Alternative polyadenylation of antisense RNAs and flowering time control

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Abstract
Flowering time is controlled by precision in gene regulation mediated by different pathways. Two Arabidopsis thaliana components of the autonomous flowering pathway, FCA and FPA, function as genetically independent trans-acting regulators of alternative cleavage and polyadenylation. FCA and FPA directly associate with chromatin at the locus encoding the floral repressor FLC, but appear to control FLC transcription by mediating alternative polyadenylation of embedded non-coding antisense RNAs. These findings prompt the re-examination of how other factors control FLC expression, as it is formally possible that they function primarily to control alternative processing of antisense RNAs. As co-expressed sense and antisense gene pairs are widespread in eukaryotes, alternative processing of antisense RNAs may represent a significant form of gene regulation.

Precision in gene regulation controls the switch to flower development
Plants control the time at which they flower in order to ensure they reproduce in favourable conditions. This fundamental developmental transition is controlled in response to environmental signals such as day length and temperature and is integrated with an endogenous programme of development. This quantitative trait is underpinned by precision in gene regulation mediated through genetically separable pathways [1,2].

The autonomous flowering pathway in Arabidopsis thaliana limits the expression of mRNA encoding a potent repressor of flowering: the transcription factor FLC. This genetically defined pathway comprises a combination of components implicated in RNA processing and chromatin modification [1,2]. Recent findings reveal that RNA-binding proteins within the autonomous pathway can control RNA 3′-end formation and affect alternative polyadenylation of antisense RNAs at the FLC locus [3,4].

Regulators of flowering time control alternative cleavage and polyadenylation of RNA
The characterization of late-flowering mutants of the autonomous flowering pathway has revealed that the genes disrupted in two of these mutants, fca and fpa, encode RNA-binding proteins that regulate RNA 3′-end formation [3,5], whereas another mutant, fy, encodes a core component of the RNA cleavage and polyadenylation complex [6].

FCA
FCA is an apparently plant-specific protein comprising two RRs (RNA recognition motifs) and a protein interaction WW domain [7]. Evidence that FCA controls RNA 3′-end formation came from the discovery that it autoregulated its expression by controlling the alternative polyadenylation of FCA pre-mRNA [5].

FY
FY was first identified as a late-flowering mutant [8], revealing that it is required to promote flowering. It was therefore something of a surprise when the gene disrupted in fy mutants was found to encode a protein highly conserved in eukaryotes [6]. FY comprises seven highly conserved WD repeats at the N-terminus. The C-terminal region is not conserved, except for two proline-rich (PPLPP) repeats.
FPA and FCA control RNA 3′-end formation genetically independently

Although genetic evidence suggested a close relationship between FCA and FY, which was borne out by the physical interaction between these two proteins, this is less clear with respect to FCA and FPA. An fca fpa double-mutant flowers much later than either single mutant [12] and overexpression of FPA in an fca-mutant background can suppress the otherwise late flowering of fca mutants [16]. However, complete down-regulation of FLC by FCA appears to require FPA [4]. FCA is not required for alternative polyadenylation of FPA pre-mRNA and likewise, FPA is not required for alternative polyadenylation of FCA pre-mRNA [3]. Thus FCA and FPA can control RNA 3′-end formation genetically independently and on specific pre-mRNAs.

FPA and FCA control the 3′-end formation of antisense RNAs at the FLC locus

The ultimate target of FPA, FCA and FY activity in flowering time control is the expression of the transcription factor FLC; a double-mutant for flc with fpa, fca or fy abolishes the late-flowering phenotype of these plants [9, 17]. As FCA, FY and FPA control RNA 3′-end formation, an obvious hypothesis was that they might control alternative polyadenylation of FLC pre-mRNA. However, no differences in FLC mRNA 3′-end formation have so far been found in backgrounds with different FCA or FPA activities. Indeed, it appears that FPA and FCA control the expression of FLC at the transcriptional, not post-transcriptional level [18]. Microarray analysis of RNA expression in fca and fpa mutants did not reveal evidence that these proteins controlled an intermediate factor that regulates FLC [19, 20]. Instead, ChIP analysis suggests that both FCA and FPA are physically associated with FLC chromatin in vivo, indicating that they regulate FLC directly [3, 18, 21]. How then do these regulators of RNA 3′-end formation control FLC? The answer appears to lie in antisense RNAs that are embedded within the FLC locus.

Whole-genome tiling array analysis revealed widespread expression of antisense RNAs in A. thaliana, including at the FLC locus [22]. Two antisense RNAs were reported at FLC: the first, referred to as class I, was cleaved and polyadenylated at a promoter proximal site antisense to FLC intron 6, whereas the other, referred to as class II, was cleaved and polyadenylated at a distal site antisense to the FLC promoter [21]. Further characterization has revealed that there are more than two RNA isoforms and that these are capped at the 5′ end, transcribed from multiple start sites, alternatively spliced and alternatively polyadenylated [3, 21, 23]. Intron position

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**Figure 1** Summary of RNA expression and processing at FLC locus in wild-type and mutant backgrounds

Black lines represent introns, rectangles represent exons. Grey boxes are polyadenylation sites. Black arrows show FLC expression, grey arrows show antisense RNAs (asRNA).

FPA affects RNA 3′-end formation and, as with FCA, the evidence for this first came from the finding that FPA autoregulates its expression through alternative polyadenylation of its pre-mRNA [3]. FPA appears to function directly in this processing event as ChIP (chromatin immunoprecipitation) assays revealed FPA to be associated with FPA chromatin downstream of the regulated polyadenylation site [3].

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Found in homologues in other plant species [9]. FY closely resembles Saccharomyces cerevisiae Pfs2p, an essential protein required for cleavage and polyadenylation of mRNA [10] and is the homologue of the recently identified human CPSF (cleavage and polyadenylation specificity factor) component WDR33 (WD repeat domain 33) [11].

**FCA and FY interact**

Genetic analysis suggested a close relationship between FCA and FY: fca fy double-mutants did not flower later than either single mutant [12] and the overexpression of FCA failed to accelerate the late-flowering phenotype of fy mutant plants [6]. In fact, FCA and FY physically interact via the WW domain of FCA and the PPLPP repeats of FY [6, 9]. This interaction is required for FCA to promote proximal polyadenylation site selection in FCA pre-mRNA and to regulate FLC [6]. Interestingly, natural variation in the flowering response of A. thaliana accessions to changing light was that they might control alternative polyadenylation of FCA pre-mRNA [4, 21, 22]. Further characterization has revealed that there are more than two RNA isoforms and that these are capped at the 5′ end, transcribed from multiple start sites, alternatively spliced and alternatively polyadenylated [3, 21, 23]. Intron position
within \textit{FLC} sense and antisense RNAs is distinct, and splicing of antisense pre-mRNA introns involves splice sites that match conventional U2-type intron consensus sequences [3]. The antisense RNAs are of low abundance, but can be detected by conventional RNA blot hybridization if seedlings are first treated with cold, as the promoter driving expression of the antisense RNAs is cold-responsive [23].

Alternative polyadenylation of \textit{FLC} antisense RNAs correlated with FPA activity [3]. When FPA is overexpressed, detectable antisense RNAs appear to be cleaved and polyadenylated at the promoter proximal class I site, whereas in loss-of-function \textit{fpa} (and \textit{fca}) mutants, there is increased read-through detectable at the class II site [3]. This suggests that the function of FPA and FCA is to control proximal polyadenylation site selection of antisense RNAs at the \textit{FLC} locus. Consistent with the somewhat redundant role these proteins play in flowering time control, the overexpression of FPA in an \textit{fca} mutant background suppresses the otherwise late flowering of \textit{fca} mutants [3] and correlates with suppression of class II read-through antisense RNAs [3].

**Mechanism of alternative RNA 3′-end formation**

It seems likely that FCA binds RNA close to the polyadenylation sites it regulates. ChIP results indicate that FCA closely associates with the class I polyadenylation site of \textit{FLC} antisense RNAs [21]. Since FCA absolutely requires FY and other components of the 3′-end RNA processing complex to regulate \textit{FLC}, it probably functions to tether the cleavage and polyadenylation machinery to a weak polyadenylation site in order to enhance its selection [6]. In contrast, the peak ChIP signal of FPA is somewhat downstream of the polyadenylation sites it controls [3]. This may reflect an association with RNA polymerase II, as human cleavage and polyadenylation factors also peak downstream of polyadenylation sites in ChIP analysis [24]. Alternatively, it may reflect a direct association with RNA downstream, similar to the brain specific protein Nova (neuro-oncological ventral antigen), which binds RNA downstream of the polyadenylation sites it promotes [25]. Thus the mechanism by which FPA promotes polyadenylation site selection remains to be determined.

**Regulation of \textit{FLC} expression by FCA requires \textit{FLD}, a homologue of human LSD1 (lysine-specific demethylase 1)**

Genetic analysis indicates that FCA requires another component of the autonomous pathway, \textit{FLD}, in order to regulate \textit{FLC} expression [21]. Likewise, complete down-regulation of \textit{FLC} in plants overexpressing FPA depends on \textit{FLD} [16]. \textit{A. thaliana} \textit{FLD} is related to human LSD1, which demethylates the mono- and di-methylated Lys4 of H3 (histone H3), H3K4me and H3K4me2, but not the tri-methylated form (H3K4me3). It has been suggested that cleavage of antisense RNA triggers silencing of \textit{FLC} by recruiting \textit{FLD} [4]. Consistent with this idea, increased levels of H3K4me2 are found at the \textit{FLC} locus downstream of the class I polyadenylation site in \textit{fld} mutants [21]. An alternative possible explanation, proposed by Hornyik et al. [3], is that FLD-mediated histone modification affects alternative processing of antisense RNAs, which is consistent with recent studies demonstrating that histone modifications can affect alternative processing of pre-mRNA [26–28]. A third possibility is that the function of FLD in flowering time control is independent of its histone demethylase activity as human LSD1 controls methylation of proteins other than histones, such as p53 [29], and is also required for interchromosomol interactions that occur in interchromatin granule clusters [30].

**Do other autonomous pathway components act primarily on \textit{FLC} antisense RNA?**

Another presumed RNA-binding protein, the KH domain-containing protein FLK, is a core component of the autonomous flowering pathway [31,32]. Furthermore, the RRM-containing glycine-rich protein AtGRP7 [33], the regulator of alternative splicing SR45 [34] and the \textit{A. thaliana} homologue of the splicing factor PRP39-1 [35] all limit \textit{FLC} expression and so can be defined as autonomous pathway components. The mechanism by which these RNA-processing factors control \textit{FLC} is not yet known.

In addition to FLD, the autonomous pathway includes FVE and REF6 that are also thought to be involved in chromatin modification. FVE is a homologue of yeast MSI (multicopy suppressor of IRA1) and mammalian RbAp (retinoblastoma-associated protein) 46/48, which are found in several histone-modifying complexes [36]. REF6 possesses a JMJC domain, which is found in human histone demethylases like human JHDM (jumonji C domain-containing histone demethylase) 1 and JHDM2A, specific for H3K36me and H3K9me respectively [37].

As the autonomous pathway is defined by a combination of RNA processing and chromatin-modifying components [2] it may be a manifestation of the documented connections between chromatin modification and alternative processing of RNA [26–28,38], acting to regulate processing of \textit{FLC} antisense RNAs. Multiple RNA-processing and chromatin-modifying factors have also been identified that are required to promote \textit{FLC} expression [1]. It remains formally possible that some of these factors and autonomous pathway components antagonize each other’s function to influence processing of \textit{FLC} antisense RNAs.

**How can alternative polyadenylation of antisense RNAs affect sense-strand transcription?**

The alternative polyadenylation of antisense RNAs correlates with \textit{FLC} sense-strand transcription levels. It is important to remember that the available results represent only a correlation and much remains to be done to dissect the mechanisms involved. This correlation is set in the context
that: (i) FCA and FPA both control RNA 3'-end formation; (ii) that they are directly associated with the FLC locus; (iii) that in their absence, sense-strand transcription increases dramatically; and (iv) that alternative polyadenylation of antisense RNAs changes in a reciprocal manner with backgrounds either lacking or over-expressing FCA and FPA activity.

Readthrough of RNA antisense to the FLC promoter in fpa and fca mutants results in increased FLC expression. This appears counter-intuitive to examples from yeast where antisense RNA expression leads to down-regulation of sense-strand expression [39]. However, co-expression of sense and antisense RNAs is widespread in A. thaliana [40–42]. How can read-through antisense RNA result in increased sense-strand expression? A relevant precedent may come from yeast: low level transcription of RNA through the promoter of S. cerevisiae PHO5 leads to chromatin modification that enhances the rate of activation of sense-strand expression [43]. Therefore the act of transcription through the FLC promoter may modify chromatin and rearrange nucleosomes favouring enhanced activation of FLC. Another aspect of gene expression to consider is loop formation between the 5'- and 3'-ends of genes [44–46]. Gene looping requires components of the RNA 3'-end processing machinery [45] and functional polyadenylation signals [46]. It is therefore interesting to ask what happens at loci like FLC where there is alternative polyadenylation? FCA and FPA promote cleavage and polyadenylation at proximal sites antisense to the terminal intron of FLC. In the absence of FPA and FCA, 3'-end formation occurs at the distal class II sites antisense to the FLC promoter. Therefore the FLC promoter may be engaged in a gene loop only in the absence of FCA or FPA, and this may facilitate recruitment of positive factors to the FLC promoter.

Why should FLC be regulated in this way? We speculate that this form of gene regulation provides poised control of transcription. This is important because the autonomous pathway limits, but does not silence, FLC expression as FLC controls temperature-dependent germination [47] and circadian clock function [48], as well as flowering.

Is alternative polyadenylation of antisense RNAs a widespread mechanism for regulating sense-strand gene expression? Co-expression of sense transcripts and polyadenylated antisense RNAs is widespread in A. thaliana [40] and mammalian genomes [49] where conserved sense/antisense gene pairs have been associated with alternative polyadenylation [50]. It is therefore conceivable that control of sense-strand expression by alternative cleavage and polyadenylation of antisense RNAs is not restricted to FLC, but has the potential to be a significant form of gene regulation. One implication is that disease-causing mutations that map to specific loci may actually disrupt processing of non-coding antisense RNAs rather than the processing or coding potential of the sense-strand gene itself, with consequences for understanding the cause and treatment of a particular disease.

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