RNA Splicing of FLC Modulates the Transition to Flowering

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Flowering is a critical stage of plant development and is closely correlated with seed production and crop yield. Flowering transition is regulated by complex genetic networks in response to endogenous and environmental signals. FLOWERING LOCUS C (FLC) is a central repressor in the flowering transition of Arabidopsis thaliana. The regulation of FLC expression is well studied at transcriptional and post-transcriptional levels. A subset of antisense transcripts from FLC locus, collectively termed cold-induced long antisense intragenic RNAs (COOLAIR), repress FLC expression under cold exposure. Recent studies have provided important insights into the alternative splicing of COOLAIR and FLC sense transcripts in response to developmental and environmental cues. Herein, at the 20th anniversary of FLC functional identification, we summarise new research advances in the alternative splicing of FLC sense and antisense transcripts that regulates flowering.

Keywords: alternative splicing, FLOWERING LOCUS C, COOLAIR, flowering transition, splicing factor, vernalization

HIGHLIGHTS
FLOWERING LOCUS C (FLC) is a key repressor in flowering transition. The alternative splicing of FLC sense and antisense transcripts regulated by external and internal cues modulates flowering transition.

INTRODUCTION
RNA splicing is a critical step in the post-transcriptional regulation of gene expression. This process occurs by removing intronic sequences and joining exons by spliceosome and numerous splicing factors (Jurica and Moore, 2003; Wahl et al., 2009; Matera and Wang, 2014; Vosseberg and Snel, 2017). Spliceosome is a highly dynamic ribonucleoprotein complex that catalyses RNA splicing and is composed of five small nuclear ribonucleoprotein (snRNP) particles (U1, U2, U4/U6 and U5) (Will and Lührmann, 2011; Fica et al., 2019). Splicing factors are one of the key determinants as accessory non-snRNP proteins regulating RNA splicing (Cho et al., 2011; Liu et al., 2016; Long et al., 2019; Wang et al., 2019; Xiong et al., 2019a; Xiong et al., 2019b). A pre-mRNA may undergo different splicing patterns, creating various mature transcripts that encode distinct functional proteins (Samach et al., 2011; Wang et al., 2015; Zhu et al., 2017; Lockhart, 2018; Okumoto et al., 2018). This phenomenon is called alternative splicing (AS).

AS fulfils important biological functions in plants, such as flowering transition and flower development (Wang et al., 2014; Melzer, 2017; Rodriguez-Cazorla et al., 2018; Park et al., 2019a; Park et al., 2019b; Wang et al., 2019). AS is also implicated in plant response to circadian rhythm regulation (Filichkin and Mockler, 2012), phytohormone (Hrtyan et al., 2015; Wang et al., 2015; Zhu...
et al., 2017; Xiong et al., 2019a), ambient temperature (Verhage et al., 2017) and abiotic and biotic stresses (Lyons and Kazan, 2016; Huang et al., 2017; Mei et al., 2017; Shang et al., 2017; Laloum et al., 2018). These cues are all important for flowering transition; therefore, AS plays multiple roles in flowering by integrating endogenous developmental and exogenous environmental signals. The flowering inhibitor gene flowering locus c (FLC) encodes a MADS-box transcription factor and is a key regulator of vernalisation and autonomous pathways in Arabidopsis and related species (Michaels and Amasino, 1999; Sheldon et al., 1999; Sheldon et al., 2000; Chen et al., 2019; Coupland, 2019). FLC inhibits flowering by repressing the expression of a subset of key genes in promoting flowering, such as flowering locus t (FT), suppressor of overexpression of constans 1 (SOC1) and target of ftc and svp1 (TFS1) (Helliwell et al., 2006; Searle et al., 2006; Luo et al., 2019; Richter et al., 2019). Thus, FLC regulation is central for flowering at the transcriptional, post-transcriptional and post-translational levels (Michaels et al., 2003; Lempe et al., 2005; Li et al., 2016; Kwak et al., 2017; Whittaker and Dean, 2017; Xiong et al., 2019a). The AS of FLC sense and antisense transcripts is required for flowering transition in Arabidopsis and other dicots (Michaels and Amasino, 1999; Sheldon et al., 1999; Helliwell et al., 2006; Yuan et al., 2009; Wu et al., 2012).

Twenty years ago, the works of Michaels and Amasino (1999), and Sheldon et al. (1999) provided a first glimpse of the central functions of FLC in flowering and the molecular basis of FLC in vernalisation (Coupland, 2019). Subsequent research works have demonstrated the regulation of FLC at the transcriptional and post-transcriptional levels, especially the epigenetic silencing of FLC by histone methylation in vernalisation, and the splicing regulation of FLC sense and antisense transcripts by splice factors (Bastow et al., 2004; Liu et al., 2007; Liu et al., 2010; Marquardt et al., 2014). In this review, we describe the current understanding of the AS of FLC sense and antisense transcripts in modulating flowering time and the splice factors involved in these processes.

**AS OF FLC SENSE TRANSCRIPTS MEDIATES FLOWERING TIME**

Arabidopsis accessions exhibit markedly different flowering behaviour from different environments. The AS of FLC sense transcripts in these accessions generate multiple splice isoforms (Figure 1A). The naturally occurring splice variants of FLC are related to different vernalisation responses of various Arabidopsis accessions (Bloomer and Dean, 2017). Arabidopsis ecotype Bur-0 is late flowering and vernalisation insensitive. FLC cDNA from Bur-0 contains 64 bp of intron sequence immediately upstream of exon 7, causing a mutation at the final position of intron 6. The 64 bp intron retention causes a frame shift and a premature stop codon in FLC cDNA. Thus, the encoded FLC lacking the C-terminal 33 amino acid residues is a null function protein in Bur-0 (Werner et al., 2005). Similarly, in variations of Cen-0 and Cal-0, alternative splice acceptor sites in the last exon and last intron are used, respectively. These aberrant splicing forms all lead to a frame shift of cDNA sequences and severely compromise protein function (Lempe et al., 2005). In Col-0 Arabidopsis, there are several additional splicing variants from FLC locus besides the canonical transcript (Severing et al., 2012; Park et al., 2019a); however, their roles in flowering transition remains to be investigated. An additional shorter transcript from FLC locus is induced after vernalisation treatment for 15 days in Est-0 and Le-0 ecotypes, which is not observed at normal temperature (Caicedo et al., 2004). Whether this short transcript is involved in vernalisation-induced flowering in Arabidopsis is unknown.

In other plant species, the naturally occurring splice variants of the FLC locus also reveal that FLC AS is important for the control of flowering time. For example, four different transcripts of the FLC homologs in Brassica rapa BrFLC1 and BrFLC2 have been identified from naturally occurring splicing mutations (Yuan et al., 2009; Zhao et al., 2010; Wu et al., 2012). These different splice types of BrFLCs are significantly associated with a natural variation of flowering time in different germplasms of B. rapa. Additionally, differential splicing variants of BrFLC A3b, a FLC homolog in B. napus, have been observed in leaves at the seedling stage between winter cultivar Tapidor and semi-winter cultivar Ningyou7. The transcripts from Tapidor are usually spliced canonically, but numerous incompletely spliced transcripts have been identified in Ningyou7, causing decreased functional transcripts (Zou et al., 2012). In tetraploid Capsella bursa-pastoris, splice site polymorphisms in the FLC loci create different transcripts which are nonfunctional (Slotte et al., 2009). These findings partially explain the differential flowering times of C. bursa-pastoris from different districts. A natural splicing site mutation in the BrpFLC1 gene causes early flowering in the cultivated variety of purple flowering stalk (B. campestris L. ssp. chinensis var. purpurea) compared with that of pakchoi (B. campestris ssp. chinensis var. comminus) (Hu et al., 2011). Additionally, in an early flowering trifoliate orange (Poncirus trifoliata) mutant, five alternatively spliced transcripts of PtFLC have been identified; furthermore, their abundances are variable at the juvenile and adult stages, suggesting that the AS of PtFLC is related to flowering time (Zhang et al., 2009). Therefore, the AS of FLC is a target of natural selection for flowering regulation under natural conditions.

**AS OF FLC ANTISENSE TRANSCRIPTS REGULATES FLC EXPRESSION**

COOLAIR is a set of long noncoding RNAs expressed at the FLC locus in the antisense direction that was first identified in Arabidopsis (Liu et al., 2007; Swiezewski et al., 2007; Swiezewski et al., 2009; Jeltsaart et al., 2012). COOLAIR RNAs functionally repress FLC sense expression at an early stage of cold exposure (vernisation) via different ways, such as by directly associating with FLC chromatin or affecting H3K36me3 and H3K27me3 levels (Corbana et al., 2014; Berry and Dean, 2015; Rosa et al., 2016; Pajoro et al., 2017; Whittaker and Dean, 2017; Tian et al., 2019) (Figure 1B). The AS of antisense transcripts generated from the FLC locus produces two main classes of COOLAIR isoforms, terminating at proximal (sense intron 6, class I) and distal (sense promoter, class II) sites of FLC locus (Liu et al., 2007; Swiezewski et al., 2009; Marquardt et al., 2014). Each class contains several
subclasses produced by AS associated with the usage of alternative 3′ polyadenylation in spliced variants (Figure 1A). These multiple spliced variants with alternative polyadenylation are linked to different FLC expression states. Class I COOLAIR RNAs with proximal polyadenylation are associated with FLC repression (Liu et al., 2007; Swiezewski et al., 2009; Liu et al., 2010; Hornyik et al., 2010; Csorba et al., 2014). By contrast, class II COOLAIR transcripts are associated with high FLC expression levels. These COOLAIR RNAs with distal polyadenylation affect the capping of FLC nascent transcripts (Li et al., 2015).

The efficient splicing of class I intron promotes proximal polyadenylation in the antisense transcripts of FLC. As a result, this proximal polyadenylation inhibits the transcription of FLC through triggering H3K4me2 demethylation in the FLC locus (Marquardt et al., 2014). A few 3′-end processing factors containing RNA-recognition motifs, such as flowering control locus a (FCA) and FPA, glycinine-rich rna-binding protein7 (GRP7), cleavage stimulation factor 64 (CstF64), CstF77, and nuclear speckle RNA binding protein a (NSRa), affect the splice site selection and polyadenylation site usage of COOLAIR transcripts, leading to an altered ratio of proximal–distal spliced variants (Liu et al., 2007; Streitner et al., 2008; Liu et al., 2010; Hornyik et al., 2010; Streitner et al., 2012; Xiao et al., 2015; Bazin et al., 2018). Loss-of-function mutations in these factors decrease or increase the usage of COOLAIR proximal polyadenylation site, leading to upregulated or downregulated FLC transcription.

The AS of COOLAIR is altered by environmental conditions and natural intronic polymorphisms. Cold exposure influences COOLAIR splicing (Swiezewski et al., 2009). For example, class I COOLAIR RNAs increase more rapidly than do class II ones during vernalisation (Csorba et al., 2014; Berry and Dean, 2015; Xiao et al., 2015; Rosa et al., 2016). The class II COOLAIR transcripts with distal polyadenylation influence the degree of capping of the FLC nascent transcripts (Li et al., 2015).

![Diagram of splicing patterns of FLC sense and antisense transcripts, and AS of FLC antisense transcripts regulating FLC expression.](image-url)
a downstream distal splice acceptor site and the inclusion of an internal exon. This change of splicing site produces isoforms with altered secondary structure and upregulates FLC expression (Li et al., 2015; Hawkes et al., 2016).

**SPLICING FACTORS REGULATE THE PROCESSING OF FLC TRANSCRIPT**

Splice sites, including 5′ donor splice site, branch point site, polypyrimidine tract and 3′ acceptor splice site, in pre-mRNA introns are precisely recognized by some splicing factors. Numerous splicing factors, such as BRR2, SC35 and SC35-like (SCL), RZ-1B and RZ-1C, are involved in the splicing of FLC in *Arabidopsis* flowering transition (Table 1). BRR2, an ATP-dependent RNA helicase, is an integral component of the U5 snRNP that is required for the activation of the spliceosome complex (Raghnunath and Guthrie, 1998). A missense mutation in *Arabidopsis* **BRR2a** results in defective FLC splicing and reduced FLC transcript levels (Mahrez et al., 2016). SC35 is a serine/arginine-rich (SR) protein that functions in the selection of splice sites (Valcárcel and Green, 1996). SC35 and SC35-like (SCL) proteins in *Arabidopsis* simultaneously modulate the splicing and transcription of FLC (Yan et al., 2017). *Arabidopsis* **RZ-1B** and RZ-1C, two heterogeneous nuclear ribonucleoproteins (hnRNPs), regulate FLC splicing and transcription by directly interacting with the SR protein (Wu et al., 2016). Interestingly, the retention of FLC introns 1, 5 and 6 in the **brr2a** mutant increases and the splicing efficiency of FLC intron 1 decreases in **rz-1b** **rz-1c** double mutants. These findings suggest that BRR2a, RZ-1B and RZ-1C promote the splicing of FLC introns. By contrast, the splicing efficiency of intron 1 in FLC increases compared with that in wild-type seedlings in quintuple mutants of SC35 and SCL genes, indicating that SC35 and SCL proteins inhibit the splicing of the first intron of FLC. The splicing efficiency of FLC introns is also inhibited by KHZ1 and KHZ2, two RNA-binding proteins containing CCCH zinc-finger and K homology (KH) domain (Yan et al., 2019).

U2 auxiliary factor (U2AF) regulates flowering via modulating FLC splicing. U2AF65, a large subunit of U2AF in mammals, recognises and binds to the 3′ polypyrimidine tract of introns (Wang et al., 2008; Shao et al., 2014). The binding site of U2AF65 with RNA is regulated and shifted in noncanonical introns (Shao et al., 2014; Howard et al., 2018). The genes **AtU2AF65a** and **AtU2AF65b** encode the U2AF large subunit in *Arabidopsis* (Jang et al., 2014). AtU2AF65b plays roles in regulating flowering transition by splicing the introns 1 and 6 of FLC (Xiong et al., 2019a) (Table 1). AtU2AF65b expression is responsive to ABA, by which AtU2AF65b is involved in ABA-regulated flowering. AtU2AF65a is also implicated in FLC splicing (Park et al., 2019a). Strikingly, the loss-of-function mutants of AtU2AF65a and AtU2AF65b display opposite flowering phenotypes. Mutations in **atu2af65a** cause late flowering, whereas **atu2af65b** mutants exhibit early flowering (Park et al., 2019a; Xiong et al., 2019a). The differences in the noncanonical splicing variants between **atu2af65a** and **atu2af65b** null mutants (Park et al., 2019a) indicate that AtU2AF65a and AtU2AF65b might recognise different FLC introns.

In addition, some proteins play roles in RNA splicing by interaction with splicing factors. For example, the mRNA cap-binding complex (CBC) is involved in modulating pre-mRNA splicing activities via interaction with the splicing factors that recognise the 5′ splice site of the cap proximal intron (Izaurralde et al., 1994; Lewis et al., 1996). The CBP80/ABA Hypersensitive 1 (ABH1) and CBP20 are the large and small subunits of CBC protein complex in *Arabidopsis*, respectively (Hugouvieux et al., 2001). Knockout mutants of **ABH1/CBP80** and **CBP20** showing early-flowering phenotypes result from the defective splicing of FLC introns, especially the large first intron (Kuhn et al., 2007) (Table 1). In the **abhl** knockout mutant, the most prominent products are the splice intermediates containing the first intron, causing the downregulation of FLC transcript and the early-flowering phenotypes (Kuhn et al., 2007). Similarly, CBP20 null mutation also results in increased unspliced–spliced ratio of FLC introns and low FLC mRNA levels (Geraldo et al., 2009).

To date, little is known about the splicing regulation of **COOLAIR** transcripts mediated by splicing factors. Only one splice factor, PRP8, a core spliceosome component, is found to interact with function in modulating **COOLAIR** RNAs splicing (Marquardt et al., 2014) (Table 1). **PRP8** is specifically required for the splicing of antisense transcripts **COOLAIR** but not for that of **FLC** sense transcripts. Single-base mutations of **PRP8** reduce the splicing efficiency of **COOLAIR** introns, especially class li introns. The decreased splicing efficiency of **COOLAIR** class li reduces proximal poly(A) site usage, leading to increased H3K4me2 and the transcriptional upregulation of **FLC** expression (Liu et al., 2010; Marquardt et al., 2014). **NSR**s, as a nuclear speckle RNA binding protein, modules flowering time through regulation of

| Proteins | Type of Proteins | Splicing | References |
|----------|-----------------|----------|------------|
| **U2AF65a, U2AF65b** | Subunits of U2 auxiliary factors | **FLC** introns | Park et al., 2019a; Xiong et al., 2019a |
| **SC35, SCL28,30,30A,33** | SR proteins | **FLC** intron 1 | Yan et al., 2017 |
| **BRR2a** | U5 snRNP | **FLC** introns 1, 5 and 6 | Mahrez et al., 2016 |
| **RZ-1B, RZ-1C** | CAP-binding complex subunits; Interaction proteins of splicing factor | **FLC** intron 1 | Wu et al., 2016 |
| **ABH1/CBP80, CBP20** | **FLC** introns | Geraldo et al., 2009; Kuhn et al., 2017 |
| **KHZ1, KHZ2** | RNA-binding proteins | **COOLAIR** introns | Yan et al., 2019 |
| **PRP8** | Core spliceosome component | **COOLAIR** introns | Marquardt et al., 2014 |
| **NSR** | Nuclear speckle RNA binding protein | | Bazin et al., 2018 |
the COOLAIR AS (Table 1). Only distal variants are decrease in the nsra mutant. This change in relative variant usage of proximal-distal RNAs leads to a down-regulation of FLC mRNA and an early flowering phenotype of nsra mutant (Bazin et al., 2018).

CONCLUSION AND PERSPECTIVE

FLC is a key inhibitor in flowering transition in Arabidopsis and other dicots (Michaels and Amasino, 1999; Sheldon et al., 1999; Helliwell et al., 2006; Yuan et al., 2009; Wu et al., 2012). Therefore, the regulation of FLC is central to the transition to flowering in these plants. The AS of FLC sense and antisense transcripts is a critical step for FLC expression regulation. In Arabidopsis, multiple spliced variants of FLC sense and antisense transcripts have been determined (Kuhn et al., 2007; Severing et al., 2012; Li et al., 2015; Hawkes et al., 2016; Park et al., 2019a); however, we know little about functions in vivo of these spliced isoforms, especially, the COOLAIR-mediated regulation of switching of chromatin states at FLC and processing of FLC sense transcripts (Figure 1B). The further investigation will focus on how regulation of COOLAIR AS is linked to FLC chromatin modifications in response to external and internal influences.

Alternative splicing is regulated by splicing factors; however, only few splicing factors have been identified to be involved in the AS of FLC, especially those in the AS of COOLAIR transcripts. The identification of splicing factors and their functions is important for understanding the AS regulation of FLC sense and antisense transcripts in flowering transition. Additionally, the patterns of FLC AS is altered in response to environmental and signal stimuli (Swiezewski et al., 2009; Hornýk et al., 2010; Liu et al., 2010; Xiong et al., 2019a). Thus, the mechanisms by which the activities of splicing factors are regulated in response to external and internal cues must be investigated to study the AS of FLC sense and antisense transcripts in flowering regulation.

In agriculture, flowering is a prerequisite for crop production. Changes in the splicing patterns of FLC sense and antisense transcripts have enabled adaptation in response to changing environment for Arabidopsis accessions. Moreover, cold-induced sense and antisense RNAs of FLC are evolutionarily conserved in Arabidopsis perennial relatives and sugar beet (Reeves et al., 2007; Castaings et al., 2014; Li et al., 2015; Hawkes et al., 2016). Therefore, the molecular dissection of the diversity in splicing patterns of FLC across natural populations of Arabidopsis provides an important insight into how splicing regulation influences the switch from vegetative to reproductive growth. These findings propose a possible application for cultivating new varieties and augmenting the control of flowering time to adapt the environmental changes via modulating FLC expression in some crops, such as Brassicaceae.

AUTHOR CONTRIBUTIONS

X-LW designed the concept, organized and drafted the text. H-DQ performed the meta-analysis, drafted text, and prepared figures. H-DQ and YL edited the manuscript with help of Q-PR, Y-YW and FX.

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