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REVIEW

The circadian clock goes genomic

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Abstract

Large-scale biology among plant species, as well as comparative genomics of circadian clock architecture and clock-regulated output processes, have greatly advanced our understanding of the endogenous timing system in plants.

Introduction

Plants rely on an endogenous timekeeper to optimally prepare for the recurrent cycles of day and night, light and darkness, energy production and energy consumption, activity of pollinators, as well as seasonal changes that tell them when to flower or shed their leaves [1,2]. The 'circadian' clockwork (from Latin *circa diem*, about one day) is entrained to the periodic light regime of the environment: plants use this information to control internal processes so that they take place at the most appropriate time of day for maximal output and performance. This global system works at various genomic levels.

The core clockwork consists of negative feedback loops through which clock proteins sustain their own 24-h rhythm [3-6]. In the model plant *Arabidopsis thaliana*, the Myb-type transcription factors LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) oscillate with a peak around dawn (Figure 1a). LHY and CCA1 activate the expression of four *PSEUDO-RESPONSE REGULATOR*s (*PRR*s) that are sequentially expressed, starting with *PRR9* in the morning, followed by *PRR7*, *PRR5* and *TOC1*/*PRR1*. This activation occurs indirectly via inhibition of the evening complex (EC), which is a repressor of the *PRR*s (Figure 1b); three proteins, LUX ARRHYTHMO (LUX)/PHYTOCLOCK1 (PCL1) and the plant-specific proteins EARLY FLOWERING 3 (ELF3) and ELF4, interact to form the EC. The PRRs induce the EC in the late evening, whereas CCA1 and LHY repress EC expression. The EC, in turn, indirectly activates *CCA1* and *LHY* by directly inhibiting the repressive PRRs. These and other clock proteins regulate rhythmic molecular and biochemical processes in the cell (Figure 1c) (see section 'From a single oscillating mRNA to the rhythmic transcriptome'). These molecular-genetic events have been integrated into quite sophisticated systems models (reviewed at a systems level in Bujdoso and Davis [7]).

Overall, the principles of rhythm generation in plants are the same as in mammals or *Drosophila*, but the components involved are largely different, pointing to independent origins of the timekeeping mechanisms. In mammals, the core loop comprises the transcription factors CLOCK and BMAL1, which activate the expression of *Cryptochrome* and *Period* genes. The PERIOD/CRYPTOCHROME complex, in turn, represses BMAL1/CLOCK-mediated transcription of their own genes. Additional feedback loops consisting of transcriptional activators and repressors interlock with this central loop to regulate the expression of the core clock genes (for a detailed description, see Zhang and Kay [8], Staiger and Köster [9], and Dibner *et al.* [10]).

In this review, we summarize recent insights into the blueprint of the circadian clock and the function of clock proteins based on genomic studies in *Arabidopsis* and other plant species (Figure 2). Furthermore, we describe how large-scale biology has greatly advanced our understanding of how timing information is translated into rhythmic processes in the plant cell.

From a single oscillating mRNA to the rhythmic transcriptome

Chronobiology, the discipline of endogenous timekeeping, went molecular with the first demonstration of mRNAs in pea plants that appeared at sunrise and disappeared at sunset, and continued to cycle with a 24-h rhythm even in the absence of a light-dark cycle [11]. It was difficult to appreciate these circadian experiments as they were not just a 'minus light' sample compared with a 'plus light' sample, but required processing of many samples harvested around the clock. A major advance in this sort of approach was to move beyond a gene-by-gene examination. The first circadian microarray study was

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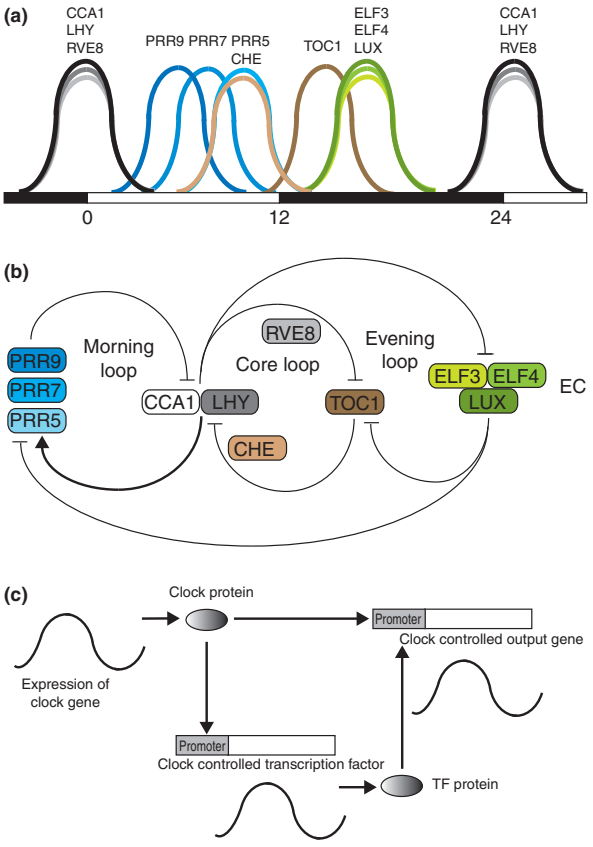

Figure 1. Circadian oscillations in clock gene expression lead to a global rhythm of large parts of the transcriptome. (a) Oscillations of the *Arabidopsis thaliana* clock genes across the day. The open bar refers to day, the dark bar refers to night. The numbers indicate hours after lights on. Please note that the amplitude of the oscillations is arbitrarily chosen to be equal for all transcripts. **(b)** Blueprint of the circadian oscillator in *Arabidopsis thaliana*. The core loop consists of the Myb-type transcription factors CCA1 and LHY, and the Pseudo response regulator TOC1, which reciprocally regulate each others' oscillations. Interconnected with the core loop are the morning and the evening loops. In the morning loop, CCA1 and LHY activate *PRR5*, *PRR7* and *PRR9*, which in turn leads to inhibition of *CCA1* and *LHY*. In the evening loop, the evening complex (EC), a protein complex consisting of ELF3, ELF4 and LUX, inhibits expression of *PRR9* and perhaps other *PRR*s. EC components are themselves rhythmic through repression by CCA1 and LHY. Additional transcription factors, such as RVE8 and CHE, modulate these interconnected loops. **(c)** Oscillations in the output genes can be accomplished through direct binding of rhythmically expressed clock proteins to phase modules in their promoters or via intermediate transcription factors (TF). In this way, transcripts are directed to different times of the day. As one example, components involved in metabolizing sugars produced through photosynthesis peak early in the day, and components involved in starch degradation, in turn, peak in the middle of the night [12].

opportunely performed just after the compilation of the *Arabidopsis* genome [12,13]. Cycling gene clusters could thus be linked to nearby non-coding DNA, and conserved elements in the upstream regions revealed phase-specific promoter elements [12,14-16]. These studies provided valuable insights into the genome-wide mechanism of clock outputs for the first time. Groups of genes that are co-ordinately directed to certain times of the day pointed to entire pathways that were not previously known to be clock-regulated, such as the phenylpropanoid pathway [12].

Subsequently, many homologous genes were found to be clock-regulated and phased to similar times of day in poplar and rice, as they are in *Arabidopsis* [17]. Furthermore, the same three major classes of *cis*-regulatory modules of *Arabidopsis* were found in poplar and rice. The morning module consists of the morning element (CCACAC), which confers expression at the beginning of the day, and a ubiquitous G-box (CACGTG) regulatory element associated with regulation by light and by the phytohormone abscisic acid. The evening module consists of the evening element (AAAATATCT), which confers expression at the end of the day, and the GATA motif, which is associated with light-regulated genes. The midnight modules come in three variants, ATGGCC (PBX), AAACCCT (TBX) and AAGCC (SBX). This points to a strong conservation of clock-regulated transcriptional networks between mono- and dicotyledonous species [17]. As shown in Figure 1c, oscillations of the output genes can be accomplished through direct binding of rhythmically expressed clock proteins to phase modules in the promoters of output genes, or via intermediate transcription factors.

The information from numerous microarray experiments conducted under different light and temperature regimes by the community were assembled into the easy-to-use DIURNAL database [18]. This site is widely consulted to check for rhythmic transcript patterns, reflecting the growing awareness of the importance of temporal programs in gene expression [18].

Rhythmically expressed genes in *Arabidopsis* were found to be over-represented among phytohormone- and stress-responsive pathways. This revealed that endogenous or environmental cues elicit reactions of different intensities depending on the time-of-day [15,19]. This so-called 'gating' is thought to optimize the response to a plethora of stimuli impinging on the plant, and may be of particular relevance for sessile organisms [2]. An example of this is how the PRR5, PRR7 and PRR9 proteins contribute to the cold stress response [20]. These PRRs also contribute to coordinating the timing of the tricarboxylic acid cycle [21]. In this way, one set of regulators directly link global gene expression patterns to rhythmic primary metabolism and stress signaling.

A similar systems-based approach identified the circadian clock as a key player in other facets of metabolism, since CCA1 regulates a network of nitrogen-responsive genes throughout the plant [22]. CCA1 also

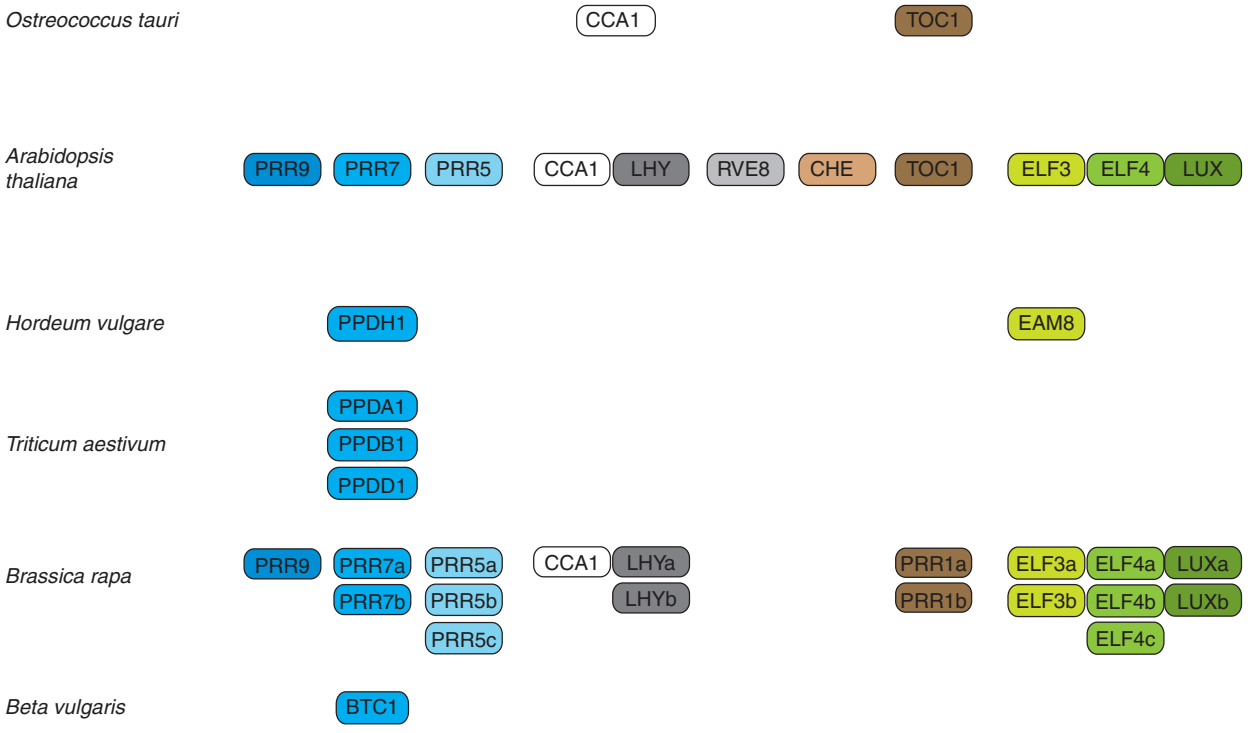

Figure 2. Genes of the circadian oscillator in *Arabidopsis thaliana* and homologs in other plant species discussed in this review. *Ostreococcus tauri* contains single homologs of CCA1 and TOC1, respectively [71]. The PRR ortholog PPD, most similar to PRR7, in *Hordeum vulgare* (PPDH1) [72] and *Triticum aestivum* (PPDA1, PPDB1 and PPDD1, designated after the location they derive from) [73] is important for flowering time control. The PRR7-like BvBTC1 in beet (*Beta vulgaris)* regulates bolting time [74]. *Hordeum vulgare* contains an ELF3 ortholog, EAM8 [75]. *Brassica rapa* retains a suite of clock genes after polyploidization and subsequent gene loss [80].

has a role in coordination of the reactive oxygen species response that occurs each day as part of light harvesting for photosynthesis and the reaction to abiotic stress, such as the response to high salt [23]. Another clock-optimized process is the regulation of plant immunity. The defense of *Arabidopsis* against *Pseudomonas syringae* or insects depends on the time-of-day of pathogen attack [24-26]. Furthermore, genes that are induced upon infection with the oomycete *Hyaloperonospora arabidopsidis*, which causes downy mildew disease, have more CCA1 binding sites in their promoters than expected [27]. *cca1* mutants show reduced resistance when infected at dawn. Since *lhy* mutants are not impaired in disease resistance, this points to a specific effect of the CCA1 clock protein rather than a general effect of the clock [27]. Similarly, the RNA-binding protein *At*GRP7 (*Arabidopsis thaliana* glycine-rich RNA binding protein 7), which is part of a negative feedback loop downstream of the core oscillator, plays a role in immunity [28-30].

Microarray analysis has also contributed to the question of whether there is one clock for all parts of the plant. Plants, unlike animals, do not have their circadian system organized into a master clock situated in the brain and 'slave' clocks in peripheral organs [31]. However, the differential oscillatory patterns of core clock genes in *Arabidopsis* shoots and roots point to a distinct clock in roots that runs only on the morning loop [32].

Post-transcriptional control contributes to rhythms of the transcriptome

Soon after discovering the effect of the clock on transcription, it became apparent that clock-controlled promoter activity does not always lead to detectable oscillations in mRNA steady-state abundance. This was attributable to a long half-life of the transcripts [33]. In *Arabidopsis*, a global search for short-lived transcripts identified a suite of clock-controlled transcripts. For some of these, the mRNA stability changes over the circadian cycle [34]. Corresponding factors that may coordinately regulate the half-life of sets of transcripts are yet to be identified, although candidates include RNA-binding proteins that themselves undergo circadian oscillations [35].

A prominent role for post-transcriptional control in circadian timekeeping was suggested by the long period phenotype of the *prmt5* mutant defective in PROTEIN ARGININE METHYLTRANSFERASE 5 [36-38]. Among the protein substrates of PRMT5 are splicing factors, and

thus PRMT5 has a global impact on splicing. Alternative splicing of the clock gene *PRR9* is affected by loss of PRMT5 and the transcript isoform encoding functional *PRR9* is barely detectable in *prmt5* mutants, suggesting that the circadian defect may partly be caused by changes in *PRR9* splicing [36]. Additional splicing factors that affect circadian rhythms are SPLICEOSOMAL TIMEKEEPER LOCUS1, the SNW/Ski-interacting protein (SKIP) domain protein SKIP, and the paralogous RNA-binding proteins *At*GRP7 and *At*GRP8 [39-41]. Notably, *At*GRP7 and *At*GRP8 form a feedback loop through unproductive alternative splicing and decay of transcript isoforms with a premature termination codon, associating for the first time nonsense-mediated decay with the circadian system [42,43].

In another approach, a high-resolution RT-PCR panel based on fluorescently labeled amplicons was used to systematically monitor alternative splicing of the core oscillator genes [44]. Alternative splicing events were observed 63 times, and of these, at least 13 were affected by low temperature. This suggested that alternative splicing might serve to adjust clock function to temperature changes. More recently, RNA-Seq analyses identified alternative splicing of many clock genes, and an event leading to the retention of an intron in *CCA1* was conserved across different plant species [45]. In the future, a systematic comparison of alternative splicing networks (both for core clock genes and clock output genes) to the corresponding transcriptional programs will unravel the contribution of alternative splicing to the rhythms in transcript and protein abundance.

To date, the extent to which proteins undergo circadian oscillations in the plant cell has not been systematically studied. An initial proteomic study in rice revealed a difference in expression phases between mRNAs and proteins, suggesting regulation at the post-transcriptional, translational and post-translational levels [46]. Uncoupling of protein rhythms from mRNA rhythms has also been observed in mouse liver, where 20% of soluble proteins show a rhythm in protein abundance but only half of them originate from rhythmic transcripts [47].

Noncoding RNAs and the plant clock - a not-so-well defined connection

A prominent class of small noncoding RNAs are microRNAs (miRNAs), which are 19 to 22 nucleotide long single-stranded RNAs that base-pair with mRNA targets and thereby control the level of target transcripts or the level of translation of these mRNAs [48]. miRNAs that oscillate across the circadian cycle have been widely described in mammals and *Drosophila*. In these organisms, miRNAs target clock components and play a role in entrainment or regulation of clock output [49,50].

In *Arabidopsis*, a suite of miRNAs was interrogated for rhythmic expression. Using tiling arrays, miR157A, miR158A, miR160B and miR167D were found to be clock-controlled [51]. On the other hand, miR171, miR398, miR168 and miR167 oscillate diurnally but are not controlled by the clock [52]. The functional implications of these mRNA oscillations are not yet clear. Based on the prominent role miRNAs play in modulating the circadian clock in *Drosophila* or mammals, such a function is to be expected in plants, where miRNAs so far have a demonstrated role only in clock output, such as seasonal timing of flowering [53].

Another class of noncoding RNAs is naturally occurring antisense transcripts (NATs). In *Arabidopsis*, rhythmic NATs were detected for 7% of the protein coding genes using tiling arrays [51]. Among these were the clock proteins LHY and CCA1, TOC1, PRR3, PRR5, PRR7 and PRR9. In the bread mold *Neurospora crassa*, NATs have been implicated in clock regulation. Suites of large antisense transcripts overlap the clock gene *frequency* in opposite phase to sense *frq*. These NATs are also induced by light and thus appear to play a role in entrainment by light signals [54]. A causal role for noncoding RNAs in the plant circadian system has yet to be established.

Forward and reverse genetics to define the core oscillator mechanism

Forward genetic screens of mutagenized plants carrying clock-controlled promoters fused to the LUCIFERASE reporter for aberrant timing of bioluminescence were instrumental to uncover the first clock genes, *TOC1*, *ZEITLUPE* and *LUX/PCL1* [55-58]. Likely because of extensive redundancy in plant genomes, most other clock genes were identified by reverse genetic approaches and genome-wide studies. In fact, up to 5% of transcription factors have the capacity to contribute to proper rhythm generation [59]. A yeast one hybrid screen of a collection of transcription factors for their binding to the *CCA1/LHY* regulatory regions revealed CIRCADIAN HIKING EXPEDITION (CHE) as a modulator of the clock [60].

These CHE studies attempted to bridge TOC1 with the regulation of CCA1/LHY, but failed to fully explain the effect of TOC1 on *CCA1/LHY* expression. Subsequently, chromatin immunoprecipitation (ChIP)-Seq showed that TOC1 directly associates with the *CCA1* promoter, and this interaction is not dependent on CHE [61,62]. Thus, while CHE is not generally seen as a core clock component, its analysis revealed that genomic approaches can feasibly interrogate the capacity of a given transcription factor to modulate clock performance. Genome-wide analysis of *cis*-elements in clock-controlled promoters should identify the motifs that control rhythmic RNA expression of a clock-controlled gene, and this facilitates the identification of the *trans* factors that create such rhythms (Figure 1c).

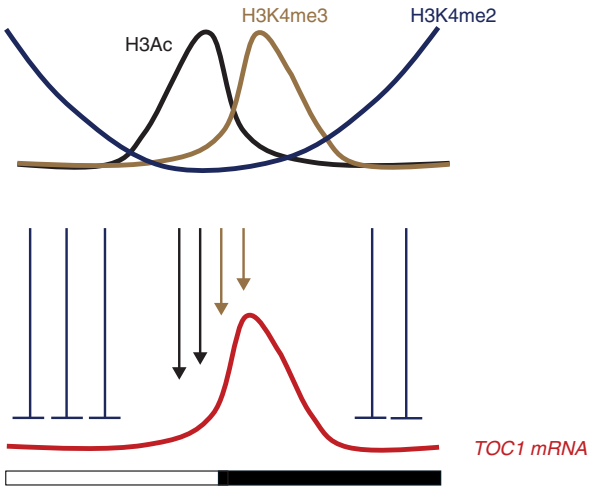

Figure 3. Scheme of the dynamics of chromatin marks and *TOC1* gene expression across the day. The marks for active chromatin, H3Ac and H3K4me3 (top), sequentially correlate with peaks in clock gene mRNA accumulation (bottom), whereas the mark for repressive chromatin, H3K4me2 (top), inversely correlates with *TOC1* mRNA accumulation (bottom). The open bar refers to day, the dark bar refers to night.

ChIP-Seq revealed that PRR5 functions as a transcriptional repressor to control the timing of target genes [63]. It can be expected that the global DNA-binding activity of all core-clock components will be rapidly assembled and this will be associated with the roles of each factor in regulating global transcription, accounting for up to 30% of all transcripts [64].

Epigenetic regulation - a facilitator to rhythmic gene expression?

Rhythmic clock gene transcription is accompanied by histone modification at the 5' ends. For example, in mammals transcriptional activity of the promoters of the *Period* clock genes coincides with rhythmic acetylation of histone H3 lysine 9 that is dependent on the histone acetyltransferase activity of CLOCK [65]. In *Arabidopsis*, it was shown that acetylation of H3 at the *TOC1* promoter is rhythmically regulated, and this positively correlates with *TOC1* transcription [66]. Later, the chromatin of other clock genes, including *CCA1*, *LHY*, *PRR9*, *PRR7* and *LUX*, was additionally found to be rhythmically modulated by multiple types of histone modification [67,68] (Figure 3). The level of the transcription activating marks, acetylation on H3 (H3ac) and tri-methylation on H3 lysine 4 (H3K4me3), increases when these clock genes are actively transcribed, whereas the level of the transcription repressing marks H3K36me2 and H3K4me2 reach their peak when the genes are at their trough [67,68]. These histone modifications are found to be dynamically controlled such that H3 is sequentially changed as H3ac→H3K4me3→H3K4me2 within a rhythmic period [68]. The level of other chromatin marks such as H4Ac, H3K27me3, H3K27me2 and H3K9me3 at the clock gene promoter region does not change rhythmically [67,68].

So far, a number of clock components have been shown to be required to modify histones at the appropriate time. For example, CCA1 antagonizes H3Ac at the *TOC1* promoter [66]. In contrast, REVEILLE8 (RVE8), a MYB-like transcription factor similar to CCA1 and LHY, promotes H3Ac at the *TOC1* promoter, predominantly during the day [69]. However, it is unclear if CCA1 and RVE8 cause the histone modification at the *TOC1* promoter, or if histone modification allows CCA1 or RVE8 to actively participate in regulation of *TOC1* transcription, respectively. The underlying molecular mechanism of the temporal histone modification and components involved are currently elusive. Furthermore, it remains to be shown whether other histone modifications, such as phosphorylation, ubiquitination or sumoylation [70], also contribute to the clock gene expression and change across the day.

Comparative genomics

The availability of an ever-increasing number of sequenced plant genomes has made it possible to track down the evolution of core clock genes. The *Arabidopsis* core oscillator comprises families of proteins that are assumed to have partially redundant functions [1,3]. The founding hypothesis was that the higher-land-plant clock derived from algae. The green alga *Ostreococcus tauri*, the smallest living eukaryote with its 12.5 Mb genome (10% of *Arabidopsis*) has only a *CCA1* homolog, forming a simple two-component feedback-loop with a *TOC1* homolog, the only *PRR*-like gene found in *Ostreococcus* [71]. This supported that the hypothesis that the CCA1-TOC1 cycle is the ancestral oscillator (Figure 2).

Recent efforts to clone crop-domestication genes have revealed that ancient and modern breeding has selected variants in clock components. The most notable examples include the transitions of barley and wheat as cereals and alfalfa and pea as legumes from the Fertile Crescent to temperate Europe. This breeding and seed trafficking was arguably the greatest force in Europe leading the transition from nomadic to civilized lifestyles. It is known that ancestral barley and wheat are what are now called the winter varieties. The common spring varieties arose as late flowering cultivars, which profit from the extended light and warmth of European summers over that of the Middle East. That occurred from a single mutation in barley (*Hordeum vulgare*) in a *PRR* ortholog most similar to *PRR7* termed *Ppd-1* (*Photoperiod-1*) (Figure 2) [72]. In wheat (*Triticum aestivum*), since it is polyploid and recessive mutations rarely have any phenotypic impact, breeders selected promoter mutations at *PPD* that led to

dominant late-flowering [73]. Interestingly, in the beet *Beta vulgaris*, a *PRR7*-like gene named *BOLTING TIME CONTROL1* (*BvBTC1*) is involved in the regulation of bolting time, mediating responses to both long days and vernalization [74]. Evolution at *PRR7* is thus a recurrent event in plant domestication.

As barley (*Hordeum vulgare*) moved north, early flowering was selected in a late-flowering context due to the presence of the spring allele at *ppdh1.* Mutations in the barley *ELF3* ortholog, termed *EAM8* (Figure 2), were selected [75]. Interestingly, the migration of bean and alfalfa to temperate Europe also coincided with *ELF3* mutations [76]. In Asia, rice varieties in domestication have also mapped to the *ELF3* locus [77]. It will be intriguing to assess the genome-wide population structure of clock gene variation as a possible driving force in species migration over latitude and altitude. Genome-wide efforts to explore this show that such studies have merit [78].

One identifying feature of plants within clades of multicellular organisms is the possibility of fertile polyploids. It is speculated that, over evolutionary time, all higher-land plants were at one time polyploid, and indeed, it has been estimated that up to 80% of extant plant species are in a non-diploid state [79]. This raises several confounding features on the genome. For one, in autopolyploids, derived from an expansion of genomes derived from one species, the process of going from 2× to 4× obviously increases the copy number of all genes by twofold. One report to examine this comes from the comparison of the *Brassica rapa* oscillator repertory [80]. On average, it is possible for this species to have threefold more of an individual gene over *Arabidopsis*. However, this is not always the case, as gene loss of these redundant copies has occurred at numerous loci [81]. By examining the probability of gene presence, it has been shown that the retention of clock genes has been more highly favored than the retention of genes randomly sampled from the genome [81]; this was not a linkage disequilibrium effect, as even the neighboring genes, as known by synteny, were retained at a lower rate. Thus, *Brassica rapa* has gained fitness by keeping additional copies of clock genes (Figure 2). Why that is awaits testing.

In allopolyploids that arise from the intercrossing of species, the clock confronts allele choice issues between the potentially conflicting parental genomes. Allopolyploids are common in nature, are often easy to recreate in the lab, and are often more vigorous than the parents. Using a newly generated allopolyploid, the role of the clock in providing a genome-wide fitness was assessed [75,76]. Epigenetic modification at two morning clock genes was found to associate with vigor through regulation of metabolic processes [82]. In subsequent studies, this was further related to stress response pathways in a genome-wide analysis of mRNA decay [83]. Thus, genome-wide polyploidy acts early on clock genes to partition metabolism and stress signaling.

Outlook

High-throughput approaches have greatly advanced our understanding of the pervasive effect of the clock on the transcriptome and molecular underpinnings of rhythms in promoter activity. However, our knowledge of rhythms in protein abundance conferred by subsequent layers of regulation and of small RNA regulation in the plant circadian system is underdeveloped. Comparative genomics among different plant species have pointed to divergences in clock-output processes, and perhaps in the clock mechanism itself. Relating the orthologous function of a given clock protein across the function of the plant genomes will undoubtedly continue to require large-scale genomics.

Abbreviations

*At*GRP, *Arabidopsis thaliana* glycine-rich RNA binding protein; CCA1, circadian clock associated 1; CHE, circadian hiking expedition; EC, evening complex; ELF, early flowering; LHY, late elongated hypocotyl; LUX, lux arrhythmo; NAT, naturally occurring antisense transcript; PRMT5, protein arginine methyltransferase 5; PRR, pseudo-response regulator; RVE8, reveille 8; TOC1, timing of CAB expression 1.

Competing interests

The authors declare that they have no competing interests.

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