Roles of Alternative Splicing for the Circadian Clock Control in *Arabidopsis*

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Abstract: Alternative splicing plays an important role in regulating gene functions and enhancing the diversity of the proteome in plants. Most of the genes are interrupted by introns in *Arabidopsis*. About one half of the intron-split genes involved in multiple biological processes including the circadian clock are alternatively spliced. In this review, we focus on the involvement of alternative splicing in the circadian clock regulation.

Keywords: Alternative splicing, Arabidopsis, pre-mRNA splicing, regulation of the circadian clock, spliceosome, the circadian clock.

INTRODUCTION

The circadian clock, an internal timing system generating rhythms with a period about 24 hours, functions as a biochemical oscillator composed of multiple interlocked regulatory feedback loops in *Arabidopsis*, including the central, morning and evening loops [1]. The first identified oscillator is the central loop, in which two morning-expressed Myb transcription factors, CIRCADIANCLOCK-ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), repress the expression of evening-phased gene, *TIMING OF CAB EXPRESSION1* (*TOC1*) through binding to the Evening Elements in its promoter region [2, 3]. Recent discoveries reveal that the expression of TOC1 in the evening suppresses the accumulation of *CCA1*/*LHY* through associating directly with the TOC1 morning element (T1ME) located in their promoters [4, 5]. TOC1, a member of PSEUDO-RESPONSE REGULATOR (PRR) protein family with a PSEUDO-RECEIVER (PR) and a CONSTANS (CO), CO-like, TOC1 (CCT) domain, functions as a general transcriptional repressor and possesses DNA-binding activity [5]. The DNA binding and the transcriptional repression activity of TOC1 are mediated through the CCT domain at the C terminus and PR domain at the N terminus respectively (Fig. **1**) [5-7]. In the morning loop, CCA1/LHY enhances the mRNA abundance of *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*) and *PSEUDO-RESPONSE REGULATOR 9* (*PRR9*). Conversely, PRR7 and PRR9 repress the accumulation of *CCA1*/*LHY* through directly binding [1, 8, 9]. The evening loop consists of GIGANTEA (GI), TOC1, EARLY FLOWERING 3 and 4 (ELF3 and ELF4) and LUX ARRHYTHMO (LUX) [10, 11]. The evening complex (EC) formed by the combination of ELF3, ELF4 and LUX suppresses the expression of *LUX*, *ELF4*, *GI*, *TOC1* and *PRR9* [12-14]. Taking together, the interlocked central, "morning" and "evening" loops build up the fundamental structure of the circadian clock in *Arabidopsis*.

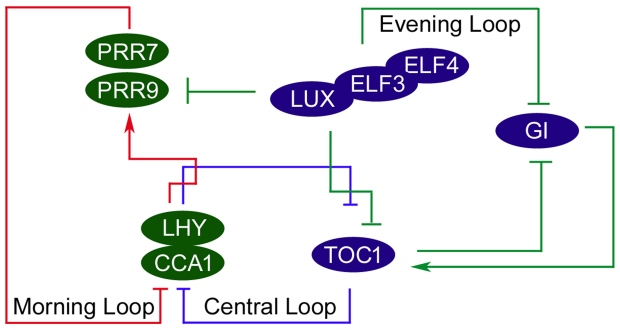

Fig. (1). Architecture of the circadian clock in *Arabidopsis*. In the central loop, CCA1 and LHY repress the expression of *TOC1*; in turn, TOC1 down-regulates the expression of *CCA1* and *LHY*. In the morning loop, CCA1 and LHY accelerate the expression of *PRR7* and *PRR9*; on the contrary, PRR7 and PRR9 repress the mRNA abundance of *CCA1* and *LHY*. In the evening loop, TOC1 represses the expression of *GI*; GI up-regulates the expression of *TOC1*. ELF3, ELF4 and LUX form the evening complex to suppress the expression of *TOC1*, *GI*, *PRR9*, *et al.*

Posttranscriptional regulation, including 5' capping, splicing and 3' polyadenylation, is becoming an important principle in fine-tuning the clock-related gene expression in *Arabidopsis* [15-18]. In this review, we briefly summarize some aspects of splicing mechanisms before turning to our main topic of the roles for alternative splicing in regulating the circadian clock.

Pre-mRNA SPLICING MACHINERY, SPLICEOSOME

The mRNA is synthesized as a precursor mRNA (pre-mRNA) during transcription in the nucleus [19]. There, it undergoes a series of processing steps before being

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transported to the cytoplasm where it serves as a template for protein biosynthesis and is eventually degraded [19]. One of the processing steps is the exclusion of introns from the intron-containing pre-mRNAs, which is termed pre-mRNA splicing. In eukaryotes, pre-mRNA splicing is one of the fundamental processes in constitutive and regulated gene expression as most of the genes typically contain multiple introns [15].

The removal of introns from the pre-mRNA is involved in sequential phosphodiester transfer reactions which are catalyzed by the spliceosome, a large ribonucleoprotein (RNP) complex [20]. Spliceosomes is one of the most complex machines in the cell [21-23], consisting of five Uridine-rich (U-rich) small nuclear RNAs (snRNAs U1, U2, U4, U5, and U6), five small nuclear RNPs (snRNPs), and a multitude of non-snRNP splicing factors, such as serine/arginine-rich (SR) proteins [24-26]. Spliceosome assembly anew at each intron guided by consensus sequences located in the pre-mRNA is a highly ordered and dynamic reaction [19].

During splicing, exon and intron sequences have to be effectively recognized and appropriate 5'- and 3'-splice sites (5'-SS and 3'-SS) have to be selected prior to the catalytic step [20]. Three conserved *cis*-acting elements in introns of the pre-mRNAs include the 5'-splice site (5'-SS) with a conserved GU dinucleotide, the 3'-splice site (3'-SS) with a conserved AG dinucleotide, and the branch point sequence (BPS) with a conserved UACUAAC sequence in yeast, but little conserved BPS in other higher eukaryotes located about 18-40 nucleotides upstream of the 3'-SS [20]. These elements are recognized by the splicing complexes and participate in regulating the splicing reactions.

The assembly of spliceosome is highly dynamic by forming several intermediate complexes, referred to E, A, B, and B* [24]. The U1 snRNP interacts with the conserved 5'-SS forming the E complex or early pre-splicing complex. Subsequently, the U2 snRNP interacts with the pre-mRNA's BPS stably, leading to the formation of the A complex or pre-spliceosome dependent on the hydrolysis of ATP. Finally, the preformed U4/U6.U5 tri-snRNP particle joins the A complex and forms the spliceosomal B complex, which contains a full set of U snRNAs in a pre-catalytic state. After a series of conformational and compositional changes, including the release of the U1 and U4 snRNPs, the catalytic activities of the spliceosomal B complex are activated and give rise to the formation of the B* complex, so-called activated spliceosome to perform the sequential phosphodiester transfer.

Splicing is catalyzed by a two-step mechanism [24, 27]. During the first step the 5'-SS is cleaved, and the 5'-end of the intron is covalently linked to the BPS forming a lariat structure. During the second step of splicing, the 3'-SS is cleaved, releasing the intron, and the 5'- and 3'- of the exons are ligated to form the mRNA [24]. Upon disassembly of the spliceosome, both the pre-mRNA splicing products and the components of the spliceosome are ultimately released, and the individual subunits of the spliceosome take part in subsequent rounds of splicing.

The composition of the spliceosomes might be similar to the animal spliceosome because many components of the spliceosomes in animal are present in plants, indicating the basal mechanisms in plants is similar to other organisms [28, 29]. The 5'- and 3'-SS in all introns of *Arabidopsis* and rice analyzed are very similar to humans, but the noncanonical splice sites occur in only 0.7% of all splice sites, slightly lower than that in animals [30]. Furthermore, the branch point sequence (CURAY) is not obvious in plants because of the variation in the position of the branch point in different introns, suggesting that the mechanisms involved in splice site recognition likely differ in these organisms [30].

SIGNIFICANCE OF ALTERNATIVE SPLICING IN PLANTS

Pre-mRNAs with multiple introns often undergo alternative splicing (AS) to generate multiple splicing isoforms containing different combinations of exons from the same gene [20, 31]. Alternative splicing can affect the stability and translatability at the RNA level and produce truncated or extended proteins with altered activity, cellular localization, regulation, and/or stability [20]. Multiple transcripts from a single gene can be produced by exon skipping, retention of introns and/or selection of an alternative 5'- or 3'-SS [30].

Alternative splicing is also an important mechanism for regulating gene function and enhancing the coding potential of a genome in plants [28, 30]. Alternative splicing can be spatially and developmentally regulated and is frequently associated with environmental stress [18, 32, 33]. Alternative processing of the *N* gene in tobacco and *RPS4* gene in *Arabidopsis*, which confer resistance to TMV and *Pseudomonas syringae* pv tomato strain DC3000, is required for the normal function of these genes [34, 35]. The alternative splicing of *FLOWERING TIME CONTROL LOCUS* A (*FCA*) results in four transcripts (α, β, γ and δ), which is important in the autoregulation of its own expression and the control of the floral transition [36-38]. Impairing the function of SERINE/ARGININE-Rich 45 (SR45), the plant-specific protein, results in a splicing defect, later floral transition and aberrant leaf morphology phenotypes in *Arabidopsis* [39, 40]. Abiotic stresses, such as heat and cold, strongly alter the alternative splicing of the most of SR genes. Altered ratios of splice variants in response to stresses may have a role in the adaptation of plants to these stresses [32].

ALTERNATIVE SPLICING IN THE CIRCADIAN CLOCK

Roles of alternative splicing in regulating the clock gene expression have been discovered recently. More data in *Arabidopsis* reveal the significance of alternative splicing in the control of the clock [41].

Clock regulated *PROTEIN ARGININE METHYL TRANSFERASE 5* (*AtPRMT5*) gene encodes a type II protein arginine methyltransferase that catalyzes the methylation of diverse substrates [42]. Mutations in *Atprmt5* reduce the methylation of components of the spliceosome, such as AtSmD1 and AtLSm4, causing the splicing defects in genes involved in multiple biological processes [42, 43]. The circadian period are lengthened by the *atprmt5* mutations [44]. Defects in the alternative splicing of *PRR7* and *PRR9* in *atprmt5-5* are responsible for the elongated period of the

clock, first linking the alternative splicing to the clock (Fig. **2**) [43].

Other two splicing factors such as Ski-interacting protein (SKIP) and SPLICEOSOMAL TIMEKEEPER LOCUS1 (STIPL1) are involved in the regulation of the circadian clock in *Arabidopsis* [45, 46]. Mutations in the two splicing factors have dramatic effects on the circadian clock. The period of the circadian clock is elongated by the *skip-1* and *stipl1* mutations. The capacity for temperature compensation of the clock is also impaired by *skip-1*. Consistent with the role of SKIP in both mammals and yeast (Prp45), AtSKIP encodes a conserved SNW domain-containing protein and acts as a component of the spliceosome through associating with SR45 [45, 47, 48]. The alternative splicing defects in *PRR7* and *PRR9* partially contribute to the lengthened period of the clock in the *skip-1* mutant (Fig. **2**) [45]. STIPL1 is the another splicing factor associated with the regulation of the circadian clock, which encodes a homolog of TUFTELIN-INTERACTING PROTEIN11 (TFIP11) in humans and Ntr1p in yeast involved in spliceosome disassembly [46, 49]. The altered expression of *CCA1*, *LHY*, *PRR9*, *GI*, and *TOC1* caused by the aberrant splicing is the contributor to the circadian defects in *stipl1* mutant (Fig. **2**) [46]. These findings suggest that the splicing factors, including AtSKIP and STIPL1 are required for the correct splicing of the circadian clock-related genes and for the normal function of the circadian clock.

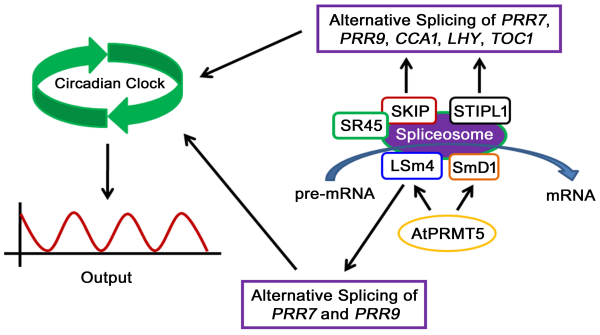

Fig. (2). Splicing Factors linking alternative splicing to the circadian clock in *Arabidospsis*. SKIP is component of the spliceosome through interacting with SR45. STIPL1is also a splicing factor. Both of SKIP and STIPL1 are involved in regulating the circadian clock through splicing the pre-mRNAs of *PRR7*, *PRR9*, *CCA1*, *LHY* and *TOC1* alternatively. AtPRMT5 is required for the normal function of the clock through regulating the expression of splicing factors, LSm4 and SmD1 and the alternative splicing of *PRR7* and *PRR9*. These findings add another layer of regulation, posttranscriptional regulation to the circadian clock.

After the detection of the two *CCA1* transcripts, *CCA1α* and *CCA1β*, their functions in regulating the circadian clock have been recently uncovered [50]. The abundance of the *CCA1β* alternative splicing isoform with retained 4th intron of *CCA1* increases under strong light intensity but decreases in the cold (Fig. **3**) [18]. The protein of CCA1β has a dimerization domain but lacks the DNA binding MYB motif [50, 51]. It has been known that the homo- and heterodimerization of CCA1α and LHY are required for their function in regulating circadian rhythms [52]. The CCA1β competes with CCA1α to form nonfunctional CCA1α/CCA1β and CCA1β/LHY complexes and to disrupt the functions of CCA1α and LHY in the clock, revealing the regulatory role of alternative splicing of CCA1 in the clock [50]. Thus, auto-regulation of the transcription factors by generating competitive inhibitors through alternative splicing may be a common mechanism in their expressions. Furthermore, the characterization of CCA1β gives an explanation on the involvement of central circadian oscillators in freezing tolerance. Under cold conditions, because the expression of *CCA1β* is decreased, CCA1α activity is released [18, 50, 53, 54]. The enhancement of CCA1α expression leads to the induction of cold tolerance-related gene expression , including C-repeat/dehydration-responsive element binding factors [50]. Therefore, the self-regulation of CCA1 through alternative splicing is crucial for plant to adapt to the cold conditions.

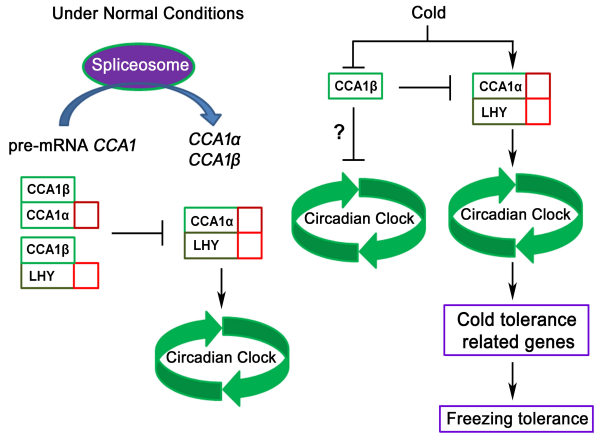

Fig. (3). Roles for the alternative splicing of *CCA1* in regulating the circadian clock and cold response in *Arabidopsis*. Under normal conditions, two alternative splicing isoforms, which are the full-spliced form, *CCA1α* and 4th intron retention form, *CCA1β*, are detected. *CCA1β* encodes partial protein of CCA1α containing the dimerization domain, but lacking the DNA binding domain. CCA1β competes with CCA1α interacting with LHY to self-regulate the function of CCA1 required for the normal function of the clock. Under cold conditions, the expression of *CCA1β* is decreased and the suppression of *CCA1α* is released inducing the expression of cold tolerance related genes causing freezing tolerance. The involvement of CCA1β in the circadian clock is obscure. Frame in green is the dimerization domain of CCA1α and CCA1β; Frame in dark red is the DNA binding domain of CCA1α; Frame in dark green is the dimerization domain of LHY; Frame in red is the DNA binding domain of LHY.

The circadian clock is an essential mechanism in plants to synchronize the endogenous biological and biochemical processes with the cues of the local day/night cycles. Though alternative splicing is essential for the normal function of the circadian clock, how alternative splicing regulates the circadian clock is far from clear. Not only *CCA1*, but *LHY*, *TOC1*, *PRR3*, *PRR5*, *PRR7*, *PRR9*, *ZTL*, *GI* and other circadian clock-related genes as well are subject to alternative splicing in *Arabidopsis* [43, 50, 55]. However, the molecular principles of them in regulating the circadian clock are obscure. The exploration on how the alternative splicing of *LHY*, *PRR7*, *PRR9*, *GI* and *TOC1* genes functions in regulating the circadian clock will shed light on the regulatory mechanisms of the circadian clock.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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