Review

Circadian clock function in *Arabidopsis thaliana*: time beyond transcription

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The past decade has seen a remarkable advance in our understanding of the plant circadian system, mostly in *Arabidopsis thaliana*. It is now well established that *Arabidopsis* clock genes and their protein products operate through autoregulatory feedback loops that promote rhythmic oscillations in cellular, metabolic and physiological activities. This article reviews recent studies that have provided evidence for new mechanisms of clock organization and function. These mechanisms include protein–protein interactions and the regulation of protein stability, which, together, directly connect light signalling to the *Arabidopsis* circadian system. Evidence of rhythmic changes in chromatin structure has also opened new and exciting ways for regulation of clock gene expression. All of these mechanisms ensure an appropriate synchronization with the environment, which is crucial for successful plant growth and development.

Introduction

The rotation of the earth around its axis leads to environmental changes in light and temperature that predictably define the 24-h day–night cycle. Throughout evolution, organisms have evolved to coordinate their life cycle in anticipation of these environmental fluctuations. Accordingly, most organisms have 'learned' to keep track of time and to use environmental cues to synchronize their cellular activities to the most appropriate times of the day–night cycle [1]. It is now well accepted that both the measurement of time and synchronization with the environment are achieved by an internal time-keeping mechanism, or 'biological clock', that precisely measures the passage of time and generates rhythms (see Glossary) with a periodicity of ~24-h [2]. The biological clock can thereby be considered as a mechanism that translates environmental signals into temporal information to rhythmically coordinate metabolism and physiology [3].

Various fundamental properties of clock function exist: the persistence of rhythms in the absence of environmental cues; the synchronization or entrainment (see Glossary) to environmental signals; the maintenance of period (see Glossary) over a range of physiologically permissible temperatures; and the rhythmic regulation of many important cellular, physiological and developmental activities within the life cycle of the organism (Box 1). In this sense, biological clocks enable organisms to separate incompatible metabolic processes and to coordinate phase-sensitive cellular events so that they occur at a biologically beneficial time of the day or year (e.g. DNA replication occurring at night minimizes possible DNA mutations due to exposure to ultraviolet light) [1]. This underscores the importance of precisely timed rhythmic activities, which seem to confer an adaptive advantage compared with randomly occurring activities [1].

Historically, plants have played a very important role in the study of biological clocks. As early as 1727, the French astronomer de Mairan reported that rhythms in leaf movement persisted even in the absence of environmental cues [4]. These studies provided the first experimental evidence that biological rhythms might be driven by an endogenous cellular mechanism. From these initial steps, extensive research efforts have conclusively confirmed the existence of a biological clock and its pervasive role in the regulation of plant biology [5]. Some examples of processes tightly regulated by the plant clock include the movement of leaves, cotyledons and petals, the subcellular localization of chloroplasts, the growth of the embryonic stem (hypocotyl), the opening and closing of the stomata, and the photoperiodic regulation of flowering time [5,6]. Endogenous oscillations in gene and protein expression, nucleocytoplasmic partitioning, and protein phosphorylation and degradation underlie all these physiological rhythms [5]. This review outlines some recent advances towards the cellular and molecular characterization of circadian clock (see Glossary) function in *Arabidopsis thaliana*.

Glossary

Amplitude: difference between mean value and maximum or minimum of a sinusoidal oscillation.
Biological rhythm: oscillatory changes in a biological variable that persist with a similar pattern in a recurrent interval or period.
Circadian clock: a timing mechanism composed of a central oscillator that is entrained by environmental cues to generate 24-h biological rhythms.
Circadian rhythm: a biological rhythm with a period of approximately 24 h.
Day-length: the duration of the illuminated part of a light–dark cycle.
Entrainment: the adjustment of rhythms to match the 24-h solar cycles.
Free-running rhythms: self-sustained oscillations under constant conditions.
Gating: differential regulation of clock responsiveness to synchronizing cues depending on the time of day.
Oscillator: the endogenous timekeeper responsible for the generation of rhythmicity.
Period: duration of one complete rhythmic cycle.
Phase: the state of a rhythm relative to another reference rhythm (e.g. the day–night cycle).
Phase-shift: a displacement in the timing phase of an oscillation.
Photoperiodic response: the biological response to changes in day-length. Usually, it is associated with seasonal adaptations.

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Box 1. Fundamental properties of circadian clock function

Biological clocks are ubiquitous in nature and are found at various levels of organization and complexity, suggesting that they must provide an adaptive advantage. The biological clock generates self-sustained rhythms that are synchronized with the daily fluctuations in the environment. In the absence of environmental cues, the clock is able to maintain the rhythmicity, but its free-running period (i.e. the time required to complete a cycle) is close to, although not exactly, 24 h (hence the use of the Latin terms circadian, *circa* [approximately] and *dies* [day]; see Glossary for common terms used in circadian biology). Although the oscillations persist in the absence of environmental transitions, the circadian clock does not run in isolation from the environment. The clock includes a mechanism by which it is synchronized every day to the correct time. The environmental fluctuations in light and temperature synchronize the expression and activity of key clock components that ultimately define the period, phase and amplitude of output rhythms. Another property of the circadian function is its capacity to maintain a constant period over a range of physiological temperatures. This might act as a buffering system against changes in cellular metabolism.

Box 2. Circadian oscillations of small metabolites and signalling molecules in plants

The concentration of calcium ($[Ca^{2+}]$) oscillates diurnally, with peaks during the day and minimum concentration at night [77,78]. Given that Ca^{2+} is a signalling intermediate participating in the regulation of many physiological responses, the daily Ca^{2+} oscillation was proposed to encode circadian information [77]. Recent studies in *A. thaliana* have also provided evidence that cyclic adenosine diphosphate ribose (cADPR) concentration peaks early in the day [79]. This oscillation was disrupted in plants with defective clock function, indicating that it is controlled by the clock. Furthermore, decreasing concentrations of cADPR lengthened the period of circadian gene expression [79]. On the basis of these and other observations, the authors proposed that cADPR and Ca^{2+} signalling define a new feedback loop at the core of the oscillator.

The functional relevance of rhythmic changes in chromatin structure, and oscillations in gene expression and protein stability, in addition to the mechanisms linking the circadian clock with plant development are the major focus of this review. Particular aspects of plant circadian biology are covered in more detail by several excellent reviews, and the reader is encouraged to consult them [5–11].

The clock-signalling network

To work as an effective time-keeping device, the circadian clock must compartmentalize functions and establish close connections among the different functional modules. Some of the proposed modules include 'input components', which perceive the environmental signals and transmit this information to the 'central oscillator' (see Glossary), which is responsible for generating rhythms through multiple 'output pathways' [12]. This lineal pathway is overly simplified, because the clock in turn controls many input components, and some output components feed back to modulate clock function (Figure 1). The biological clock also incorporates small metabolites and intermediate signalling molecules, which confer additional complexity to the circadian system (Box 2). Furthermore, the different free-running periods of various outputs (see Glossary) suggest the existence of multiple oscillators that might share common elements and mechanisms in distinct locations [5]. Overall, a realistic view of the clock-signalling network would include differentially entrained, overlapping and interconnected oscillators that ultimately would define the period, phase and amplitude (see Glossary for these terms) of the overt rhythmicity (Figure 1). In any case, the input–oscillator–output model has proven to be conceptually useful for explaining clock function in diverse organisms, and it is also the model used to study the circadian system in plants.

Mechanisms of clock progression: transcriptional feedback loops

Genetic and molecular approaches have unambiguously identified conserved regulatory mechanisms underlying clock function in mammals, insects, fungi and plants

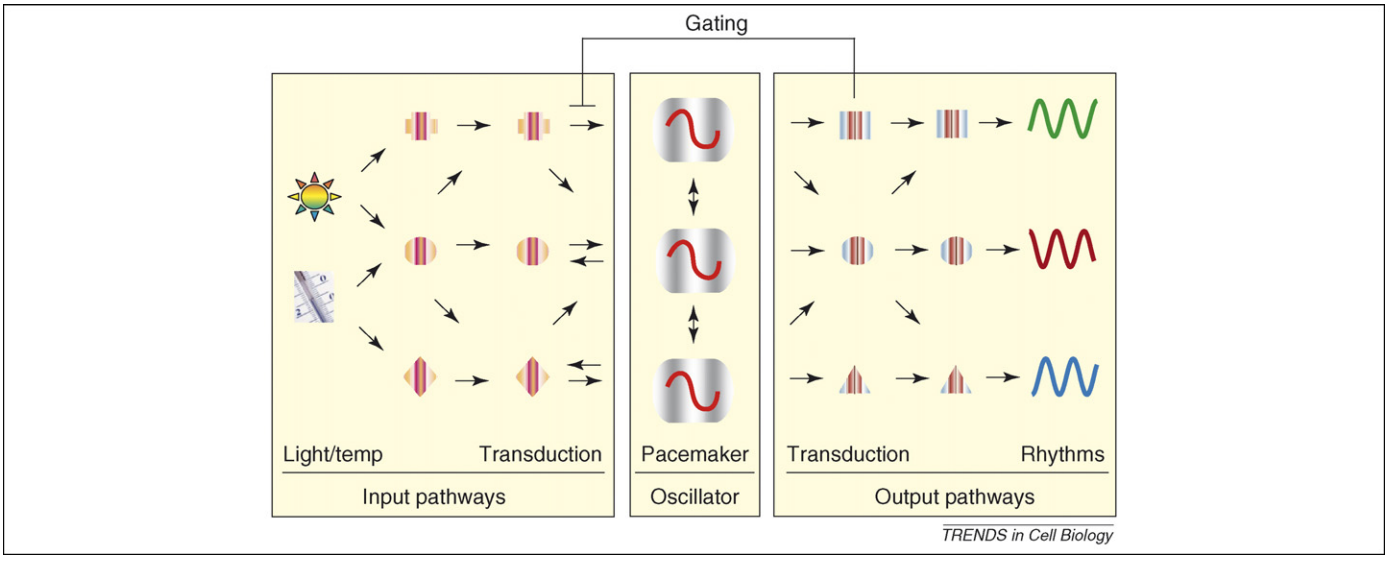

Figure 1. Schematic representation depicting the circadian clock-signalling pathways. The circadian system has been divided into three main functional units: input pathways, central oscillators and output pathways. The input components perceive environmental cues (e.g. changes in light and temperature) and synchronize multiple central oscillators. The oscillators generate and transmit the rhythmicity to molecular and physiological outputs. Some clock components are able to modulate the sensitivity of the oscillator to the environmental cues, in a process known as gating (see Glossary). The expression and/or activity of some input components can, in turn, be rhythmically modulated by the clock. The various shapes (ovals, triangles and diamonds, etc) represent different components of the signalling transduction pathways.

[12]. With the possible exception of the cyanobacteria oscillator, which might rely on circadian patterns of protein phosphorylation [13] and ATPase activity [14], the common mechanism involves transcriptional feedback loops at the core of the oscillator, with positive and negative components that control their own expression by regulating that of the other oscillator components [15]. In *Arabidopsis*, the generation of rhythmicity appears to be based on mechanisms similar to those described for other organisms. However, different, non-homologous molecular components are recruited to perform these functions [3]. *Arabidopsis* clock components include the single MYB transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) [16] and LATE ELONGATED HYPOCOTYL (LHY) [17]. In plants constitutively overexpressing either gene, the clock is unable to work, leading to arrhythmia, whereas loss-of-function *CCA1* or *LHY* mutations retain rhythmicity, albeit with a shortened period [16–19]. Detailed characterization of *TIMING OF CAB EXPRESSION 1* (*TOC1*) mutant plants [20] revealed that this pseudo-response regulator might be also an essential component of the *Arabidopsis* oscillator. TOC1 protein, also known as PSEUDO-RESPONSE REGULATOR 1 (PRR1), contains a receiver domain similar to that found in plant response regulators [20]. However, the phospho-accepting aspartate residue present in *bona fide* response regulators is absent in TOC1, suggesting that it does not function in a canonical phosphor-relay mechanism [21]. *TOC1* mutant plants exhibited a shortened period phenotype for clock-controlled gene expression in addition to a day-length-insensitive flowering phenotype [20]. In a similar fashion to overexpression of *CCA1* or *LHY*, the constant and high expression of *TOC1* caused arrhythmia in several clock outputs [22,23]. Analysis under different degrees of quality and quantity of light provided evidence of unpredicted roles for TOC1 as a molecular link between the environmental signals and the circadian and photo-morphogenic outputs [23]. Further studies provided a functional connection between CCA1, LHY and TOC1, enabling the reciprocal regulation of these core components to be proposed as one of the transcriptional loops regulating rhythmicity in *Arabidopsis* [24]. According to this model, the partly redundant transcription factors CCA1 and LHY [19,25] function as negative components that participate in the repression of *TOC1* [24] by directly binding to the Evening Element (EE) motif present in the *TOC1* promoter [26]. Increased *TOC1* expression was predicted to close the feedback loop by activating the transcription of *CCA1* and *LHY* [24].

Although the CCA1, LHY–TOC1 reciprocal regulation is important for clock function, this single loop cannot explain all the rhythmicity in *Arabidopsis*. Therefore, recent experimental research and computer modelling studies [27,28] have focused on the characterization of new core components that participate in additional loops essential for clock function (Box 3). The proposed core genes include the additional members of the TOC1 family, known as *PSEUDO RESPONSE REGULATORS 3, 5, 7 and 9* (*PRR3, PRR5, PRR7* and *PRR9*) [21]. Among others, the EARLY FLOWERING 4 (ELF4) [29,30], the GARP (GOLDEN2, ARR-B, Psr1)-MYB-domain transcription factor LUX ARRHYTHMO (LUX) or PHYTOCLOCK 1 (PCL1) [31,32], and *GIGANTEA (GI)* [33,34] closely associate with the circadian system and contribute to the regulation of *CCA1* and *LHY* rhythmic expression (Figure 2). Despite all the substantial progress, we are still far from a complete understanding of the different regulatory loops at the core of the oscillator. Identifying new clock components and the interactions among them will be crucial for understanding how the biological clock generates the 24-h rhythms.

Box 3. Interconnected morning and evening feedback loops at the core of the *Arabidopsis* oscillator

Similar to what has been proposed in the mammalian circadian system [80], the *Arabidopsis* oscillator is thought to comprise morning and evening oscillators. Following a newly described three-loop network [27,28], light activates the expression of *GIGANTEA* (*GI*), which participates in the induction of *TOC1*. The loop would be closed by TOC1 function as a negative component participating in the repression of *GI*. A hypothetical component 'X' was included in the model as a functional linker mediating the TOC1-dependent activation of LHY and CCA1 expression. The model proposes that *GI* expression is also repressed by LHY and CCA1 (Figure 2). By contrast, CCA1 and LHY positively regulate the expression of *PRR7* and *PRR9* by direct binding to a CCA1-binding site in the *PRR7* and *PRR9* promoters. The *prr7;prr9* double mutation delays the period of *CCA1* and *LHY* expression, suggesting that the PRRs feedback to negatively regulate *CCA1* and *LHY* expression. The model thus proposes a new basis for understanding the circadian circuitry, including coupled morning (LHY and CCA1–PRRs) and evening (TOC1–GI) oscillators. Within the plant circadian system, functions still need to be assigned to other clock-related genes that most probably form part of the oscillator (Figure 2). New experimental and computational data will help us to complete the intricate puzzle of the plant autoregulatory circadian network.

Post-transcriptional mechanisms of clock regulation

The transcriptional mechanisms of clock regulation have been extensively studied. However, fewer details are known about post-transcriptional regulation within the plant circadian system. Transcripts of *Arabidopsis* clock-related genes were shown to be highly unstable, and this was associated with the presence of the destabilizing downstream (DST) element in the 3′ untranslated region (3′UTR) of these genes [35,36]. Experiments with a mutant that was defective in DST-mediated decay revealed that the circadian regulation of transcript half-life was affected at a whole-plant level [36]. These results suggest that a sequence-specific mRNA degradation pathway might, at least in part, regulate plant circadian rhythms (see Glossary).

More-specific studies focused on *CCA1* have shown that light regulates *CCA1* transcript stability [37]. *CCA1* mRNA is stable in the dark but has a short half-life in red and blue light. The use of chimeric *CCA1* constructs revealed that the regions responsible for *CCA1* transcript instability were most probably located in the coding region [37]. The authors proposed that light regulation of *CCA1* transcription and mRNA degradation is important for accurately synchronizing the clock with the environment. The RNA-binding protein AtGRP7 (*A. thaliana* GLYCINE-RICH RNA-BINDING PROTEIN 7) illustrates another example of post-transcriptional regulation. Constitutive overexpression of *AtGRP7* (also known as *COLD- AND*

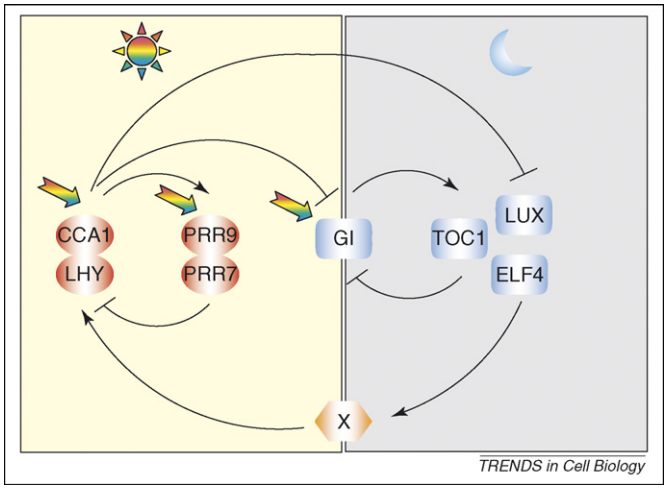

Figure 2. Interconnected feedback loops at the core of the *A. thaliana* oscillator. Mathematical modelling and experimental evidence predict the possible existence of morning and evening oscillators. In the morning oscillator, light (coloured arrows) together with CCA1 and LHY activate the expression of the pseudo-response regulators *PRR7* and *PRR9*, which, in turn, participate in the repression of CCA1 and LHY. In the evening oscillator, light activates GI function, which promotes *TOC1* expression. TOC1, in turn, represses *GI*. The morning and evening loops are interconnected by an as yet unknown component, X, which is required for the TOC1-mediated activation of *CCA1 and LHY*. At dawn, transcripts of evening-expressed genes such as *TOC1, LUX* and *ELF4* are maintained at low abundance by negative regulation of *CCA1 and LHY*. Arrows denote transcriptional activation, and lines ending in perpendicular dashes indicate repression. Adapted from [27] with permission from Macmillan Publishers Ltd.

CIRCADIAN-REGULATED 2 [*CCR2*]*)* promoted a splicing change in the *AtGRP7* mRNA [38], whereas the use of a cryptic 5′ splice site in the middle of the intron led to a short-lived splice variant. It was proposed that AtGRP7 regulates its own transcript oscillation by a post-transcriptional negative feedback mechanism. The AtGRP7 protein specifically binds to two elements present in its mRNA [38], suggesting that the negative autoregulation might occur through direct physical contact between the protein and its own pre-mRNA.

Post-transcriptional regulation by alternative splicing is not exclusive to the *Arabidopsis* circadian system [39]. For example, alternative splicing of the *Drosophila* transcript *PERIOD* (*PER*) within its 3′ UTR gives rise to two transcripts [40]. Low temperatures lead to intron removal, which promotes an earlier rise of transcript during the circadian cycle [40]. It is thought that these changes influence behavioural rhythms during the cold days in winter. Alternative splicing of the *Neurospora* clock gene *FREQUENCY* is also differentially regulated by temperature, and this regulation contributes to robust rhythmicity in a wide range of temperatures [41]. Similar regulations involving the 3′UTR in *PER1*, and *PER3* mRNA decay have been described in the mouse circadian system [42,43]. All these levels of post-transcriptional regulation might impose a delay in the cycles such that they occur with a 24-h period, characteristic of circadian rhythmicity. Other possible functions of post-transcriptional regulation include the maintenance of robust cycling amplitude in addition to buffering the clock mechanism against abrupt changes [44].

An interesting area of new research involves the study of microRNAs (miRNAs) as silencers of clock gene expression by mRNA degradation or translational repression. A recent study has functionally characterized the role of two miRNAs in the light-induced phase resetting of the mammalian clock [45]. The plant circadian system might be regulated by similar mechanisms involving rhythmic or acutely induced plant miRNAs. This is an open area of research for which further exploration is worthwhile.

Chromatin dynamics at the core of the oscillator

Transcriptional and post-transcriptional regulation of clock gene expression is important, but recent studies have shed some light upon new mechanisms of clock progression, including changes in chromatin structure [46]. Eukaryotic chromatin is organized in a highly complex nucleoprotein structure that controls several nuclear processes, including regulation of gene expression and DNA repair [47]. Chromatin architecture dynamically fluctuates between a condensed and a decondensed state, and controlling this fluctuation has been associated with the regulation of specific cellular functions. A variety of remodelling activities has been correlated with changes in chromatin structure by inducing post-translational modifications of the N-terminal tails of histones [47]. It is proposed that these modifications modulate the contacts with the DNA, providing a mechanism to regulate the accessibility of the genome. In this context, histone hyperacetylation has been associated with relaxed chromatin fibres and gene transcriptional activation [48]. Conversely, a hypo-acetylated state of histones has been correlated with condensed chromatin architecture and the transcriptional repression of genes [48].

A recent study has shown that the dynamic changes in chromatin structure are tightly connected to the *Arabidopsis* circadian clock [46]. Chromatin immunoprecipitation (ChIP) assays revealed that the transcriptional state of the *TOC1* gene relies on the dynamic modulation of chromatin remodelling, which precisely regulates the 24-h rhythmic oscillation of *TOC1* (Figure 3). The transcriptional activation of the *TOC1* gene follows a clock-controlled pattern of histone 3 (H3) acetylation in addition to the binding of chromatin remodelling factors to the *TOC1* promoter. Oscillatory rhythms in transcriptionally permissive chromatin structures were antagonized by the circadian binding of CCA1, which contributed to repression by impeding histone acetylation at the *TOC1* promoter. This study [46] suggests that decreased CCA1 binding throughout the circadian cycle might enable histone acetyltransferase (HAT) activities to acetylate histones at the *TOC1* locus, thus facilitating the accessibility of the transcriptional machinery and activators. This study also suggests that histone deacetylation facilitates the switch to repressive chromatin structures at the *TOC1* promoter, supporting the condensation of nucleosomal fibres and/or facilitating the binding of repressive factors (e.g. CCA1). Interestingly, the experiments showed that *TOC1* mRNA oscillation was distinctively modulated by day-length (see Glossary) or photoperiod (i.e. longer photoperiods correlated with higher amplitude and a delayed phase of *TOC1* expression). This photoperiod-dependent regulation of *TOC1* expression specifically associated with a distinct pattern of H3 acetylation in each photoperiod. Therefore, all these findings indicate that the oscillatory waveform of *TOC1*

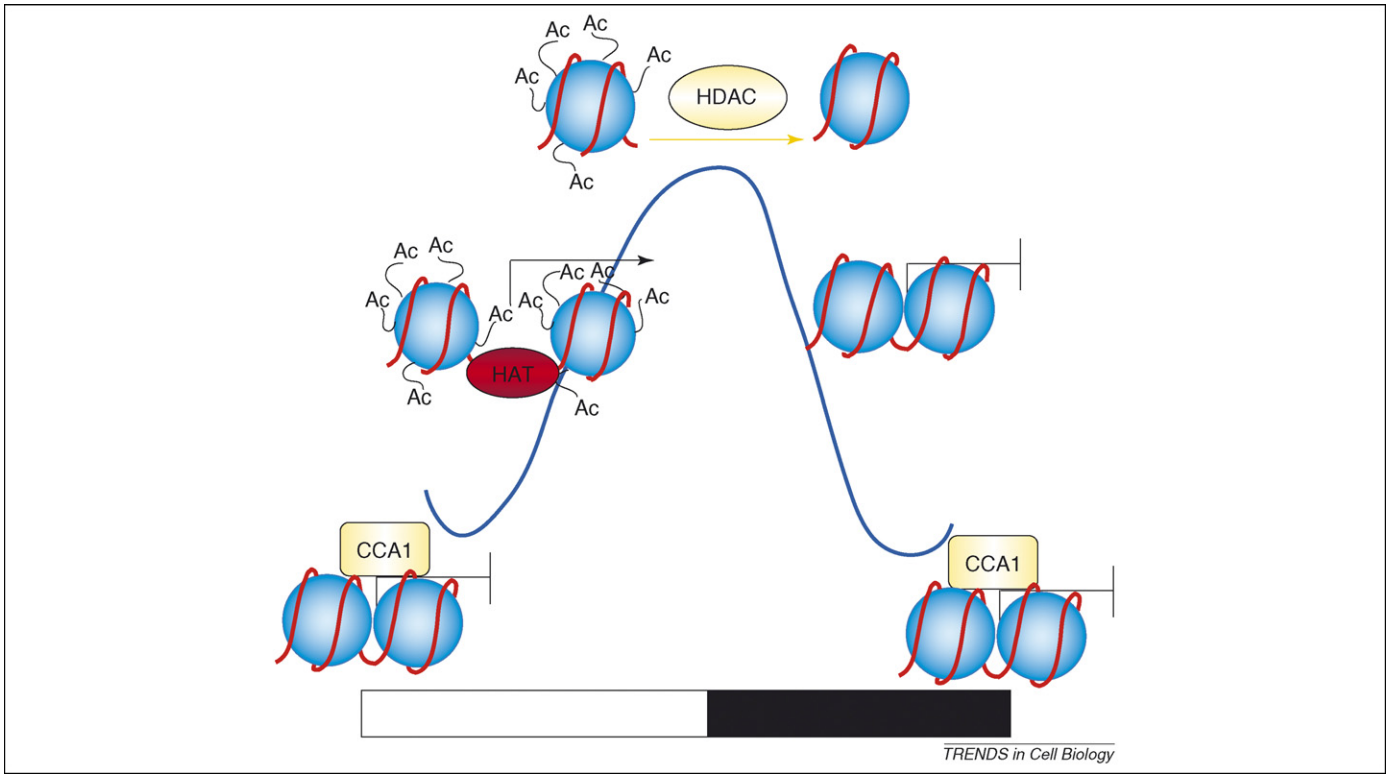

Figure 3. Schematic representation illustrating the rhythmic regulation of *TOC1* expression. The circadian expression of *TOC1* is controlled by changes in chromatin structure at the *TOC1* locus. *TOC1* repression at dawn depends on circadian binding of CCA1. Decreased CCA1 binding throughout the day enables transcriptional activation by rhythmic cycles of histone acetylation, which favours the formation of transcriptionally permissive chromatin structures. Histone deacetylase activities after *TOC1* peak of expression facilitate the switch to repressive chromatin structures and contribute to the declining phase of *TOC1* waveform around dusk. Different photoperiodic conditions distinctively modulate these chromatin remodelling activities, defining a mechanism by which plants might synchronize the phase of the biological clock. Nucleosomes are shown as blue circles, with the H3 N-terminal tails as curved lines coloured in pale blue; the dark blue line represents the waveform of *TOC1* mRNA expression; black arrows indicate transcriptional activation, whereas lines ending in perpendicular dashes indicate repression. White and black boxes indicate the day (light period) and night (dark period), respectively. Abbreviations: HAT, histone acetyltransferases; HDAC, histone deacetylases. Adapted from [46] by permission from ASPB. Copyright American Society of Plant Biologists (www.plantcell.org).

expression is regulated by the state of chromatin structure, which, in turn, is modulated by day-length or photoperiod [46].

The biological relevance of this regulation was dissected in studies exploring how dysfunctional regulation of chromatin structure at the *TOC1* promoter affects plant physiology and development [46]. These studies showed that the timing of clock-regulated processes such as hypocotyl elongation and the initiation of flowering depended on the adequate photoperiodic modulation of chromatin architecture at the *TOC1* promoter. Together, these findings illustrate the importance of histone acetylation–deacetylation for day-length measurement and for regulation of cellular and physiological processes in plants. The chromatin-dependent amplitude and phase of *TOC1* expression under different photoperiods might function as a day-length sensor, providing a mechanism by which plants perceive the photoperiodic information and consequently adjust their physiology and development.

The link between chromatin structure and clock function pervades other circadian systems, including the mammalian circadian clock. Recent studies have shown that the CLOCK protein, an essential component of the mammalian circadian system, is a histone acetyltransferase [49]. Furthermore, the expression of several mammalian clock genes was shown to be associated with changes in histone acetylation [50–53]. These results emphasize the idea that the chromatin-dependent regulation of clock gene expression is common to both plant and mammal circadian systems.

Post-translational mechanisms of clock function

In mammals, insects, fungi and bacteria, kinase and phosphatase signalling cascades have emerged as key mechanisms modulating the activity and stability of clock components [54,55]. Similarly, the CASEIN KINASE 2 (CK2) regulatory subunits CKB3 [56–58] and CKB4 [59,60] have been closely associated with the *Arabidopsis* circadian clock. CKB3 interacts with and phosphorylates CCA1 [57]. Furthermore, *Arabidopsis* plant extracts contain a CK2-like activity that affects the formation of a DNA–protein complex containing CCA1, suggesting that CK2 can modulate CCA1 activity *in vivo*. By using plants constitutively expressing a mutated version of *CCA1* that could not be phosphorylated by CK2, it was demonstrated that CK2-mediated phosphorylation of CCA1 was important for clock function [56]. As with *CKB3* overexpression, overexpression of *CKB4* results in period shortening of clock genes that peak at different times in the circadian cycle [58,59]. The short-period defect of *CKB4*-overexpressing (*CKB4*-ox) plants was shown to shift the phase (see Glossary) of clock gene expression, which correlated with an altered day-length-dependent regulation of developmental outputs [60]. It was postulated that the alteration

in the expression of the core components *TOC1* and *CCA1* was responsible for the short-period phenotype and for the improper matching of the clock period with the environment. The pervasive alterations of many clock outputs and the changes in oscillator expression in *CKB4*-ox plants suggest that CKB4 is very closely associated to the oscillator [60].

Another possible connection between the *Arabidopsis* clock and phosphorylation cascades is provided by the interaction with, and phosphorylation of, PRR3 by the protein kinase WNK1 (With No K) [61]. Eight members of the WNK family of protein kinases have been described in *Arabidopsis*, and the expression of WNK1, WNK2, WNK4 and WNK6 is controlled by the biological clock. Further experiments are required to demonstrate the relevance and biological implications of the phosphorylation of PRR3 by WNK1.

Post-translational regulation of protein stability through the proteasome pathway is also essential for circadian clock function. In *Arabidopsis*, members of the ZEITLUPE (ZTL) protein family, including ZTL [62], LOV KELCH PROTEIN2 (LKP2) [63] and FLAVIN BINDING KELCH F-BOX1 (FKF1) [64], have been closely associated with the circadian system. In addition to a Light-Oxygen-Voltage (LOV) motif and six Kelch repeats, these proteins contain an F-box domain, which suggests that they function as components of a Skp1–Cullin–F-box (SCF) complex. Indeed, ZTL was shown to be involved in the dark-dependent degradation of TOC1 protein [65]. The physical interaction of TOC1 with ZTL was abolished by the *ztl-1* mutation, resulting in constitutive levels of TOC1 protein expression. The ZTL-mediated degradation of TOC1 occurs mainly in the dark, and this regulation is responsible for the accurate control of circadian period by the clock [65]. The TOC1–ZTL interaction might be modulated by PRR3 in the vasculature, providing a mechanism for tissue-specific regulation within the circadian circuitry [66]. Regulation of protein stability through the proteasome pathway appears to be a mechanism shared among a diverse set of plant clock proteins. Clock components regulated by proteasomal degradation include LHY [67], ZTL [68], GI [69], CBK4 [59], PRR5 [70], PRR7 [71] and PRR9 [72]. Proteasomal regulation of circadian proteins has been also described in other circadian systems. Such proteins include the *Drosophila* F-box proteins SLIMB (SLMB) and JETLAG (JET), which regulate the clock proteins PERIOD (PER) and TIMELESS (TIM). In the *Neurospora* clock, the F-BOX–WD-40 REPEAT-CONTAINING PROTEIN1 (FWD-1) targets the protein FREQUENCY (FRQ) for degradation, and the β-TRCP (Beta-Transducin Repeat Containing Protein) regulates the PER homolog in human cells [54]. Altogether, these findings reflect the conservation of post-translational regulation of clock components among diverse organisms and illustrate the importance of precisely timed regulation of protein stability in the generation of circadian rhythms.

Relevance of post-translational regulation in the circadian control of photoperiodic flowering

The accurate perception of changes in day-length or photoperiod is essential in the regulation of photoperiodic responses (see Glossary), including the initiation of flowering [9]. Several cellular and molecular genetic approaches have confirmed that the biological clock is the mechanism responsible for day-length measurement, which enables plants to initiate flowering when light coincides with a sensitive phase of the diurnal cycle [11]. In *Arabidopsis*, the light-inducible phase relies on the transcriptional oscillation and changes in protein stability and activity of the flowering time component *CONSTANS* (*CO*), which enable it to activate its target *FLOWERING LOCUS T (FT)* [73]. Thus, the coincidence of light and maximum CO activity was proposed as the basis for the photoperiodic regulation of flowering in *Arabidopsis* [73]. Interestingly, a recent study has provided additional clues into the light-sensing mechanism responsible for the control of flowering

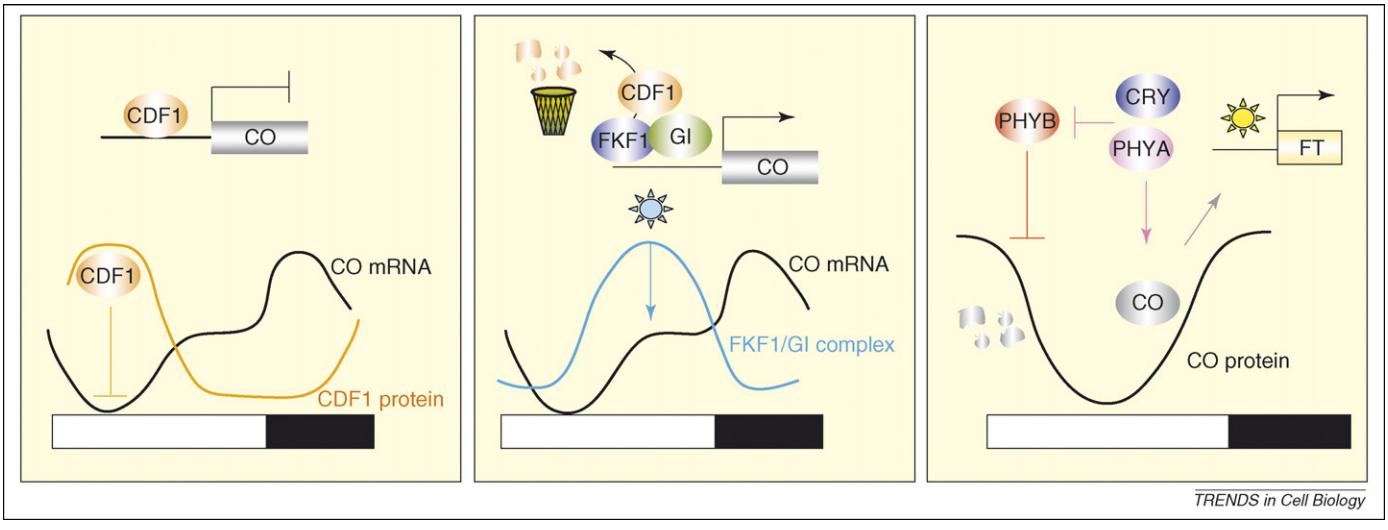

Figure 4. A model illustrating *CONSTANS* regulation under long-day conditions. CDF1 functions as a transcriptional repressor of *CO* early in the morning. The circadian and light-induced FKF1 interaction with GI enables the formation of the FKF1–GI complex in the late afternoon. On the *CO* promoter, CDF1 is degraded by FKF1, facilitating the daytime peak of *CO* expression. Blue and far-red light stabilizes CO protein through the action of the photoreceptors CRY and PHYA, whereas PHYB destabilizes CO protein in the morning. The combination of these regulatory activities favours the light-mediated induction of *FT* expression by CO, which results in flowering under conditions of long days. White and black boxes at the bottom of the schemes indicate the day (light period) and night (dark period), respectively. Adapted from [74] with permission from American Association for the Advancement of Science (AAAS).

time by the biological clock [74]. It was already known that PHYTOCHROMES (PHY) and CRYPTOCHROMES (CRY) participate as light-input components essential for clock entrainment and for regulation of flowering time [10]. The LOV domain of the ZTL, FKF1 and LKP2 proteins was proposed to function as a blue-light-sensing receptor that mediates the input of light to the clock [74,75]. The combination of LOV plus F-box and Kelch motifs in these proteins suggests a functional role mediating the degradation of key clock and flowering proteins. Indeed, as mentioned above, ZTL directly controls the stability of the TOC1 protein, and FKF1 regulates the degradation of CYCLING OF DOF FACTOR 1 (CDF1), a transcriptional repressor of flowering [76]. Recent studies have focused on the late-flowering phenotype under inductive long-day conditions of the *FKF1* mutant plants, a phenotype also caused by mutations in *GI* [74]. Interestingly, blue-light signals trigger the physical interaction between FKF1 and GI [74]. The blue-light-dependent FKF1–GI association coincided with the rapid induction of *CO*, the transcription of which is impaired by the flowering repressor CDF1 (Figure 4). By using ChIP assays, an FKF1–GI–CDF1 complex was detected on the *CO* promoter, which suggests that the association between FKF1 and GI affects the CDF1-mediated repression of *CO* [74]. These results provide the mechanistic basis for explaining how photoperiodic flowering might be controlled by the coincidence of light and circadian timing controlled by the biological clock (Figure 4). A parallel study shows that GI also interacts with ZTL in a blue-light-dependent manner [75]. Mutations within the LOV domain of ZTL diminish the interaction with GI and lead to reduced ZTL abundance. The authors propose that the interaction between GI and ZTL cooperatively stabilizes both proteins, thereby increasing their accumulation. Given that TOC1 protein is a substrate of ZTL, the interaction and stabilization of ZTL by GI might help to sharpen the oscillatory waveform of TOC1 protein [75]. This study proposes that this regulatory mechanism might contribute to the robustness of the circadian oscillations.

Conclusions

A circadian system able to generate biological rhythms with a 24-h period is ubiquitously found in organisms ranging from cyanobacteria to mammals. Circadian rhythms enable biological processes to occur at the most appropriate times during the day–night cycle, which confers a selective advantage to organisms. The studies described in this review illustrate the complexity of the mechanisms governing the plant circadian system. The *Arabidopsis* oscillator seems to comprise multiple interlocked feedback loops. However, we are still far from a complete understanding of the transcriptional circuitry regulating rhythmicity at the core of the oscillator

Future research should focus on the identification of new core components as well as on the elucidation of the regulatory interactions that operate within this circadian network. Recent studies on post-translational processing of clock proteins have provided mechanistic explanations for circadian function, including the use of environmental information to synchronize the photoperiodic initiation of flowering. The finding that *TOC1* expression is regulated by circadian changes in chromatin structure opens up several exciting avenues of research. These include regulatory studies aimed at deciphering the cellular, molecular and structural determinants of chromatin connections with the clock. Advances in understanding the mechanisms regulating the circadian system will help us to elucidate the intricacies of clock function and will contribute to our general understanding of plant physiology and metabolism.

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