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An expanding universe of circadian networks in higher plants

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Abstract

Extensive circadian clock networks regulate almost every biological process in plants. Clock-controlled physiological responses are coupled with daily oscillations in environmental conditions resulting in enhanced fitness and growth vigor. Identification of core clock components and their associated molecular interactions has established the basic network architecture of plant clocks, which consists of multiple interlocked feedback loops. A hierarchical structure of transcriptional feedback overlaid with regulated protein turnover sets the pace of the clock and ultimately drives all clock-controlled processes. While originally described as linear entities, increasing evidence suggests that many signaling pathways can act as both inputs and outputs within the overall network. Future studies will determine the molecular mechanisms involved in these complex regulatory loops.

The circadian clock

The circadian clock is an endogenous mechanism that allows an organism to coordinate the temporal organization of biological processes to specific times of the day or night. Clock function is widely distributed among species since most organisms adapted their physiology and behavior to the periodic oscillations in external signals that take place throughout each diurnal cycle [1,2]. These signals entrain or set the pace of the clock, which in turn has the capacity to drive self-sustained oscillations with a period ~ 24 hours [1,2]. Clock-generated biological rhythms allow an organism to anticipate the onset of environmental changes providing an adaptive advantage that result in increased fitness and survival [3–5].

Because of their non-motile nature, plants strongly rely on the ability to interact with their environment. The circadian clock provides a critical mechanism that integrates multiple external signals and adjusts plant physiological responses accordingly. Indeed, the pervasiveness of the clock extends to nearly every aspect of plant growth and development [6–8]. In this article we review recent progress on the mechanisms underlying clock function in plants. The accumulated data point to an expansive regulatory network that integrates in a reciprocal fashion with many major signaling modules.

Transcriptional circuits within the circadian oscillator

In most organisms studied to date, the circadian clock is proposed to function as a biochemical oscillator built on multiple interlocked regulatory loops [1,2,9,10]. Despite this common architecture, specific clock components and biochemical processes are encountered among

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different species [1,11]. The molecular characterization of clock mutants in *Arabidopsis* (*Arabidopsis thaliana*) revealed that the circadian oscillator in plants is defined by a core feedback loop that connects morning- and evening-phased circuits (Figure 1). The core feedback mechanism is based on the reciprocal regulation between the two morning-expressed MYB transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and the evening-phased pseudoresponse regulator TIMING OF CAB EXPRESSION 1 (TOC1) [12–15]. CCA1 and LHY are partially redundant [16,17] and directly repress *TOC1* by binding a motif within its promoter region, called the EVENING ELEMENT (EE) [12,18]. This motif is frequently found in clock-regulated gene promoters and is sufficient to drive robust evening-phased rhythms when multimerized [19]. In addition, it was recently reported that the EE can also mediate cold responses [20]. The mechanism by which overlapping signals converge at the EE possibly involves the binding of alternative transcription factor complexes. This is supported by the ability of CCA1 and LHY to form homo- and hetero-dimers [21,22], as well as the presence of protein-EE complexes even in the absence of CCA1 and LHY [19].

On the other arm of this core feedback loop, TOC1 regulates the expression of both *CCA1* and *LHY* transcripts [12,23]. While some data suggest that TOC1 functions as a transcriptional activator of *CCA1* and *LHY* [12,24,25], either reduced or elevated *TOC1* levels lead to low *CCA1* and *LHY* mRNA expression [12,23]. This observation and the lack of known DNA-binding domains in TOC1 protein [14] indicate a complex mechanism for the regulation of *CCA1* and *LHY* by TOC1. Recently, we identified a TCP (TB1, CYC, PCFs) transcription factor [26] called CCA1 HIKING EXPEDITION (CHE) that binds to the *CCA1* promoter [27]. Both CHE and CCA1 directly repress each other's expression, establishing an additional module to the core CCA1-LHY/TOC1 feedback loop [27]. Importantly, we found that CHE and TOC1 proteins directly interact and bind to the same region of the *CCA1* promoter, and that the long period caused by increased *TOC1* levels is shortened by the overexpression of *CHE* [27]. Thus, CHE appears to be part of the mechanism by which TOC1 regulates the expression of *CCA1*. It is possible that TOC1 antagonizes CHE, and probably other transcription factors, to eliminate the repression on *CCA1* promoter by the end of the night [28]. As CHE does not appear to bind the *LHY* promoter, this hypothesis predicts that other TOC1 binding partners might be involved in the regulation of *LHY* expression. An additional level of regulation within the core feedback loop is provided by the ability of CCA1 and LHY to inhibit their own and each other's expression [13,15]. Consistent with the repressor activities of CHE and LHY, we found that they are redundant in the negative regulation of *CCA1* [27].

Based on experimental observations and mathematical modeling, two additional phase-specific feedback loops were proposed [25,29,30]. One of these circuits is a morning loop where the expression of two TOC1 homologs, *PSEUDORESPONSE REGULATOR 7* and *9* (*PRR7* and *PRR9*), is directly activated by CCA1 and LHY [25,29,30]. In turn, *PRR7* and *PRR9* are partially redundant to inhibit the expression of *CCA1* and *LHY* [29]. While the mechanism of this regulation is unknown, extrapolation of the relationship between CHE and TOC1 suggests *PRR7* and *PRR9* might interact with transcription factors bound to the *CCA1* or *LHY* promoters. The other feedback loop involves the induction of *TOC1* by an unknown (generically named Y) evening-phased clock component or function. In this evening loop, Y was also predicted to be light inducible and negatively regulated by TOC1, CCA1 and LHY [24]. These requisites are partially fulfilled by a protein named GIGANTEA (GI), however, additional redundant factors likely contribute to the predicted functions of Y [24,25].

Despite the transcriptional nature of these feedback loops, CHE, CCA1 and LHY are the only transcription factors among the identified core clock components. An additional Myb-transcription factor named LUX ARRHYTHMO (LUX) (also known as PHYTOCLOCK 1 - PCL1) is likely a component of the circadian oscillator [31,32]. However, because its molecular

targets are not yet determined, the exact contribution of LUX within the clock network is unknown. Our ability to identify transcription factors associated to clock gene-promoters seems to be limited not only by the recurrent nature the clock regulatory circuits, but also by the high level of redundancy among plant transcription factors [33,34]. Consistent with these redundancy issues, while most clock components were identified in loss-of-function genetic screens; CHE, CCA1 and LHY were respectively identified by alternative means like the yeast one-hybrid system [27], hybridization to a phage cDNA library [35] and a gain-of-function mutant screen [13]. CHE belongs to a 13-member subclass of TCP-transcription factors [26, 36] that, because their close homology at the DNA-binding domain, likely share target site specificity. In fact, a reduction in *CCA1* expression was recently observed in plants overexpressing a translational fusion between TCP20 and the ERF-associated amphiphilic repression (EAR) motif [37], indicating that other TCP-transcription factors might be redundant to CHE on the regulation of *CCA1*. Also, since TOC1 interacts with the CHE DNA-binding domain [27] it is possible that TCP20 and other TCP-transcription factors participate of the mechanism by which TOC1 regulates the *CCA1* promoter activity. [27]. CCA1 and LHY belong to a subfamily of Myb-transcription factors also encompassing REVEILLE 1 to 8 (RVE1 to 8) [38,39]. All but RVE5 were shown to bind the EE in protein microarrays [40], suggesting they could be redundant to CCA1 and LHY. Recent characterization of RVE1, CIRCADIAN 1 (CIR1/RVE2) and EARLY PHYTOCHROME RESPONSIVE 1 (EPR1/RVE7) revealed that their expression patterns are similar to *CCA1* and *LHY*, peaking close to dawn in subjective days [38,41,42]. This circadian expression is dependent on CCA1 and LHY and is likely mediated by EE or EE-like motifs in their promoters. CIR1 and EPR1 were also shown to regulate their own expression [38,42], but only CIR1 appears to have a role in the core clock [42]. On the other hand, EPR1 and RVE1 have been associated with clock output pathways. While EPR1 was proposed to be part of a slave oscillator, RVE1 was associated to the control of circadian oscillations in auxin levels [38,41]. Interestingly, the reported phenotypes were observed under the constitutive overexpression of *RVE1*, *CIR1*, or *EPR1* and only subtle or no phenotypes were observed in the respective single mutants [38,41,42]. These observations suggest their activity could be redundant with other transcription factors or, as it seems for RVE1, could be tissue-specific [38,41,42].

The lack of phenotypes in single mutants can also be explained by the capacity of several transcription factors to form heterodimers. For example, CHE-related TCP-transcription factors can interact with each other [36] and, as mentioned, CCA1 and LHY can heterodimerize [21,22]. Since the interaction between CCA1 and LHY appears to occur through their DNA-binding domains, other combinations between homologous Myb-subfamily members might be possible [21,22]. CCA1 also interacts with the bZIP-transcription factor HY5 [43], suggesting that heterodimerization between transcription factors of different families should be further investigated. While the biological significance of these interactions has not been yet explored, heterodimerization of transcription factors would provide additional regulatory mechanisms to the clock network.

Daily changes in the chromatin structure also regulate the transcription of clock genes. Increased marks for gene activation such as histone acetylation (H3K9Ac and H3K14Ac) and dimethylation (H3K4Me2) at the *CCA1*, *LHY*, *GI* and *TOC1* promoters positively correlates with expression levels [18,44]. In addition, a rhythmic pattern of histone acetylation at the *TOC1* promoter precedes the peak of *TOC1* expression [18], suggesting the presence of clock-controlled changes in the chromatin structure. Finally, as demonstrated for *CCA1* [45], post-transcriptional control of mRNA stability might contribute to the regulation of transcript abundance of other clock genes as well.

Regulated protein turnover within the circadian oscillator

In addition to the transcriptional landscape described above, the current model for *Arabidopsis* circadian oscillator includes the post-translational regulation of protein levels and function. Stability of many clock components is regulated by proteasomal degradation. In this mechanism, target proteins are recognized and ubiquitinated by E3 ubiquitin-ligases and subsequently degraded by the 26S proteasome complex. Skp/Cullin/F-box (SCF) complexes are a special type of E3 ubiquitin-ligases, whose substrate specificity is defined by distinct F-box proteins [46]. In the circadian clock the proteasomal degradation of TOC1 and its homolog PSEUDORESPONSE REGULATOR 5 (PRR5) is mediated by the interaction with the F-box protein ZEITLUPE (ZTL) [47–51]. Two ZTL homologs, FLAVIN BINDING KELCH F-BOX 1 (FKF1) and LOV KELCH PROTEIN 2 (LKP2), also interact with TOC1 [50]. However, distinct clock phenotypes were observed in *ZTL*, *FKF1* or *LKP2* knock-out or overexpression backgrounds [51–54], suggesting that these F-box proteins target alternative clock-specific substrates. ZTL, FKF1, and presumably LKP2, function as blue light photoreceptors [55,56]. Light dependent interaction between ZTL and GI stabilizes ZTL and TOC1 protein levels throughout the day [55]. TOC1 is further stabilized by PSEUDORESPONSE REGULATOR 3 (PRR3), which directly binds to TOC1 preventing its interaction with ZTL at the beginning of the night [47,57]. Thus, TOC1 degradation is the result of a complex interplay among TOC1, ZTL, GI and PRR3. Other clock components such as LHY [58], PRR7 [59], PRR9 [60] and GI [61] are also subject to proteasomal degradation. Whereas the stability of GI appears to be regulated by a RING-type E3-ubiquitin-ligase during the night [62], mechanisms for the other proteins are still unknown. Interestingly, as noted for transcription factors, our ability to identify clock-related E3-ubiquitin-ligases is likely limited by genetic and functional redundancies. The F-box protein family is substantially more expanded in plants compared to other eukaryotes (~700 are encoded in *Arabidopsis* but only ~60 in humans) and; as ZTL, FKF1 and LKP2; many plant F-box protein subfamilies exhibit high domain conservation [63,64]. Similarly, although not as drastically expanded as F-box-containing proteins, the RING-type E3-ubiquitin-ligase protein family is more populated in *Arabidopsis* than humans [65].

Protein phosphorylation also regulates activity and abundance of clock components. The overexpression of either CKB3 or CKB4 CASEIN KINASE2 (CK2) β -subunits leads to altered circadian rhythms likely by modifying the phosphorylation status of oscillator components [66–68]. For example, CKB3-mediated phosphorylation of CCA1 modulates its ability to form homodimers and to interact with the DNA [67–69]. A circadian phosphorylation pattern was also observed for TOC1, PRR3, PRR5, PRR7 and PRR9 [47,59]. Whereas the identity of their respective kinases is unknown, a rhythmic kinase activity is supported by the circadian regulation of CKB4 protein levels [66]. TOC1 and PRR3 phosphorylated forms exhibit enhanced interaction affinity suggesting that the regulation of TOC1 stability by its competitive interaction with PRR3 or ZTL is modulated by their phosphorylation status [47].

The oscillator's input and output circuits

Outside the oscillator, the circadian system encompasses input pathways that set the pace of the clock and output pathways that regulate the expression of clock-controlled genes. Although originally defined as a unidirectional path, increasing evidence suggests that many inputs are subject to clock regulation and that outputs often feedback to modulate clock function. Light and temperature, the most studied clock-entrainment signals, regulate the abundance of clock components by multiple mechanisms. Phytochrome and cryptochrome photoreceptor signaling pathways mediate the induction of *CCA1* [15], *LHY* [70], *PRR9* [25,29] and *GI* [24] transcripts [71,72]. At the post-transcriptional level, light also controls *CCA1* mRNA stability [45], LHY translation rate [73] and TOC1 protein abundance [55]. Interestingly, light input pathways are reciprocally regulated by the clock. This phenomenon involves the activity of factors that

attenuate light signaling during the day or night, such as EARLY FLOWERING 3 (ELF3) [74,75], EARLY FLOWERING 4 (ELF4) [76], TIME FOR COFFEE (TIC) [77], LIGHT INSENSITIVE PERIOD 1 (LIP1) [78], FAR-RED ELONGATED HYPOCOTYL 3 (FHY3) [79] and XAP5 CIRCADIAN TIMEKEEPER (XCT) [80]. The circadian regulation of *ELF3* and *ELF4* gene expression, and likely LIP1 and XCP protein levels [74,76,78,80,81] evidence that the clock modulates light signaling by transcriptional and post-translational mechanisms. Although neither TIC nor FHY3 oscillate at mRNA or protein levels [79,82], it is possible the clock directly or indirectly regulate their activity. This is supported the additive phenotypes observed in *tic-elf3* double mutant seedlings [77]. Besides their role in setting the pace of the oscillator, these proteins also modulate other light signaling pathways [74,83] providing a mechanism by which the clock controls different light responses.

Whereas light is a major resetting signal, temperature cycles are sufficient to drive circadian rhythms [84–87]. Core clock components such as *CCA1*, *TOC1*, *LHY* and *PRR7* exhibit rhythmic expression after temperature entrainment [86] and altered expression after short or long cold exposures [88–90]. These observations, and the circadian defects encountered after temperature entrainment in loss-of-function alleles of clock genes (*lhy-20*, *ztl-4*, *prr3-1*, *prr5-3*, *prr7-3* and *prr9-1*) [86], suggest that circadian oscillations after light or temperature cycles are driven by the same clock components. However, distinct mechanisms seem to regulate the expression of clock components by light or temperature signals. Cold treatment induces transcriptional changes largely mediated by the redundant AP2-transcription factors C-REPEAT/DRE BINDING FACTOR 1 to 3 (CBF1, CBF2 and CBF3) [91]. Thus, it is possible that temperature input occurs through the CBF-dependent regulation of core clock-gene transcript levels. Although this is a suitable hypothesis, other mechanisms such as the cold regulation of cytosolic calcium concentration might modulate clock function by temperature as well [92]. Similar to light input signals, responses to low temperature are reciprocally regulated by the circadian clock. Because of this feedback, a large subset of genes is more effectively regulated by low temperatures at the beginning of the day [88,93]. Although the mechanism is unknown, the induction of *CBFs* and other transcription factors among these genes, likely trigger a transcriptional regulatory cascade that results in the differential expression of cold-responsive genes [88,93]. In addition, the clock regulates the circadian expression *CBF3* which results in higher transcript levels at the beginning of the day and possibly contributes to the enhanced response to cold temperatures at dawn [7,94]. Induction of cytosolic calcium by low temperature also exhibits a clock-regulated differential sensitivity throughout the day [95], thus suggesting that multiple mechanisms mediate the clock regulation of cold responses. Consistent with this hypothesis, *CBF*-dependent and - independent mechanisms regulate the enhanced freezing tolerance in *prr5-7-9* triple and *gi* single mutant seedlings, respectively [89,96].

By controlling the expression of at least one third of the *Arabidopsis* transcriptome, the clock appears to regulate circadian rhythms of virtually every plant biological process [6,7,97]. Essential mechanisms such as the regulation of seed dormancy and germination [98], hormone responses (reviewed in [99]), hypocotyl growth [100], onset of flowering (reviewed in [101]) and responses to environmental stresses [96,102] or nutrient availability [103] are regulated by the circadian clock. Intracellular events, such as calcium [104] and small GTPase signaling pathways [78] or chloroplast function [105,106] also exhibit clock-controlled circadian oscillations. In an interesting turn, recent reports suggest that most of these circadian-regulated mechanisms also feedback on the clock function. As observed for cold treatments, the clock regulates circadian rhythms in the expression of many stress-responsive genes [107]. High tolerance to salt and osmotic stresses was observed in the arrhythmic *prr5-7-9* triple mutant [96] and the opposite effect in *cca1-lhy* double mutant seedlings [102]. While this indicates that salt and osmotic stress responses are clock-outputs, elevated salt concentrations or osmotic pressure induce *CCA1* and *LHY* levels [102,107], suggesting these signals are clock inputs as

well. Clock-regulated gene expression is also encountered in every hormone pathway [6,7, 108,109]. Circadian emission of ethylene and abscisic acid- or auxin-regulated responses are modulated by the circadian clock [41,98,110–112]. Reciprocally, endogenous reduction in hormone activity or the external application of phytohormones changes period, phase or amplitude of circadian oscillations [111,113,114]. Calcium and nitrogen responses also have their own feedback loops [92,103]. Clock-controlled rhythms in cyclic adenosine diphosphate ribose (cADPR) levels likely drive circadian oscillations of cytosolic calcium; in turn, core clock components (*GI*, *PRR5*, *PRR7*, *CCA1* and *LHY*) are regulated by cADPR [92,104]. Similarly, whereas transcript levels of nitrogen assimilation genes are directly regulated by *CCA1*, addition of nitrogen nutrients produce phase shifts in *CCA1* expression [103].

Altogether these data support a robust reciprocal relationship between input- or output-signaling pathways and the circadian oscillator. Little is known about the molecular mechanisms involved in these additional regulatory loops. However, the large proportion of circadian-expressed genes in *Arabidopsis* [6,97] and the identification of phase- and response-specific regulatory motifs in clock-controlled promoters [8] suggest that transcriptional regulation plays an important role.

Conclusions and perspectives

The circadian clock provides a robust mechanism to coordinate the delicate interplay between plants and their environment. The core mechanism of this biochemical oscillator composed of interlocked regulatory circuits and functionally overlapping components. molecular wiring is mainly based on the transcriptional regulation of gene expression and post-translational control of protein levels. However, proteins specific to these mechanisms are clearly underrepresented among the clock components identified so far. This is possibly a consequence of the redundant nature of clock mechanisms and the expansion of protein families in plants. Thus, identification of the missing factors will require creative strategies to bypass these limitations. Clock function is mainly driven by light and temperature cycles, but additional environmental, organismal and intracellular clock-input signals have been identified. Single environmental cues possibly trigger the activation of different input pathways to generate a set of overlapping clock-entraining signals that contribute to the fine-tuning and robustness of the circadian clock. The reciprocal regulation between the circadian clock and input-output pathways suggests a heavily integrated system where each signaling pathway could impact the performance of any other through the core clock (Figure 2). Further characterization of the mechanisms underlying these multiple connections will ultimately result in a better understanding of how clock function enhances plant fitness and performance.

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Figure 1.

Model of the circadian clock in *Arabidopsis*. Transcriptional and post-translational mechanisms define the basic architecture of *Arabidopsis* clock. The core feedback loop consists of two Myb-transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which negatively regulate the expression of TIMING OF CAB EXPRESSION 1 (TOC1). TOC1 been proposed to activate the expression of CCA1 and LHY. An additional module within this loop includes the reciprocal repression between CCA1 and CCA1 HIKING EXPEDITION (CHE). TOC1 likely antagonizes CHE through a direct protein interaction. Two additional phase-specific feedback loops have been proposed. In the morning loop CCA1 and LHY activate the expression of *PSEUDORESPONSE*

REGULATORS 7 and 9 (PRR7 and PRR9), which in turn repress *CCA1* and *LHY*. In the evening loop *TOC1* represses an unknown component generically named *Y* (*GIGANTEA* (*GI*) appear to be part of *Y*), which in turn activates the expression of *TOC1*. *TOC1* levels are controlled by proteasomal degradation mediated by the F-box protein *ZEITLUPE* (*ZTL*). This mechanism is modulated by *GI* and the competitive interaction between *ZTL* and *PSEUDORESPONSE REGULATOR 3* (*PRR3*) with *TOC1*. The interaction between *TOC1* and *PRR3* is likely favored by the phosphorylation of these proteins. For clarity, clock components that cannot be placed with confidence to these feedback loops were omitted. Further details on the regulatory mechanisms shown here can be found throughout the text.

Figure 2.

Emerging model of the plant circadian system. Although initially outlined as three modules (clock-input pathways, circadian oscillator, and clock-output pathways) organized in a unidirectional path, accumulated experimental evidence indicates that the circadian system constitutes an expansive regulatory network where the circadian oscillator and major plant signaling modules (red circles) are regulated in a reciprocal fashion (black curved arrows, thicker lines denote the higher importance of these pathways as clock inputs). Main input signals such as light and temperature are also subject to clock regulation and many clock-output pathways often feedback to modulate the function of the circadian oscillator. This mechanism and the multiple interconnections between signaling modules (red dotted arrows) provide an integrated system to sense environmental cues (black dashed frame and arrows) and control plant physiology accordingly.