Regulation of the circadian clock through pre-mRNA splicing in Arabidopsis

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Received 21 October 2013; Revised 3 January 2014; Accepted 28 January 2014

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. More than 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 regulation of the circadian clock.

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

Introduction

The circadian clock, an internal timing system generating rhythms with a period of ~24 h, functions as a biochemical oscillator. It is composed of multiple interlocked regulatory feedback loops in Arabidopsis, including the central, morning, and evening loops (Harmer, 2009).

The first identified oscillator in Arabidopsis is the central loop, in which two morning-expressed Myb transcription factors, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), repress the expression of the evening-phased gene, TIMING OF CAB EXPRESSION 1 (TOC1), through binding to the evening elements in its promoter region (Alabadi et al., 2001; Green and Tobin, 2002). 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 (Gendron et al., 2012; Huang et al., 2012). TOC1, a member of the 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 (Gendron et al., 2012). The DNA binding and the transcriptional repression activities of TOC1 are mediated through the CCT domain at the C-terminus and the PR domain at the N-terminus, respectively (Fig. 1) (Tiwari et al., 2010; Gendron et al., 2012; Pokhilko et al., 2012). In the morning loop, CCA1/LHY enhances the mRNA abundance of PSEUDO-RESPONSE REGULATOR 7 (PRR7) and PRR9. Conversely,
Fig. 1. Architecture of the circadian clock in Arabidopsis. In the central loop, CCA1 and LHY repress the expression of TOC1 (Alabadi et al., 2001); in turn, TOC1 down-regulates the expression of CCA1 and LHY (Gendron et al., 2012; Huang et al., 2012). In the morning loop, CCA1 and LHY accelerate the expression of PRR7 and PRR9; in contrast, PRR7 and PRR9 repress the mRNA abundance of CCA1 and LHY (Farre et al., 2005; Nakamichi et al., 2010). In the evening loop, TOC1 represses the expression of GI; GI up-regulates the expression of TOC1 (Pruneda-Paz and Kay, 2010). ELF3, ELF4, and LUX form the evening complex (EC) and suppress the expression of TOC1, GI, and PRR9 (Helfer et al., 2011; Herrero et al., 2012). Purple, red, and green lines represent the central, morning, and evening loops, respectively. Arrows and T-bars indicate activation and suppression, respectively. Green and purple filled oval represent genes in the loops.

The assembly of the spliceosome is highly dynamic by forming several intermediate complexes, referred to as E, A, Manley, 2009; Di Giammartino et al., 2011; Ji et al., 2011). The third step involved in mRNA maturation is splicing, through which the introns are removed from the mRNA precursors and the adjacent exons are connected in the complex and dynamic spliceosome (Moore and Proudfoot, 2009; Bessonov et al., 2010). Many biological processes of plants are regulated by splicing (Reddy, 2007). Alternative splicing (AS) of pre-mRNA is becoming an important principle in regulating the clock-related gene expression in Arabidopsis (Cheng et al., 1998; Colot et al., 2005; Moore and Proudfoot, 2009; Filichkin et al., 2010). In this review, we briefly summarize some aspects of splicing mechanisms before turning to our main topic of the roles of AS in regulating the circadian clock.

Pre-mRNA splicing machinery: the spliceosome

mRNA is synthesized as a precursor mRNA (pre-mRNA) during transcription in the nucleus (Wahl et al., 2009). There, it undergoes a series of processing steps before being transported to the cytoplasm where it serves as a template for protein biosynthesis and is eventually degraded (Wahl et al., 2009). 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 genes typically contain multiple introns (Moore and Proudfoot, 2009).

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 (Wang and Burge, 2008). The spliceosome is one of the most complex machines in the cell (Zhou et al., 2002; Jurica and Moore, 2003; Nilsen, 2003), 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 (Deckert et al., 2006; Belhadjnia et al., 2007; Bessonov et al., 2008). The spliceosome assembly which begins anew at each intron guided by consensus sequences located in the pre-mRNA is a highly ordered and dynamic reaction (Wahl et al., 2009).

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 (Wang and Burge, 2008). Three conserved cis-acting elements in introns of the pre-mRNAs include the 5′-SS with a conserved GU dinucleotide, the 3′-SS with a conserved AG dinucleotide, and the branch point sequence (BPS) with a conserved UACUAA sequence in yeast, but little conserved BPS in other higher eukaryotes located about 18–40 nt upstream of the 3′-SS (Wang and Burge, 2008). These elements are recognized by the splicing complexes and participate in regulating the splicing reactions.

Many aspects of mRNA metabolism, including transcription termination by RNA polymerase II, mRNA stability, mRNA export to the cytoplasm, and the efficiency of translation are all dependent on 3′ processing (Richard and
B, and B* (Deckert et al., 2006; Will and Luhrmann, 2011). The U1 snRNP interacts with the conserved 5′-SS, forming the E complex or early pre-splicing complex. At this time, the U2 snRNP is loosely related to the pre-mRNA (Das et al., 2000; Deckert et al., 2006). Subsequently, the U2 snRNP stably interacts with the BPS of the pre-mRNA, leading to the formation of the A complex or pre-spliceosome dependent on the hydrolysis of ATP (Deckert et al., 2006). Finally, the pre-formed 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 (Deckert et al., 2006; Wahl et al., 2009). 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, the so-called activated spliceosome, to perform the sequential phosphodiester transfer (Smith et al., 2008; Wahl et al., 2009).

Splicing is catalysed by a two-step mechanism (Deckert et al., 2006; Smith et al., 2008). 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′ ends of the exons are ligated to form the mRNA (Deckert et al., 2006). 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 that of the animal spliceosome because many components of the spliceosomes in animal are present in plants, indicating that the basal mechanisms in plants is similar to that of other organisms (Lorkovic et al., 2000; Reddy, 2004). The 5′- and 3′-SS in all introns of Arabidopsis and rice analysed are very similar to those of humans, but the non-canonical splice sites occur in only 0.7% of all splice sites, slightly lower than the percentage found in animals (Reddy, 2007). 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 probably differ in these organisms (Reddy, 2007).

**Significance of alternative splicing in plants**

AS is a process to generate two or more different transcripts from the same gene (Johnson et al., 2003; Wang and Burge, 2008). At least 42–61% of intron-containing genes in Arabidopsis are alternatively spliced (Filichkin et al., 2010; Marquez et al., 2012). AS is an essential post-transcriptional regulatory mechanism that can regulate gene expression, and extend transcriptome plasticity and proteome diversity in eukaryotes (Stamm et al., 2005). AS can affect mRNA stability and translatability, and produce truncated or extended proteins with altered activity, cellular localization, regulation, and/or stability (Wang and Burge, 2008). 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 (Reddy, 2007). The prevalence of intron retention events in plants has been confirmed by several studies (Ner-Gaon et al., 2004; Wang and Brendel, 2006; Reddy, 2007; Filichkin et al., 2010).

The different types of AS events have distinguishable fates or functions. Events of AS from exon skipping or alternative 5′- or 3′-SS may lead to functional changes of the translation products by in-frame addition or deletion of a functional domain of the protein. These rearrangements can alter the binding properties, activity, stability, or subcellular localization of the protein encoded (Kim et al., 2007; Reddy, 2007; Barbazuk et al., 2008).

Intron retention events often produce mRNAs with premature termination codons (PTCs) (Maquat, 2004; Reddy, 2007). The PTCs in the transcripts may result either in mRNA degradation through a nonsense-mediated mRNA decay (NMD) mechanism or in the translation of truncated proteins (Barbazuk et al., 2008; Kalyna et al., 2012; Mastrangelo et al., 2012). AS coupled to NMD represents a mechanism to regulate the abundance of the functional transcripts, which may be considered as an mRNA surveillance mechanism to prevent accumulation of truncated and potentially harmful proteins (Lewis et al., 2003; Maquat, 2004). Furthermore, mRNAs with PTCs can be translated into truncated proteins, lacking some active domains that are present in the full-length protein. These truncated proteins can contribute to the control of the amount of functional protein that is produced, which has been demonstrated for many development- and disease resistance-related genes in plants (Dinesh-Kumar and Baker, 2000; Zhang and Gassmann, 2003; Seo et al., 2012). Genome-wide mapping of AS in Arabidopsis recently revealed that alternatively spliced isoforms with PTCs comprised ~45–78% of alternatively spliced transcripts, indicating that AS coupled to NMD is the important mechanism to regulate gene expression in the cell and may serve as an essential regulatory mechanism in plants even though the roles of NMD in plants are far from known (Wang and Brendel, 2006; Filichkin et al., 2010; Kalyna et al., 2012).

It is clear that not all of the thousands of AS events are biologically functional (Wang and Brendel, 2006). The aberrant splicing inevitably happens during the dynamic splicing processes, most of which are removed by the NMD mechanism. Some splicing variants are fixed as functional AS events to regulate gene expression or define the functions of certain proteins in plants, though the functions of only limited AS events have been uncovered in plants (Wang and Brendel, 2006).

AS is an important mechanism for regulating gene function and enhancing the coding potential of a genome in plants (Lorkovic et al., 2000; Reddy, 2007). It plays important roles in regulating plant development and tolerance to biotic and abiotic stresses. In contrast, AS is also regulated by developmental and environmental stresses (Brett et al., 2002; Palusa et al., 2007; Filichkin et al., 2010).
Alternatively spliced transcripts that include the plant resistance (R) genes, pathogenesis-related (PR) genes, and defence-related genes, are induced in response to pathogen attacks (Dubrovina et al., 2013). Alternative processing of the N gene in tobacco, which confer resistance to Tobacco mosaic virus (TMV), is required for the normal function of the N gene (Dinesh-Kumar and Baker, 2000). The disease resistance gene in Arabidopsis, RPS4, produces multiple transcripts via AS. The ratios of different alternatively spliced isoforms of RPS4 are functional in response to pathogen attack (Zhang and Gassmann, 2003, 2007).

AS of FLOWERING TIME CONTROL LOCUS A (FCA) results in four transcripts (α, β, γ, and δ), which are important in the autoregulation of its own expression and the control of the floral transition (Macknight et al., 1997; Quesada et al., 2003; Simpson et al., 2003; Manzano et al., 2009). Among the four transcripts of FCA, the γ transcript encodes the only protein that functions in the control of flowering time (Macknight et al., 2002).

SR proteins are the components of the spliceosome which modulate the AS pathway either in a tissue-specific or a development-dependent manner. There are 19 SR genes in the Arabidopsis genome, 15 of which undergo AS and produce two or more transcripts (Palusa et al., 2007). SR1 is a plant homologue of the human general/alternative splicing factor SF2/ASF (Lazar et al., 1995). One of the five SR1 transcripts detected encodes the full-length protein, while the other four are different variants of the essential arginine-serine-rich domain (Lazar and Goodman, 2000). Overexpression of SR1 can affect AS of several genes (Caceres et al., 1994; Iida et al., 2004). Impairing the function of SR45, a plant-specific protein, results in a splicing defect, later floral transition, and aberrant leaf morphology phenotypes in Arabidopsis (Tanabe et al., 2009; Zhang and Mount, 2009).

AS of most SR genes is strongly altered by abiotic stresses. Alternatively spliced forms of SR1 are regulated by high temperature (Lazar and Goodman, 2000). Splicing variants of SR1 with a long exon 11 are induced under cold stress (Iida et al., 2004). Transcripts of other SR genes or splicing factors, including SR30, SR34/SR1, RS31, RS40, etc., show variations under stress conditions (Iida et al., 2004; Palusa et al., 2007). The AS patterns of SR genes differ among tissues or organs including SR34b, RS31a, RS40, RSZ33, and SC35, and among developmental stages, including SR30, RS31, and SR33 (Palusa et al., 2007).

Large-scale genomic data show that profiles of AS in Arabidopsis are significantly affected by environmental stresses (Iida et al., 2004; Palusa et al., 2007; Filichkin et al., 2010). The splicing variation of 13 out of 33 genes annotated as splicing factors is detected (Iida et al., 2004). The changes of AS and transcriptional regulation of splicing factors contribute to the alteration of AS profiles under cold stress (Iida et al., 2004). It is also found that the relative abundance of unproductive isoforms with PTCs of some essential regulatory genes can be regulated by the NMD surveillance machinery under abiotic stress (Sunkar et al., 2007). Altered ratios of splice variants in response to stresses may have a role in the adaptation of plants to these stresses (Palusa et al., 2007).

Regulatory roles of alternative splicing in the circadian clock

Roles of AS in regulating the clock gene expression have been discovered recently. More data in Arabidopsis reveal the significance of AS in the control of the clock (Wang and Ma, 2013).

The clock-regulated PROTEIN ARGinine METHYLTRANSFERASE 5 (AtPRMT5) gene encodes a type II protein arginine methyltransferase that catalyzes the methylation of diverse substrates (Deng et al., 2010). 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 (Deng et al., 2010; Sanchez et al., 2010). The circadian period is lengthened by atprmt5 mutations (Hong et al., 2010). Defects in the alternative splicing of PRR7 and PRR9 in atprmt5-5 are responsible for the elongated period of the clock, first linking AS to the clock (Fig. 2) (Sanchez et al., 2010).

Two other splicing factors, Ski-interacting protein (SKIP) and SPLICEOSOMAL TIMEKEEPER LOCUS 1 (STIPL1), are involved in the regulation of the circadian clock in Arabidopsis (Fig. 2; Jones et al., 2012; Wang et al., 2012). 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 (Fig. 2; Gahura et al., 2009;}

![Fig. 2](image-url)
The alternative splicing defects in PRR7 and PRR9 partially contribute to the lengthened period of the clock in the skip-1 mutant (Fig. 2) (Wang et al., 2012). STIPL1 is another splicing factor associated with the regulation of the circadian clock, which encodes a homologue of TUFTELIN-INTERACTING PROTEIN 11 (TFIP11) in humans and Ntr1p in yeast involved in spliceosome disassembly (Tamnukit et al., 2009; Jones et al., 2012). The altered expression of CCA1, LHY, PRR9, GI, and TOC1 caused by the aberrant splicing is the contributor to the circadian defects in the stipl1 mutant (Fig. 2) (Jones et al., 2012). 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).

After the detection of the two CCA1 transcripts, CCA1α and CCA1β, their functions in regulating the circadian clock have recently been uncovered (Seo et al., 2012). The abundance of the CCA1β AS isoform with the retained fourth intron of CCA1 increases under strong light intensity but decreases in the cold (Fig. 3) (Filichkin et al., 2010). The CCA1β protein has a dimerization domain but lacks the DNA-binding MYB motif (Daniel et al., 2004; Seo et al., 2012). It is known that the homo- and heterodimerization of CCA1α and LHY are required for their function in regulating circadian rhythms (Lu et al., 2009). CCA1β competes with CCA1α to form non-functional CCA1α-CCA1β and CCA1β-LHY complexes and to disrupt the functions of CCA1α and LHY in the clock, revealing the regulatory role of AS of CCA1 in the clock (Seo et al., 2012). Thus, autoregulation of the transcription factors by generating competitive inhibitors through alternative splicing may be a common mechanism in their expression. Furthermore, the characterization of CCA1β provides an explanation of the involvement of central circadian oscillators in freezing tolerance. Under cold conditions, because the expression of CCA1β is decreased, CCA1α activity is released (Espinoza et al., 2010; Filichkin et al., 2010; Dong et al., 2011; Seo et al., 2012). The enhancement of CCA1α expression leads to the induction of cold tolerance-related gene expression, including C-repeat/dehydration-responsive element-binding factors (Fig. 3; Seo et al., 2012). Therefore, the self-regulation of CCA1 through AS is crucial for plants to adapt to the cold conditions.

AS not only regulates the functions of the circadian clock or the clock-related genes, but also is under the control of the circadian clock (Staiger and Green, 2011). About 25–33% of the protein-coding genes are regulated by the circadian clock at the whole-genome scale of Arabidopsis (Covington et al., 2008; Hazen et al., 2009). About 499 transcripts with rhythmic introns are observed by genome-wide tiling arrays (Hazen et al., 2009). Among them, 213 of the rhythmic introns in genes oscillate in phase with adjacent exons, which may produce transcripts encoding a truncated protein. About 290 genes without evidence of rhythmic expression of exons contain an intron exhibiting a circadian rhythmicity. The expression of this type of AS product, termed 'gated intron inclusion' transcripts, is controlled by the oscillating introns, which determine that a protein will exert its function at a specific time of day (Hazen et al., 2009). Several other genes also show >4 h phase differences between oscillating introns and exons of the same transcription unit. Some genes have two splice variants, with those regulated by a cycling intron (Hazen et al., 2009). Two transcripts of PRR9 are revealed to have a different phase. The ratio of the abundance of the two PRR9 AS isoforms is altered by atprmt5 mutation. The changes of PRR9 AS isoforms contribute to the circadian defects in the atprmt5 mutant (Sanchez et al., 2010). Taken together, the circadian variations of AS are prevalent, which is important in governing the level, the form, or the time of day-dependent appearance of a specific form of proteins to perform their functions in Arabidopsis (Hazen et al., 2009; Staiger and Green, 2011).

In addition to AS, microRNA (miRNA)-mediated gene silencing is also emerging as a regulatory mechanism to control the function of the circadian clock. MiRNAs are an abundant group of endogenous, short (20–22 nt) single-stranded non-coding RNAs that act as post-transcriptional regulators of gene expression through sequence-specific cleavage or translational repression of their target mRNAs in plants and animals (Bartel, 2004; He and Hannon, 2004). The double-stranded precursor of miRNAs (pre-miRNAs) are processed in two sequential steps to generate 20–22 nt mature miRNA (Bartel, 2004). The mature miRNA is incorporated into an RNA-induced silencing complex (RISC), which is subsequently guided to target the mRNAs that contain the
recognition cis-elements located in their 3′-untranslated region (3′-UTR) for that particular miRNA (He and Hannon, 2004). The targeted miRNAs will be negatively regulated by inhibiting their translation or stimulating their degradation (He and Hannon, 2004).

It has been suggested that each miRNA is capable of controlling hundreds of target genes in humans, and >60% of the protein-coding genes are the targets of miRNA (Friedman et al., 2009). The expression of miRNAs is regulated by the circadian clock (Cheng et al., 2007; Xu et al., 2007; Yang et al., 2008; Hazen et al., 2009). In the meanwhile, miRNAs control the functions of the circadian clock through regulating the expression of the components in the oscillators, in the input pathways, and in the output pathways in mammals, Drosophila, or Arabidopsis (Mehta and Cheng, 2013).

A number of miRNAs controlled by the circadian clock have been identified in Arabidopsis by using whole-genome tilling arrays, including miR-160b, miR-167d, miR-156a, and miR-157a miRNAs in Arabidopsis (Hazen et al., 2009). In Arabidopsis, photoperiodic flowering control is one of the circadian clock outputs that has been shown to be under miRNA regulation (Schwab et al., 2005; Jung et al., 2007). The core components in the photoperiodic control flowering pathway, such as GI, COSTANS (CO), and FLOWERING LOCUS T (FT), exhibit circadian rhythmicity (Hayama and Coupland, 2003). miR-172 has been isolated as the miRNA responds to daylength in Arabidopsis (Schmid et al., 2003). The response of miR-172 to daylength is regulated by GI. The miR-172 targets the TARGET OF EAT I (TOE1) for degradation to promote the expression of FT and accelerate flowering (Schwab et al., 2005; Jung et al., 2007). miR-172 defines a CO-independent pathway for GI to promote flowering (Jung et al., 2007).

**Perspective**

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 AS is essential for the normal function of the circadian clock, how AS regulates the circadian clock is far from clear. Not only CCA1, but also LHY, TOC1, PRR3, PRR5, PRR7, PRR9, ZTL, GI, and other circadian clock-related genes are subject to AS in Arabidopsis (Sanchez et al., 2010; James et al., 2012; Seo et al., 2012). However, the molecular principles of their regulation of the circadian clock are still obscure. An investigation into how the AS 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.

The spliceosome is one of the most complicated protein complexes which is highly conserved in plants (Reddy, 2007). More than 100 components have been purified from mammals (Deckert et al., 2006). However, only a small proportion of them have been functionally deciphered. It will be of great interest to identify the circadian phenotypes of lacking the splicing factors or regulators of AS and to discover their functions in the circadian clock.

In addition to AS, alternative polyadenylation (APA) is becoming noted as being an important post-transcriptional regulatory mechanism in gene expression and the fates of miRNA (Di Giammartino et al., 2011). Like AS, APA is the process of generating multiple mRNA transcripts from a single gene, but in some case this extends the mRNA coding potential; in other case, APAs only alter the 3′-UTR length, influencing the availability of RNA-binding protein sites and microRNA-binding sites (Di Giammartino et al., 2011). The APAs of FCA and FPA play important roles in regulating the flowering time of Arabidopsis (Hornyik et al., 2010; Sonmez et al., 2011; Duc et al., 2013). However, less has been known about the functions of APAs in modulating the circadian clock in Arabidopsis. Exploring the genes involved in the APA of the circadian clock-related genes will be an attractive field of plant biology.

Although great efforts have made in determining the regulatory roles of miRNA in the clock in the last two decades, less is known about the functions of the miRNAs in regulating the circadian clock in Arabidopsis. Whether the known components of the oscillators are the targets of miRNAs and if the expression of the regulatory components in the clock is controlled by miRNAs are the questions remaining in Arabidopsis.

**Acknowledgements**

This work was supported by the National Natural Science Foundation of China (NSFC) [grant no. 31371222] and the Research Fund for the Doctoral Program of Higher Education of China [grant no. 20132103110004].

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