection leads to silencing of *FY* and associated yellowing of leaf tissues. Whole shoot (C) and leaf (D) phenotypes of *Nicotiana* plants infected with TRV-FY. (E) RNA was extracted from *Nicotiana* infected with either TRV-FCA, TRV-FY and TRV-00 (empty vector) and used to generate cDNA. The cDNA was then analysed by RT-PCR for expression of endogenous *FCA*, *FY* and *ACTIN* mRNA. The number of PCR cycles used is indicated above the gel and the final lane (–RT) is a minus RT control.

Young leaves of *N. benthamiana* plants were innoculated with *Agrobacterium* carrying the TRV binary vectors. Transformation and expression of the inserts leads to systemic infection of the plant with TRV, and VIGS-derived phenotypes in newly emerging leaves (Ratcliff et al., 2001). Plants were infected with TRV-00 empty vector as a negative control (Ratcliff et al., 2001). The TRV-00-infected plants showed no obvious phenotypes and infection was largely asymptomatic. The TRV-FCA infected plants were similar to the TRV-00 plants and lacked any obvious phenotype (Fig. 6A,B). The TRV-FY-infected plants exhibited strong phenotypes around 14 days post inoculation (dpi). The systemically infected, emerging leaves displayed yellowing and were distorted (Fig. 6C,D). After a further week of growth, the stature of TRV-FY-infected plants was reduced. These phenotypes are consistent with a reduction in cell viability in the TRV-FY-infected tissues. This supports the conclusion that *FY*, but not *FCA*, is required generally for plant growth and development.

Although silencing of *FY* in *Nicotiana* induced deleterious phenotypes, the infected plants continued to grow and produce leaves and flowers. However, VIGS does not completely eliminate target mRNAs and residual levels of *FY* mRNA may be sufficient for continued growth (Lu et al., 2003; Ratcliff et al., 2001). To investigate the extent of silencing induced by TRV infection, total RNA was extracted from systemically infected tissue and analysed for target gene expression using RT-PCR (Fig. 6E). Expression was assayed relative to TRV-00 infected plants and *ACTIN* mRNA levels, used as an internal control. Both the TRV-FY and TRV-FCA viruses induced silencing of their target endogenous gene, though in both cases residual levels of mRNA remained (Fig. 6E). The remaining *FY* mRNA may provide sufficient FY expression to support the continued growth.

FY expression pattern reflects flowering time and essential functions

The failure of seed development in *fy-4* mutants reveals that *FY* is required for embryogenesis. To determine whether this reflects expression of *FY* during seed development, a reporter transgene was constructed. 1.3 kb of genomic promoter sequence was used to generate a *FY::GUS* transgene. Transformation with *FY::GUS* was performed in the Col background and homozygous lines were analysed for GUS expression. *FY::GUS* siliques were harvested at progressive stages of maturity and seeds histochemically stained for GUS expression (Fig. 7A-C). At globular stage, strong GUS staining was evident throughout the embryo, endosperm and surrounding maternal seed tissues (Fig. 7A). From heart-stage of embryogenesis onwards GUS expression was more restricted to the embryo (Fig. 7B,C), Additionally, the funiculus connecting the seed to the silique showed strong staining, which extended into the chalazal base of the seed (Fig. 7B). To confirm the *FY::GUS* expression pattern we also performed in situ hybridization experiments. Sectioning of embryos and hybridization with an antisense *FY* probe revealed expression in globular, heart and torpedo stage embryos (Fig. 7K-M). No signal was detected when a sense probe was used for hybridization (Fig. 7N). Therefore, consistent with the failure of embryo and endosperm development in *fy-4* mutants, the *FY* promoter is active in these tissues.

The function of FY in regulating flowering time also leads

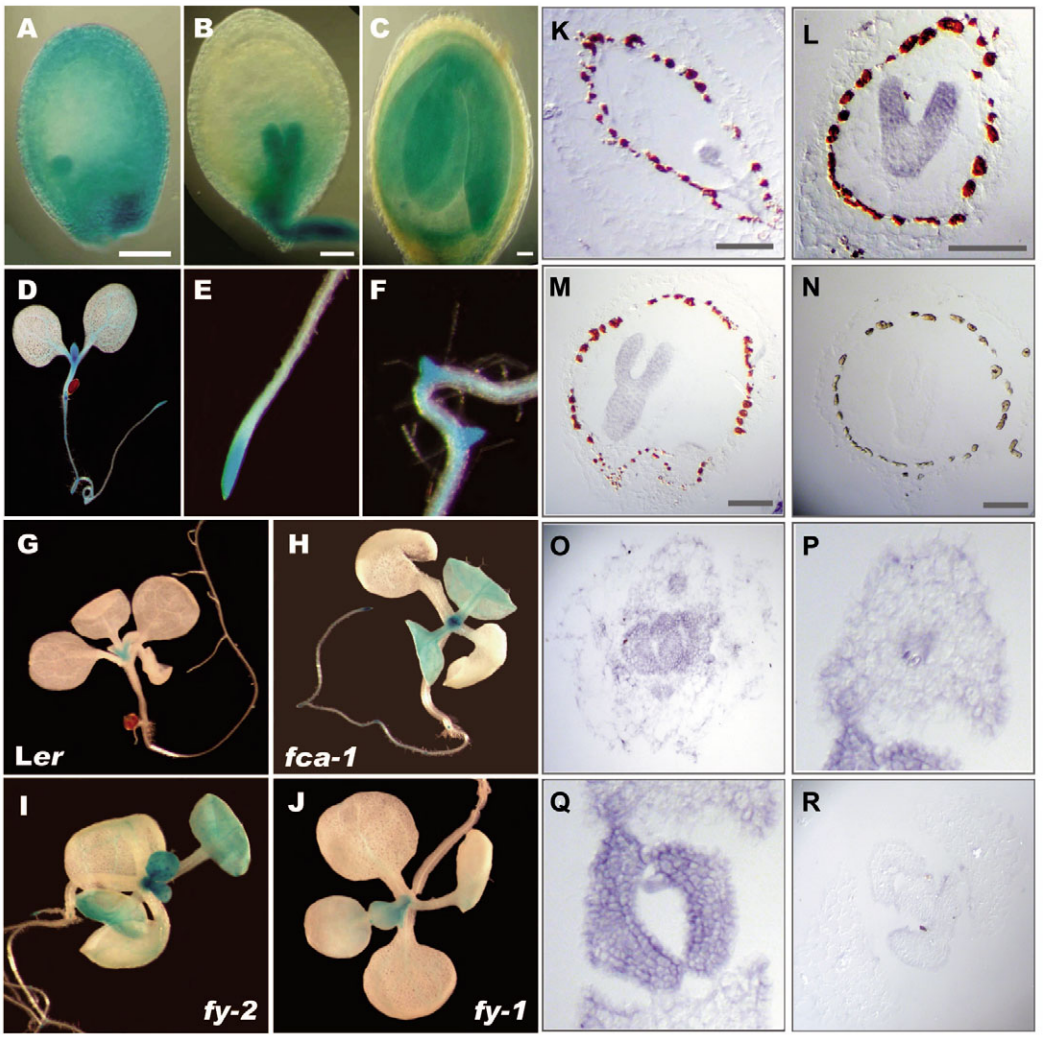

Fig. 7. Expression pattern of *FY*. (A-C) *FY::GUS* expression during seed development. Seeds were histochemically stained for GUS activity which appears as a blue precipitate. Seed were stained at globular (A), heart (B) and mature (C) stages of embryogenesis. Scale bars: 50 µm. (D-F) *FY::GUS* expression in 12-day-old seedlings. Whole seedlings show GUS staining around the shoot meristem and in young leaves and in the vascular tissue of the cotyledons (D). The roots also show GUS staining in the meristematic regions. In roots, both the root apical meristem (E) and emerging lateral root meristems (F) show GUS expression. (G-J) Expression of the P_{FCA}–$FCA_{\text{to exon5}}$:*GUS* reporter transgene in L*er* (G), *fy-1* (J), *fy-2* (I) and *fca-1* (H) backgrounds. 12-day-old seedlings were grown on plates and histochemically stained for GUS expression. (K-R) *FY* expression was confirmed using in situ hybridization. Hybridization with an antisense *FY* probe to sectioned seed (K-M) or mature seedlings (O-Q) showed expression as blue staining. Scale bars: 50 µm. Hybridization with a sense probe to seeds or mature tissue shows no staining (N and R).

to an expectation of post-embryonic expression. *FY::GUS* expression was analysed in 12-day-old seedlings. Strong GUS expression was evident in the meristematic regions of the shoot and the root (Fig. 7D-7F). High expression was clear in the shoot meristem and the adjacent young leaves (Fig. 7D). In cotyledons and older leaves GUS expression was only evident in the vasculature (Fig. 7D). In situ hybridization using an antisense *FY* probe confirmed expression in shoot apical meristems, young leaves and vasculature (Fig. 7O-Q), while no signal was detected when a sense *FY* probe was used (Fig. 7R). In the root, both the apical and emergent lateral meristems showed strong expression (Fig. 7E,F). The *FY::GUS* line with strongest levels of expression also showed staining in differentiated, non-proliferating tissues (data not shown). However, expression was still much weaker relative to meristematic and vascular regions. This expression pattern is closely related to the majority of genes defined to regulate vernalization requirement and response, including *FLC* (Macknight et al., 2002; Schomburg et al., 2001; Sheldon et al., 2002; He et al., 2003) (C. Lister and C. D., unpublished data).

FY expression in meristematic regions may reflect general functions in proliferating tissue or developmental control of its target gene, *FLC*. In addition to regulating *FLC*, *FCA* and *FY* also mediate *FCA* negative autoregulation (Quesada et al., 2003; Simpson, 2003). The P_{FCA}–$FCA_{to\ exon5}$:*GUS* transgene consists of the *FCA* gene with *GUS* inserted downstream of intron 3 (Macknight et al., 2002). FCA-mediated polyadenylation within P_{FCA}–$FCA_{to\ exon5}$:*GUS* intron 3 leads to an absence of *GUS* expression. Thus, the expression of this transgene can be used as an in vivo reporter of *FCA* autoregulation and FCA/FY activity (Macknight et al., 2002; Quesada et al., 2003). In a L*er* background P_{FCA}–$FCA_{to\ exon5}$:*GUS* expression is restricted to the meristematic regions of the shoot and root and is upregulated 6 days after germination (Macknight et al., 2002; Quesada et al., 2003) (Fig. 7G). In *fca-1*, P_{FCA}–$FCA_{to\ exon5}$:*GUS* expression is earlier and in a broader pattern (Fig. 7H), which led to the conclusion that FCA normally limits its own expression through intron 3 autoregulation, but this mechanism is inefficient in meristematic regions (Quesada et al., 2003). Crossing P_{FCA}–$FCA_{to\ exon5}$:*GUS* into *fy* mutant backgrounds should also lead to spatial and temporal upregulation of transgene *GUS* expression. P_{FCA}–$FCA_{to\ exon5}$:*GUS* was crossed into the *fy-1* and *fy-2* mutants and *GUS* expression analysed. The P_{FCA}–$FCA_{to\ exon5}$:*GUS fy-2* line showed pronounced GUS staining in a pattern similar to P_{FCA}–$FCA_{to\ exon5}$:*GUS fca-1* (Fig. 7I). The P_{FCA}–$FCA_{to\ exon5}$:*GUS fy-1* line also showed

increased GUS staining relative to P_{FCA}–$FCA_{to\ exon5}$:*GUS* L*er*, though to a lesser extent than that seen in *fy-2* and *fca-1* backgrounds (Fig. 7J). The weak effect of *fy-1* on P_{FCA}–$FCA_{to\ exon5}$:*GUS* expression reflects its effects on *FLC* and flowering time (Fig. 3C and Table 1). Hence, FY functions in differentiated cells to promote *FCA* alternative polyadenylation. However, in meristematic regions this regulation is inhibited, allowing higher levels of FCA expression.

Discussion

FY was originally identified during a screen for late-flowering mutants (Koornneef et al., 1991). We demonstrate here that *FY* also plays a role in cell viability and these functions are performed differentially by distinct domains of FY. The N terminus of FY is highly conserved with the essential yeast polyadenylation factor Pfs2p and disruption of this domain in *fy-4* causes embryo lethality. In contrast, the plant-specific FY C-terminal domain appears to be required for more limited functions and from a phenotypic analysis just for control of flowering time. The C terminus contains the binding sites for FCA and thus its disruption probably prevents FCA/FY interaction in vivo. The *fy-3* substitution allele demonstrates that intact WD repeats are also required for repression of *FLC*. WD repeats consist of four β-sheets connected by loop regions, with each WD repeat forming a propeller blade (Smith et al., 1999). The glycine (G141) residue affected in *fy-3* is predicted to occur in a structural residue of the B-β-strand in the first propeller blade (Smith et al., 1999). However, the stability of mutant *fy-3* protein makes it unlikely that its effect is via misfolding and destabilisation (Holm et al., 2001; McNellis et al., 1994). Therefore, the G141S substitution may have a specific effect on FY-WD interactions, which reduces *FLC* repression. A direct test of this hypothesis awaits the identification of FY-WD interacting proteins.

The homology of FY to the Pfs2p polyadenylation factor presents a hypothesis for the cause of *fy-4* lethality. Polyadenylation factors are generally essential proteins in eukaryotes and the lethality of such mutations in yeast reflects an inability to correctly process 3′ ends of transcripts (Zhao et al., 1999; Dheur et al., 2003; Ohnacker et al., 2000; Wang et al., 2005). Additionally, loss of the *Drosophila* Su(f) polyadenylation factor leads to cell-autonomous defects in proliferation and viability (Audibert and Simonelig, 1999). FY essential functions may reflect a conserved role in general RNA 3′-end processing. However, although the WD repeats of FY and Pfs2p are highly homologous, *FY* fails to complement *PFS2* function in *S. cerevisae* (I.R.H. and C.D., unpublished data). Hence, some aspects of their function appear to have diverged. An alternative possibility is that FY could function to regulate a subset of RNAs, one or several of which are essential.

Although FY may function generally in RNA processing, it is possible that it performs a more specialised role in regulated 3′-end processing. In vitro study of polyadenylation has commonly used constitutively utilised 3′-end processing signals (Zhao et al., 1999). However, there is abundant evidence that regulated 3′-end processing occurs in vivo (Beaudoing and Gautheret, 2001; Edwalds-Gilbert et al., 1997). The cis signals and trans factors mediating alternative polyadenylation are poorly understood, though polyadenylation site choice during *FCA* autoregulation represents one instance (Macknight et al., 2002; Quesada et al., 2003). With respect to FY function in constitutive versus regulated polyadenylation it is important to consider potential redundancy within 3′-end processing complexes (Keller and Minvielle-Sebastia, 1997). Pfs2p and a second polyadenylation factor, CstF-50, are proposed functional orthologues based on their domain organisation and similar protein interactions (Ohnacker et al., 2000; Takagaki and Manley, 1992). *S. cerevisae* is unusual relative to other eukaryotes in encoding only a Pfs2p-like protein. The presence of both CstF-50-like and Pfs2p-like proteins in other eukaryotes may have facilitated functional divergence of Pfs2p-like proteins. Indeed, Pfs2p-like proteins sequenced from eukaryotes other than *S. cerevisae* display unusual features. The acquisition of distinct C-terminal domains is evident in many FY/Pfs2p homologues (Fig. 1). By analogy, these domains may function as binding sites for trans-regulators of 3′-end processing, similar to FCA. A developmental function for the mammalian FY/Pfs2p homologue, WDC146, is probably the result of its specific expression pattern during spermatogenesis and its absence from constitutive polyadenylation complexes (Ito et al., 2001; Zhao et al., 1999). The functions of FY/Pfs2p proteins in regulated polyadenylation may be addressed by searching for proteins interacting with these C-terminal domains. It will be interesting to determine whether distinct FY polyadenylation complexes mediate flowering time and essential functions, and furthermore whether this reflects different roles in regulated versus constitutive RNA 3′-end processing.

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