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Transcriptional and post-transcriptional control of the plant circadian gene regulatory network

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Abstract

The circadian clock drives rhythms in multiple physiological processes allowing plants to anticipate and adjust to periodic changes in environmental conditions. These physiological rhythms are associated with robust oscillations in the expression of thousands of genes linked to the control of photosynthesis, cell elongation, biotic and abiotic stress responses, developmental processes such as flowering, and the clock itself. Given its pervasive effects on plant physiology, it is not surprising that circadian clock genes have played an important role in the domestication of crop plants and in the improvement of crop productivity. Therefore, identifying the principles governing the dynamics of the circadian gene regulatory network in plants could strongly contribute to further speed up crop improvement. Here we provide an historical as well as a current description of our knowledge of the molecular mechanisms underlying circadian rhythms in plants. This work focuses on the transcriptional and post-transcriptional regulatory layers that control the very core of the circadian clock, and some of its complex interactions with signaling pathways that help synchronize plant growth and development to daily and seasonal changes in the environment.

Keywords: Circadian clock; transcriptional regulation; ChIP-seq; post-transcriptional regulation; alternative splicing.

Abbreviations: Transcriptional-translational feedback loop (TTFL), High-throughput Yeast one hybrid (HT-Y1H), Chromatin Immunoprecipitation (ChIP), Alternative splicing (AS).

1. Introduction

Plants have played an important historical role in the study of circadian rhythms [1]. In fact the first report of a circadian rhythm, i.e. a biological rhythm with an approximately 24 hour period under constant environmental conditions, corresponds to the landmark observation by De Mairan in 1729 of *Mimosa* leaf movements in constant darkness [2]. In addition to the morphological rhythms, plants exhibit numerous circadian oscillations at the physiological and molecular levels. Processes as diverse as stem elongation, stomatal opening and closure and many enzymatic activities are under circadian control [3-6]. Most of these rhythms are now known to be tightly linked to strong oscillations in gene expression in most organisms, a phenomenon that was first characterized at a genome-wide level in plants [7].

Interestingly, evidence that circadian oscillations are genetically encoded was also first obtained in plants, when Bünning reported the heritability of circadian period among the progeny from crosses of parents with distinct period lengths in *Phaseolus* [1]. The modern era of molecular chronobiology, however, began with genetic studies performed in animals several decades later, with the identification of the first mutants with altered circadian rhythms, the *period* (*per*) mutants in *Drosophila melanogaster* [8], and with the cloning and characterization of the *PER* gene [9, 10]. Although plant morphological rhythms are robust, they were not very suitable for high-throughput genetic screens and, therefore, the molecular-genetic era of plant chronobiology was delayed until the development of promoter::luciferase fusions that allowed researchers to simultaneously monitor rhythms in gene expression in real time and *in vivo* in thousands of individual plants. This approach led to the identification of the first plant clock mutant, the *Arabidopsis timing of cab1 expression* (*toc1*) mutant [11]. In parallel, two *Arabidopsis* MYB transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) were identified through the molecular and genetic characterization of light regulated biological processes that are known to be controlled by the circadian clock, and were characterized as core-clock genes [12, 13]. Later on, the *PSEUDORESPONSE REGULATOR 1 (PRR1)/TOC1* gene was cloned and, in 2001, the initial characterization of the mutual interactions between that gene and the two MYB transcription factors, *CCA1* and *LHY,* helped shape the first molecular model of the plant circadian clock [14, 15]. In the past 15 years, we have witnessed a myriad of new discoveries in several aspects of the plant circadian clock, ranging from new components constituting multiple interconnected feedback loops operating at different regulatory layers [16], to mechanistic insights into the organization of the circadian network at the whole organismal level [17-21].

In this review we describe how the frontier of knowledge associated with the plant circadian gene regulatory network has expanded in recent years through the advent of new technologies in the fields of genomics (high throughput yeast one-hybrid screens and ChIP-seq), transcriptomics (first microarrays and more recently RNA-seq) and proteomics (mass spectrometry) [22], in combination with the early adoption of systems biology approaches and mathematical modeling [23]. We apologize to colleagues whose work has not been included in this review due to space constraints.

2. Transcriptional Regulation within the Plant Circadian Network

2.1. Transcriptional-translational regulatory circuits operating at the core of the clock

The classical genetic nature of the foundational studies on circadian clocks, coupled with the observation that many of the first clock genes identified regulated their own expression [12-14], led to the assumption that circadian rhythms were the result of a simple transcriptional-translational feedback loop (TTFL) mechanism, composed of the products of a few rhythmically expressed genes [24]. Nowadays, this concept has evolved and circadian oscillators are believed to be composed by multiple interlocked TTFLs, creating a much more complex scenario [25]. In fact, this already complex scenario is getting even more complex due to the growing evidence that post-transcriptional as well as non-transcriptional regulatory layers are strongly associated with circadian timekeeping [26-28]. Finally, an additional layer of complexity is added by the finding that "peripheral" clocks present in each cell can specifically process environmental cues and regulate individual physiological responses, but at the same time, they can be modulated by "master" clocks present in specific tissues [17, 20, 21]. In mammals, the master clock lies within the suprachiasmatic nucleus and through neuro-humoral signals it can modulate the peripheral clocks present in other tissues throughout the body [29]. In plants, recent evidence points to the shoot apical meristem as the tissue were the master clock resides [20].

2.2. A journey to the center of the clock: an historical account on the dissection of transcriptional regulation of the plant core clock genes

The first molecular insights of clock controlled transcription were made in the late 80's, when several groups described the circadian fluctuations in the levels of mRNAs encoding the light-harvesting chlorophyll a/b-binding proteins (CAB) of photosystem II [30, 31]. At that time, there was clear evidence indicating that the circadian clock regulated the expression of *CAB* genes at the transcriptional level, but there was no hint as to the molecular nature of the plant circadian clock itself [31].

As described above, it was not until the discovery and characterization of CCA1 and LHY, and the pseudo-response regulator TOC1/PRR1 that the initial model of the plant circadian clock was proposed. This early model consisted of a single negative feedback loop in which CCA1 and LHY (whose levels are light regulated and peak in the morning) act as direct repressors of *TOC1* by directly binding to a 9bp "Evening Element" (EE, AAAATATCT) present in the *TOC1* promoter. In turn, TOC1 (whose expression peaks in the early evening) was proposed to promote *CCA1* and *LHY* expression through an unknown mechanism (Figure 1A) [15]. Although the initial model of the plant circadian clock explained many of the observations regarding the pattern of expression of

these core clock genes in wild type plants, in plants overexpressing *LHY* and *CCA1*, as well as in the *toc1* mutant, further studies suggested that the model was incomplete. In particular, while *cca1;lhy* double mutants appeared to be arrhythmic after two days in continuous white-light conditions, plants carrying a loss-of-function *TOC1* allele retained significant rhythmicity (although with a shortened period) under the same conditions, suggesting that other genes besides *TOC1/PRR1* were involved in the generation of the oscillations [32-34]. This observation stimulated the analysis of the role in circadian rhythmicity of other genes of the pseudo-response regulator family: *PRR3*, *PRR5*, *PRR7* and *PRR9* [35]. The analysis of the PRR family members quickly bore fruits, and the combination of additional experimental studies and mathematical modeling gave rise to a new feedback loop called the "morning loop". According to this expanded model, a new TTFL was established early in the morning in which CCA1 and LHY directly bind to the promoters and activate the expression of *PRR7* and *PRR9*, which encode repressors of *CCA1* and *LHY* [36, 37]. This loop was further supported by subsequent mathematical modeling, predicting in fact a third loop, called the "evening loop", composed by TOC1 and GIGANTEA (GI) [38, 39].

In addition to the MYB transcription factors and pseudo-response regulators that made up the initial plant clock model, two additional core clock components, EARLY FLOWERING 3 (ELF3) and EARLY FLOWERING 4 (ELF4), were identified in genetic screens aimed at identifying new flowering time regulators [40-42]. The proteins encoded by these two genes are plant specific and exhibit no known functional domains, and the corresponding mutants not only flower early irrespective of photoperiodic conditions, but are also arrhythmic and have long hypocotyls [41-43]. Likewise, LUX ARRHYTHMO (LUX, also called PHYTOCLOCK 1), a single-MYB domain DNA binding protein that belongs to the GARP protein family, was identified as a novel clock component in a genetic screen for long hypocotyl mutants with extensively altered circadian rhythms [44, 45]. The similar phenotypes of the *elf3*, *elf4* and *lux* mutants, coupled with the observation that the corresponding genes display similar expression profiles, peaking in the evening, prompted a detailed analysis of their relationships. A series of very elegant experiments revealed that these three proteins assemble into a large protein complex called the Evening Complex (EC) [46-48], which directly binds through LUX to the *PRR9* promoter and to the *LUX* promoter itself, and functions as a transcriptional repressive complex that defines an additional loop at the core of the clock [49, 50].

2.3 Revisiting the model

The inclusion of the EC to the circadian clock core in 2011 established a new clock model based on three TTFLs. However, further studies on TOC1 activity described in 2012, imposed a major shift to this model. Previous models of the plant circadian clock proposed that TOC1 was an activator of *CCA1* and *LHY.* This was based on the reduced mRNA levels of these genes observed in the strong *toc1-2* mutant allele [15]. Nevertheless, the analysis of TOC1 overexpressing plants also revealed reduced rather than enhanced levels of *CCA1* and *LHY* [51, 52]. Furthermore, overexpression of TOC1 was also found to severely repress *PRR9* mRNA levels, suggesting that the TOC1 protein might have a negative transcriptional regulatory effect on gene expression [51]. In agreement with this last possibility, a repressive role of PRR5 over *CCA1* and *LHY* expression was later demonstrated [53]. Thus, it was concluded that the progressive decline in *CCA1* and *LHY* expression from midday to the late afternoon is the result of the repressing activities of PRR9, PRR7 and PRR5, which are rhythmically expressed with a slight phase delay in their peak expression (*PRR7* expression peaks a few hours later than *PRR9* and *PRR5* later than *PRR7*). A thorough and detailed analysis conducted more recently finally proved that TOC1 binds to the promoters of several clock genes, including *CCA1*, *LHY* and *PRR9*, and is indeed a transcriptional repressor. This was confirmed with a combination of approaches that included chromatin immunoprecipitation (ChIP), ChIP followed by deep sequencing (ChIP-seq) and gene expression analysis using hormone and ethanol-inducible *TOC1* expressing plants, leading to the observation that TOC1 actually prevents the activation of morning expressed genes at night [54-56]. In fact the experiments that led to the revision of TOC1 function had profound bearings on our current understanding of the clock regulatory network.

As a result of the insights resulting from the TOC1 characterization, the inclusion of the EC as a key component of the circadian core-clock network, and the establishment of the morning loop composed by PRR7 and PRR9, a new model known as the “three-component repressilator” was built. This model was based on three repressive elements: CCA1/LHY, the EC, and the family of PRRs (TOC1/PRR5/PRR7/PRR9) [56] (Figure 1B).

Recently the hypothesis that claims that CCA1 and LHY act as activators of *PRR9* and *PRR7* expression has also been challenged. Indeed, transcriptional profiling of plants in which the levels of CCA1 or LHY proteins were transiently increased using an inducible system clearly indicates that these proteins repress rather than induce *PRR9* and *PRR7* [57, 58]. The apparent positive effects of CCA1 and LHY on *PRR9* and *PRR7* expression described previously are therefore likely to be the

indirect result of their repressive effects on the transcription of members of the EC, such as *ELF4* and *LUX*, which in turn repress *PRR9* and *PRR7* expression.

The final demonstration that TOC1 acts as a repressor rather than as an activator of *CCA1* expression constitutes an excellent lesson revealing the challenge of delineating the precise wiring of a complex transcriptional regulatory network with multiple interconnected loops. In particular, it is very clear that the wiring of complex gene regulatory networks, such as those associated with circadian rhythms, cannot be deduced only through the analysis of changes in the steady state mRNA levels of individual network components in mutant and/or overexpressing plants that also affect the levels of other key components. To be useful, this information must be complemented and integrated with the analysis of protein-DNA interactions and with studies evaluating the immediate effects of the controlled induction of these components on the transcriptome

2.4. Overcoming the "dearth of activators": The emergence of transcriptional positive elements in the clock

Although the repressilator model performed well in fitting the available experimental data, it was likely an oversimplification due to the absence of positive factors contributing to the generation and sustenance of circadian rhythms [59, 60]. This apparent gap in the model was recently filled by the observation that REVEILLE 8 (RVE8), REVEILLE 6 (RVE6) and REVEILLE 4 (RVE4), three of the eleven members of the CCA1/LHY/RVE family of single MYB transcription factors, act as activators of evening clock genes. Other members of this gene family, such as *RVE1*, *RVE2* and *RVE7*, in contrast, affect clock outputs including the regulation of cell elongation, but not the clock itself [61-63]. The first evidence that RVE8 could be involved in clock regulation resulted from the observation that *rve8* mutants displayed period lengthening, while RVE8 overexpression shortened the circadian period [64, 65]. This, coupled with the evidence obtained through a combination of proteomic and biochemical approaches, revealed that the RVE8 protein binds to the EE in the promoters of evening-expressed genes in the afternoon, strongly suggesting that RVE8 could be acting by promoting the expression of evening phased clock genes [64]. Indeed, a direct role for RVE8 as an activator of evening clock genes was finally demonstrated using an inducible RVE8 system, which revealed hundreds of evening expressed genes with an EE element in their promoters, including *PRR5* and *TOC1*, whose expression was enhanced upon activation of the RVE8 protein in the absence of protein synthesis [66]. Interestingly, the use of a proteomic approach and a protein microarray containing 802 transcription factors , had already revealed that

RVE4, RVE6, together with RVE3 and RVE5 also bind to the EE motif, thereby raising the possibility of functional redundancy among members of the RVE family [64, 67]. This hypothesis was later corroborated by the observation that, in contrast to the modest 1 h period lengthening in single *rve8* mutants, triple *rve4;rve6;rve8* mutants display a 4 h period lengthening of rhythms [66].

More recently, in an effort to find components that link the photoreceptors to the circadian clock, two novel night light-inducible and clock regulated (LNK) transcriptional regulators were discovered: LNK1 and LNK2. Light perceived by the phytochrome photoreceptors regulates the expression of both *LNK1* and *LNK2* in the morning. These genes then promote the expression of a subset of afternoon genes, including the core clock genes *PRR5*, *TOC1* and *ELF4*. In turn, PRR9, PRR7, PRR5 and TOC1 bind to the LNK's promoters to block their expression from noon to the early evening [68, 69]. Thus a negative feedback loop is hereby established between the LNKs and the PRRs, in which the LNKs act as transcriptional activators of some PRRs (*PRR5* and *TOC1*) and the PRRs feedback to repress the expression of LNKs. Furthermore, the LNKs are not only involved in transducing the light signals to the clock; they also play an important role mediating temperature effects. *LNK1*, in addition to being a night light-induced gene, is also induced by warm temperatures at night through the EC in a similar manner to *PRR7* and *GI*, which are also night light-inducible genes. This evidence points to *LNK1*, *PRR7* and *GI* as possible integrators of light and temperature signals at the core of the circadian clock to help keep track of seasonal changes in photo and thermo-cycles [70].

Although LNK1 and LNK2 were proposed to act as transcriptional activators, whether this activity was direct or indirect was not known [69]. Interestingly, LNKs do not exhibit any known DNA binding domain. LNK1 and LNK2 interact with the Myb transcription factors CCA1, LHY, RVE4 and RVE8 [71, 72] and ChIP assays demonstrated that LNK1 is recruited to the *PRR5* and *TOC1* promoters via interactions with RVE8 and RVE4, two *bona fide* DNA binding proteins. Furthermore, the fact that an increase in *PRR5* mRNA abundance was still observed (although to a lesser extent) after RVE8 induction in the *lnk1;lnk2* background, suggested that additional co-activators should interact with RVE8 to regulate *PRR5* expression [71]. With the discovery of the RVEs, a new model of the *Arabidopsis* circadian clock was proposed, a four-component quadripressilator circuit where RVE8 acts as an activator [23]. We added the activator role of the LNKs to this model, thereby getting a ring of four repressors with RVEs and LNKs as activators (Figure 1C).

It is worth noting that an antagonistic role between RVE8 and LNKs in the anthocyanin biosynthesis regulation was also reported recently. While RVE8 up-regulates anthocyanin biosynthesis gene expression by directly associating to the promoters of these genes at dawn, this up-regulation ceases at midday by the repressive activity of the LNK proteins. In this case, chromatin immunoprecipitation assays demonstrated that binding of RVE8 to target promoters is precluded by LNK proteins [72]. This evidence shows that the regulatory activity of RVE8-LNK interaction switches from a synergistic co-activating role of the regulation of core oscillatory genes to a repressive antagonistic role in the regulation of circadian clock outputs such as anthocyanin biosynthesis.

Other proteins with a potential activating role within the plant circadian clock network are LIGHT-REGULATED WD1 (LWD1) and LWD2. The *Arabidopsis lwd1;lwd2* double mutants display early flowering, a shortened circadian period and many clock genes exhibit alterations in their expression phases [73]. Further analysis demonstrated that LWD1 associates with the promoters of *PRR9*, *PRR5* and *TOC1* in vivo, and a positive feedback loop was proposed between LWD1 and PRR9, even though a direct interaction between PRR9 and the LWD1 promoter has yet to be demonstrated [74].

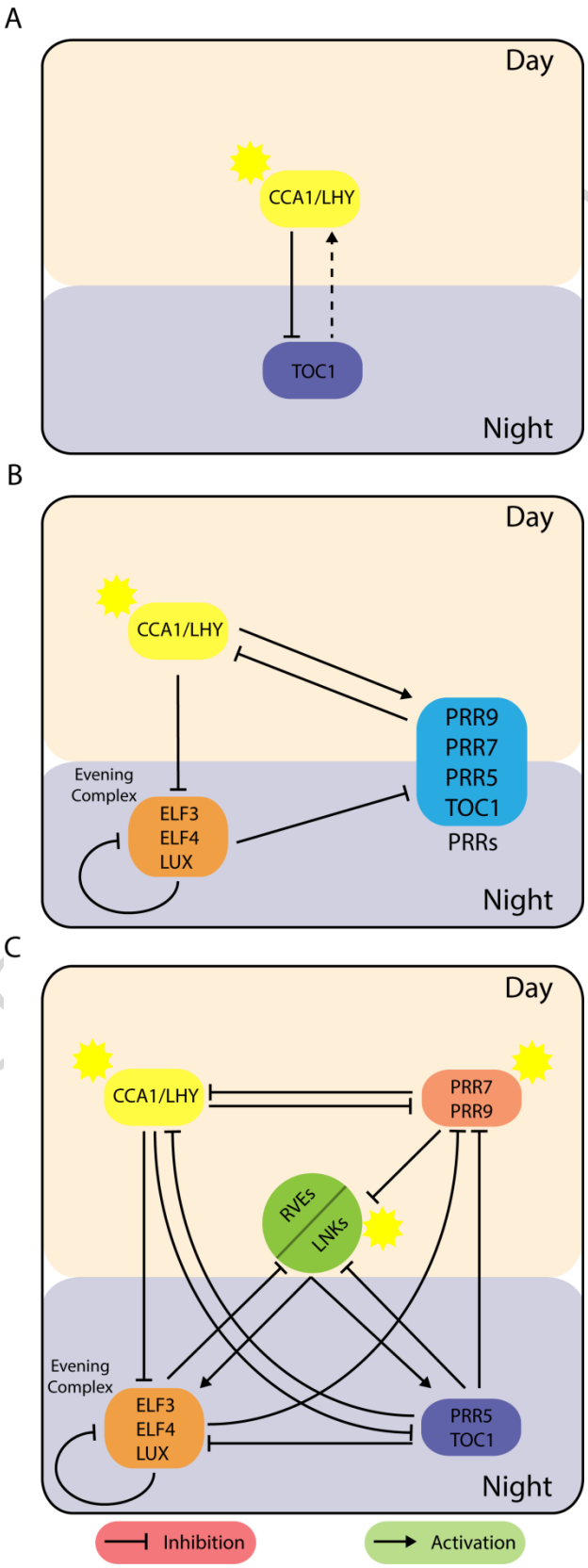


Figure 1. Evolution of the circadian clock model. A) The first *Arabidopsis* circadian clock based on a simple TTFL mechanism, proposed in 2001 [15]. B) The 2011 Arabidopsis circadian clock circuit modeled as a three component repressilator [56]. C) An updated model based on the four-component quadripressilator clock circuit [23]. This model exhibits a ring of four repressors, with RVEs and LNKs as activators. The sun icon indicates light regulation of gene transcription.

2.5 Expanding the frontiers of the clock: the impact of high- throughput technologies in the study of the plant circadian gene regulatory network

Forward genetic screens were essential to identify key clock gene components in *Arabidopsis*. The use of bioluminescent reporters coupled with the characterization of clock-regulated phenotypes such as hypocotyl growth and flowering time, paved the way to the identification of more than 30 clock-associated genes. However, the genetic redundancies that confer robustness to the clock architecture had largely hindered new discoveries using this kind of approaches [75, 76]. High-throughput genomic techniques, combined with reverse genetic approaches, have revealed new components and new layers of regulation within the plant circadian clock network.

To better understand gene regulatory networks associated with the circadian clock, an attempt to uncover direct regulators of core clock genes, high-throughput yeast one-hybrid (HT-Y1H) was developed [77]. This approach allows the characterization of the repertoire of transcription factors (TFs) that can bind a single DNA region of interest (Figure 2A). This approach, termed "Promoter Hiking", was first used to evaluate TFs binding to regulatory regions within the *CCA1* promoter and resulted in the discovery of CCA1 HIKING EXPEDITION (CHE), a TCP transcription factor that represses *CCA1* expression [78]. Furthermore, CCA1 also binds to the *CHE* promoter to repress transcription thereby forming a novel reciprocal feedback loop. In addition to CHE, this approach also identified a bHLH transcription factor, FLOWERING BASIC HELIX-LOOP-HELIX 1 (FBH1), which binds *in vivo* to the *CCA1* promoter [77]. FBH1 was found to have a positive effect on the amplitude of *CCA1* oscillations and to modulate warm temperature effects on the clock, and FBH1 overexpression causes period shortening of *CCA1* expression upon temperature changes [79]. Yeast and *in planta* assays demonstrated that FBH1 binds to a non-canonical E box-like motif, CACTAG, present in the *CCA1* promoter and, in turn, CCA1 also binds and regulates *FBH1* expression, constituting an additional feedback loop [77, 79]. When the same Y1H technique was applied using the *PRR7* promoter as bait, it was found that HsfB2b, a member of the Heat Shock Factor (HSF) family, binds and negatively regulates *PRR7* through its binding to two of the nGAAn Heat Shock Elements (HSE) present in the *PRR7* promoter. Remarkably, this factor appears to

mediate heat and salt stress signals into the clock. The loss of HsfB2b under those conditions results in a short circadian period phenotype and, interestingly, HsfB2b is also involved in modulating temperature compensation and temperature resetting of the clock [80]). Finally, the application of the HT-Y1H approach with the *LUX* promoter sequence as bait revealed that the cold-associated transcription factor CBF1/DREB1b transcriptionally regulates *LUX* by binding to a cold-inducible C-repeat (CRT)/drought-responsive element (DRE) present in the *LUX* promoter, thus integrating cold signals into clock function. Through the analysis of different genetic constructs based on the *LUX* promoter, the authors also found that the EE and the CRT element are sufficient to recapitulate the expression of the endogenous *LUX* gene in the cold. Furthermore, CBF1 overexpression results in the upregulation of *LUX* expression. Freezing tolerance is disrupted in *lux* mutants, revealing that these interactions define new transcriptional mechanisms through which temperature modulates clock function and the ability of plants to withstand abiotic stress conditions [81].

Another high-throughput genomic approach used to expand our understanding of the wiring of the circadian transcriptional network in plants is genome-wide chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) (Figure 2B). In contrast to the HT-Y1H approach that facilitates the characterization of the transcription factors that recognize a specific DNA region, this approach allows the identification of all the genomic regions bound, directly or indirectly, by a specific protein. The first clock protein for which ChIP-seq analysis was conducted was TOC1 [55]. As a result of that study, direct targets of TOC1 were identified among the morning phased clock genes *CCA1*, *LHY*, *PRR7* and *PRR9*, and evening phased clock genes such as *PRR5, GI*, *ELF4, LUX* and *TOC1* itself. Analysis of TOC1-bound sequences in the promoters of clock genes identified two significantly enriched motifs: a GBox-expanded, (A/C)C(A/T/G)CG(T/C), and an EE-like motif, (A/T/G)AA(T/G)ATC(T/G/C), which were then confirmed by ChIP-qPCR screening. In order to further inspect the role of TOC1 in clock output control, the reported targets were analyzed for GO enrichment and genes associated to diverse processes, including abiotic/biotic stimulus, response to stress, development, and nucleic acids metabolism were found.

A similar approach was then used to study the targets of PRR5 and PRR7 [68, 82]. The study by Liu et al of the PRR5 targets led to the finding that PRR9, PPR7 and PRR5 constitute a genetic network that directly regulates the timing of expression of key transcription factors to coordinate multiple plant physiological processes [68]. Interestingly, this study also provided further evidence for the

interaction between PRR5 and the circadian clock positive element RVE8. The PRR7 ChIP-seq analysis resulted in the identification of 113 putative targets, whose expression levels peak around dawn in antiphase to PRR7 protein levels, and are repressed by PRR7. This stressed the important role of PRR7 as a transcriptional repressor of morning-expressed genes. Among the PRR7 targets, abiotic stress response genes and oxidative stress response genes, such as cold regulated genes, drought and ABA responsive genes, and genes involved in iron excess adaptation were found [82]. ChIP-seq analysis of PRR9 targets allowed the development of a global picture of the PRR family targets. This study showed that the different PRRs share a large number of binding sites and associate with conserved *cis*-regulatory regions in open chromatin. Most PRR binding regions are shared by at least two PRRs. This broadly correlates with the similarity in gene expression phases found among the different PRR's targets. PRR binding regions were highly enriched in a G box related motif, each slightly different for TOC1, PRR5, PRR7 and PRR9, and it was proposed that this motif is necessary for transcriptional regulation by the PRRs. Nevertheless there is no evidence that PRRs are able to directly bind to G-box motifs, opening up the possibility that PRRs interact with other factors to regulate gene expression [83]. In support of this idea, Yamashino et al found several years ago that TOC1 interacted with PIF3, PIF4, PIL1 PIL2, PIL5 and PIL6, which are basic helix-loop-helix (bHLH) transcription factors that belong to the PHYTOCHROME INTERACTING FACTOR (PIF) family [84]. Additionally, very recently Soy et al, showed that the TOC1 interaction with the PIFs repressed their transcriptional activation activity, antagonizing PIF-induced growth. These studies constitute solid evidence indicating that the PRRs interact with transcription factors to regulate gene expression [85]. Also, it is worth noting that several target genes were non-cycling. This lack of rhythmicity could be explained by phase diversity among different tissues or could also imply a much larger scope of action of these clock components as modulators of downstream signaling processes. Previous work using a microarray approach demonstrated that PRRs are involved in many biological processes, including circadian rhythms, biotic stimuli, response to cold stress, drought stress, blue, red and far red light response, development, protein metabolism, signal transduction and electron transport. The triple *prr5;prr7;prr9* mutant used in that study was found to be resistant to cold, salt and drought stress and had previously been shown to be arrhythmic, exhibit late flowering under long day conditions and extremely hyposensitive to red light [86], which is in accordance to the microarray results [87]. The work by Liu et al found that over 33% of the PRR9 putative targets were affected in the *prr5;prr7;prr9* triple mutant [83]. Taking advantage of the existence of these new datasets we compared the

microarray data with the combined targets of PRR5, PRR7 and PRR9 from each ChIP-seq report, and were able to identify 94 gene targets in common, which were involved in circadian rhythms, response to light, response to radiation, regulation of transcription, RNA biogenesis.

More recently, two independent studies have reported the results of a CCA1 ChIP-Seq experiment [57, 88]. In the report by Nagel et al, more than 1000 genomic regions were identified as direct targets of CCA1 in CCA1p::CCA1-GFP expressing WS plants. These targets included genes involved in cellular metabolic processes, such as nucleotide metabolism, nitrogen compound metabolism, regulation of transcription and gene expression, sugar metabolism, and also abiotic and biotic stress response. Remarkably, although many targets contained an EE element and were evening phased, as expected, many targets did not cycle and a significant subset was morning expressed, revealing new targets of CCA1 regulation. Additionally, three new motifs were found to be associated with the morning subset, one of which was highly similar to the G and E Box motifs: N(C/A/T)(C/A/G)(A/T)(C/A)(T/G/A)T(G/A)(T/G)(C/A/T), suggesting interactions with bHLH transcription factors. The more recent report by Kamioka et al was performed in *cca1;lhy* mutant (Col-0 background) plants expressing a CCA1p:CCA1-FLAG construct. This report found that CCA1 associated with at least 449 loci, of which 254 were also found in the Nagel dataset. It also found the existence of a morning subset. Furthermore, this report also demonstrated that CCA1 directly determines the repression state of PRR5 by direct binding to three distinct regions in the promoter of this PRR, and that this regulation is epistatic to LNK1 activity [57].

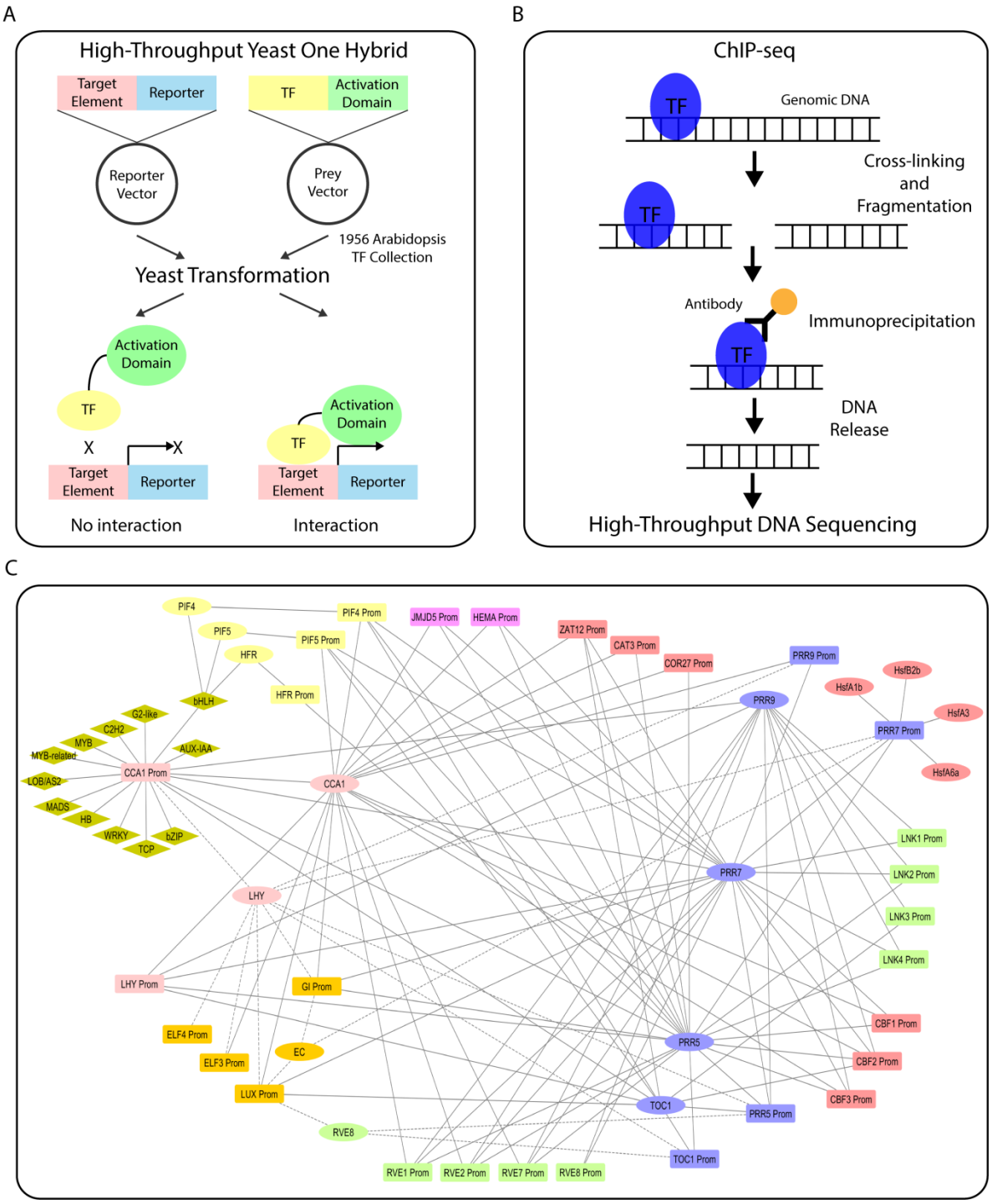


Figure 2. Impact of high-throughput technologies in the study of the plant circadian gene regulatory network. A) Scheme of the High-Throughput Yeast One Hybrid system (HT-Y1H) used to discover interactions between a promoter of interest (Target Element) and transcription factors (TFs). A comprehensive collection of *Arabidopsis* TFs is analyzed [77]. B) Scheme of the ChIP-seq (Chromatin Immunoprecipitation followed by high throughput DNA sequencing) used to discover direct interactions between a TF and its target DNA sequences. C) Circadian clock interaction

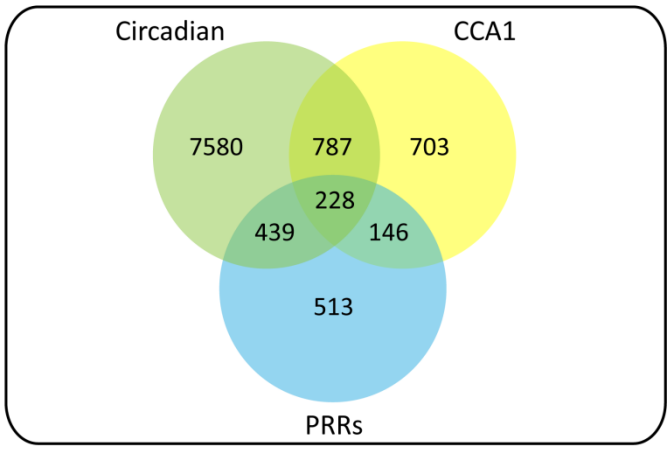
network built from the HT-Y1H and ChIP-seq avalilable data. The gene promoter regions are showed as rectangles while the proteins are displayed as geoids. Brown diamonds: Trancription factors that bind the *CCA1* promoter, Pink: clock core MYB- transcription factors, Blue: Pseudoresponse regulators family, Orange: Evening loop components, Green: Circadian clock proposed activators, Yellow: light signaling related genes, light red: stress response related genes, Violet: clock related genes with unknown roles within the circadian system [89] . Full line: Confirmed interaction by HT-Y1H or ChIP-seq evidence [55, 57, 68, 77, 80-83, 88]. Dotted line: ChIP interaction evidence [58, 64, 90].

We integrated the data of high-throughput experiments reported to date into a network that displays the wiring diagram of the circadian transcriptional network in plants (Figure 2C). It shows the multiple TTFLs that interlock the circadian clock core components and also helps us visualize its connections to several circadian clock outputs, such as light signaling and stress related genes. At the same time, it highlights the fact that further work is needed to unveil new EC, RVEs and LNKs targets. Additionally, we searched for each of the target genes described in all of these ChIP-seq reports in a comprehensive circadian dataset combined from two publicly available datasets [91, 92] and found that several coincide with clock-controlled outputs (Figure 3A). When considering all PRR's targets we found that little over 50% of these genes are also circadian regulated. This percentage is a little higher, 54.45%, when considering the combination of both CCA1 available datasets (Figure 3B). It is worth noting that phase analysis of the targets using Phaser (http://phaser.mocklerlab.org/) revealed, as expected, that most of the clock-controlled targets of PRRs are close-to-dawn phased and that CCA1 targets are evening phased. Also, 228 genes were found to be circadian regulated and bound by both PRRs and CCA1. The phase of these genes appears to be evenly dispersed along the circadian cycle.

It would be very interesting to perform ChIP-seq analyses of the rest of the circadian clock elements and perform transcriptome correlation analysis, co-regulated genes identification, validation and discovery of novel *cis* elements. As we have described above, MYB domain containing factors modulate transcription mainly through binding to the EE element, and PRRs act through interaction to G-box elements, although the ChIP-seq evidence points out to other *cis*-elements being also involved. However this interaction may be indirect, so further experiments are needed to study whether the MYB domain factors or PRRs directly bind to the novel elements or act indirectly through interaction with other proteins that bind the *cis* elements. Also, little is known on downstream secondary regulation cascades that involve clock-regulated transcription

factors that do not affect clock function. The study of these cascades will enhance our current understanding of clock-controlled processes.

A



B

	Unique Targets	Circadian Regulated	Percentage of total (%)	Reference
TOC1	772	374	48.45	Huang et al, 2012
PRR5	518	271	52.32	Nakamichi et al, 2012
PRR7	113	82	72.57	Liu et al, 2013
PRR9	132	94	71.21	Liu et al, 2016
Combined PRRs	**1326**	**667**	**50.30**	This work
CCA1 (DS1)	1306	776	59.42	Nagel et al, 2015
CCA1 (DS2)	863	436	50.52	Kamioka et al, 2016
Combined CCA1	**1864**	**1015**	**54.45**	This work

Figure 3. Circadian regulated binding targets of CCA1 and PRRs. A) Venn diagram comparing circadian regulated genes (union of Covington + Edwards and Hsu datasets [91-93]), the combined PRRs targets and the combined CCA1 targets. B) The table summarizes the number of unique targets, number of circadian regulated targets and the percentage of circadian regulated targets of each ChIPseq experiment, encompassing the PRRs and CCA1 datasets (DS1: Data Set1 [88], DS2: Data Set 2 [57]).

2.6 Molecular mechanisms underlying transcriptional regulation: the role of chromatin remodeling and histone modifications.

Gene expression is regulated at several levels in eukaryotic organisms. Chromatin remodeling factors modulate the availability of promoter regions, allowing their recognition by transcription factors that recognize specific *cis*-acting elements and then recruit general transcription factors as well as the RNA polymerase II (Pol II) to the transcription start site of their target genes. Most ChIP-seq analysis conducted with plant core-clock transcription factors reveals that these proteins

are indeed enriched in the vicinity of transcription start sites. One way clock-associated transcription factors could further modulate transcription is by recruiting additional co-activators and/or chromatin regulators to control rhythmic transcription. Indeed, the expression of many core-clock genes in plants appears to be modulated by alterations in the chromatin state, which is achieved, in part, by post-translational modifications of histone proteins present in their promoter regions, which in turn modulate the accessibility of transcriptional regulatory proteins. For example, histone acetylation at the *TOC1* promoter exhibits a circadian regulated pattern, and it has been proposed that CCA1 binding to the *TOC1* promoter at dawn blocks the accessibility of histones acetyltransferases (HATs) to this region. During the day the binding of CCA1 decreases and HATs are recruited to the *TOC1* promoter enhancing its expression through histone acetylation [94, 95]. Interestingly, a role for RVE8 in the acetylation state of the *TOC1* promoter has also been demonstrated. RVE8 binds to the *TOC1* promoter during the rising phase of *TOC1* expression, facilitating histone acetylation. While CCA1 favors histone hypoacetylation at the *TOC1* promoter, RVE8 leads to H3 hyperacetylation, thus antagonizing CCA1 repressing activity [65]. Although the precise mechanism through which CCA1 and RVE8 modulate histone acetylation at the *TOC1* promoter is not known, the PRRs have been recently shown to repress circadian expression of *CCA1* by interacting with TOPLESS/TOPLESS RELATED (TPL/TPR) proteins, members of the Groucho/Tup1 corepressor protein family, which then form a larger protein complex that includes the PRRs and HISTONE DEACETYLASE 6 (HDA 6) [96]. Oscillatory histone marks associated with the regulation of gene expression are not confined to *TOC1.* In fact, histone acetylation (H3K56ac and H3K9/14ac) and methylation (H3K4me3) are involved in the rhythmic expression of *LHY*, *CCA1*, *TOC1*, *PRR9*, *PRR7* and *LUX*, and the histone methyltransferase SDG2 has been proposed to mediate at least some of these effects [97-99]. Besides lysine methylation, arginine methylation is also known to modulate gene expression in many organisms. Protein Arginine Methyltransferase 5 (PRMT5) is a member of a protein family that catalyzes arginine methylation, a common post-translational modification that occurs in eukaryotes and regulates a myriad of processes through its effects on proteins involved in the modulation of chromatin structure, transcription, RNA processing, signal transduction and cellular differentiation, among other processes [100-105]. PRMT5 has been shown to modulate circadian rhythms in both plants and flies. Although most of the effect of PRMT5 on circadian rhythms appears to be linked to its role on alternative splicing (see below for more details), the possibility that part of its effect occurs through histone methylation cannot be excluded [106-108]. Finally, in addition to histone

methyltransferases, the histone demethylase Jumonji C domain- containing protein D5 (JMJD5) is also involved in circadian clock control. The expression of *JMJ30/JMJD5* is regulated by direct binding of CCA1 and LHY to its promoter, displaying a peak of expression at dusk. At the same time, JMJ30/JMJD5 promotes the expression of *CCA1* and *LHY*. Furthermore, the human and the *Arabidopsis* JMJ30/JMJD5 orthologs rescue the circadian phenotypes of the mutants in both plants and human U2OS cell lines, suggesting a common and conserved function [109-111].However, whether the effects of JMJD5 homologs on the plant circadian clock depend on its histone demethylase activity remains to be determined.

When the architecture of the circadian gene regulatory network was studied in the mouse liver at a genome scale through the characterization of time-dependent patterns of transcription factor binding, RNA pol II recruitment, RNA expression, and chromatin states, only 22% of mRNAs of cycling genes were found to be driven by de novo transcription, suggesting that both transcriptional and post-transcriptional mechanisms underlie the mammalian circadian clock [112]. Although similar genome-wide studies have yet to be conducted in plants, there is plenty of evidence indicating that post-transcriptional regulation plays a significant role in modulating the operation of the plant circadian gene regulatory network.

3. Post-transcriptional regulation

Once transcription has been initiated, the nascent pre-mRNA experiences a series of processing steps to finally generate a mature mRNA [113]. Quickly after transcription initiation takes place, the nascent pre-mRNA receives the 7-methylguanosine cap at its 5’ end to protect the mRNA against degradation. The pre-mRNA is also processed by splicing to remove introns, i.e. sequences that will not appear in mature mRNA. The possible usage of alternative splice sites can give rise to different transcripts from a single pre-mRNA [114, 115]. Then, polyadenylation signals determine the specific position for cleavage and addition of the poly(A) tail that protects the mRNA from being degraded from its 3’ end [116]. Finally, the mature mRNA is exported from the nucleus to the cytoplasm for translation. All these post-transcriptional processing steps are suitable for regulation and have been extensively studied in plants [117, 118]. There is an overwhelming amount of information linking the regulation of these post-transcriptional processes to the proper function of the plant circadian clock [26, 119, 120].In addition to the regulation of these processing steps, non-protein coding RNAs (ncRNAs), in particular long ncRNAs, and microRNAs (miRNAs) are known to be major regulators of gene expression. In plants, some lncRNAs have as their natural

antisense targets CCA1, LHY, TOC1, PRR3, PRR5, PRR7 and PRR9 [121]. Furthermore, 114 annotated *Arabidopsis* miRNAs were shown to display circadian rhythmicity [122]. This suggests a greater level of post-transcriptional regulation of the plant circadian clock.

3.1 The role of alternative splicing

The process of pre-mRNA splicing is catalyzed by a molecular complex called the "spliceosome", a dynamic complex formed by five small nuclear ribonucleoproteins particles (snRNPs):U1, U2, U4, U5 and U6; along with a variety of auxiliary proteins [123]. The spliceosome assembles on exon-intron boundary sequences known as 5' donor splice site (5'SS) and 3' donor splice site (3'SS). Auxiliary RNA-binding proteins interact with the different sequence motifs present in the pre-mRNAs allowing or inhibiting the recruitment of the spliceosome to neighboring splice sites [114, 115]. During this process, not all the splicing sites are used constitutively. In fact, splice site usage is variable, and the remaining exons may be joined in many different ways through alternative splicing (AS). Variations in the splicing pattern of a single pre-mRNA gives rise to proteins presenting different combinations of domains, thus AS considerably increases the coding capacity of a genome [114]. At the mRNA level, AS can generate aberrant isoforms that are target for degradation. The retention of sequences changes the open reading frame and this, in turn, can produce the inclusion of a premature termination codon (PTC). Indeed, many PTCs are recognized by the nonsense-mediated decay (NMD) mechanism, which entails the degradation of the transcripts [124, 125]. In essence, this means that through use of the NMD mechanism, transcript levels may be regulated via AS [126].

The landmark observation of a significant role for feedback loops based on post-transcriptional regulation in the plant circadian system was originally made for the *Arabidopsis thaliana* genes *GLYCINE-RICH RNA BINDING PROTEIN 7* (*GRP7*) and *GLYCINE-RICH RNA BINDING PROTEIN 8* (*GRP8*). An elevated level of GRP proteins promotes the autoregulation of an AS event that makes use of a cryptic 5'SS. The resulting isoform harbors an intron which includes a PTC that triggers its own degradation via NMD. These proteins are clock regulated and form a feedback loop through which both proteins autoregulate and reciprocally crossregulate their own transcript levels by coupling alternative splicing to NMD [127, 128]. Furthermore, it was demonstrated that GRP7 and GRP8 also regulate the AS and the abundance of several other clock-controlled transcripts. It has been proposed that the feedback loops established by these two RNA binding proteins constituted a conduit to convey timing information from the circadian clock core to its outputs [129-131].

The first insights of crosstalk between AS and the core of the circadian clock in plants were reported in 2010, through the characterization of a loss of function mutant of *PRMT5* that displayed a long period circadian phenotype [106]. In *prmt5* mutant plants, *PRR9* levels were significantly higher than in wild type plants, but the long circadian period phenotype of *prmt5* mutants did not fit with the well-established observation that PRR9 over-expressing plants display a short period clock phenotype [132]. A detailed characterization of post-transcriptional processing events associated with *PRR9* revealed that *prmt5* mutant plants displayed drastic changes in AS of the *PRR9* pre-mRNA, leading to the accumulation of non-functional transcripts. These transcripts, which resulted from the usage of an alternative 5´SS at the end of exon 2 and increased retention of intron 3, accumulated at the expense of the functional isoform. These observations, coupled with an epistatic analysis, suggested that the long period circadian phenotype of *prmt5* mutants could be due, at least in part, to the altered splicing of *PRR9* transcripts [106]. This hypothesis was reinforced through the evidence that the RNA processing ribonucleoproteins SmD1, SmD3 and the SM-like (LSM) LSM4 protein are targets of PRMT5 [133]. Furthermore, subsequent studies on LSM genes, which encode core components of the spliceosomal U6 small nuclear ribonucleoprotein complex, determined that members of this family also regulate circadian rhythms in plants and mammals, and the expression of several genes encoding LSM proteins is clock regulated. Both *lsm4* and *lsm5* mutant plants displayed a long period phenotype as well as aberrant splicing of several clock genes such as *CCA1* and *TOC1*, but not *PRR9,* suggesting the effect of PRMT5 on the clock is not simply due to its effect on LSM4 [134]. Interestingly, mutations in other spliceosome components, such as the splicing factors SNW/Ski-interacting Protein (SKIP) and SPLICEOSOMAL TIMEKEEPER LOCUS 1 (STIPL1), also led to alterations in clock function. *Arabidopsis* plants harboring nonfunctional alleles of both *SKIP* and *STIPL1* possess long-period phenotypes [135, 136]. The SKIP mutation lengthens circadian period in a temperature-sensitive manner, and it was reported that SKIP associates to the pre-mRNA of several clock genes, such as *PPR7* and *PRR9*, and is necessary for the regulation of their AS. Furthermore, genome-wide studies revealed that SKIP regulates the AS of a significant number of genes and this regulation would be through the recognition or usage of specific 5'SS and 3'SS [135]. STIPL1 has been described as the plant homolog of the human spliceosomal protein TFP11 and the *Saccharomyces* spliceosomal protein Ntr1p, which are involved in spliceosome disassembly [136, 137]. The mutation of STIPL1 produces an alteration in the accumulation of circadian clock-associated transcripts such as *CCA1*, *LHY1*, *TOC1*, *PRR9* and *GI*, and this may

contribute to the observed clock phenotype. Very recently, another splicing factor emerged as a link between circadian clock, temperature compensation, and AS. The spliceosomal small nuclear ribonucleoprotein assembly factor GEMIN2 displayed an important role in the modulation of low temperature effects on a large subset of pre-mRNA splicing events. In particular, GEMIN2 modulates the AS of several clock genes, such as *TOC1* and other *PRRs*, and attenuates the effects of temperature on the circadian period [138].

3.2 Environmental regulation of AS at the core of the plant circadian clock

The observations linking temperature, alternative splicing and circadian rhythms are not surprising. Most organisms, including plants, do not control their own body temperature and, therefore, have evolved mechanisms ensuring that biological processes are robust to temperature changes. Circadian rhythms maintain a relatively constant period over the broad range of temperatures resulting from seasonal fluctuations, a property known as temperature compensation. Splicing, on the other hand, involves interactions between spliceosomal snRNAs and pre-mRNAs, which are temperature sensitive [139]. Our observation that GEMIN2, a conserved spliceosomal snRNP assembly factor, attenuates low temperature effects on a large subset of pre-mRNA splicing events, including the AS of several clock genes, and also attenuates the effects of temperature on the circadian period in *Arabidopsis*, suggests that GEMIN2 is one component of a mechanism that helps plants keep time accurately irrespective of temperature changes at different times of the day and year [138].

Indeed, a large number of core clock genes have been reported to undergo changes in AS in response to temperature changes [138, 140-144]. High throughput technologies once again played a major role improving our current understanding of the role that environmental modulation of AS plays in the plant circadian system. RNA-seq and high resolution RT-PCR analysis of the *Arabidopsis thaliana* transcriptome revealed that several clock genes undergo AS when plants are subjected to different environmental conditions, including different steady-state temperatures or temperature transitions [138, 140, 142-145]. Some of these changes might be the unavoidable consequence of the temperature sensitivity of biochemical reactions. Others, on the other hand, may have a role helping plants adjust their growth and development in anticipation to daily and seasonal changes in temperature conditions. For example, during cold acclimation the core clock gene *CCA1* exhibits an AS event that leads to a truncated version of the full-length transcript (*CCA1α*), named *CCA1β*. This AS isoform retains the 4th intron, which would give rise to a truncated protein due to the

presence of a PTC if translated. Overexpression of *CCA1β* interferes with the formation of CCA1α-CCA1α and LHY-LHY homodimers, as well as CCA1α-LHY heterodimers, by forming nonfunctional heterodimers with reduced DNA binding affinity [141]. Whether the CCA1*β* protein isoform exists *in-vivo* remains to be determined [146]. Furthermore, LHY itself experiences a change in AS in response to low temperatures. An isoform that possesses a PTC and is a substrate for NMD is produced by AS through retention of the 5th intron. Thus, low temperatures reduce the levels of LHY protein [143]. PRRs transcripts also experience AS in response to low temperatures. Recently, retention of the 4th intron of TOC1 was shown to increase as result of exposure of *Arabidopsis* plants to 10°C, although the functional significance of this events has yet to be elucidated [138, 143, 144]. *PRR7* also undergoes AS producing nonfunctional isoforms; thus, at low temperatures PRR7 protein level are decreased [143]. Alternative splicing in the circadian clock core genes has also been observed in response to high temperature. It has been reported that transcripts of the EC protein LUX, TIME FOR COFFEE (TIC) and LOV KELCH PROTEIN 2 (LKP2) experience AS at high temperatures; nonetheless the biological roles of these AS events, if any, are still unknown [142].

Despite the fact that the effect of light input over the circadian clock has been described a while ago [147-151], it was not until recently that its effect on the AS of circadian clock genes was addressed. As a response to red light activation of the phytochromes, changes in the AS patterns of the *LHY* and *PRR7* transcripts were described in etiolated *Arabidopsis* seedlings [152]. Another work used the same light treatment that allowed the identification of the LNK gene family, in combination with RNA-seq. The AS of the clock related genes *LHY*, *RVE8*, *TIC*, *JMJD5* and *CASEIN KINASE II BETA CHAIN 3* (*CKB3*) was affected by the light treatment. Interestingly, some of these events are exclusively regulated by light at the post-transcriptional level and do not show changes in steady state mRNA levels. Also, it is worth to mention that there is evidence pointing out that some light regulated AS events are not mediated by the canonical well known photoreceptors [153, 154]. Whether any of these light-regulated AS events associated with clock genes play a role in the modulation of clock entrainment remains to be determined.

Concluding remarks, open questions and future perspectives

Impressive progress has been achieved in characterizing the plant circadian gene regulatory network over the last three decades. The field has moved a long way from the initial observation that circadian rhythms in photosynthetic activity were associated with clock regulation of mRNA levels of *CAB* genes, to the current knowledge of thousands of genes that mediate clock effects on

plant growth and development and whose expression cycle with a 24 hour period. Furthermore, genetic and genomic approaches revealed more than 30 genes encoding core-clock components that regulate the pace of the clock itself, and more than half of them are strongly regulated at the transcriptional level and are themselves transcriptional regulators. High throughput genomic techniques currently allow a detailed characterization of the protein-DNA interactions that underlie the circadian gene regulatory network, and the identification of phase specific *cis*-elements bound by clock components that orchestrate clock regulation of the circadian transcriptome. It is important to bear in mind that, besides this transcriptional regulatory network, post-transcriptional, translational, post-translational and metabolic regulatory layers are also involved in the control of the plant circadian network. Circadian, temperature and light dependent regulation of AS, mRNA stability, mRNA export, as well as protein translation, degradation or localization, have been reported for several core clock components and play a major role in the modulation of circadian control of growth and development [26, 119]. Thus, in addition to completing the characterization of the wiring of the circadian transcriptional network in plants through additional ChIP-seq, Y1H, transcriptomic and proteomic studies involving core-clock components, a detailed understanding of how oscillations in mRNA levels are connected to oscillations in post-transcriptional, translational, post-translational and metabolic processes will be required. While all these processes have often been considered independently, a challenge for the future will be finding the links interconnecting them and the Circadian Gene Regulatory Network provides an excellent case study for this purpose.

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Highlights

- An historical description of our knowledge of the plant circadian clock is reviewed.

- We focus on the transcriptional and post-transcriptional regulatory layers.

- The impact of high-throughput technologies in the field is thoroughly analyzed.