HHS Public Access

Author manuscript

Nat Struct Mol Biol. Author manuscript; available in PMC 2020 December 21.

Published in final edited form as:

Nat Struct Mol Biol. 2016 December ; 23(12): 1061–1069. doi:10.1038/nsmb.3327.

Molecular mechanisms at the core of the plant circadian oscillator

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Abstract

Circadian clocks are endogenous timekeeping networks that allow organisms to align their physiology with their changing environment and to perform biological processes at the most relevant times of the day and year. Initial feedback-loop models of the oscillator have been enriched by emerging evidence highlighting the increasing variety of factors and mechanisms that contribute to the generation of rhythms. In this Review, we consider the two major input pathways that connect the circadian clock of the model plant *Arabidopsis thaliana* to its environment and discuss recent advances in understanding of how transcriptional, post-translational and post-transcriptional mechanisms contribute to clock function.

Rhythmic oscillations in environmental conditions occur as a consequence of the earth's rotation and have a prominent effect on the metabolism, physiology and behavior of most organisms[1]. Circadian clocks have evolved as molecular timekeeping mechanisms that enable organisms to predict and anticipate these periodic changes in their surrounding environment, e.g., light–dark cycles and temperature oscillations, thereby allowing for efficient allocation of resources and enhancing fitness[2].

Despite their independent evolutionary origins, clocks in eukaryotes, in cases in which the molecular basis is known, rely on transcription- and translation-based feedback loops[3]. In animals and fungi, the central circuitry consists of heterodimeric PAS-domain-containing transcription factors that act as positive elements and promote the expression of their own transcriptional repressors, which constitute the negative elements in the loop[3]. In addition to the core circuit, additional interlocked feedback loops have been recognized over the years, thus giving rise to a more complex concept of the clock as a highly wired network. Although the nature of the individual clockwork components may differ, the overall network architecture is conserved across kingdoms[1]. A unique feature of plant clocks, however, is the prevalence of repressor elements in the system[2].

To synchronize with the environment, the central oscillator perceives and responds to external signals that provide timing cues. This information is then integrated by the clock

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

and is transferred to output networks for the regulation of a plethora of physiological processes including growth, metabolism, biotic and abiotic stress responses, and developmental transitions[4]. The proper sensing and integration of these environmental signals is especially relevant to plants, because their sessile nature necessarily restricts their ability to avoid challenging conditions. In this review, we discuss existing knowledge of how the surrounding environment sets the pace of the clock and integrate recent progress in understanding of the molecular mechanisms that shape the oscillator in the model plant *Arabidopsis thaliana*.

Integration of environmental signals with endogenous timing

Although circadian rhythms are self-sustaining, environmental signals such as light and temperature convey time information and are required to properly synchronize the clock with its surrounding environment (Fig. 1). The influence of light on the pace of the plant clock is pervasive, and multiple regulatory levels are affected by light quality and intensity. Clock transcription[5–8], mRNA stability[9], translation[9] and protein stability[10–12] are all affected by light, but how this information is transmitted to and incorporated by the central oscillator is still not fully understood. Furthermore, given that light is a major resetting signal, its perception and signaling are in turn regulated by the clock. The expression of the photoreceptors is under circadian control[13], and several clock and light-signaling components have been found to be involved in modulating clock sensitivity to light[14,15].

Light perceived by multiple photoreceptors has been shown to be involved in setting the pace of the clock[13,16–18]. Whereas the involvement of the blue-light photoreceptor ZETLUPE (ZTL) in clock protein stability is better understood (as described below), a mechanistic understanding of how other photoreceptors such as phytochromes, cryptochromes and UV RESISTANCE LOCUS 8 (UVR8) influence clock progression is less developed. All the phytochromes are dispensable for sustaining robust circadian oscillations, but they are required for period-length determination[13,16,19]. Interestingly, low and high fluence rates affect period length in opposite ways in phytochrome-null mutants, thus suggesting that the inactive and light-activated forms of the phytochromes may play antagonistic roles in determining the pace of the clock[20]. Specifically, phytochrome B (phyB) signaling in the nucleus is required for sustaining rhythms in response to red light[16]. In this context, a major mechanism through which phyB propagates light signals to transcriptional networks is the repression of the basic helix-loop-helix transcription factors PHYTOCHROME INTERACTING FACTORS (PIFs)[21], but a role of these proteins in clock function has not been confirmed. In contrast, direct binding of phyB to multiple clock proteins has been reported, and, intriguingly, these interactions appear to be enhanced or impaired in a light-dependent manner[22]. Interaction with phyB and the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) provides a direct link between the clock protein EARLY FLOWERING 3 (ELF3) and light-signaling pathways[12,23], and recent evidence has revealed that phyB plays a key role in this connection, because it mediates ELF3's interaction with further light-signaling components[24]. The physiological relevance of these interactions and their connection to light input remains an open and exciting question for future studies. The intersection between light-signaling and clock components for proper oscillator function is best exemplified by the transcriptional regulation of the

clock gene *EARLY FLOWERING 4* (*ELF4*), whose rhythmic expression is accounted for by the coordinated action of both light and the clock[15]. Three positive regulators of phyA signaling, ELONGATED HYPOCOTYL 5 (HY5), FAR RED IMPAIRED RESPONSE 1 (FAR1) and FAR RED ELONGATED HYPOCOTYL 3 (FHY3), directly bind the *ELF4* promoter and promote its expression as the day progresses[15]. This binding is later inhibited by the core clock proteins CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LONG ELONGATED HYPOCOTYL (LHY), which repress *ELF4* expression at dawn[15].

In addition to photocycles, temperature rhythms are also capable of entraining the clock, and the transcription of several clock genes is sensitive to temperature[8,25,26]. Although the oscillator components *PSEUDO-RESPONSE REGULATOR 9* (*PRR9*) and *PRR7* play an important role in temperature entrainment[26], the underlying mechanisms are not well understood. A recent study has indicated the involvement of HEAT SHOCK TRANSCRIPTION FACTOR B2B (HSFB2B) in mediating temperature resetting and heat-stress signaling into the clock through direct binding and repression of *PRR7* (ref. 27). Low temperatures, in contrast, have been shown to signal through the regulation of the clock gene *LUX ARRHYTHMO* (*LUX*; also known as *PHYTOCLOCK, PCL1*) by the cold-induced transcription factor C-REPEAT/DRE BINDING FACTOR 1 (CBF1)[28]. Interestingly, *HSFB2B* and *CBF1* are themselves regulated by the clock, thus evidencing how the clock gates its own sensitivity to external factors.

Despite the capacity of temperature input to reset and entrain the clock, a fundamental feature of circadian oscillators is their ability to maintain a relatively constant pace over a range of temperatures, buffering the effects of slight and physiologically irrelevant temperature changes[25]. Again, *PRR9* and *PRR7* are key players in this temperature compensation, in addition to *CCA1*, *LHY* and *GIGANTEA* (*GI*)[25,26]. Recently, a role of HSFB2B in this process has been described, probably through *PRR7* regulation[27]. Another transcription factor, FLOWERING BHLH 1 (FBH1), is also involved in warm-temperature compensation through direct regulation of *CCA1*, which in turn also modulates *FBH1* expression[29].

In recent years, post-transcriptional and post-translational mechanisms have emerged as relevant paths for light and temperature signal transduction to the clock. Phosphorylation of clock proteins, which directly affects clock progression, appears to be affected by temperature[30,31]. In addition, numerous clock genes undergo alternative splicing in response to temperature[32–35] and other environmental signals[36,37], including light[34,37]. Although the underlying mechanisms are still not well understood, it is notable that some of these mechanisms may not involve the canonical photoreceptors[37,38]. Hence, alternative splicing exemplifies a case in which light and temperature converge in the regulation of a biological process. Intriguingly, a recent study has proposed that cryptochromes are additional integrators of light and temperature in the regulation of the clock[39].

Molecular mechanisms at the basis of the plant oscillator

Current understanding of the circadian oscillator in plants is based on genetic and biochemical studies from the late 1990s that provided the description of the first

transcriptional loop[40]. Since then, the early model of the core oscillator as being composed of a few morning- and evening-expressed elements that reciprocally regulate one another's transcription has evolved into a much more complex and wired clock circuit. Over the past several decades, the identification of additional clock components and the description of numerous interlocked feedback loops among them has evidenced the high level of intricacy in the circadian network. Moreover, knowledge of additional molecular mechanisms that shape the plant circadian system beyond transcriptional regulation is expanding.

Transcriptional circuits

Repression feedback loops.

The first clock components characterized in *Arabidopsis* were CCA1 (ref. 5) and LHY [41], two single-MYB-domain-containing transcription factors that are expressed at dawn. These two proteins heterodimerize[6,42] and repress the expression of evening -phased genes by binding a *cis*-regulatory promoter motif called the evening element (EE) [43–45]. In addition, they also repress each other's expression[5,41]. The relevance of these repressors in accurate timekeeping is evidenced by the circadian phenotype of the respective mutants: whereas single loss of either *CCA1* or *LHY* causes a shortening of the period under free-running conditions, the *cca1;lhy* double mutant is arrhythmic[46]. The first evening clock gene shown to be repressed by CCA1 and LHY was *TIMING OF CAB EXPRESSION 1* (*TOC1*, also known as *PRR1*), a member of the PRR family whose mutation shortens the period[47]. These three proteins compose the first loop, because it has also been proposed that TOC1 may promote *CCA1* and *LHY* expression either directly or indirectly[40]. Although both the activating mechanism and the biochemical function of TOC1 had long been elusive, TOC1 has since been found to repress *CCA1* and *LHY*, and to function as a general repressor of clock-gene expression[48–50]. Subsequently, a revised understanding of the plant clock system primarily relies on negative feedback loops, wherein multiple morning and evening oscillator components are connected through their reciprocal repressive activities[2] (Fig. 2a; components listed in Table 1).

In the morning, CCA1 and LHY also transcriptionally repress other members of the PRR family in addition to *TOC1* (refs. 45,51). These PRR proteins are in turn sequentially expressed throughout the day and suppress *CCA1* and *LHY* transcription. This temporally phased repressive mechanism starts at midday with *PRR9* and is followed consecutively by *PRR7* and *PRR5* in the afternoon and finally by *TOC1* in the evening; ultimately, this mechanism contributes to the appropriate restriction of *CCA1* and *LHY* expression to a narrow window of time near dawn[52]. Another element contributing to *CCA1* repression is CCA1 HIKING EXPEDITION (CHE), a TCP transcription factor that interacts with TOC1 and directly represses *CCA1* transcription[53]. While the PRRs jointly contribute to *CCA1* and *LHY* suppression, they also repress one another's transcription[48,54–56] and, interestingly, they affect clock progression differentially. Whereas mutation of *PRR9* and *PRR7* lengthens the period[7], that of *PRR5* shortens it, similarly to *TOC1* (ref. 57). The PRRs play important roles not only in the central oscillator but also in its connection to input and output. *PRR9* and *PRR7* are involved in the transmission of light[7,58] and temperature[26,27,29] signals to the clock and, together with PRR5, also modulate multiple clock output processes, such as

flowering time, hypocotyl elongation and abiotic stress responses, by repressing the expression of key morning-phased transcription factors[54–56].

In the evening, GI, a large plant-specific protein that lacks well-characterized functional domains, is also required for circadian timekeeping[25,59]. This evening clock gene is repressed in the morning by CCA1 and LHY[60], both of which in turn appear to be induced by GI[59]. In agreement with this notion, *GI* loss-of-function mutants display a short period[59], but, given the intertwined nature of the plant oscillator, it is not known whether the activation is direct or indirect. In the evening, TOC1 and the evening complex (EC; described below) both contribute to *GI* repression[48,61]. Similarly to the PRRs, GI is involved not only in the central oscillator but also in light and temperature input[25,59], and in multiple output pathways ranging from photoperiodic flowering and growth to starch accumulation and abiotic stress responses[62].

After the PRRs and GI, three additional evening clock proteins associate into a hub termed the EC[63,64] and maintain the repression of morning and evening oscillator components. This complex comprises LUX, a MYB-like GARP transcription factor and ELF3 and ELF4, two unrelated plant-specific proteins. The EC components are repressed by CCA1 and LHY in the morning[15,30,60] and by TOC1 in the evening[48], and mutation of any of the components results in arrhythmia[64,65]. The EC functions as a transcriptional repressor and is recruited to the promoters of *PRR9*, *PRR7* and *GI*, and to the *LUX* promoter itself[61,65–67]. EC-mediated repression of *PRR9* and *PRR7* may thus indirectly promote *CCA1* expression at the end of the night. Interestingly, the closest homolog of LUX, NOX (also known as BROTHER OF LUX ARRHYTHMO or BOA), also forms a complex with ELF3 and ELF4 (ref. 63) and is required for recruitment of the EC to the *PRR9* promoter[65,67]. However, LUX and NOX are not fully redundant[65], and NOX has been reported to directly bind the *CCA1* promoter[68]. The EC DNA binding affinity and its effect on target gene transcription have been shown to be modulated by ambient temperature, thus placing the EC as an integrator of temperature and clock function[61].

Transcriptional activation within the core clock network.

Despite the prevalence of repressive interactions within the plant circadian network, several activating components have been uncovered in recent years (Fig. 2b). LIGHT-REGULATED WD 1 (LWD1) and LWD2 are both associated with activation and light input to the circadian system, and their mutation results in a shortened period[69,70]. LWD1 binds to the promoters of *CCA1*, *PRR9*, *PRR5* and *TOC1*, and is required for their expression[70,71]. In addition, LWD1 has been proposed to form a positive feedback loop with PRR9 (ref. 70). Two LWD1-interacting proteins, TEOSINTE BRANCHED1-CYCLOIDEA-PCF20 (TCP20) and TCP22, have recently been shown to be required for *CCA1* activation at dawn through direct binding to the *CCA1* promoter region, and consequently their mutation results in a shorter period[71]. Another activating component is the CCA1 and LHY homolog REVEILLE 8 (RVE8, also known as LHY-CCA1-LIKE 5 or LCL5). RVE8 is expressed in the morning but is most active in the afternoon, when it directly promotes the expression of the day- and evening-phased genes *PRR9*, *PRR5*, *TOC1*, *GI*, *ELF4* and *LUX*[72–74]. In turn, expression of *RVE8* is inhibited by binding of PRR5, PRR7 and PRR9 to its

promoter[54,56,72–74]. RVE8 activation of gene expression interestingly requires the EE, as is the case for the repressors CCA1 and LHY, albeit with an antagonistic outcome[74]. Similarly, RVE8 homologs such as RVE4 and RVE6 also bind the EE motif and are likely to be functionally redundant, because the long-period phenotype of *rve8* is accentuated in the *rve4;rve6;rve8* triple mutant[73,74]. Additional activation has recently been proposed to be provided by NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 1 (LNK1) and LNK2 (ref. 8). LNKs are expressed during the day and are thought to induce the expression of the afternoon and evening genes *PRR5*, *TOC1* and *ELF4* (refs. 8,75). Chromatin immunoprecipitation studies have shown that LNK1 is recruited to the *PRR5* and *TOC1* promoters, where it acts as a transcriptional coactivator necessary for the expression of these clock genes[75]. In turn, all the PRRs including TOC1 bind to the regulatory regions of the LNK-encoding genes and repress them[8,54,56]. However, given that mutation of the LNKs results in a longer period, the understanding of how these proteins affect the pace of the clock remains incomplete. LNKs have been suggested to function as integrators of light and temperature signals with circadian clock function. Because their expression is induced by light and repressed by the EC in a temperature-dependent manner[8,76].

Chromatin modifications.

The chromatin landscape is directly linked to the regulation of gene expression. Chemical modifications of DNA and histones affect chromatin architecture and hence the accessibility of transcriptional regulators, including clock modulators. In the case of the *TOC1* promoter, for example, it has been shown that repression of *TOC1* by CCA1 depends on a repressive chromatin environment promoted by histone H3 deacetylation[77]. By contrast, RVE8 facilitates a hyperacetylated state of H3 at the same promoter[72]. The antagonistic effects of CCA1 and RVE8 on the chromatin landscape at the *TOC1* locus may therefore underlie their effects on its expression pattern, although the precise mechanism through which these acetylation changes occur is not fully understood. Changes in H3 acetylation and methylation at the promoter regions of *CCA1*, *LHY*, *PRR9*, *PRR7*, *TOC1*, *GI* and *LUX* have been shown to correlate with their expression levels, and the histone methyltransferase SET DOMAIN PROTEIN 2 (SDG2) has been suggested to be involved in these dynamic changes either directly or indirectly[78,79]. *JUMONJI DOMAIN CONTAINING 5* (*JMJD5*, also known as *JMJ30*), an evening-phased histone demethylase that is regulated by CCA1 and LHY, is also involved in accurate timekeeping[80,81], as is the E3 ligase HISTONE MONOUBIQUITINATION 1 (HUB1), which affects H2B monoubiquitination and its associated H3 methylation mark at several circadian clock genes and alters their amplitude[82,83]. Nevertheless, further investigation is required to determine how these and other clock-controlling chromatin regulators participate in circadian rhythmicity.

Post-translational regulation

Layered onto the transcriptional circuits, post-translational relationships bring additional complexity into the circadian network. Clock proteins may dynamically interact with each other and consequently form functional complexes or may modulate one another’s activity and stability, thereby directly affecting time measurement (Fig. 3).

Protein-protein interactions.

The functional relevance of CCA1 and LHY homo- and heterodimers[6,42] is still not fully understood. Given that these proteins are only partially redundant, the different dimer compositions may have divergent functions *in vivo* that reflect their differential DNA binding affinities[84] or protein stabilities. In addition, both factors are constituent subunits of much larger protein complexes in which the additional partners may further modulate their activity[6]. Indeed, DE-ETIOLATED1 (DET1), a repressor of photomorphogenesis (light-mediated plant development), has been shown to act as an essential corepressor of CCA1 and LHY evening target genes[85].

Likewise, PRR9, PRR7 and PRR5 interact with members of the plant Groucho/Tup1 corepressor family, TOPLESS (TPL) and TOPLESS-RELATED PROTEINS (TPRs), and this interaction is required for the transcriptional repression of *CCA1* and *LHY*[86]. Furthermore, TPL-dependent recruitment of HISTONE DEACETYLASE 6 (HDA6) appears to be required for the activity of the complex[86]. Genome-wide analyses of PRR genomic targets have revealed that a large portion of them are also targets of other transcription factors[56], thus suggesting that a more complex network of partners may play a role in PRR promoter recruitment and specificity. Notably, PRRs interact with TPL through their EAR domain, which is absent in TOC1. It would therefore be interesting to explore whether and how other proteins influence TOC1's association with chromatin. Although the recruitment of TOC1 to the *CCA1* promoter has been proposed to occur through interaction with CHE[53], the underlying molecular mechanism remains uncharacterized.

LUX, ELF3 and ELF4 associate in the evening and consequently form the EC[63], which is required for sustaining circadian rhythms[64,67]. Of these three components, LUX is the only DNA-binding transcription factor [65], but the formation of the complex is necessary for LUX to become active[61,63]. ELF3 bridges the interaction between LUX and ELF4 (ref. 63), whereas ELF4 is required for the proper nuclear localization of ELF3, thus leading to the formation of distinct nuclear bodies[64]. ELF4 also influences GI nuclear dynamics[87], and physical interaction of ELF4 with GI results in GI's sequestration from the nucleoplasm and its localization into discrete nuclear bodies[87]. Because GI functions in both the nucleus and the cytoplasm, adequate nucleocytoplasmic partitioning is critical for the rhythmicity and robustness of the clock[88]. In a recent study, novel interactions between the EC and the clock proteins TOC1 and LWD1 have been observed[24]. Whereas the interaction between ELF3 and TOC1 is direct, the association with LWD1 requires the presence of the photoreceptor phyB, thus directly linking light signaling to clock protein-protein relationships[24]. The physiological relevance of these findings and their potential connection to light input to the clock remains to be explored. Interestingly, LWD1 has recently been shown to interact with TCP20 and TCP22 and to act as a coactivator required for *CCA1* activation at dawn[71].

LNK1 and LNK2 interact with CCA1, LHY, RVE4 and RVE8 (refs. 75,89). In fact, the recruitment of LNK1 to the *PRR5* and *TOC1* promoters has been suggested to occur via interaction with the bona fide DNA-binding proteins RVE4 and RVE8 (ref. 5). Although activation of *PRR5* and *TOC1* transcription by RVE8 requires LNK1 and LNK2 as transcriptional coactivators[75], LNKs antagonize RVE8 function in the regulation of anthocyanin accumulation[89]. What drives this switch in the outcome of the interaction, from

synergic to antagonistic, remains an open and intriguing question. The biological relevance of the interaction between the LNKs and CCA1 and LHY is enigmatic as well, because these proteins accumulate and are functional at different times of the day.

Protein stability and turnover.

Beyond DET1's role as a corepressor of CCA1 and LHY, it remains unclear whether it also contributes to the stability of CCA1 and LHY. Earlier work has suggested that DET1 inhibits the proteolytic turnover of LHY[90], and SINAT5 has been identified as an E3 ubiquitin ligase promoting the ubiquitination and degradation of LHY *in vitro*[91]. This degradation has been shown to be inhibited by DET1, possibly through its direct interaction with SINAT5 (ref. 91). Whether this mechanism also operates *in vivo* is unclear, because a more recent study was not able to confirm these results[85]. Two deubiquitinases, UBIQUITIN-SPECIFIC PROTEASE 12 (UBP12) and UBP13, have recently been linked to circadian period and shown to be under clock control[92], thus suggesting that deubiquitination is also important for circadian function.

Dark-induced proteasomal degradation of PRR5 and TOC1 occurs in the cytoplasm and is modulated by their interaction with the F-box protein ZTL[10,93] (Fig. 3b). ZTL is a member of the E3 ubiquitin ligase Skp–Cullin–F-box (SCF) complex and contains a blue-light-sensing light, oxygen and voltage (LOV) domain[17]. PRR9, PRR7 and PRR3 are also subject to proteasomal degradation, but despite the high homology among the PRRs they are unlikely to be targets of ZTL[57]. TOC1 degradation by ZTL increases the pace of the clock and is therefore precisely timed through several mechanisms. On the one hand, PRR3, which is temporally coexpressed with TOC1 in a tissue-specific manner, binds TOC1 and consequently hinders ZTL's access to and subsequent degradation of TOC1[57,94]. In addition, PRR5 also interacts with TOC1 and enhances its nuclear accumulation, thereby preventing cytoplasmic degradation of TOC1 by ZTL[95]. Furthermore, blue-light-dependent interaction with GI stabilizes ZTL and indirectly promotes the stability of PRR5 and TOC1, thus resulting in sharper and higher-amplitude oscillations of their protein levels[11,57]. Additionally, GI stabilizes ZTL through the same pathway as HEAT SHOCK PROTEIN 90 (HSP90), a chaperone involved in the maturation of ZTL [96]. Two ZTL homologs, FLAVIN-BINDING, KELCH REPEAT AND F-BOX 1 (FKF1) and LOV KELCH PROTEIN 2 (LKP2), also contribute to shaping PRR5 and TOC1 protein oscillations through direct interaction and degradation[95,97].

Whereas GI is essential in sustaining and modulating ZTL protein oscillations, ZTL reciprocally regulates the stability and nucleocytoplasmic partitioning of GI[98]. This GI-ZTL reciprocal co-stabilization is essential for robust circadian oscillations and proper function of the circadian system [88]. Later during the night, GI proteasomal degradation is promoted via ELF3-mediated interaction with COP1, thereby also triggering the ubiquitination and degradation of the substrate adaptor ELF3 (ref. 12) (Fig. 3b).

Phosphorylation.

Protein phosphorylation has been shown to regulate the activity, stability and complex formation of several clock components. Interestingly, the expression of many kinases and

phosphatases is controlled by the circadian clock[99], and in fact, a recent study has identified rhythmic oscillations in the phosphorylation state of a number of transcription factors and kinases[31].

Phosphorylation of CCA1 and LHY by the evolutionarily conserved protein kinase CK2 influences their dimerization and interferes with their DNA binding activity[30,100], thus directly affecting the pace of the clock[100,101]. In turn, the turnover of a regulatory subunit of CK2 is itself under clock control[101]. Physiologically, a balance between CCA1 DNA binding activity and phosphorylation by CK2, both of which are enhanced by temperature, has been proposed to contribute to temperature compensation[30]. The PRRs also undergo phosphorylation, and indeed a progressive phosphorylation pattern leading to the degradation of many PRRs has been observed[57]. Specifically for PRR5 and TOC1, increased phosphorylation enhances their binding to ZTL, thereby promoting their subsequent degradation[57]. In contrast, the phosphorylation of both PRR3 and TOC1 appears to be necessary for their interaction and the resulting protection of TOC1 from ZTL degradation, thus suggesting that TOC1 stabilization is also phosphorylation dependent[57]. Hence, a complex interplay between phosphorylation and stability appears to operate in the regulation of these clock proteins. In the case of ELF4, phosphorylation of the S45 residue has recently been found to oscillate over the course of the day in a circadian manner[31]. Functionally, this modification appears to enhance binding to ELF3 and to be involved in temperature compensation.

Beyond CK2, the kinases involved in rhythmic phosphorylation remain unknown. Identification of these kinases will be fundamental to understanding how appropriate phosphorylation patterns contribute to clock protein activity and abundance, and ultimately modulate circadian rhythmicity. In this context, phosphorylation motif analyses in a recently performed circadian phosphoproteome profiling study have yielded a number of candidates[31].

Post-transcriptional mechanisms

An additional layer of regulatory complexity is provided by post-transcriptional processes, which are key to circadian function in several organisms[9]. Some of these processes, including mRNA stability, mRNA export, translational control and noncoding RNAs, have been suggested to also operate in plant clocks[9,102]. However, deeper investigation of these mechanisms is required to elucidate their connection to the circadian network.

The most extensively studied post-transcriptional mechanism in the plant circadian system is alternative splicing (AS). Numerous clock genes have been reported to undergo AS, including *CCA1*, *LHY*, *RVE8*, PRR-encoding genes, *TOC1*, *ELF3* and *GI*[32,34,36]. In most cases, the abundance of the different splice variants appears to be regulated by temperature[32,33], but effects of photoperiod, salt stress and light have also been reported [36,37]. Growing evidence supports the idea of AS being a mechanistic link between environmental signals and clock performance. For example, the *CCA1* transcript exhibits a temperature-sensitive AS event in which the fourth intron is retained[33]. This splice variant is thought to give rise to a truncated nonfunctional version of the CCA1 protein (CCA1β) that lacks the MYB domain and can compete with full-length CCA1 (CCA1α) and LHY in the

formation of homo- and heterodimers, leading to the formation of nonfunctional complexes[33]. The production of CCA1β is suppressed under cold conditions, thus resulting in the accumulation of CCA1α, which in turn leads to the promotion of cold-induced genes. Notably, an *LHY* splice variant with a premature stop codon accumulates at low temperatures[32], thereby suggesting a role of AS in maintaining an appropriate balance between CCA1 and LHY during cold acclimation.

At the mechanistic level, the extent of spliceosome regulators that affect plant clock function is only beginning to be elucidated. The first such regulator to be characterized was PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5), a conserved methyltransferase involved in the methylation of histones, ribonucleoproteins and spliceosomal components[103,104]. Its expression is regulated by the clock, and its mutation results in period lengthening[103,104]. PRMT5 regulates the AS of *PRR9* and consequently the balance between functional and nonfunctional variants[104]. This regulation presumably occurs via PRMT5-dependent methylation of splicing factors, and in fact SM-like (LSM) spliceosomal proteins, which are targets of PRMT5 methylation, also affect circadian rhythmicity[105]. Several LSM-encoding genes are themselves clock regulated and, strikingly, they affect the AS of *CCA1* and *TOC1* but not *PRR9*, thus suggesting an unexpected regulatory complexity[105]. Further spliceosome components have also been shown to be involved in the post-transcriptional regulation of circadian clock genes. SNW/SKI-INTERACTING PROTEIN (SKIP) regulates the AS of *PRR9* and other clock genes including *PRR7*, *CCA1*, *LHY* and *TOC1*, and has been linked to temperature compensation and light input to the clock[34]. Additionally, mutation of SPLICEOSOMAL TIMEKEEPER LOCUS 1 (STIPL1), a homolog of human and yeast spliceosomal proteins involved in spliceosome disassembly, causes a long-period phenotype which is probably due to the accumulation of aberrant splice variants of *CCA1*, *LHY*, *TOC1* and *PRR9* (ref. 106). More recently, GEMIN2, another conserved spliceosomal assembly factor, has been shown to be involved in temperature compensation through the modulation of core clock-gene AS[35].

Concluding remarks and perspectives

Although enormous progress has been made toward the understanding of the molecular features and architecture of the plant circadian oscillator, key mechanistic connections for proper comprehension of the network still remain to be clarified. This is exemplified by the lack of knowledge regarding the biochemical functions of some core clock components and the increasing evidence of additional molecular mechanisms that contribute to circadian rhythmicity. Moreover, the current view of the circadian system has been derived mainly from studies performed on whole organisms (usually *Arabidopsis* seedlings) and is therefore likely to be distorted. Recent work has revealed the existence of tissue-specific clocks in plants that reciprocally regulate one another, are organized and show distinct rhythmic properties[107–110]. These findings have brought an additional complexity that had previously been overlooked into the network. The challenge now and in the future will be to decipher how these tissue-specific clocks are organized and communicate with each other, and to incorporate that knowledge into current models. Because the circadian system plays a crucial role in the connection between external environmental signals and internal

physiology, research on those connections should contribute to a more precise understanding of relevant physiological pathways and the clock's overall effects on plant fitness.

ACKNOWLEDGMENTS

We thank S. Sanchez, D. Nagel and J. Gallego-Bartolome for critical reading of the manuscript. Work at the laboratory of S.A.K. is supported by the National Institute of General Medical Sciences of the National Institutes of Health under award numbers RO1GM067837 and RO1GM056006. We apologize to our colleagues whose work could not be cited, owing to space limitations.

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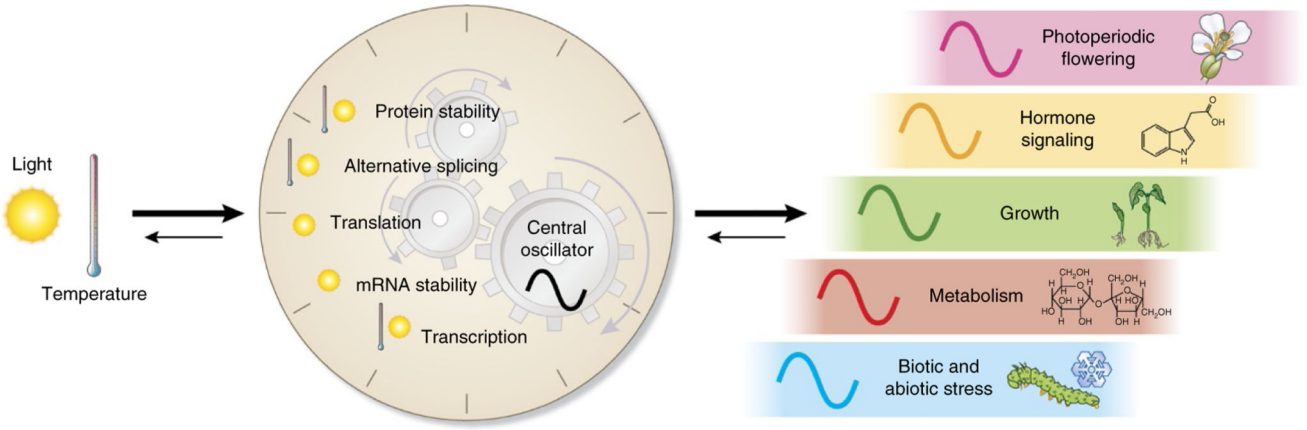

Figure 1.

Environmental signals are integrated by the central oscillator to coordinate multiple physiological processes. External signals such as light and temperature influence the pace of the clock (exemplified by the black wave in the oscillator) and entrain it by impinging on different molecular processes at the core of the oscillator. The clock then coordinates output rhythms (colored waves) accordingly. Proper clock function is required for the orchestration of multiple physiological pathways, including photoperiodic flowering, hormone signaling, growth, metabolism, and biotic and abiotic stress responses. Although the circadian system has traditionally been seen as a linear pathway, growing evidence supports the notion that it is a highly intricate network. Oscillator function is not unidirectionally regulated by external stimuli, but it also modulates its own sensitivity to them. In addition, multiple output pathways carry out feedback regulation of clock function, as is the case of hormones and metabolites. There is also extensive cross-talk among output pathways (not depicted), which can additionally be directly influenced by external conditions. Integration of these complex interconnections gives rise to a robust yet flexible network that plays an essential role in the coordination of plant physiology in natural environments.

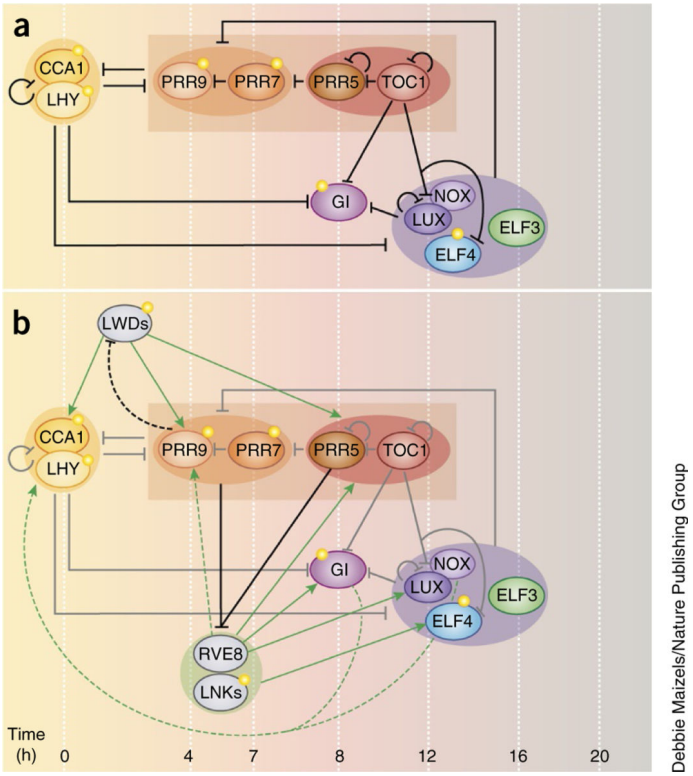

Figure 2.

Transcriptional feedback loops at the core of the circadian oscillator in *Arabidopsis thaliana*. The sequential expression of each component throughout the day is shown from left to right, and the time of activity is expressed in hours after dawn. The yellow and gray areas represent day and night, respectively. Black bars indicate repression, and green arrows indicate activation of transcription. Broken lines indicate relationships not proven to be direct or detected only under specific conditions. Ovals represent functional groups. The sun icon depicts light promotion of transcription. **(a)** At dawn, CCA1 and LHY repress the expression of the PRR-encoding genes, *TOC1*, *GI* and the EC members *LUX*, *ELF3* and *ELF4*. PRR9, PRR7, PRR5 and TOC1 are sequentially expressed and repress the expression of *CCA1* and *LHY*, as well as their own transcription. In the evening, TOC1 represses all of the previously expressed components in addition to *GI*, *LUX* and *ELF4*. Subsequently, the EC maintains the repression of *GI* and represses *PRR9* and *PRR7*. (**b**) LWD1 and LWD2 promote expression of *CCA1*, *PRR9*, *PRR7* and *TOC1*, and are probably repressed by PRR9. In the afternoon, transcriptional activation is mediated by RVE8 and the LNKs, which stimulate expression of *PRR5*, *TOC1* and the EC component *ELF4*. RVE8 additionally induces expression of *PRR9*, *GI* and *LUX*. GI appears to be required for activation of *CCA1* and *LHY*, as does an EC containing NOX.

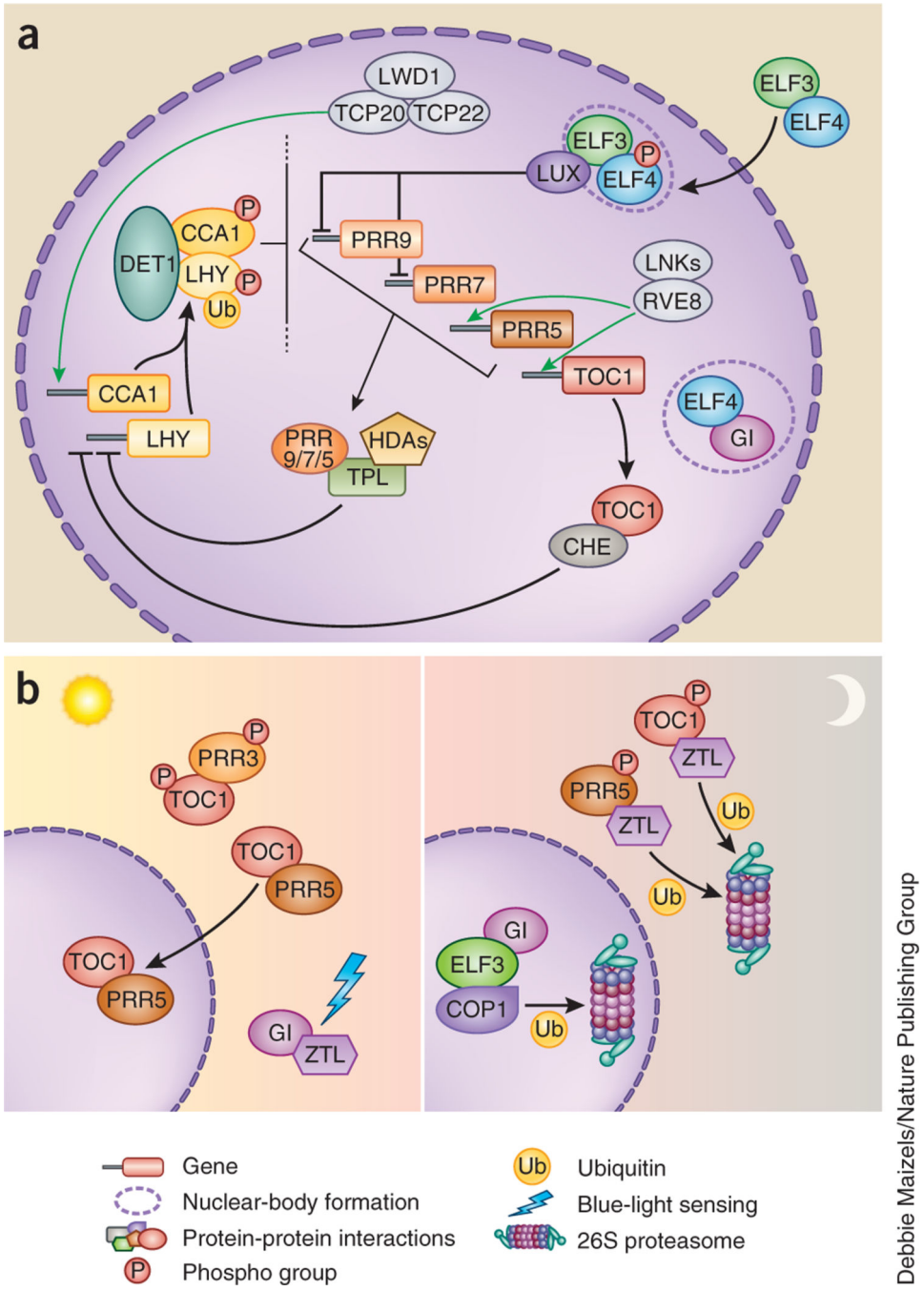

Figure 3.

Post-translational regulatory circuits within the clock of *Arabidopsis thaliana*. The purple oval depicts the nucleus; the brown area depicts the cytoplasm. (**a**) Protein-protein interactions among clock components. Activation of *CCA1* by TCP20 and TCP22 requires LWD1 as a coactivator. CCA1 and LHY homo- and heterodimerize and repress evening-phased genes by binding to a specific *cis*-regulatory motif in their promoters, the EE. To repress gene targets, they require DET1 as a corepressor. Transcriptional repression of *CCA1* and *LHY* is achieved through sequential expression of the PRRs (denoted PRR9/7/5),

which bind to the *CCA1* and *LHY* promoters and recruit TPL and HDA6, thereby inhibiting transcription. TOC1 is thought to be recruited to the *CCA1* and *LHY* promoters through interaction with CHE. Additionally, LNKs interact with RVE8 and act as coactivators inducing expression of *PRR5* and *TOC1*. ELF4 promotes nuclear translocation of ELF3, which then bridges the interaction between ELF4 and LUX, thereby forming the repressive EC. GI subnuclear localization is also modulated by ELF4. (**b**) Protein stability and turnover sets the pace of the clock. Left, in the afternoon, TOC1 is protected from ZTL-mediated proteasomal degradation through its interaction with PRR3 (which hinders ZTL access) and PRR5 (which promotes TOC1 translocation to the nucleus), as well as by blue-light-dependent GI-mediated ZTL stabilization. Right, progressive phosphorylation of PRR5 and TOC1 enhances their binding to ZTL, which promotes their degradation later in the evening. In addition, GI proteasomal degradation is promoted through ELF3-mediated interaction with COP1 in the dark; this interaction also triggers the degradation of ELF3.

Table 1

Genes functioning at the core clock network in *Arabidopsis*

Gene	AGI	Time of activity	Circadian phenotype (loss-of function)	Molecular function
CCA1	AT2G46830	Dawn	Short period	Transcription factor; partially redundant with *LHY*
LHY	AT1G01060	Dawn	Short period	Transcription factor; partially redundant with *CCA1*
LWD1	AT1G12910	Morning	Short period	Transcription regulator; redundant with *LWD2*
LWD2	AT3G26640	Morning	Short period	Transcription regulator; redundant with *LWD1*
PRR9	AT2G46790	Morning	Long period	Transcription factor; partially redundant with *PRR7* and *PRR5*
PRR7	AT5G02810	Midday	Long period	Transcription factor; partially redundant with *PRR9* and *PRR5*
RVE8	AT3G09600	Midday to afternoon	Long period	Transcription factor; partially redundant with *RVE6* and *RVE4*
RVE6	AT5G52660	Midday to afternoon (presumably)	Not obvious	Transcription factor; partially redundant with *RVE8* and *RVE4*
RVE4	AT5G02840	Midday to afternoon (presumably)	Not obvious	Transcription factor; partially redundant with *RVE8* and *RVE6*
LNK1	AT5G64170	Midday to afternoon	Long period	Transcription regulator; redundant with *LNK2*
LNK2	AT3G54500	Midday to afternoon	Long period	Transcription regulator; redundant with *LNK1*
PRR5	AT5G24470	Afternoon	Short period	Transcription factor; partially redundant with *PRR9* and *PRR7*
PRR3	AT5G60100	Evening	Short period	Putative transcription regulator; tissue specific
TOC1 (*PRR1*)	AT5G61380	Evening	Short period	Transcription regulator
CHE	AT5G08330	Evening	Not obvious	Transcription factor
GI	AT1G22770	Evening	Short period	Putative transcription regulator; clock and output interactions
NOX (*BOA*)	AT5G59570	Evening	Short period	Transcription factor
LUX (*PCL1*)	AT3G46640	Evening	Arrhythmic	Transcription factor
ELF4	AT2G40080	Evening	Arrhythmic	Transcription regulator
ELF3	AT2G25930	Evening	Arrhythmic	Transcription regulator