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Regulated RNA processing in the control of *Arabidopsis* flowering

VICTOR QUESADA[1], CAROLINE DEAN[2] and GORDON G. SIMPSON*[3,4]

[1]División de Genética and Instituto de Bioingeniería, Universidad Miguel Hernández, Campus de Elche, Elche, Spain, [2]Department of Cell & Developmental Biology, John Innes Centre, Norwich, U.K, [3]Dundee University Plant Research Unit at SCRI, Invergowrie, Dundee, U.K. and [4]Gene Expression Programme, Scottish Crop Research Institute, Invergowrie, Dundee, UK.

ABSTRACT Flowering time is controlled in order to ensure reproductive success. Molecular genetic analyses in *Arabidopsis thaliana* have identified many genes regulating this developmental switch. One group of factors which promote flowering do so by down-regulating the expression of the MADS-box floral repressor, *FLC*. RNA processing appears to play an important role in this regulation as genes within this group encode RNA binding proteins (FCA, FPA and FLK) and an mRNA 3' end processing factor (FY). FCA promotes flowering and negatively autoregulates its own expression post-transcriptionally through a mechanism that involves alternative polyadenylation. FCA physically interacts with FY and this interaction is required for the function FY performs in flowering control and in *FCA* autoregulation. Potential similarities are emerging in the molecular mechanisms controlling *FLC* expression and those controlling the floral homeotic gene, *AGAMOUS*. In addition, microRNAs have been shown to regulate plant developmental processes including the timing to flower. Together, these new data indicate that post-transcriptional regulation of gene expression plays an important role in regulating the floral transition.

KEY WORDS: *Arabidopsis thaliana, flowering time, RNA processing, RNA-binding protein, miRNA*

Introduction

Flowering time control is a complex process that involves the integration of responses to multiple environmental stimuli and endogenous signals. This complexity ensures that flowering occurs in conditions favourable for reproductive success. The use of *Arabidopsis thaliana* as a model system has helped define the mechanisms that control flowering. The molecular genetic characterization of *A. thaliana* mutants defective in flowering time control has led to the identification of many genes involved in this process. These genes comprise genetically separable pathways that quantitatively control the transition to flowering. The role of these pathways can be classified as promoting, repressing and enabling flowering or resetting the vegetative state (Boss *et al*., 2004; Fig. 1). Distinct pathways promote flowering in response to day-length, the phytohormone gibberellic acid, changes in light quality and ambient temperature. These pathways converge on the activation of an overlapping set of genes known as "floral pathway integrators" (Simpson and Dean, 2002; Fig. 1). In turn, the products of these genes activate or up-regulate floral meristem identity genes required for flower formation such as *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) and *LEAFY* (*LFY ;* see Simpson and Dean, 2002) (Fig. 1). The up-regulation of floral pathway integrators is antagonized by the MADS box transcription factor, FLC, a key repressor of flowering in *A. thaliana* (Michaels and Amasino, 1999; Sheldon *et al*., 1999). Flowering is enabled by the autonomous and vernalization response pathways which prevent the accumulation of *FLC* mRNA (Fig. 1). Vernalization is the acceleration of flowering caused by a long period of cold and is considered to be an adaptation that ensures flowering occurs only after winter (reviewed in Amasino, 2004).

Progress in dissecting the molecular basis of flowering time control has been recently reviewed (Simpson and Dean, 2002; Mouradov *et al*., 2002; Boss *et al*., 2004; Henderson and Dean, 2004; Putterill *et al*., 2004; Simpson, 2004). It is clear that a range of regulatory processes are involved in flowering time control. These include transcriptional, post-transcriptional and post-translational control mechanisms. As *A. thaliana* flowering time is controlled in such a quantitative manner, it provides a genetically tractable system to study the molecular basis of regulated gene expression in higher plants. In this review we will focus on what is known of regulated RNA processing in flowering time control. The *A. thaliana* genome encodes 196 RNA Recognition Motif (RRM) -type RNA binding proteins, more than either *Caenorhabditis*

***Address correspondence to:** Dr. Gordon G. Simpson. Dundee University Plant Research Unit at SCRI, Invergowrie, Dundee,DD2 5DA, UK. Fax: +44-1382-562-246. e-mail: g.g.simpson@dundee.ac.uk

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elegans or *Drosophila melanogaster* (Lorkovic and Barta, 2002). Around half of these RNA binding proteins are novel and specific to plants, indicating that they function in plant-specific processes. The only two plant-specific RRM-containing proteins of known function, FCA and FPA, are proteins that promote flowering. The *A. thaliana* genome also encodes 26 K-Homology (KH) domain RNA binding proteins. The function of only two of these, HUA ENHANCER 4 (HEN4) and FLK has been established and FLK also promotes flowering (Lim *et al*., 2004). In addition to RNA processing mediated by RNA binding proteins, the recent functional characterisation of *A. thaliana* microRNAs (miRNAs) has revealed that some are involved in flowering time control. Here, we review our current understanding of regulated gene expression mediated by RNA processing proteins and miRNAs in flowering time control.

The regulation of *FLC* expression is complex and involves multiple RNA processing factors

The RNA binding proteins, FCA, FPA and FLK all promote flowering by preventing the accumulation of mRNA encoding the floral repressor *FLC*. The regulation of *FLC* expression is complex. In order to understand how RNA processing regulates flowering, it is first necessary to review the mechanisms by which *FLC* expression is controlled. In this way, we identify potential targets of RNA-mediated regulation and set the context in which these RNA binding proteins function.

The study of natural variation in flowering time in *A. thaliana* showed that most vernalization-requiring winter annual accessions of *A. thaliana* flower late in the absence of vernalization because of the presence of elevated levels of *FLC* mRNA promoted by *FRIGIDA* (*FRI*). In contrast, most rapid cycling accessions analysed flower earlier in the absence of vernalization as they carry loss of function *fri* alleles (Johanson *et al*., 2000). The regulation of *FLC* by *FRI* specifically requires *FRIGIDA LIKE 1* and *2* (*FRL1* and *FRL2)* (Michaels *et al*., 2004). The mechanism by which FRI increases *FLC* expression has not been determined. However, it has recently been shown that this is associated with enhanced histone 3 trimethylation at Lysine (Lys) 4 in *FLC* chromatin (He *et al*., 2004).

A collection of factors are required for *FLC* mRNA accumulation. Prominent among these are *A. thaliana* proteins related to the Paf1 complex of yeast: VERNALIZATION INDEPENDENCE 4 (VIP4; Zhang and van Nocker, 2002), VIP5, VIP6/ EARLY FLOWERING 8 (ELF8) (Oh *et al*., 2004; He *et al*., 2004) and ELF7 (He *et al*., 2004) (see Fig. 1) with homology to budding yeast Leo1, Rtf1, Ctr9 and Paf1 proteins respectively. The yeast Paf1 complex associates with RNA polymerase II transcribing a sub-set of genes and promotes trimethylation of histone 3 (H3) on Lys4 (K4), a chromatin modification at the 5' end of genes associated with active transcription (Santos-Rosa *et al*., 2002). These proteins are required for the same chromatin modification in the 5' region of *A. thaliana FLC* (He *et al*., 2004). Trimethylation of H3-K4 in the 5' regions of genes is thought to promote gene expression by recruiting ISW1p, a yeast ATP-hydrolysing chromatin remodelling protein. PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1) is a candidate for a protein fulfilling this function in *A. thaliana* as it is related to ISW1p and loss-of-function *pie1* mutants have reduced levels of *FLC* mRNA (Noh and Amasino, 2003). In yeast, loss-of-function mutations in Paf1 complex components also results in RNA processing defects, such as shortened poly (A) tails on transcribed mRNAs (Mueller *et al*., 2004). This might be explained by changes in the phosphorylation status of the C-terminal domain (CTD) of the large subunit of RNA polymerase II that are found in these mutants which would compromise the association of RNA processing factors with the elongating polymerase. The Paf1 complex regulates the expression of a sub-set of *A. thaliana* genes that includes transcription factors, like *FLM*, that are related to *FLC* (He *et al*., 2004).

In addition to the Paf1 complex and PIE1, *FLC* expression depends on EARLY in SHORT DAYS 4 (ESD4) (Reeves *et al*., 2002) and EARLY FLOWERING 5 (ELF5) (Noh *et al*., 2004). *ESD4* encodes a nuclear protein related to proteases that are specific for SMALL UBIQUITIN-RELATED MODIFIER (SUMO) (Murtas *et al*., 2003). ELF5 is similar to a WW domain containing nuclear Npw38-binding protein, NpwBP. The mechanism by which ESD4 and ELF5 promote *FLC* mRNA expression is not yet known.

While the Paf1 complex promotes *FLC* transcription, other factors required for *FLC* mRNA accumulation may act post-transcriptionally, *ABA HYPERSENSITIVE 1* (*ABH1*), which encodes the large subunit of the eukaryotic nuclear mRNA cap-binding complex (CBP80; Hugovieux *et al*., 2002) and HUA2, a protein required for the efficient processing of *AGAMOUS* pre-mRNA intron 2 (Chen and Meyerowitz, 1999; Cheng *et al.,* 2003) are both required for normal *FLC* expression (Bezerra *et al.,* 2004; Doyle *et al.,* 2005).

CBP80 binds the cap structure attached to the 5' end of the eukaryotic transcripts produced by RNA polymerase II. The expression of only a small sub-set of *A. thaliana* transcripts appear sensitive to loss of this cap-binding protein (Hugovieux *et al*., 2002), but the reason why *FLC* should be one of them is unknown. The processing of first introns can be enhanced by exon bridging interactions between splicing factors and the cap-binding complex (Lewis *et al*., 1996). Some feature, possibly a weak 5' splice site or the large size of *FLC* intron 1 may make its processing more dependent on the cap-binding complex. Another possibility is that ABH1 bound to *FLC* mRNA modulates its interaction with *FLC* regulators (such as the RNA binding proteins of the autonomous pathway). Alternatively, the effect might be indirect and *ABH1* may affect the activity of genes required for *FLC* expression (Bezerra *et al*., 2004).

The HUA2 protein sequence contains an RPR domain, a motif found in proteins that function in RNA metabolism and HUA2 affects *AG* pre-mRNA processing in certain genetic backgrounds (Cheng *et al.,* 2003). HUA2 is required for the accumulation of *FLC* mRNA and other related MADS box transcription factors that are also repressors of flowering such as *FLOWERING LOCUS M* (*FLM*/*MAF1* ; Scortecci *et al*., 2001), *MAF2* (Ratcliffe *et al.,* 2003) and *SHORT VEGETATIVE PHASE* (*SVP* ; Hartmann *et al*., 2000) which are also regulated by the Paf1 complex (Doyle *et al.,* 2005).

The up-regulation of *FLC* mRNA by *FRI* in winter annual accessions of *A. thaliana* confers a so-called vernalization requirement. Down-regulation of *FLC* expression is a basic feature of the *A. thaliana* vernalization response. The epigenetic maintenance of *FLC* repression is mediated by chromatin modifications. Consistent with this, factors that modify chromatin at the *FLC* locus are required for the vernalization response of *A. thaliana* (Gendall *et al*., 2001; Bastow *et al*., 2004; Sung and Amasino, 2004). Vernal-

ization also apparently promotes the alternative processing of *A. thaliana FLC* pre-mRNA (Caicedo *et al.*, 2004). Following 15 days vernalization treatment (growth at 4ºC), a short transcript is detected, the putative product of alternative splicing involving the use of unprecedented non-canonical splice donor and acceptor sites within intron 1 and 6 respectively (Caicedo *et al.*, 2004). The functional significance of this transcript in the regulation of *FLC* expression or activity remains to be determined.

The autonomous pathway prevents the accumulation of *FLC* mRNA (Simpson, 2004). The autonomous pathway currently comprises seven recessive loss-of-function mutants: *fca*, *fy*, *fpa*, *fve*, *fld*, *luminidependens (ld)* (Koornneef *et al.*, 1998) and *flk* (Lim *et al.*, 2004). The genes disrupted in each of the known autonomous pathway mutants have been identified. *LD* encodes a homeodomain containing protein (Lee *et al.*, 1994). Such domains typically bind DNA, or more rarely, RNA (Dubnau and Strhul, 1996; Rivera-Pomar *et al.*, 1996). Other genes of the autonomous pathway, *FCA*, *FPA* and *FLK*, encode RNA binding proteins (Macknight *et al.*, 1997; Schomburg *et al.*, 2001; Lim *et al.*, 2004), while FY is probably a polyadenylation factor (Simpson *et al.*, 2003). *FLD* and *FVE* encode proteins that modify *FLC* chromatin. FLD is homologous to the human protein KIAA0601, a component of the histone deacetylase 1,2 (HDAC1/2) co-repressor complexes (He *et al.*, 2003), while FVE is a WD repeat-protein and homolog of the mammalian Retinoblastoma Associated Protein46 (RbAp46) and RbAp48 proteins (Ausin *et al.*, 2004). FLD and FVE are required for histone deacetylation of chromatin at the *FLC* locus, but the RNA binding proteins of the autonomous pathway, FCA and FPA, are not (He *et al.*, 2003). It is well established in many systems that histone deacetylation is associated with repression of gene expression (Cress and Seto, 2000; Yang and Seto, 2003; Tian *et al.*, 2005). The recently identified *RELATIVE OF EARLY FLOWERING 6* (*REF6*) gene, which encodes a Jumonji/zinc-finger-class transcription factor, could also be considered part of the autonomous pathway: loss-of-function *ref6* mutants show elevated levels of *FLC* mRNA, flower later than wild-type in long days (LDs) and short days (SDs) and this late flowering can be overcome by a vernalization treatment. *ref6* mutants display hyperacetylation of histone 4 (H4) in *FLC* (Noh *et al.*, 2004b). Therefore, *REF6*, like *FLD* and *FVE*, is required for histone deacetylation of chromatin at the *FLC* locus.

The potency with which FLC acts as a floral repressor differs between *A. thaliana* accessions. The relatively weak activity of the Landsberg *erecta* (L*er*) *FLC* allele is caused by the insertion of a transposable element in *FLC* intron 1 (Gazzani *et al.*, 2003; Michaels *et al.*, 2003). Small interference RNAs (siRNAs) corresponding to the transposon accumulate in L*er* and this correlates with an increase in histone 3 (H3) dimethylation on Lysine 9 (K9) within this intron (Liu *et al.*, 2004). The late flowering mutant, *hua enhancer 1-1* (*hen1-1*), shows an accumulation of *FLC* mRNA and a reduction in the levels of *FLC* intron 1 siRNAs and H3-K9 dimethylation. *HEN1* is involved in siRNA metabolism (Xie *et al.*, 2003) and is required for the accumulation of *FLC* intron 1 siRNAs (Liu *et al.*, 2004). Therefore, *HEN1* participates in the production of siRNAs homologous to *FLC* intron 1 which triggers dimethylation of H3-K9 within intron 1, leading to the silencing of *FLC* expression. This discovery establishes a clear connection between post-transcriptional and chromatin modification mechanisms in the control of *FLC* gene expression affecting *A. thaliana* flowering time control.

The regulation of *FLC* expression therefore involves many factors, some that act directly to modify chromatin at the *FLC* locus and others that are involved in RNA processing. The RNA binding proteins must either regulate *FLC* directly or antagonize

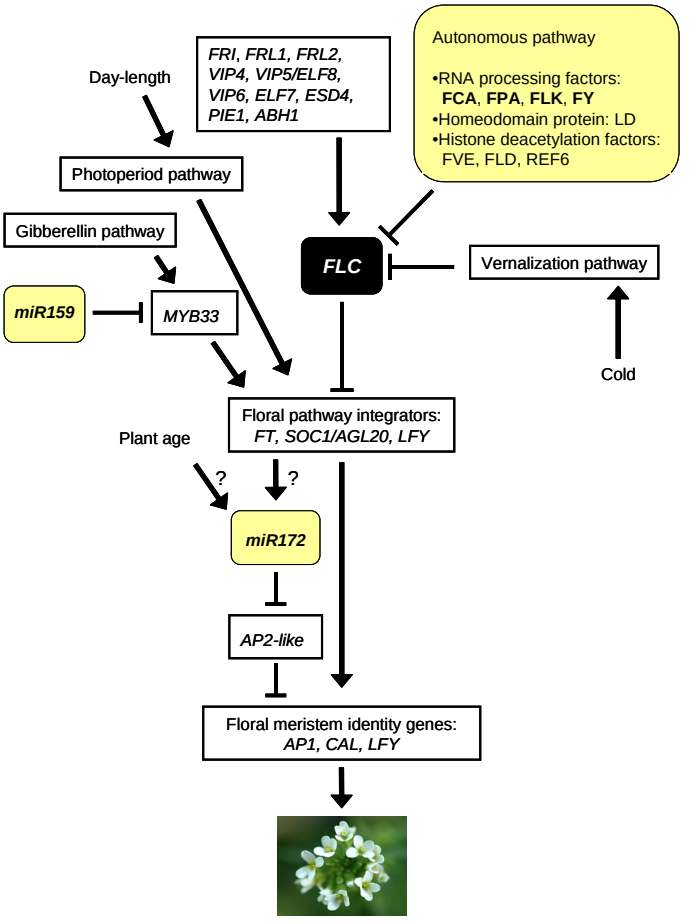

Fig. 1. Schematic representation of the principal pathways controlling flowering time in *A. thaliana*. *The photoperiod and gibberellin pathways promote the activity of the floral pathway integrator genes* FT, SUPRESSOR OF THE OVEREXPRESSION OF CO1/AGAMOUS LIKE 20 (SOC1/AGL20) *and* LEAFY (LFY)*, whereas the MADS-box transcription factor FLC represses their activity. Up-regulation of* FLC *expression depends on* FRIGIDA (FRI), FRI-like1 (FRL1) *and* 2 (FRL2), VERNALIZATION INDEPENDENCE 4 (VIP4), VIP5/EARLY FLOWERING 8 (ELF8), VIP6, ELF7, EARLY IN SHORT DAYS 4 (ESD4), PHOTOPERIOD INSENSITIVE EARLY 1 (PIE1) *and* ABA HYPERSENSITIVE 1 (ABH1)*, while* FLC *mRNA expression is inhibited by the genes of the autonomous and vernalization pathways. In the autonomous pathway,* FCA, FPA, FLK *and* FY *(bold letters) encode RNA processing factors, LD is a homeodomain containing protein and* FVE, FLD *and* RELATIVE OF EARLY FLOWERING 6 (REF6) *genes encode factors required for deacetylation of histones at the* FLC *locus. miRNAs* miR159 *and* miR172 *repress and promote flowering time respectively by negatively regulating (post-transcriptionally) the expression of* MYB33 (miR159) *and* AP2-like *floral repressors (*miR172*). The floral integrators activate the expression of a highly redundant set of genes that includes* APETALA1 (AP1), CAULIFLOWER (CAL) *and* LFY *which are required for floral meristem identity. Question marks denote that there is a current lack of consensus in the literature regarding whether* miR172 *expression is affected by plant age or* CO *and* FT. *Promoter and repressive activities are denoted by arrowheads and T-bars, respectively.*

the expression or activity of factors that promote *FLC* expression.

Post-transcriptional autoregulation of *FCA* expression controls flowering time in *A. thaliana*

FCA is perhaps the best characterized autonomous pathway component. FCA is a nuclear protein containing two RNA recognition motif (RRM)-type RNA-binding-domains and a WW protein interaction domain (Macknight, *et al*., 1997). The expression of *FCA* is complex, since four different transcripts (α, β, γ and δ) are produced as a result of alternative splicing and alternative polyadenylation events that take place at two different sites in *FCA* pre-mRNA. In transcript α, all the introns are excised with the exception of intron 3 which is retained. Transcript β results from premature cleavage and polyadenylation at a promoter-proximal poly (A) (polyadenylation) site within intron 3 (Macknight, *et al*., 1997; Fig. 2). At 3.0kb, intron 3 is unusually long compared to most *A. thaliana* introns. The alternative processing of intron 3 and its large size has been conserved in other plant species (Macknight, *et al*., 2002). *FCA* transcripts, γ and δ, lack introns but are differentiated by an alternative splicing event around intron 13 that produces the δ transcript and results in a shift in the reading frame that introduces a premature termination codon (PTC) (Macknight, *et al*., 1997). The consequence of the alternative processing of the *FCA* pre-mRNA is the formation of three transcripts (α, β and δ) encoding truncated and inactive proteins at the expense of transcript γ, the only fully spliced transcript able to encode full-length active FCA protein (Macknight, *et al*., 2002; Fig. 2).

The alternative processing of *FCA* pre-mRNA is regulated and a major regulator is FCA itself. FCA negatively regulates its own expression by ultimately promoting cleavage and polyadenylation at the promoter-proximal poly (A) site within intron 3 (Quesada *et al*., 2003; Fig. 2). This results in the formation of the truncated and inactive β transcript limiting the formation of the fully spliced, full-length and functional *FCA* γ transcript (Fig. 2). This post-transcriptional regulation is developmentally controlled, restricting in a temporal and spatial manner the pattern of expression of functional FCA protein (Quesada *et al*., 2003). However, this negative feedback either does not take place, or is less effective in meristematic regions (Macknight *et al*., 2002; Quesada *et al*., 2003).

FCA autoregulation has a functional consequence on flowering time. The expression of *FCA* from intronless transgenes, lacking the *cis*-element required for *FCA* regulation results in precocious flowering (Quesada *et al*., 2003). *FCA* autoregulation therefore provides a mechanism to fine-tune the level of functional FCA protein and in turn, *FLC* mRNA accumulation and thereby controls flowering time.

FCA interacts with the polyadenylation factor FY to control *FCA* expression and flowering time

FCA contains a WW domain that is involved in protein-protein interactions (Sudol and Hunter, 2000). The FCA WW domain interacts with another protein of the autonomous pathway, FY (Fig. 2), a WD-repeat protein highly related to the *Saccharomyces cerevisiae* RNA 3'-end-processing factor, Pfs2p (polyadenylation factor 1 subunit 2; Ohnacker *et al*., 2000). Consistent with its association with the FCA WW domain and its similarity to Pfs2p, FY is genetically required for the function FCA performs in promoting

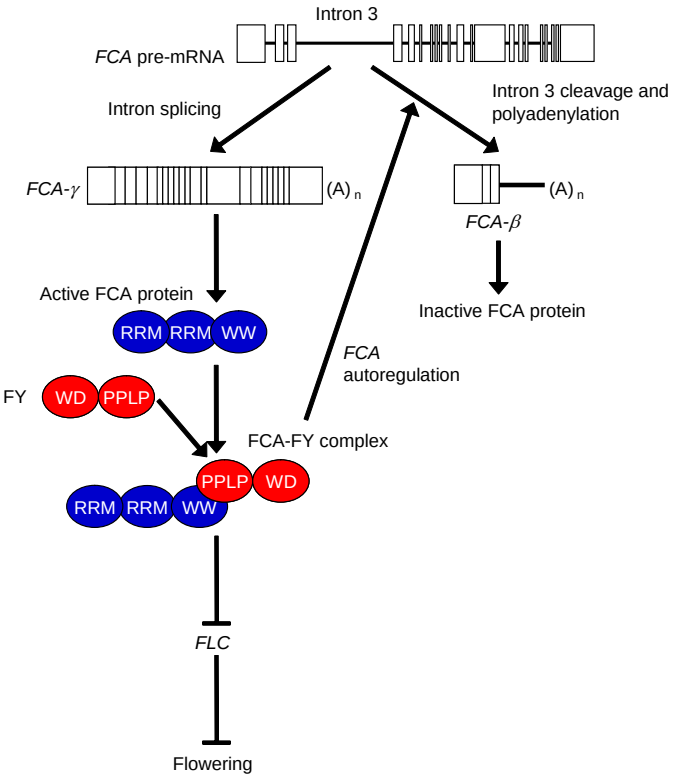

Fig. 2. *FCA* negative autoregulation and control of flowering time. *Excision of all* FCA *pre-mRNA introns results in* FCA *transcript γ which encodes a full-length and functional FCA protein. Through the WW domain FCA interacts with the PPLP motif in the C-terminal part of the 3' end processing factor FY. This may tether the 3' end formation machinery to inhibit the expression of the floral repressor gene* FLC. *It is not yet known whether this is a direct or indirect effect. The FCA-FY complex also functions to promote premature cleavage and polyadenylation within* FCA *intron 3. This might be direct, through the binding of the FCA RNA recognition motifs (RRM) to sequences in* FCA *pre-mRNA intron 3. This produces the truncated transcript* FCA-β *that encodes a non-functional FCA protein. This autoregulation mechanism sets the levels of functional FCA protein encoded by the* FCA-γ *transcript preventing precocious flowering. Exons are represented as boxes and introns by lines.*

flowering and for *FCA* autoregulation. First, the loss-of function *fy* mutation suppresses the early flowering phenotype of transgenic lines over-expressing FCA (Simpson *et al*., 2003). Second, *FY* is required for the selection of the promoter-proximal 3'-end in *FCA* pre-mRNA, since *fy* and *fca* mutants display defects in 3' end formation, with a reduced use of the promoter-proximal poly (A) site within intron 3 of *FCA* pre-mRNA and a concomitant increase in the use of the promoter-distal poly (A) site. Both autoregulation and flowering time functions of *FCA* require an intact FCA WW protein interaction domain (Simpson *et al*., 2003).

The FCA-FY complex functions to actively promote proximal poly (A) site usage in *FCA* pre-mRNA rather than inhibiting 3' end-formation at this site. This is consistent with the idea that FCA interacts with FY in order to tether the 3'-end-processing machinery to a regulated 3'-end. While direct evidence for this model is currently lacking, FCA may be described as a regulator of 3' end formation and FY may be a component of the complex required for constitutive 3' end formation. An alternative possibility is that FY

function has specialized for regulated 3' end formation. The composition of constitutive and regulatory 3' end cleavage and polyadenylation complexes may differ. The *S. cerevisiae* genome only encodes a protein related to FY (Pfs2p). However, other eukaryotic genomes encode proteins related not only to FY but also to CstF50, a polyadenylation factor identified in metazoans, which was until recently considered to be the functional homologue of *S. cerevisiae*, Pfs2p (Ohnacker *et al.*, 2000). The determination of FY-like protein–function will probably require the characterization of the protein assemblies it associates with in multicellular eukaryotes. It is notable that work on the molecular basis of regulated gene expression involved in flowering time control can raise questions about the mechanisms of gene expression control in all eukaryotes.

The interaction of FCA and FY is required for their function in the autonomous pathway to prevent *FLC* mRNA accumulation (Fig. 2). An obvious possibility is that they promote alternative 3' end formation of *FLC* pre-mRNA. However, there is currently no data to support this, nor even any evidence that they regulate *FLC* directly. FCA and FY could regulate *FLC* indirectly via one of the many factors that control *FLC* expression.

FPA and FLK are plant-specific RNA binding proteins that regulate flowering time

FPA and FLK are also components of the autonomous pathway that promotes flowering in *A. thaliana* by repressing *FLC* (Schomburg *et al.*, 2001; Lim *et al.*, 2004; Fig. 1). FPA is an RNA-binding protein that contains three RNA recognition motifs in the N-terminal region (Schomburg *et al.*, 2001) whereas FLK has three KH type RNA-binding domains (Lim *et al.*, 2004). Mutations in *FCA* or *FPA* do not perturb expression of *FLK* and the expression of *FCA* and *FPA* is normal in the *flk* mutant. This suggests that these components do not regulate the expression of each other within the autonomous pathway. Double mutant analysis suggested the existence of two epistatic groups in the autonomous pathway: one including *FCA* and *FY* and the other *FPA* and *FVE* (Koornneef, 1998) (no double mutant with *flk* has yet been reported). Taken together, this suggests that the RNA-binding proteins of the autonomous pathway function in parallel and control *FLC* expression independently.

ELF5

The recently identified floral repressor *ELF5* might be involved in RNA metabolism (Noh *et al.*, 2004a) based on sequence similarity with a nuclear human protein (NpwBP) proposed to function in RNA processing (Komuro *et al.*, 1999). If this is confirmed, it would extend the involvement of RNA processing factors in flowering time control beyond the autonomous pathway.

Similarities between *FLC* and *AG* regulation

There are some parallels between the regulation of *FLC* expression and the floral homeotic gene *AGAMOUS* (*AG*) that may provide clues about how the RNA processing proteins of the autonomous pathway (FCA, FPA, FLK and FY) function. *FLC* and *AG* encode MADS-box transcription factors involved in different aspects of plant development. *AG* is a C-class gene that specifies the identities of carpels and stamens and also controls determinacy of the floral meristem (Yanofsky *et al.*, 1990). Both genes contain unusually long introns that harbour important *cis* regulatory sequences [intron 1 of 3.5 kb and intron 2 of 3.0 kb in length in *FLC* and *AG*, respectively (Sheldon *et al.*, 2002; Hong *et al.*, 2003)]. In addition, both genes appear to be regulated epigenetically (Goodrich *et al.*, 1997; He *et al.*, 2003; Ausin *et al.*, 2004) and by proteins involved in RNA metabolism (Macknight, *et al.*, 1997; Li *et al.*, 2001; Schomburg *et al.*, 2001; Western *et al.*, 2002; Cheng *et al.*, 2003; Simpson *et al.*, 2003; Lim *et al.*, 2004). HUA1, HUA2, HUA ENHANCER2 (HEN2) and HEN4 are required for the normal processing of *AG* pre-mRNA. HUA1 has a CCCH-type zinc finger, while HEN4 has a KH-domain (Cheng *et al.*, 2003). HUA2 shares homology with metazoan RNA-processing proteins (Chen and Meyerowitz, 1999) and HEN2 is a DExH RNA helicase similar to the yeast Dob1 protein, a component of the nuclear exosome involved in the production of ribosomal RNAs and in pre-mRNA degradation (Western *et al.*, 2002). The analysis of *AG* expression in the loss-of-function triple mutants *hua1 hua2 hen2* and *hua1 hen2 hen4* showed that processing of *AG* pre-mRNA is compromised: less mature and functional *AG* mRNA and more alternative and longer RNA species accumulates in the triple mutants (Cheng *et al.*, 2003). The two longer *AG* RNA transcripts are non-functional by-products resulting from alternative polyadenylation events within the large intron 2. Therefore, the normal function of HUA1, HUA2, HEN2 and HEN4 proteins would be to promote splicing of intron 2 by repressing the use of the poly (A) sites within intron 2, or alternatively, facilitating the removal of this intron. HUA1 and HEN4 proteins physically interact and HUA1 directly binds *AG* RNA *in vitro*, suggesting that this regulation might be carried out directly by the HUA1-HEN4 complex (Cheng *et al.*, 2003).

This mechanism of *AG* expression control, involving competition between splicing and premature cleavage and polyadenylation of a large intron, is very similar to the autoregulation of *FCA.* While FCA-FY ultimately actively promotes premature polyadenylation and thus represses active *FCA* expression, in the case of *AG*, HUA1, HUA2, HEN2 and HEN4 either facilitate splicing of and/or inhibit premature cleavage and polyadenylation within intron 2 and thus promote active *AG* expression. The apparent similarities between *AG* and *FCA* regulation raises the question of whether FCA and FY control *FLC* RNA 3' end formation in a similar way by promoting premature polyadenylation within the unusually large *FLC* intron 1. However, as previously mentioned, no alternatively polyadenylated transcripts of the *FLC* gene have been detected in *A. thaliana*. This may be due to the instability of these transcripts or because *FLC* is not a direct target of FCA-FY.

The fact that HUA2 is required both for the expression of *FLC* and *AG* at first appears to underline similarities between *FLC* and *AG* regulation. However, this may not necessarily be the case. The effects of *hua2* mutations on *AG* are dependent on *hua1* mutations (Cheng *et al.,* 2003). However, *hua1* does not enhance the effects that *hua2* has on flowering time (Doyle *et al.,* 2005). Therefore, HUA2 may regulate *FLC* and *AG* by different mechanisms.

Flowering time and miRNAs

miRNAs have emerged as important regulators of developmental processes in a range of multi-cellular organisms. miRNAs are small non-coding regulatory RNAs, about 21 nucleotides in length, produced from the processing of longer precursor transcripts.

Plant miRNAs identified to date display near-complete matches with a complementary sequence in their target mRNAs and this often produces the cleavage of the mRNA at this site. In contrast these complementarities are more limited in animals and miRNAs seem to act by repressing translation of the target messages (Bartel, 2004). Nevertheless, there are exceptions, since despite strong complementarity some plant miRNAs function by inhibiting translation (Aukerman and Sakai, 2003; Chen, 2003) as well as through promoting cleavage of target mRNAs (Kasschau *et al.*, 2003; Schwab *et al.,* 2005) and some animal miRNAs direct the cleavage of their target mRNAs (Yekta *et al.*, 2004).

At least 92 *A. thaliana* miRNAs have been described, with most targeting mRNAs coding for transcription factors involved in different developmental processes (Dugas and Bartel, 2004). The biological function of several *A. thaliana* miRNAs has been established (Rhoades *et al.*, 2002; Emery *et al.*, 2003; Palatnik *et al.*, 2003). Three *A. thaliana* miRNAs, *miR172*, *miR159* and *miR156* are known to be involved in the regulation of flowering time.

The *A. thaliana* genome encodes four precursors of *mir172* : *miR172a-1*, *miR172a-2*, *miR172b* and *mir172c*. *mir172* exhibits complementarity with the floral meristem and floral organ identity gene, *APETALA2* (*AP2*) and a group of genes related to *AP2*. This group includes *TARGET OF EAT1* (*TOE1*), *TOE2*, *SCHLAFMUTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*) and At5g67190. *TOE1*, *TOE2*, *SMZ* and *SNZ* are all repressors of *A. thaliana* flowering (Aukerman and Sakai, 2003; Schmid *et al.*, 2003). The assignment of these repressors to particular flowering time pathways is currently unclear. Microarray analysis revealed that all four were down-regulated upon flowering and that this down-regulation was dependent on *FT* and *CONSTANS* (*CO*), indicating that they were repressors ultimately targeted by the photoperiod pathway (Schmid *et al.*, 2003). Consistent with this, Schmid *et al.* (2003) reported that the expression of *miR172a-2* was dependent on day-length, *CO* and *FT* (Fig. 1). In contrast, Aukerman and Sakai (2003), found that *miR172* accumulated to elevated levels with increasing plant age, but found that this accumulation was not affected by day-length or mutations in the photoperiod pathway gene, *CO* (Fig. 1). Transgenic plants expressing *35S::miR172* flowered early, but still showed a response to photoperiod (ie plants flowered earlier in long day conditions than short day conditions). *miR172* acts to block the translation, in addition to promoting the cleavage of *AP2* mRNA (Aukerman and Sakai, 2003; Chen, 2003, Kasschau *et al.,* 2003; Schwab *et al.,* 2005). *miR172* may act in a similar way to regulate *TOE1* and *TOE2.* Careful analysis by Schwab *et al.* (2005) revealed that over-expression of *mir172a* resulted in reduced steady state levels of *TOE2* mRNA, but while steady state levels of *TOE1* and *AP2* mRNA were unchanged, evidence of cleavage of *TOE1, TOE2* and *AP2* was clear. This discrepancy can be explained by feedback regulation – AP2, for example, can directly or indirectly represses its own transcription and this may be true of related factors, like *TOE1.*

A function in flowering time control has been assigned to *A. thaliana miR159* (Achard *et al.*, 2004). The hormone gibberellin (GA) promotes the accumulation of *miRNA159*. Over-expression of *miRNA159* delays flowering specifically in short day conditions. This late flowering phenotype correlates with a decrease in the level of the mRNAs of the transcription factor *MYB33* and the floral pathway integrator, *LFY* (Fig. 1). However, the expression of *SOC1,* a component of the GA-induction of flowering is not affected. *MYB33* is a direct target of *miR159* : the *MYB33* mRNA is cleaved at an miRNA target site. Therefore, *miR159* represses flowering in SD conditions by negatively regulating *MYB33*. This produces a reduction in the levels of *LFY* mRNA and a delay in flowering (Fig. 1).

The expression of a group of *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes, in particular *SPL3*, *SPL4* and *SPL5* is up-regulated by flowering and dependent on *CO* and *FT* (Schmid *et al.*, 2003). This indicates that these genes may be regulators of flowering time. Consistent with this, over-expression of *SPL3* had previously been shown to accelerate flowering (Cardon *et al.,* 1997). *SPL3*, *SPL4* and *SPL5,* have been identified as candidate targets of miRNAs (Rhoades *et al.*, 2002; Kasschau *et al.*, 2003). This hypothesis has recently been tested by Schwab *et al.* (2005) who demonstrated that over-expression of *35S::mir156b* caused late flowering and downregulated the expression of *SPL* genes with *mir156* target sites. Therefore a role for miRNAs in controlling flowering time also extends to the regulation of *SPL* genes.

Conclusions

Considerable progress has recently been made in identifying and understanding the genes and molecular processes that control flowering time. This has been achieved through the use of *A. thaliana* as a model system. The discoveries highlight the important role that RNA processing and post-transcriptional regulators play in the floral transition. This is especially significant in the case of the autonomous pathway which includes three RNA-binding proteins FCA, FPA and FLK and a polyadenylation factor, FY. FCA and FY function as a complex that controls *FCA* expression post-transcriptionally ultimately by alternative polyadenylation. The discoveries made in the control of the expression of the floral identity gene, *AG*, reveal similarities between *FCA* and *AG* regulation. This might help us to understand how the RNA processing factors of the autonomous pathway control the expression of the floral repressor *FLC*, the final target of this pathway. However, there is no evidence yet that *FLC* expression is post-transcriptionally regulated (although it has been described that *FLC* pre-mRNA is alternatively spliced).

Recent discoveries have been made in the field of RNA metabolism with the identification of miRNAs as key regulators of development in animals and plants. The number of miRNAs identified and their potential target genes continues to increase. Given this and the high proportion of genes encoding RNA-binding proteins in the *A. thaliana* genome we can speculate that in the near future the use of forward and reverse genetics approaches will identify new genes encoding RNA regulators of flowering time in *A. thaliana*. The completion of the genomic sequence of other plant species will help us discover if they function in a similar way in other plants too.

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