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REVIEW

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Normal, novel or none: versatile regulation from alternative splicing

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ABSTRACT
Pre-mRNA splicing is a vital step in the posttranscriptional regulation of gene expression. Splicing is catalyzed by the spliceosome, a multidalton RNA–protein complex, through two successive transesterifications to yield mature mRNAs. In Arabidopsis, more than 61% of all transcripts from intron-containing genes are alternatively spliced, thereby resulting in transcriptome and subsequent proteome diversities for cellular processes. Moreover, it is estimated that more alternative splicing (AS) events induced by adverse stimuli occur to confer stress tolerance. Recently, increasing AS variants encoding normal or novel proteins, or degraded by nonsense-mediated decay (NMD) and their corresponding splicing factors or regulators acting at the posttranscriptional level have been functionally characterized. This review comprehensively summarizes and highlights the advances in our understanding of the biological functions and underlying mechanisms of AS events and their regulators in Arabidopsis and provides prospects for further research on AS in crops.

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1 Introduction

In eukaryotes, the process that the introns are precisely recognized and removed from the nascent mRNAs (pre-mRNAs) and the exons are ligated in the right order to yield translatable mRNAs is called pre-mRNA splicing.[1] Splicing is catalyzed by the spliceosome, which is mainly composed of five small RNAs with their specific accessory proteins (snRNPs) and multiple splicing factors.[2]

In many cases, the splicing process is flexible enough to generate multiple mature mRNAs by alternative splice site selections from one single gene, known as AS.[3] AS regulates gene expression at the posttranscriptional level, which exaggerates the transcriptome and proteome diversities with limited gene numbers.[4] In 1977, adenovirus late mRNA synthesis involving multiple splicing during maturation was observed, representing the first characterized example of alternative modes of RNA splicing.[5] In 1989, an alternative splicing event of rubisco activase in plants was first identified.[6] With sequencing technology development, genome-wide transcriptome analysis revealed that more than 61% of all transcripts are subjected to AS under normal growth conditions in *Arabidopsis thaliana*.[7] To date, substantial research has demonstrated that AS provides versatility for gene expression, in order to fine-tune the function of regulatory factors and modulate the responses to the developmental and physiological signals in Arabidopsis.[8]

2 Alternative splicing and the spliceosome

2.1 *Basic types of alternative splicing events*

High-throughput sequencing revealed that up to 95% of intron-containing genes in humans are subject to AS.[9] In Arabidopsis, at least 61% of genes undergo AS, followed by 46% in rice.[10] In addition, extensive studies indicate that the estimated figures will increase when more RNA-seq data are available, particularly from specific tissues/cells at different developmental stages and under stressful conditions.[4] Based on splice site selection, AS can be classified into four main types: (1) intron retention (IR); (2) exon skipping (ES); (3) alternative 5'splice sites (Alt 5'ss); and (4) alternative 3'splice sites (Alt 3'ss). In humans, ES accounts for 38.4%, followed by Alt 3'ss (18.4%) and Alt 5'ss (7.9%), and IR is the least (2.8%). In contrast, IR is the most frequent AS event in plants. In Arabidopsis, IR accounts for 39.6%, followed by Alt 3'ss (15.5%) and Alt 5'ss (7.5%), and ES is the rarest (2.7%).[4]

2.2 *Characterized spliceosome components in Arabidopsis*

Alternative splicing takes place in the spliceosome. A vast number of genetic analysis and biochemical studies, especially in yeast and humans, has led to a consensus view that pre-mRNA splicing is largely conserved in all organisms.[11] The spliceosome is assembled dynamically in a stepwise manner,

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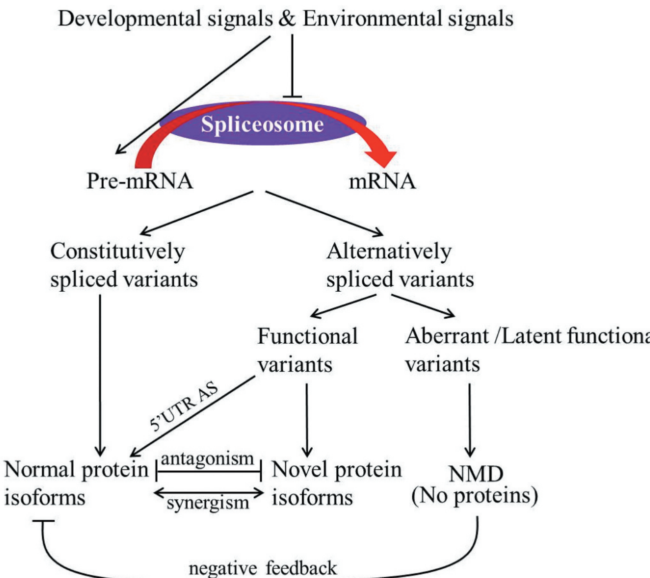

Figure 1. Pathways that alternative splicing variants are processed in Arabidopsis. Alternative splicing is catalyzed by the spliceosome. The developmental and environmental signals enhance alternatively spliced variants by dramatically increasing development- and stress-responsive pre-mRNAs which compete for the limited splicing machinery or repressing splicing machinery activity. Various mRNAs generated from one single gene by alternative splicing may be translated into normal or novel proteins or be degraded by NMD pathway. Moreover, the normal and novel proteins act in an antagonistic or synergistic manner. The 5′UTR-located alternative splicing usually has an effect on translation efficiency. The blunt end represents "repression". UTR, untranslated region; NMD, nonsense-mediated decay. Alternative splicing is catalyzed by the spliceosome. The developmental and environmental signals enhance alternatively spliced variants by dramatically increasing development- and stress-responsive pre-mRNAs which compete for the limited splicing machinery or repressing splicing machinery activity. Various mRNAs generated from one single gene by alternative splicing may be translated into normal or novel proteins or be degraded by NMD pathway. Moreover, the normal and novel proteins act in an antagonistic or synergistic manner. The 5′UTR-located alternative splicing usually has an effect on translation efficiency. The blunt end represents "repression". UTR, untranslated region; NMD, nonsense-mediated decay.

depending on multiple interactions among RNAs and/or proteins, affording the flexibility and fidelity required for splicing. Several distinct sub-complexes (i.e. E, A, B, B^{act}, B*, C, P and ILS complex), which represent spliceosome assembly, activation, splicing and disassembly stages, sequentially form along with specific proteins dissociated and recruited during the splicing process.

The spliceosome in plants is also composed of five snRNAs, consisting of U1, U2, U4, U5, and U6, which are packaged as small ribonucleoprotein particles with respective sets of proteins (snRNPs), plus a variety of non-snRNPs associated with pre-mRNAs.[12] To date, multiple sequence conserved splicing factors and regulators have been functionally characterized in Arabidopsis, which regulate many aspects of development and stress responses.[13] However, the dynamic interactions among them that are implicated in recruitment and dissociation are much less known than those in yeast and human.

3 Involvement of alternative splicing in plant development and stress response

Various mRNAs generated from one single gene via AS may be proceeded by several pathways in plants, mainly as follows: (1) the mRNAs are translated into functional or truncated proteins which display loss or gain of function, providing a means of synergistically or antagonistically fine-tuning biological processes; (2) the AS located in the untranslated region (UTR) affects the stability or translational efficiency of mRNA, showing a rapid means of controlling the protein level; and (3) the non-translated mRNAs containing premature termination codons (PTCs) are targeted for degradation by the nonsense-mediated decay (NMD) pathway, functioning as a negative feedback loop to control the amount of functional proteins (Figure 1). AS is a crucial regulatory mechanism for almost every aspect of development processes and stress responses in plant lifespan via the above mentioned pathways.

3.1 Alternative splicing in seed dormancy and germination regulation

Seed dormancy is an important adaptive trait. *DELAY OF GERMINATION 1* (*DOG1*), which is the first major QTL associated with seed dormancy, was cloned and identified to be seed-specifically expressed in Arabidopsis.[14] The accumulation of DOG1 protein was positively associated with the depth of seed dormancy.[15] The homologs of DOG1 in other crops were also subsequently identified to be required for seed dormancy. In Arabidopsis, *DOG1* is alternatively spliced and generates five transcripts, namely *DOG1-α*, *DOG1-β*, *DOG1-γ*, *DOG1-δ*, and *DOG1-ε*, in which *DOG1-β*, *DOG1-γ*, and *DOG1-ε* encode the same protein.[14] Genetic analysis indicates that the expression of single *DOG1* variants driven by the 35S promoter leads to protein accumulation to fully complement the seed dormancy defects,[16] suggesting that each protein could function properly *in vivo*. In contrast, the overexpression of a single *DOG1* variant driven by the endogenous promoter resulted in a lack of DOG1 protein and failed to restore the dormancy phenotype, while the simultaneous expression of at least two variants from the endogenous promoter accumulated DOG1 protein and largely rescued the dormancy phenotype. These genetic analyses collectively suggest that a single variant is functional, but requires two or more isoforms to protect the DOG1 protein from degradation,[16] suggesting the synergistic roles of DOG1 AS variants. Interestingly, the expression of *DOG1* is also regulated by alternative polyadenylation and antisense at the posttranscriptional level.[17,18]

Seed germination is the starting point in the plant life cycle, which is tightly linked with seed dormancy in terms of physiological state. *PHYTOCHROME INTERACTING FACTOR 6* (*PIF6*), highly expressed in late stage of seed maturation, yields two transcripts by AS, namely *PIF6-α* and *PIF6-β*, respectively. *PIF6-α* encodes the full-length protein, including a phytochrome-binding motif and a bHLH motif. In contrast, *PIF6-β* produces a truncated protein lacking the bHLH motif because of the skipping of the third exon. Overexpression of *PIF6-α* phenocopied with wild type regarding the germination ratio, while *PIF6-β* overexpressors enhanced the germination potential,[19] indicating an antagonistic effect for *PIF6* AS variants on seed germination with an unknown mechanism. AS is regulated

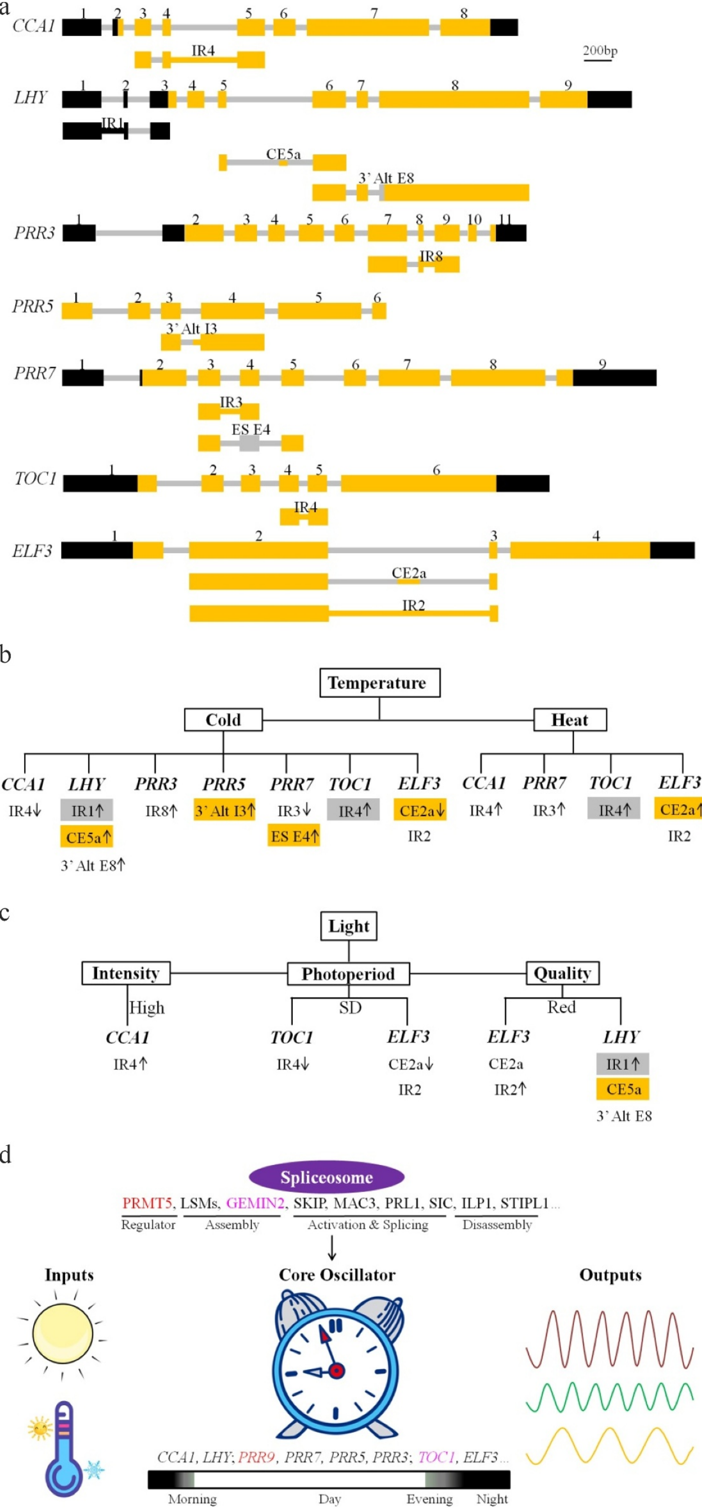

Figure 2. Alternative splicing control of the circadian clock. (a) Alternative splicing of circadian clock genes, including *CCA1, LHY, PRR3, PRR5, PRR7, TOC1*, and *ELF3*. Reported alternative splicing events are shown below the gene structure. The black box and bar represent UTRs; the yellow box and bar indicate the exons; the gray box and bar denote the introns. The figure at the top of the gene structure indicates the order of the exon. The alternative splicing events are named according to the alternative splicing types and positions. (b) and (c) Alternative splicing events occurring under different conditions, such as temperatures (b) and lights (c). The arrow indicates the up- or down-regulated alternative splicing event at the indicated conditions. The gray and yellow box represent the intron and exon subjected to degradation via nonsense mediated decay pathway, respectively. (d) The identified splicing factors and regulators in spliceosome conferring circadian clock regulation via alternative splicing of clock genes. The same color indicates the corresponding splicing factor and its specific target as reported, and the other splicing factors affect the alternative splicing of all the clock genes.

by spliceosome components. PTB1 and PTB2, which are two closely related polypyrimidine tract-binding proteins in Arabidopsis, are key splicing regulators that influence widespread AS. Interestingly, PTB misexpression results in an increase of *PIF6-β*, coinciding with the altered rates of ABA-dependent seed germination. In addition, *PIF6-β* was elevated in *sc35-scl* quintuple mutant (*scl28 scl30 scl30a scl33 sc35*), resulting in a higher seed germination rate.[20]

3.2 Alternative splicing in circadian clock control

Biological rhythms with a period of about 24 hours are regulated by circadian clock, which not only confers a daily rhythm in growth and metabolism but also interacts with signaling pathways involved in plant responses to the environment to provide plants with an adaptive advantage.[21] Circadian clock regulation can be divided into input signals, core oscillators, and output pathways. The core oscillator is mainly composed of feedback regulation loops, including morning factors *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), later-expressed *PSEUDO RESPONSE REGULATOR 9* (*PRR9*) and *PRR7*, and evening factor *TIMING OF CAB EXPRESSION 1* (*TOC1*).[21] Recently, extensive researches have indicated that AS makes an important contribution to the circadian regulation of gene expression. Clock genes such as *CCA1, LHY, TOC1, PRRs*, and *EARLY FLOWERING 3* (*ELF3*) are subjected to AS[22–24] (Figure 2a). Temperature and light are important environmental factors modulating clock function partly by AS (Figure 2b, 2c). Low temperature induces AS events of *LHY, PRR3* and *PRR5*, but suppresses those of *CCA1* and *ELF3*, while *PRR7* and *TOC1* AS events are accumulated at both low and high temperatures[23,24] (Figure 2b). The photoperiod, light quality, and light intensity also influence the AS of *TOC1, ELF3, CCA1*, and *LHY*[24–27] (Figure 2c). Plants use circadian clock to monitor the daylength in response to seasonal changes.[28] The AS of *TOC1* and *ELF3* was suppressed during short days,[24] indicating the photoperiod influences AS patterns of clock genes. In the case of *LHY*, an inclusion of exon5a introduces a PTC and is predicted to reduce the amount of functional LHY at low temperature, and the intron retention in 5′UTR is robustly elevated when exposed to red light.[26] For *CCA1*, the fourth intron retention variant *CCA1-β* decreased at low temperature but accumulated under strong light intensity,[25,29] but the CCA1-β protein was induced to accumulated in the cytoplasm under high temperature.[30]

CCA1-α is necessary for proper biological rhythm regulation, while the CCA1-β protein contains a dimerization domain, but lacks a Myb DNA-binding domain at the N terminus. CCA1-β competitively interacts with LHY to antagonistically attenuate the association with CCA1-α, thus abolishing the ability of DNA binding activity.[31] In addition to the antagonistic roles of AS in circadian control, multiple abnormal mRNAs produced by circadian clock genes, including *PRR5, PRR7, TOC1, LHY*, and *ELF3*, were identified to be targeted by the NMD pathway when exposed to environmental factors, such as temperature fluctuations and photoperiod changes[22,23] (Figure 2b, 2c). Despite the fact that the dominant roles in circadian clock control for *CCA1* and *LHY* at different temperatures can be explained at least partly explained by antagonistically AS,[32] the functional modes of the considerably validated AS events of circadian clock genes are poorly understood. However, the AS regulation of circadian clock-regulated genes *GLYCINE-RICH RNA BINDING PROTEINS 7* (*GRP7*) and *GRP8* was well studied.[33,34] Both *GRP7* and *GRP8* are negatively auto-regulated and reciprocally regulated by directly binding to their own and mutual mRNAs to promote the alternative usage of a cryptic intronic 5′ splice site at the expense of their fully spliced transcripts. The yielding of alternative transcripts with PTC, which are linked to NMD, leads to a decrease in the abundance of *GRP7* and *GRP8* .[35,36]

During the last decade, increasing evidences have indicated that spliceosome components are implicated in the circadian rhythm by regulating the AS of core circadian clock genes at the posttranscriptional level (Figure 2d). Mutations in *PROTEIN ARGININE METHYLTRANSFERASE 5* (PRMT5), which methylates arginine in Sm and LSM spliceosomal proteins, were first identified to lengthen the clock period by the disturbed AS of *PRR7* and *PRR9*.[37] However, the lengthened clock in *lsm4* and *lsm5* might be associated with the AS of other clock genes, because the splicing of *PRR9* is normal in *lsm4* and *lsm*5.[38] Subsequently, mutants in several spliceosome-associated genes, including *SKI-INTERACTING PROTEIN* (*SKIP*), SPLICEOSOME TIMEKEEPER LOCUS 1 (*STIPL1), INCREASED LEVEL OF POLYPLOIDY1–1D* (*ILP1), GEMIN2, SIC, PLEIOTROPIC REGULATORY LOCUS 1* (*PRL1*), and *MODIFIER OF snc1 4* (*MOS4*)*-ASSOCIATED COMPLEX 3* (*MAC3*), were identified. Interestingly, *skip, stipl1, ilp1, sic, prl1, and mac3* confer a long period by altering the AS of multiple circadian clock genes, while the *gemin2* mutant shortened the period partly by altering the AS of *TOC1*. Moreover, SKIP, SIC, and GEMIN2 control the circadian clock by AS in a temperature responsive manner. Mutations in SKIP confer long periods at low temperatures, but not at high temperatures.[39] Deficiency in *SIC* impaired temperature compensation.[40] In contrast, the short period resulting from the loss of function of *GEMIN2* is most evident at high temperatures.[31] Notably, a recent study on PRE-mRNA PROCESSING FACTOR 40 (PRP40) suggests that not all the spliceosome components are involved in circadian clock control.[41] Overall, the Arabidopsis circadian clock requires spliceosome activity to mediate the AS of circadian clock transcripts to establish the period, albeit by regulating the AS of different clock genes.

3.3 Alternative splicing in photomorphogenesis

PIF3 acts as a light response repressor in Arabidopsis,[42] of which expression regulation has been well studied in the transcriptional and translational level. Recently, the AS of PIF3 at the posttranscriptional level, which results in an intron retention in 5′UTR, was identified to be phyB-dependent under a short-day light cycle.[43] The AS of UTR influences the coding capacity of the genome and translational efficiency, especially 5′UTR, by producing mRNA variants with divergent upstream open reading frame (uORF) or riboswitches.[44] Further analysis indicated that the AS in 5′UTR inhibits PIF3 protein translation *in vitro* and *in vivo*. Consistently, a complementation assay indicated that the intron retention variant failed to rescue the elongated hypocotyl phenotype under the red light.[43] Moreover, a considerable number of genes were regulated by uORF-mediated translation inhibition with respect to the light response,[45] albeit with an unknown biological function. PIF3 is also demonstrated to confer freezing tolerance by stabilizing the phyB thermosensor,[46] but the role of the AS in PIF3 in freezing tolerance remains uncovered.

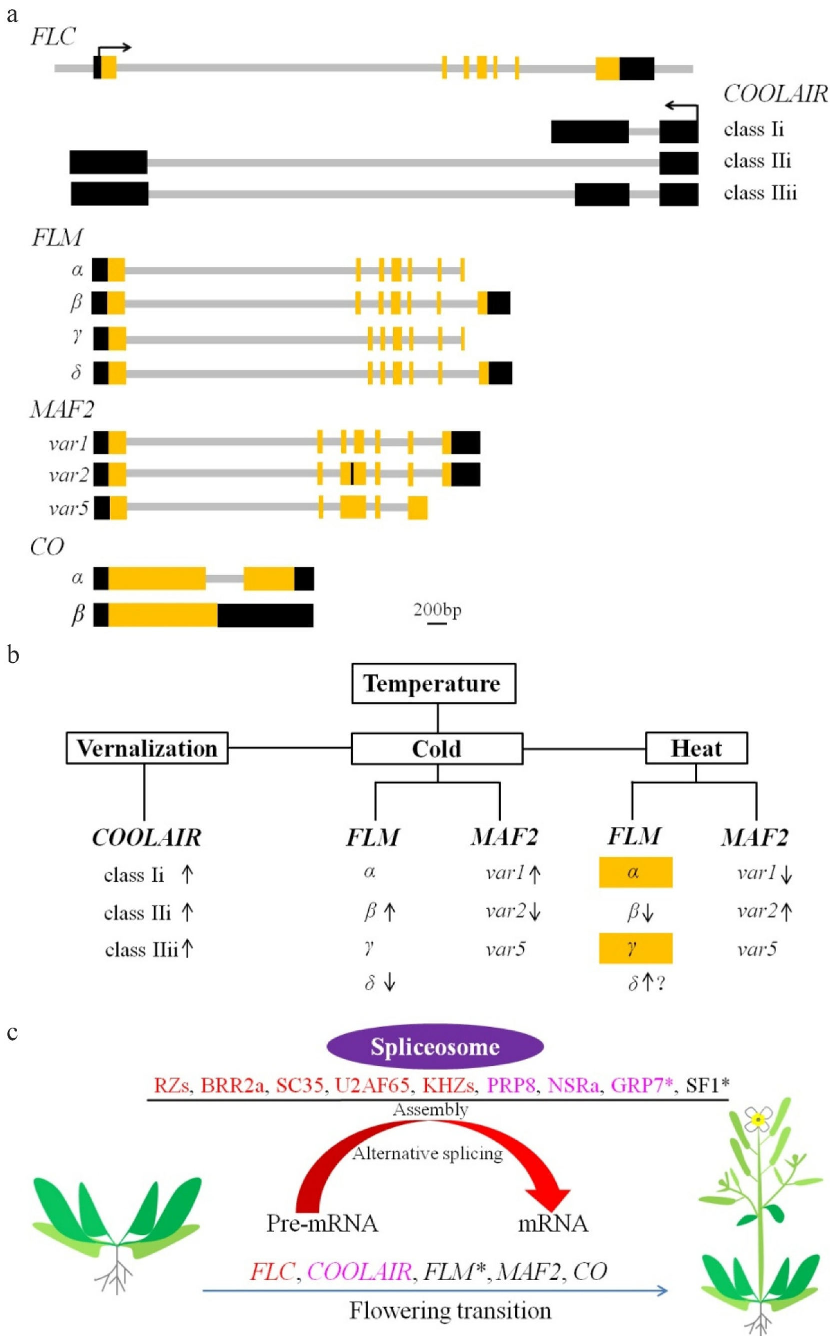

Figure 3. AS events and their regulators involved in flowering time control. (a) Alternative splicing of flowering-related genes, including *FLC, COOLAIR, FLM, MAF2*, and *CO*. Reported alternative splicing events are shown. The yellow box indicates the exon; the gray bar denotes the intron; the black box represent UTR or long non-coding RNA. (b) Alternative splicing events occurring under different temperatures. The arrow indicates the up- or down-regulated alternative splicing event at the indicated conditions. The yellow box represent the variants subjected to degradation by NMD. (c) The identified splicing factors and regulators in spliceosome conferring flowering time control via alternative splicing of flowering-related genes. The same color or the star indicate the corresponding splicing factor and its target as reported, and the splicing factors involved in the alternative splicing of *MAF2* and *CO* are unknown.

3.4 Alternative splicing in flowering time control

The appropriate timing of flowering is crucial for plants' reproductive success. Arabidopsis accessions exhibit different flowering time, partly by the various usage of alternative splice sites of *FLOWERING LOCUS C* (*FLC*),[47] which is the central floral repressor integrating the endogenous and environmental pathways in the flowering time control network. In Col-0 accession, *FLC* is alternatively spliced,[48] but the significance of the AS variants in flowering is unknown. However, *FLC* is the best-characterized gene which is involved in the cross-regulation of the sense and antisense transcript.[49] The *FLC* antisense transcript *COOLAIR* extends beyond the end of the sense transcript, which functionally repressed *FLC* expression at the early stage of cold induction[50,51] (Figure 3a). Due to the AS plus with alternative 3′polyadenylation, the antisense transcript produces two

main classes of *COOLAIR* isoforms: the proximally polyadenylated class I (terminating at the sixth intron of *FLC* gene locus) and the distally polyadenylated class II (terminating at the promoter of *FLC* gene locus)[50,51] (Figure 3a). Vernalization influences *COOLAIR* splicing with a consequence of an increase of class I/class II ratio[51] (Figure 3b), implying their different effects on *FLC* expression level. Recently, much progress has been achieved in the identification of splicing factors (Figure 3c), including RZ-1B and RZ-1 C, BAD RESPONSE TO REFRIGERATION 2a (BRR2a), SC35 and SC35-like, U2AF65, KHZs, which target *FLC* sense transcript to favor the splicing efficiency, but with the usage of unnatural splice sites. Interestingly, SKIP mediates *FLC* expression by the splicing of *SERRATED LEAVES AND EARLY FLOWERING* (*SEF*) pre-mRNA to control flowering time.[52] Another evolutionarily conserved splicing factor PRP8 (Figure 3c), in which mutations lead to flowering time delay, was identified to repress *FLC* expression by favoring class I isoform production,[53] suggesting that splicing of *COOLAIR* is functionally important. In agreement, loss of GRP7 reduces class I/class II ratio but upregulates *FLC* expression[54] (Figure 3c). In contrast, class I isoform is increased in *nsra* mutant (Figure 3c), resulting in an early flowering phenotype.[55] Taken together, these results suggest that class I is associated with *FLC* repression, but class II is linked with high *FLC* expression levels.

The flowering time is regulated by endogenous factors and environmental signals. Among environmental cues, little is known about how flowering is controlled in response to ambient temperature.[56] FLOWERING LOCUS M (FLM), an FLC-clade member, interacts with floral repressor SHORT VEGETATIVE PHASE (SVP) to repress flowering.[40,57] *FLM* undergoes AS, yielding four AS isoforms, namely *FLM-α, -β, -γ*, and *-δ*[58] (Figure 3a). *FLM-β* and *FLM-δ* were the dominant isoforms by selectively skipping the second exon and third exon, respectively, which encodes part of the MIKC intervening (I) region for protein–protein interaction.[58] *FLM-β* dominates in cool temperature (16°C) and *FLM-δ* accumulates in warm temperature (27°C) (Figure 3b). Initially, it was widely accepted that *FLM* functions in a temperature-dependent manner by the ratio of *FLM-β/FLM-δ*, because SVP-FLM-δ heterodimer abolished the DNA binding activity compared with SVP-FLM-β, thereby releasing the downstream floral activator to promote flowering at a warm temperature.[40,57] Most recently, several lines of evidences have suggested that a high temperature accelerates flowering, predominantly by the down-regulation of *FLM-β* expression via producing the NMD-sensitive variants (Figure 3b), which is supported by the fact that the thermal induction of flowering is not abolished in *flm-δ*, but in *upf* mutants.[59,60] Collectively, early flowering under an elevated temperature is only caused by the repression of *FLM-β*, rather than the decrease of the *FLM-β*/*FLM-δ* ratio.[40,57,59,60] FLM AS is regulated by splicing factors (Figure 3c), for example, inactivation of SF1 decreased the ratio of *FLM-β*/*FLM-*δ,[61] and overexpression of *GRP7* selectively favors the formation of *FLM*-β.[62]

Similarly, another FLC-clade member called MADS-AFFECTING FLOWERING 2 (MAF2), which functions redundantly with FLM,[63] is also subjected to AS in a temperature-dependent manner.[64,65] Three main transcripts are generated from the MAF2 gene locus, namely MAF2*var1*, *-var2*, and *-var5*[64,65] (Figure 3a). MAF2var1, acting as a repressor in flowering by interacting with SVP to regulate downstream gene expression, is induced by low temperature (16°C) (Figure 3b). When the temperature reaches 27°C, the *MAF2var2* isoform predominates (Figure 3b). MAF2var2 lacks part of the K-domain and all of the C-domain by introducing the third intron, which disrupts the interaction with SVP. Further genetic analysis indicates overexpression of *var2* flowers at the same time with wild type.[64] Therefore, at low temperatures MAF2 represses flowering in parallel with FLM by interacting with SVP, providing an additional input for the control of the ambient temperature-mediated flowering. Although ectopic expression of the third isoform *MAF2var5* leads to early flowering, the endogenous *MAF2var5* exhibits a very low expression level at all tested temperatures *in vivo*, suggesting a weak contribution to the flowering time in Col.[65] However, the biological significance of the rare variants of *FLM* and *MAF2* is required to be determined in specific accessions.

Apart from the central flowering repressors mentioned above, CONSTANS (CO), the flowering activator in the photoperiod pathway,[66] also undergoes alternative splicing. CO produces two protein variants: the full-length COα that is equivalent to the well-known CO protein for flowering induction,[67] and the truncated COβ lacking the DNA-binding domain (Figure 3a). COβ interacts with COα to attenuate its function by competitively compromising the formation of COα-HAP5a dimers,[68] thereby affecting the DNA-binding activity on *FT* promoter. Furthermore, COβ, resistant to E3-mediated protein degradation, induces the interactions between CO-α and CO-destabilizing E3 ligase but inhibits the association between CO-α and CO-stabilizing E3 ligase, ensuring its diurnal accumulation dynamics during photoperiodic flowering.[69] In spite of the identifications of these AS events involved in flowering time control, the regulators of these AS events are poorly understood, and not all splicing factors regulate flowering by splicing the same target (Figure 3c). Therefore, the characterizations of splicing regulators or factors will provide a new layer to establish the flowering regulation network.

3.5 Alternative splicing in floral organs regulation

SR proteins are crucial regulators in constitutive and alternative splicing and other aspects of mRNA metabolism.[70] More interestingly, most *SRs* are subjected to alternative splicing, although the biological functions are largely unknown.[71] *SR45*, encoding a unique plant SR protein, was identified to yield the two transcripts of *SR45.1* and *SR45.2*, which have distinct roles during normal plant development and salt stress.[72,73] Compared with SR45.1, SR45.2 replaces eight amino acids (TSPQRKTG) with a single arginine by the alternative usage of 21 nucleotides in 3′ splice site. Loss-of-function mutant *sr45–1*, which affects both isoforms, shows several developmental defects, including defects in petal development, root growth, and salt tolerance. Genetic analysis revealed that SR45.1-GFP complements the flower petal

and salt-sensitive phenotypes, but not the root growth.[72,73] Conversely, SR45.2-GFP only rescues root growth, and not others.[73] Interestingly, both isoforms are able to rescue the hypersensitivity to glucose and ABA.[74] Collectively, the conclusion can be drawn that both isoforms are functional, albeit with diverse roles.

Auxin response factors (ARFs) mediate multiple cellular responses to auxin by activating or repressing downstream developmental genes.[75] Different splice variants have been identified for several ARFs. In the case of ARF8, the full-length ARF8.1 contains 14 exons and ARF8.2 is alternatively spliced, leading to a premature stop codon four nucleotides 'GTAA' downstream of the 3′ end of exon 13. ARF8.3, which lacks exon1 and 34 nucleotides of exon 2, was annotated in Araport11 without function annotation.[76] Recently, a third splice variant ARF8.4, the eighth in-frame intron retention version compared with ARF8.2, was identified to be specifically expressed during late stages of stamen development. Genetic and molecular analysis revealed that ARF8.4 controls filament elongation and endothecium lignification by directly regulating the expression of AUX/IAA19 and MYB26.[77] In contrast, ARF8.1 and ARF8.2 have no or a minor effect on stamen elongation. In addition, ARF8.2 and ARF8.4 collaboratively regulate anther dehiscence, while ARF8.1 is not involved.[77]

3.6 Alternative splicing in gametogenesis

ARF5, also known as MP, is an important transcription factor in auxin signaling and activate downstream gene expression with elevated auxin levels to regulate diverse developmental processes, including embryo development, primary and lateral root formation, vasculature patterning, shoot apical meristem maintenance and floral and ovule initiation. Most recently, MP is identified to be expressed in ovule primordia with auxin minima to activate the expression of direct target genes by AS.[78] MP is subject to AS with the 11th intron retention mainly in inflorescences, which is designated as *MP11ir* and only accounts for approximately 6% of total transcripts. *MP11ir* encodes MP isoforms lacking C-terminal PB1 domain, which is required for the interaction between ARFs and AUX/IAA proteins,[79] thereby functioning independently of auxin levels and AUX/IAA. Complementation analysis indicated that MP11ir is not essential for the complementation of lateral branching, flower and pistil morphology. In terms of ovule development, both MP and MP11ir partially restores *mp* mutants, but MP11ir is more efficient,[78] suggesting MP11ir is tissue- and developmental-specific in ovule and both MP isoforms synergistically act in Arabidopsis development.

Besides the roles for AS of MP in ovule, AS of *CYCLIN-DEPENDENT KINASE G1*(*CDKG1*) is implicated in pollen development in response to ambient temperature.[80] Two AS events resulted from *CDKG1* via the selective intron mainly encodes two protein isoforms CDKG1L (long) and CDKG1S (short). The long isoform exclusively localized in the nuclear, but the short isoform lacking two out of four SR domains and NLS localized in both the nuclear and cytoplasm. At low temperature, mostly the long isoform is produced, while at high temperature, both long and short sioforms are produced. Therefore, the ratio between the long and short isoforms is regulated by temperature.[81] Moreover, inactivation of CDKG1 results in male sterility by meiosis and pollen wall defects at high temperature, but is fertile at low temperature,[82,83] suggesting the long isoform is required for pollen development at high temperature. In agreement, the long isoform could restore the *cdkg1* mutant defect in terms of the male sterility at high temperature, while the short isoform could not.[80] The above mentioned results indicate CDKG1L and CDKG1S is regulated by temperature to antagonistically control pollen development. However, on the other hand, CDKG1L and CDKG1S synergistically mediate the correct splicing of *U2AF65A* in response to fluctuating ambient temperatures.[81]

3.7 Alternative splicing in phytohormone signaling

As sessile organisms, plants have evolved sophisticated mechanisms including AS to fight against unfavorable stimuli. The ABSCISIC ACID (ABA) signal take vital actions against stress, in which many advances has been achieved in the PYL-PP2C-SnRK2 regulatory module.[84] In the absence of ABA, the PP2Cs interact with SnRK2s to inhibit its kinase activity by dephosphorylation, resulting in ABA signaling off. ABA binds to its receptor PYLs to promote the interactions with PP2Cs and inhibit their phosphotase activity, thereby releasing SnRK2s from the PP2C-SnRK2 complex.[85,86] The released SnRK2s phosphotase the downstream effectors and activate ABA signaling.[85] HYPERSENSITIVE TO ABA 1 (HAB1), a component of Group A PP2C, undergoes AS and yields two major isoforms, namely HAB1.1 and HAB1.2.[87,88] HAB1.2 differs from HAB1.1 in terms of retaining the third intron to yield a truncated protein by a premature stop codon which abolishes the phosphatase activity. However, the truncated protein HAB1.2 still physically interacts with, but fails to dephosphorylate SnRK2.6.[87] In the presence of ABA, HAB1.2 displays increased expression level and the ratio of HAB1.1 to HAB1.2 is decreased, thereby enhancing ABA signaling. Therefore, HAB1.2 isoform antagonistically functions with HAB1.1 as a dominant-negative regulator in ABA signaling.[87] RNA BINDING PROTEIN 25 (RBM25), an evolutionarily conserved splicing factor, has been identified to mediate the AS of HAB1.[88] The inactivation of RBM25 increases the ratio of HAB1.2 to HAB1.1, especially in the presence of ABA. Consistently, the *rbm25* mutant exhibits substantially increased sensitivity to increasing concentrations of ABA in terms of seed germination and cotyledon greening. Furthermore, solid evidences have indicated that overexpression of *HAB1.1* completely complements ABA-sensitive phenotypes of the *rbm25* mutant in genetic analysis and RBM25 is capable of binding HAB1 pre-mRNA at a molecular level, suggesting that RBM25 directly regulates the AS of HAB1 to fine-tune ABA signaling.[87,88]

JASMONATE ZIM-domain (JAZ) transcription factors act as repressors in JASMONATE (JA) signaling, which is induced by abiotic and biotic stresses.[89] JAZ proteins contain two highly conserved sequence motifs, including that the Jas domain and the ZIM domain. The Jas domain for interacting with E3 to destabilize the repressors and the ZIM/TIFY domain for JAZs interactions are key features of JAZ proteins.[90] JAZ10 was experimentally identified to produce three alternative

splicing variants, namely JAZ10.1, JAZ10.3, and JAZ10.4. JA10.3 weakly interacts with the E3 ligase COI1 due to lacking seven amino acids from the C-terminal end of the Jas domain,[91] while JAZ10.4 abolishes the entire Jas domain, thereby being resistant to degradation.[90] Overexpression of JAZ10.3 confers partial insensitivity to JA,[91] and the ectopic expression of JAZ10.4 shows more severe JA-insensitive phenotypes,[90] which is consistent with the biochemical results. The Jas intron-dependent AS of *JAZ* transcription factors was identified to be a general mechanism to desensitize or deactivate JA responses, including JAZ5, JAZ6 and JAZ9. Moreover, recent research suggested that MED25 recruits splicing factors PRP39a and PRP40a to prevent JAZ AS-mediated excessive desensitization of JA signaling.[92]

Another case is BRI-EMS-SUPPRESSOR1 (BES1), which is the downstream transcription factor in BR signaling. *BES1* regulates the expression of thousands of target genes in response to BRs in the nucleus.[93] BES1 shows two variants: *BES1-S* and *BES1-L*. Compared with the canonical and widely used short *BES1-S, BES1-L*, with two extra exons and one intron instead of *BES1-S* 5′UTR, encodes an N-terminal bipartite nuclear localization signal domain, which enables *BES1-L* to exclusively localize in the nucleus.[94] In contrast, *BES1-S* localizes in both the nucleus and cytoplasm, and nuclear localization is accumulated by BR treatment. Interestingly, the nuclear-localized *BES1-L* promotes the nuclear localization of *BES1-S* and BZR1 via dimerization. Further phenotypic analysis of the overexpression of *BES1* variants indicated that *BES1-L* is more important in BR signaling in Arabidopsis.[94] Intriguingly, *BES1-L* is a recently evolved isoform because the *BES1-L-like* transcript is present in the closer relative *A. lyrata*, but absent in distant relatives *Capsella rubella* and *Thellungiella salsuginea*,[94] suggesting a special and essential role for BES1-L in Arabidopsis.

3.8 Alternative splicing in abiotic stress response

AS also affects protein subcellular localization. ZINC-INDUCED FACILITATOR-LIKE 1 (ZIFL1) is a paralog of major facilitator superfamily (MFS) transporter ZIF1 that is localized in tonoplasts and required for zinc tolerance.[95] The *ZIFL1* gene generates three distinct transcripts, namely *ZIFL1.1-ZIFL1.3. ZIFL1.1* encodes the full-length protein containing 12 membrane-spanning regions, while *ZIFL1.2* uses an alternative start codon in-frame in the fourth exon, resulting in a protein lacking two first N-terminal membrane-spanning regions. The third transcript *ZIFL1.3* causes frame shift by an alternative 3′ splice site selection in the fourteenth intron, abolishing the two last N-terminal membrane-spanning regions by PTC.[96] Genetic and complementation analysis indicated that *ZIFL1.1* is specifically required for cellular auxin efflux during shootward auxin transport at the root tip by regulating PINs abundance and *ZIFL1.3* exclusively confers drought tolerance by modulating stomatal closure, which can be explained by the divergent subcellular localization, with *ZIFL1.1* in the tonoplast membrane of root cells and *ZIFL1.3* in plasma membrane of stomatal guard cells.[96,97] Interestingly, no roles in auxin transport or drought tolerance for *ZIFL1.2* were observed, suggesting that the two first N-terminal membrane-spanning regions are essential.

HOT SHOCK TRANSCRIPTION FACTOR A2 (HSFA2) is a key regulator in both basal and aquired themotolerance.[98,99] The HSFA2 locus consists of two exons and one intron. The alternative splicing of *HSFA2* is temperature-dependent.[100] At 22°C, *HSFA2* generates the full-length transcript. At 37°C, another nonfunctional variant named *HSFA2-II*, which contains a premature termination codon by retaining a 31 bp cryptic miniexon from the intron, is generated and subsequently degraded by nonsense-mediated pathway. When the temperature reaches 42°C, HSFA2 will generate a third transcript *HSFA2-III*, which retains the upstream region of the intron splited by the miniexon to encodes the short HsfA2 protein (referred to as S-HsfA2) with 129 amino acids in length. As the temperature increases to 45°C, *HSFA2-II* disappears, but *HSFA2-III* accumulates. S-HSFA2, which is derived from *HSFA2-III*, directly binds to its own promoter to activate the fully spliced *HASFA2* expression to confer thermotolerance.[100] Notably, not only *HSFA2* but also other heat shock transcription factors, including *HSFA7b, HSFB1, HSFB2a* and *HSFA4c*, undergo AS at 42°C,[100] indicating that AS for *HSFs* is a general mechanism underlying heat stress. Interestingly, loss of function of *SF1* promotes the production of productive *HSFA2*, leading to increased resistance to heat stress.[61]

ZINC-INDUCED FACILITATOR 2 (ZIF2) encodes a functional transporter that is Zn-induced. 5′UTR of *ZIF2* generates two splice variants, namely *ZIF2.1* and *ZIF2.2*, which encode the same protein. Compared with *ZIF2.1, ZIF2.2* is 139 nucleotides longer in length due to retaining the first intron in the 5′UTR to favor translation,[101] which is a conserved but poorly understood mechanism. The regulation of translation efficiency driven by *ZIF2.2* 5′UTR largely depends on a predicted stable stem loop upstream of the start codon which is excised in the *ZIF2.1*. Therefore, *ZIF2.2* confers greater Zn tolerance under excess Zn than *ZIF2.1* in a Zn-responsive manner by efficiently enhancing protein translation.[101]

4 Prospects

In the last decades, many AS events that are developmentally regulated or stress induced and their corresponding biological functions with different underlying mechanisms have been well studied in the model plant Arabidopsis (Figure 4). However, AS events functioning in fertilization and embryogenesis have not been uncovered yet. It will be of great interest to intensively explore novel AS events that play vital roles during these processes, thereby establishing elaborate AS-regulatory mechanisms during Arabidopsis life cycle from seed germination to embryogenesis.

Additionally, extensive studies have uncovered numerous alternative splicing events, especially in unfavorable conditions via developed sequencing technology in crops in recent years. However, compared with those from Arabidopsis, only several genes in crops have been characterized. Whether AS event regulation in polyploidy crops, such as wheat and oilseed rape, shares the same mechanism

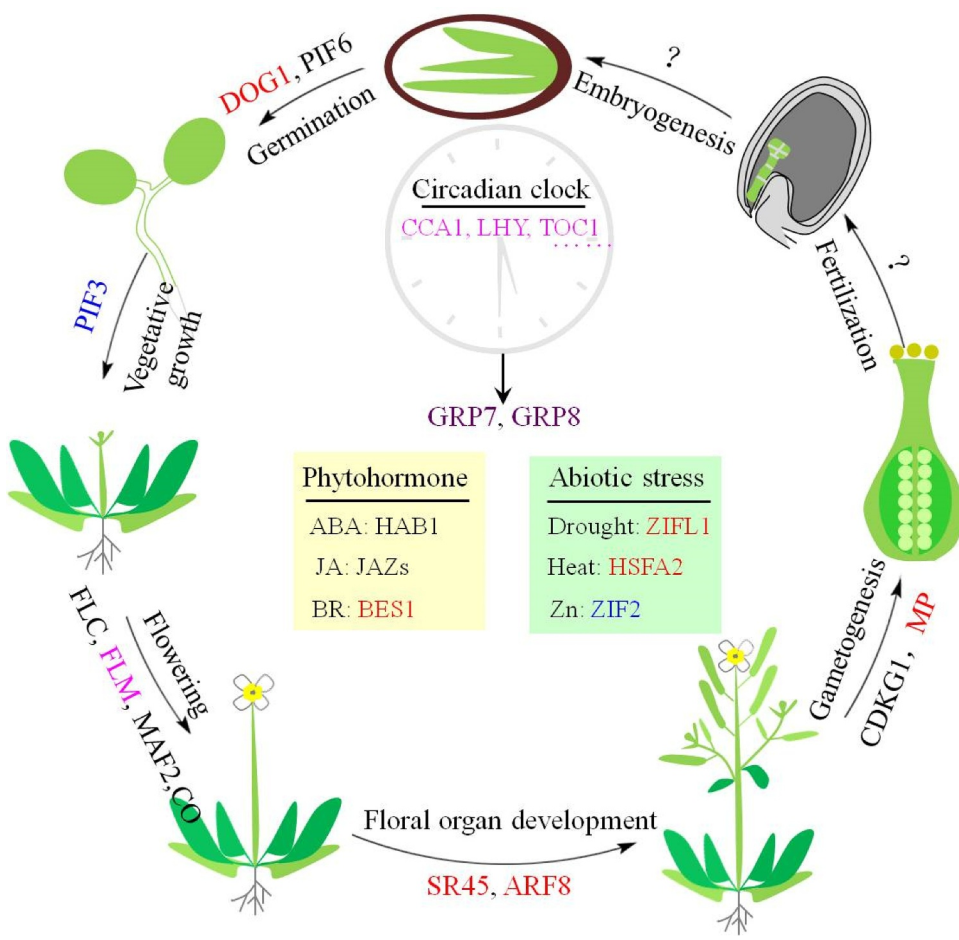

Figure 4. Development-regulated and stress-induced alternative splicing events in Arabidopsis. Alternative splicing is a general mechanism in which gene expression is subtly regulated. During Arabidopsis life cycle, the well-studied alternatively spliced genes that regulate plant development and confer stress tolerance are shown at the corresponding positions. The question mark represents the unknown alternative splicing events. The genes that generate alternative splicing variants playing potentially antagonistic or synergistic roles, altered translation efficiency, and linked to NMD are represented black, red, blue and purple, respectively. The genes in magenta indicate the alternative splicing variants are proceeded by combined functional modes.

as that in Arabidopsis remains unclear. The characterization and functional analysis of the AS events for specific tissues, time or conditions will help us to better understand the mechanisms of plant development and adaptation to the fluctuating environments, thereby promoting application for genetic improvements in crops.

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