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Unraveling the circadian clock in *Arabidopsis*

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The circadian clock is an endogenous timing system responsible for coordinating an organism's biological processes with its environment. Interlocked transcriptional feedback loops constitute the fundamental architecture of the circadian clock. In *Arabidopsis*, three feedback loops, the core loop, morning loop and evening loop, comprise a network that is the basis of the circadian clock. The components of these three loops are regulated in distinct ways, including transcriptional, post-transcriptional and posttranslational mechanisms. The discovery of the DNA-binding and repressive activities of TOC1 has overturned our initial concept of its function in the circadian clock. The alternative splicing of circadian clock-related genes plays an essential role in normal functioning of the clock and enables organisms to sense environmental changes. In this review, we describe the regulatory mechanisms of the circadian clock that have been identified in Arabidopsis.

Introduction

Circadian rhythms, daily oscillations in gene expression and activity, have been observed in almost all organisms, from cyanobacteria to mammals.[1,2] These rhythms are generated by an internal timing system, the circadian clock, which integrates environmental input to permit the organism to anticipate dawn and dusk, to phase its biological activities to specific times of the day, and to synchronize different physiological processes with each other as a means of controlling essential physiological and biochemical processes.[1] In *Arabidopsis*, multiple aspects of plant growth and development are regulated by the circadian clock, including photoperiod-dependent flowering time control, stem and hypocotyl elongation, leaf movement, stomata movement and gene expression.[3-5] Expression of about 30% of the genes in *Arabidopsis* is under circadian control.[3,6,7] Correct matching of the periodicity of the endogenous circadian clock with local day/night cycles enhances growth and survival, and confers advantages in terms of fitness to higher plants.[4,8-10]

The molecular architecture of the circadian clock in most organisms consists of multiple, interlocked regulatory loops that are responsible for integrating input and generating overt rhythms.[4,11,12] The regulation of the circadian clock in *Arabidopsis* has been studied extensively.[13] Here, we review recent progress in understanding the diverse regulatory mechanisms of the circadian clock in *Arabidopsis*.

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Repressive Activity of TOC1 in Regulating the Clock

In *Arabidopsis*, three interlocked transcriptional-translational regulatory feedback loops have been identified, and significant progress has been made in understanding their role in the circadian clock.[10,14]

The best-characterized loop and the first to be identified, the central, or core loop is comprised of two morning-expressed Myb transcription factors, *CIRCADIANCLOCK-ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), and an evening-expressed gene, *TIMING OF CAB EXPRESSION1* (*TOC1*). In this loop, the morning expression and functional redundancy of CCA1/LHY represses the expression of *TOC1* by directly binding to Evening Elements in the *TOC1* promoter region, which make up the negative arm of the core loop in *Arabidopsis*.[15,16] Conversely, the accumulation of TOC1 in the evening was initially thought to activate *CCA1*/*LHY* through an unknown mechanism.[17,18] This central loop interlocks with the "morning" and "evening" loops, forming the basic architecture of the plant circadian clock. In the morning-phased loop, CCA1 and LHY expression increases the expression of *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*), *PSEUDO-RESPONSE REGULATOR 9* (*PRR9*) and *PSEUDO-RESPONSE REGULATOR 5/NIGHT INHIBITOR* (*PRR5/NI*); *PRR5/NI, PRR7* and *PRR9* subsequently downregulate the expression of *CCA1* and *LHY* by binding to their promoters.[4,19-21] GIGANTEA (GI), a large plant-specific protein, TOC1 and other proteins comprise the evening-phased loop.[13,14,20,22-25] Recent results have shown that EARLY FLOWERING 3 and 4 (ELF3 and ELF4), as well as LUX ARRHYTHMO (LUX, also called PHYTOCLOCK1), form the evening complex (EC), which binds to the promoters of several target genes, including those encoding LUX, ELF4, GI, TOC1 and PRR9, thereby suppressing their expression.[26-30]

Given the interplay among clock activators and repressors, how TOC1 regulates the expression of *CCA1*/*LHY* remains an open question. Recent studies have shown that TOC1 does not function as an activator of *CCA1*/*LHY*, but rather as a general repressor of oscillator gene expression.[31-33]

TOC1 is a PEUDO-RESPONSE REGULATOR (PRR) family protein with two domains: a pseudoreceiver (PR) domain

that mediates protein interactions at the N terminus and a CCT [CONSTANS (CO), CO-like, TOC1] domain responsible for DNA-binding activity at the C terminus.[25,34-39] TOC1 is required for normal functioning of the circadian clock in *Arabidopsis*.[14,40] Changes in TOC1 rhythmic expression through chromatin modification and transcriptional or post-translational regulation can affect the functioning of the clock.[18,41-44] As a regulator of transcription, TOC1 also controls the expression of a suite of clock-related genes. Recent results have shown that TOC1 acts as a general transcriptional repressor that negatively regulates *CCA1*/*LHY* and a group of genomic targets involved in critical plant functions.[31-33] The repressive activity of *TOC1* lies in its PR domain, but it relies on the presence of a functional CCT domain to negatively regulate its targets.[33]

Furthermore, biochemical and molecular data show that TOC1 is a transcriptional repressor with DNA-binding activity. Full-length TOC1 binds three motifs directly through its CCT domain: TOC1 morning element (T1ME, TGTG), which is part of the CO response element [TGTG(N2-N3)ATG], morning element (ME, GTGTGG) and hormone upregulated at dawn (HUD, CATGTG).[33,38,39,45] The binding of TOC1 to T1ME, a *cis*-element located in the *CCA1* and *LHY* promoters, in vivo and in vitro indicates that TOC1 binds directly to the *CCA1*/*LHY* promoter region to repress the expression of those genes.[31-33]

Genome-wide screening has resulted in the identification of three additional *cis*-elements that are enriched in genes regulated by TOC1: a G-box (CACGTG); a class I TCP binding site (GGNCCCAC), which binds TOC1 through an interaction with phytochrome interacting factors and CCA1 HIKING EXPEDITION, a suppressor of *CCA1*; and a GA motif (AGARRGARRRAGADR) recognized by BASIC PENTACYSTEINE transcription factors.[32,33,46-48]

The discovery of the DNA-binding and repressive activities of TOC1 provides a mechanism for the regulatory role of TOC1 in the central loop, and it offers an explanation for experimental data that are inconsistent with an activating role for TOC1, such as in the *ztl* mutant in which TOC1 accumulation is accompanied by reduced *LHY* and *CCA1* expression.[32,36,49]

Thus, the conclusion can be made that the suppressive activity of TOC1 is exerted either through direct binding to T1ME or similar sites located in the promoter region of its targets mediated by its CCT domain, or by being recruited to the promoter region of its targets by interacting with other DNA-binding proteins.[33,46,48]

Fine-Tuning of Protein Activity and Degradation in the Circadian Clock

In addition to the transcriptional regulatory feedback loops described above, key components of the clock are subject to post-translational control.[50-52] Phosphorylation plays a pivotal role in regulating the activity and abundance of clock components. The abundance of some circadian clock-associated proteins, including LHY, CASEIN KINASE 2B4 (CKB4) and XAP5 CIRCADIAN TIMEKEEPER, is modulated by phosphorylation. The phosphorylation of CCA1, mediated at least in part by CK2, is required for its function.[53,54] A circadian phosphorylation pattern has also been observed for TOC1, PRR3, PRR5, PRR7 and PRR9, although the responsible kinases are unknown.[55] Phosphorylated TOC1 and PRR3 exhibit enhanced affinity for each other, suggesting that the regulation of TOC1 stability through its competitive interaction with PRR3 or ZEITLUPE (ZTL) is modulated by their phosphorylation status.[37,55] Posttranslational degradation in the regulation of circadian components was discovered after the identification of ZTL, which contains both an F-box domain and a blue light-sensing LOV domain, and which functions as part of a Skp/Cullin/F-box (SCF) E3 ubiquitin ligase complex.[36,56,57] Degradation of TOC1 and its homolog PRR5 is triggered by a direct interaction with ZTL through the 26S proteasome.[36,57,58] The interaction of GI with ZTL, which is mediated by blue light, stabilizes both proteins and prevents ZTL from targeting its substrates TOC1 and PRR5 for degradation throughout the day.[57] Similarly, PRR3 binds directly to TOC1, blocking the recruitment of TOC1 to the SCF complex via ZTL at the beginning of the night to prevent TOC1 degradation.[37]

Roles for Alternative Splicing in the Clock

Gene expression is also subject to post-transcriptional regulation in the form of pre-mRNA processing, including 5' capping, 3' polyadenylation and intron removal or splicing, which not only affects the mature mRNA level, but is important for both transcription itself and downstream mRNA metabolic events such as mRNA export and turnover.[59] Pre-mRNA splicing is an essential step in eukaryotic gene expression that takes place within the spliceosome. Components of the splicing complex include several snRNPs, numerous serine/arginine-rich (SR) proteins and other non-snRNP proteins.[60-62] The spliceosome is highly dynamic during splicing progression, guided by consensus sequences in the pre-mRNA to form sequential complexes.[63]

As in other eukaryotes, alternative splicing is an essential mechanism for modulating gene expression and increasing transcriptome plasticity and proteome diversity in plants.[64,65] More than 95% of the genes split by introns in humans undergo alternative splicing.[66] It has been reported that about 42% of intron-containing genes in *Arabidopsis* are alternatively spliced; these genes are involved in growth and development and in responses to environmental changes and biotic or abiotic stress.[65,67-71] Alternative processing of the tobacco *N* gene and of *Arabidopsis FCA* pre-mRNA is important in the control of disease resistance and the floral transition.[72-75] Dysfunction of the plant-specific protein SR45 leads to a splicing deficiency, late flowering and an abnormal leaf morphology.[76-79]

Alternative splicing is also emerging as an important mechanism in the regulation of clock gene expression. Two *CCA1* transcripts, *CCA1*α and *CCA1*β, have been detected through whole-genome sequencing and RT-PCR.[67,80] The alternative splicing variant of *CCA1*, designated *CCA1*β, in which intron 4 is retained, is conserved in at least four plant species; moreover, *CCA1*β accumulates under high light conditions but shows decreased accumulation in the cold.[67] Protein domain predictions for the alternatively spliced isoforms of CCA1 suggest that CCA1β has a dimerization domain, like CCA1α, but that it lacks the N-terminal MYB

motif, which is involved in DNA binding.[15,54] The homodimerization and heterodimerization of CCA1α and LHY are important for the ability of these proteins to regulate circadian rhythms.[54,81,82] The splice variant *CCA1*β represses *CCA1*α and *LHY* activity by competitively forming nonfunctional CCA1α/CCA1β and CCA1β/LHY heterodimers, creating a self-regulatory circuit for CCA1 and LHY through the alternative splicing of *CCA1*.[80] Thus, transcription factors can auto-regulate their expression by generating competitive inhibitors through alternative splicing.

Furthermore, the alternative splicing of *CCA1* is suppressed by low temperatures.[67] CCA1α activity is derepressed by cold because of the significantly reduced production of CCA1β at low temperatures,[80] which explains the involvement of central circadian oscillators in freezing tolerance.[21,83-87] Under cold conditions, the alternative splicing of *CCA1* is suppressed. This enhances CCA1α activity, which contributes to arrhythmicity of the clock and the induction of freezing tolerance by amplitude regulation of the expression of C-repeat/dehydration-responsive element binding factors and GI.[80] Thus, the regulation of CCA1 activity by alternative splicing is important for plant adaptation to cold conditions.

Not only *CCA1*, but other circadian clock-related genes, including *LHY*, *TOC1*, *PRR3*, *PRR5*, *PRR7*, *PRR9*, *ZTL* and *GI*, undergo alternative splicing in *Arabidopsis*, mostly in a temperature-dependent manner.[80,88,89]

The role of PROTEIN ARGININE METHYL TRANSFERASE 5 (AtPRMT5) in regulating the alternative splicing of *PRR9* was discovered recently.[89] AtPRMT5 is a type II protein arginine methyltransferase that methylates diverse substrates and affects pre-mRNA splicing in a global fashion. AtPRMT5 methylates various nonhistone substrates, including RNA processing factors, hnRNPs, U snRNP, AtSmD1, D3 and AtLSm4.[90] The expression of AtPRMT5 is under the control of the circadian clock, and mutations in *AtPRMT5* elongate the circadian period.[91] Mutations in *Atprmt5* reduce the methylation of AtSmD1 and AtLSm4, leading to splicing defects in hundreds of genes that are involved in multiple processes, probably by regulating 5' splice site recognition.[89,90] Defects in the alternative splicing of *PRR9* in *atprmt5-5* lengthen the circadian period, indicating the regulatory role of alternative splicing in the clock.[89]

The involvement of splicing factors such as Ski-interacting protein (SKIP) and SPLICEOSOMAL TIMEKEEPER LOCUS1 (STIPL1) in the regulation of the circadian clock in *Arabidopsis* has also been demonstrated recently.[92,93] Mutations in *AtSKIP* and *STIPL1* increase the period length of the circadian clock. The capacity for temperature compensation is disrupted in *skip-1*. AtSKIP is a conserved SNW domain-containing protein and confirmed component of the spliceosome that associates with the splicing factor SR45; this is consistent with the role of SKIP in both mammals and yeast (Prp45).[62,92,94-96] As a splicing factor, AtSKIP exhibits splicing activity and can complement the defects in the cell cycle, temperature sensitivity and alternative splicing seen in the yeast mutant *prp45(1-169)*; moreover, mutations in *AtSKIP* can compromise splice site selection and alter genome-wide splicing patterns.[92,96] AtSKIP is also known to play a pivotal role in the pre-mRNA splicing of several circadian oscillator genes, including *PRR7* and *PRR9*, through direct pre-mRNA binding.[92] Defects in the alternative splicing of *PRR7* and *PRR9* partially contribute to lengthening of the circadian period in the *skip-1* mutant.[92] STIPL1 is a homolog of the spliceosomal proteins TUFTELIN-INTERACTING PROTEIN11 (TFIP11) in humans and Ntr1p in yeast, which are involved in spliceosome disassembly.[93,97] The mutation of *STIPL1* causes less efficient splicing of most of the introns analyzed.[93] The altered accumulation of circadian clock-associated transcripts, including *CCA1*, *LHY*, *PRR9*, *GI* and *TOC1*, may contribute to the long circadian period phenotype of the *stipl1* mutant.[93] These findings indicate the roles of the splicing factors *AtSKIP* and *STIPL1* in regulating alternative splicing and the period length of the circadian clock.[92,93]

A similar phenomenon has been discovered in other organisms. The *period* gene, which encodes a key component of the circadian oscillator in *Drosophila melanogaster*, generates two transcripts, type A and type B', in vivo, which differ by an alternatively spliced intron in their 3'-UTRs. The abundance of type A and type B' transcripts varies in head and body tissues and is temperature-dependent.[98,99] The *frequency* (*frq*) gene is a central component of the circadian clock in *Neurospora crassa*.[100] Two FRQ proteins, LFRQ and SFRQ, are generated from two alternative initiation codons in a temperature-dependent manner.[101] The relative levels of the two alternatively spliced transcripts are highly thermosensitive, leading to temperature-dependent changes in the FRQ protein population, suggesting that the temperature-influenced alternative splicing of *frq* pre-mRNA plays a significant role in temperature sensing.[102-104]

Taken together, the alternative splicing of oscillator genes is involved in the determination of circadian period length, temperature perception and temperature compensation of the clock in Drosophila, *Neurospora*, and *Arabidopsis*, suggesting that alternative splicing is a general regulatory mechanism in the clock.

The circadian clock is an important part of the interaction between plants and their environment. Interlocked transcriptional-translational feedback loops form the basis for regulation of the clock. This basic architecture is complicated by the interplay of clock-associated components. Thus, dissecting the cellular, molecular and biochemical mechanisms of the clock system is an ongoing challenge. Much effort will be required to explore new components associated with known factors. Though recent studies have linked alternative splicing to the circadian clock, the role of alternative splicing in modulating the clock are far from clear. Is there specificity for the spliceosome, and what are the determinants of that specificity? Are there clock-related gene-specific splicing factors? How do the alternatively spliced isoforms of clock-related genes (e.g., *LHY*, *PRR7*, *PRR9* and *TOC1*) perform their functions? Addressing these questions will enhance our understanding of the circadian clock.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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