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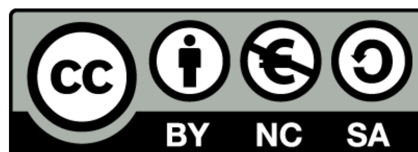
Characterization of the functional connection between the BROMODOMAIN and EXTRATERMINAL DOMAIN PROTEIN 9 (BET9) and the circadian clock in *Arabidopsis thaliana*

Aida Maric



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UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

Directora: Paloma Mas Martínez

CHARACTERIZATION OF THE FUNCTIONAL CONNECTION BETWEEN THE BROMODOMAIN AND EXTRATERMINAL DOMAIN PROTEIN 9 (BET9) AND THE CIRCADIAN CLOCK IN *ARABIDOPSIS THALIANA*

AIDA MARIC 2021

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BIOTECNOLOGÍA

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Memòria presentada per Aida Maric per optar al títol de doctor per la universitat de Barcelona

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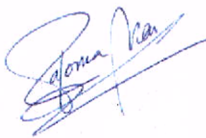	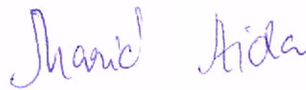	FERRER PRATS ALBERTO - 36557987Q Firmado digitalmente por FERRER PRATS ALBERTO - 36557987Q Fecha: 2021.09.29 10:24:51 +02'00'
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INTRODUCTION

1. Circadian clocks: a general overview

Life on Earth is influenced by rhythmic changes in environmental conditions that regulate physiology, reproduction and behavior of most living organisms (Rijo-Ferreira & Takahashi, 2019). In order to anticipate and adjust to these periodic environmental changes, most living organisms have developed an internal mechanism known as circadian clock. The circadian clock is able to generate rhythms that are endogenously generated and externally entrained (Patke et al. 2019). External synchronization occurs every day by the changes in environmental cues known as *Zeitgebers* (from German *zeit*: time, *geber*: giver) (Patke et al. 2019). The most important *Zeitgebers* are the light-dark cycles and the temperature changes that occur during the day and night. The circadian clock allows organisms to anticipate to the environmental changes and coordinate accordingly biological processes, thus improving their fitness and survival (Hozer et al. 2020).

The first written records of circadian rhythms came in 1729 when a French scientist Jean Jacques d'Ortous De Mairan noticed that the leaves of *Mimosa* plants opened and closed every day. This daily rhythm continued even under constant dark conditions. Rhythmicity in the absence of external cues suggested that the leaf rhythms were generated by an endogenous mechanism. Although the field did not develop much after these findings, the 1900s saw a rapid progress of the circadian clock research (**Table 1**) (Dunlap 1999). Final proof of the endogenous origin of these rhythms was provided by the results showing that *Neurospora crassa* maintained rhythmic growth in the absence of changes in rhythmic cues (Sulzman et al. 1984).

Table 1. A brief overview of early milestones in circadian research. Modified from (Kumar 2017; Roenneberg and Merrow 2005).

Year	Milestone in Circadian Clock Research
1729	De Mairan described endogenous nature of *Mimosa* leaf movement.
1880	Darwin described the advantages of daily leaf movement, which protects the plants from irradiation.
1935	Erwin Bunning and Hans Kalmus independently described that the circadian rhythms in *Drosophila melanogaster* were inherited even under constant dark conditions, i.e. in the absence of external cues.
1959	Franz Halberg used the term "*circadian"* to describe the biological rhythms with periods ~24h.
1960	Jurgen Aschoff introduced the term "*zeitgeber"* and described the correlation of animal activity and light, subsequently named the *Aschoff's rule*.
1971	First clock mutant identified in *Drosophila melanogaster*.
1994	First circadian clock mutant identified in mammals.
1995	First circadian clock mutants identified in higher plants.

1.1. Main properties of the circadian clock

Convergent evolution independently gave rise to circadian clocks at least four times across different life kingdoms (Takahashi, 2017; Young & Kay, 2001). Despite their independent origins, there is a high degree of similarity of the circadian clock function among different organisms (Nohales and Kay 2016; Turek 2016) sharing essential properties: (i) *Duration*: the rhythms are around 24 hours long, hence the term *circadian* (Latin *circa*: around, *diem* = day); (ii) *Self-sustainability*: rhythms are maintained even without environmental cues

like light-dark and temperature cycles (i.e. free-running conditions). Self-sustainability provides evidence that circadian rhythms are endogenously generated and not just a response to environmental cues. (ii) *Plasticity and Robustness:* The circadian clock is able to be resynchronized to different light:dark or temperatures regimes (something that we experience when we recover from the jet-lag after time-zone travelling). Also, the circadian systems are robust enough to buffer changes in temperature so that the speed of the clock is not importantly affected by these changes. This property is known as 'temperature compensation'.

Classical studies have organized the circadian system into three main components although this simple view is clearly an over-simplification of the circadian system (Saini et al. 2019). The three main components include: the *input pathways*, a set of molecular components and mechanisms that translate the environmental cues such as light and temperature to entrain the oscillator. *The central oscillator*, which receives environmental information from the input pathways and generates circadian rhythms of multiple *output pathways*, which are the rhythmic biological processes controlled by the clock (Saini et al. 2019). This *input-oscillator-output* lineal model has been used to explain the circadian clock function (**Figure 1**). However, and as mentioned above, the circadian organization is much more complex than a unidirectional pathway. Output pathways feedback into the central oscillator and can also be directly regulated by the external cues or regulate the input pathways (de Montaigu et al. 2010; Saini et al. 2019). The central oscillator can modulate its *Zeitnehmer* (from German *zeit*: time, *nehmer*: taker) (Roenneberg et al. 2013) response to input signals. *Zeitnehmer* refers to the rhythmic regulation of the input pathway by the central oscillator (McWatters et al., 2000; Roenneberg et al., 2013). This enables the central oscillator to "*gate"* its response to a stimulus to a specific time of day, in a process known as circadian gating (Kinmonth-Schultz et al. 2013).

Generation of the oscillations relies on the rhythmic expression and activity of the clock components, which is regulated at multiple levels from chromatin modifications to transcription and protein turnover (Chen & Mas, 2019; Seo & Mas, 2014). The multiple regulation allows efficient and precise adjustment to the rhythmic environmental changes and the generation of 24 hour rhythms.

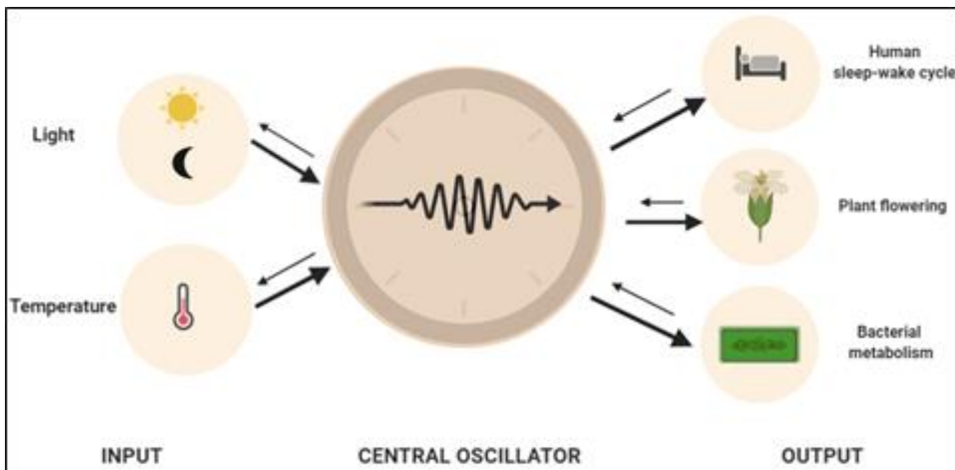


Figure 1. Schematic drawing depicting a simplified view of the main components of the circadian system. Environmental cues like light and temperature act as input signals resetting the central oscillator every day. The central oscillator perceives the input information to generate circadian rhythm of a wide range of the biological processes, known as outputs. (Modified from Nohales & Kay, 2016).

The circadian rhythms generated by the clock are sinusoidal waveforms (**Figure 2**) that can be mathematically defined by their "*phase*", which is the state of a rhythm (usually the peak or though) relative to a reference rhythm (usually the day–night cycle) (Vitaterna et al. 2001; Bell-Pedersen et al. 2005). The time required to complete one cycle is defined as *"period"* (usually measured from peak to peak), while "a*mplitude"* is defined as the distance from a peak or a through to the midpoint of the waveform (Golden and Canales 2003; Vitaterna et al. 2001).

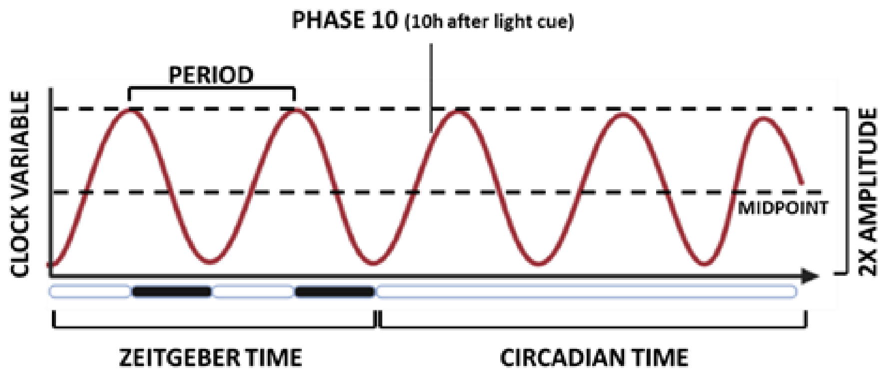


Figure 2. Schematic representation of circadian rhythm represented by sinusoidal wave. *Phase* represents a time point of the circadian waveform relative to a synchronizing rhythm such as the rhythmic environmental changes. *Period* is the time needed for the clock to complete one cycle. *Amplitude* is the distance expressed as halfway from through to peak. Modified from Saini et al., 2019. White and black boxes indicate day and night, respectively; while constant light conditions are indicated by the uninterrupted white box.

Nearly every cell contains its own circadian oscillator (Bell-Pedersen et al. 2005; Lakin-Thomas and Brody 2004). Thus, in multicellular organisms, robust circadian oscillation entails different levels of coordination between cells and tissues as well as their coordination at the whole organism level (Bell-Pedersen et al. 2005). This coordination is hierarchical in mammals and plants (Honma 2018; Nohales 2021). In mammals, the highly coupled clocks in neurons at the Suprachiasmatic Nucleus (SCN) form the master oscillator that synchronizes the slave oscillators of different peripheral tissues (Ralph et al. 1990). Homologous organization is described in plants (Takahashi et al., 2015) in which the highly coupled clocks at the shoot apex are able to synchronize clocks of distal organs like roots (Chen et al., 2020; Takahashi et al., 2015).

2. The circadian clock in plants

Due to their sessile nature, the circadian clock function is particularly relevant in plants. Studies on the plant model *Arabidopsis thaliana* have shown that many aspects of plant growth, physiology and development are under the control of the circadian clock (Greenham & McClung, 2015). The pervasive influence of the circadian clock is also evidenced by transcriptomic studies, which report that a significant fraction of *Arabidopsis* genes are regulated by the circadian clock.

Similar to other organisms, the circadian function in plants was initially studied following the input - central oscillator - output model. Over the years, it has become increasingly clear that the plant circadian system is far more complex. Output pathways feedback to regulate the central oscillator and the central oscillator regulates its sensitivity to input cues (Sanchez et al., 2020) (**Figure 3**). Although the central oscillator has been well studied (Sanchez and Kay 2016; McClung 2019), the molecular mechanisms governing its interaction with input and output pathways are less well described.

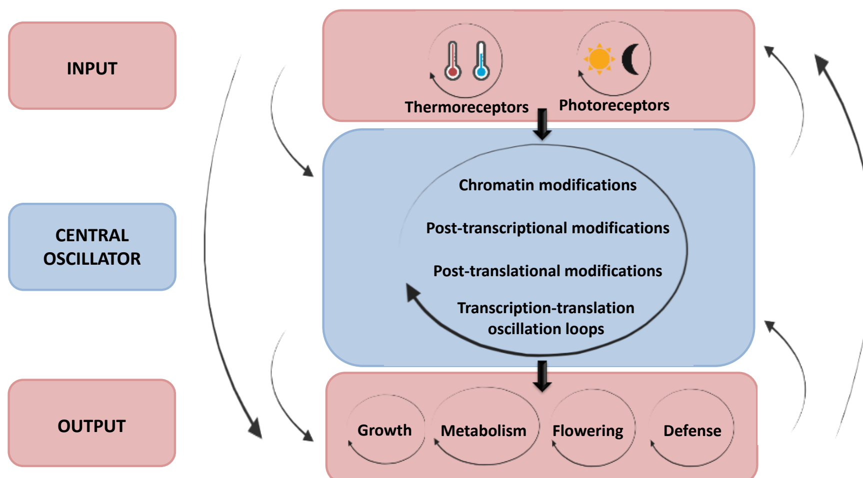


Figure 3. Simplified scheme representing the organization of the plant circadian clock. Most important input cues are daily and seasonal changes in light and temperature. They are perceived by a battery of thermoreceptors and photoreceptors that relay the information to the central oscillator. Plant oscillator is highly regulated at

different levels, from chromatin modifications to transcriptional control. The output processes regulated by the clock are numerous and include growth, metabolism, flowering or defense.

2.1. The central oscillator of *Arabidopsis thaliana*

A precise regulation of clock gene and protein expression is essential for proper function of the clock. The regulation relies on multiple mechanisms, from chromatin modifications to the control of transcription and translation. The following sections describe the components and the regulatory network at the core of the Arabidopsis circadian clock.

2.1.1. Components of the *Arabidopsis* central oscillator

The core of the oscillator in Arabidopsis is composed of morning- and evening-expressed clock components that regulate each other to define their time-of-day peak of expression (**Figure 4**). The first described morning-expressed clock components were *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) (Green & Tobin, 1999; Schaffer et al., 1998; Wang & Tobin, 1998). *CCA1* loss-of-function mutation results in shorter circadian period compared to WT, while plants over-expressing CCA1 abolished rhythmicity (Green & Tobin, 1999; Wang & Tobin, 1998). The output phenotypes of CCA1 miss-expression are reflected in short hypocotyl and early flowering of *cca1* mutant, while plants over-expressing CCA1 have long hypocotyl and late flowering (Mizoguchi et al., 2002; Wang & Tobin, 1998).

Similarly, the *lhy* loss-of-function mutation results in shorter circadian period, short hypocotyl and early flowering (Mizoguchi et al. 2005). In turn, over-expression of LHY leads to arrhythmic expression of clock genes, elongated hypocotyl and late flowering (Schaffer et al. 1998). CCA1 and LHY are partially redundant proteins (Mizoguchi et al. 2002). They can heterodimerize and function synergistically to regulate the circadian oscillation of genes by binding

to a conserved, nine-nucleotide motif named the evening element (EE) (Harmer et al. 2000; Lu et al. 2009; Nagel et al. 2015). The *cca1/lhy* double mutant plants have advanced expression phase of core clock genes (Mizoguchi et al. 2002). Moreover, they work together to regulate output phenotypes of the clock (Fujiwara et al., 2008; Mizoguchi et al., 2002).

TIMING OF CAB2 EXPRESSION 1 (*TOC1 or PSEUDO RESPONSE REGULATOR 1/PRR1)* is a core clock gene with a peak of expression at dusk (Strayer et al. 2000). TOC1 belongs to a small family of PRR proteins containing a distinctive feature at the N-terminal, similar to a receiver domain found in some response regulators (Strayer et al. 2000) but lacking the aspartic residue present in canonical response regulators. The *toc1* mutant plants have advanced phase and short circadian period (Millar et al., 1995; Somers et al., 1998; Strayer et al., 2000), while TOC1-ox plants abolish rhythmicity under constant light conditions (Más et al., 2003a). Analysis of transgenic lines with different levels of TOC1 protein unveiled the direct effect of TOC1 controlling circadian period (Más et al., 2003b). Moreover, the clock-related output phenotypes are also regulated by TOC1. The *toc1* mutant plants show longer hypocotyl phenotype, whereas TOC1-ox seedlings are extremely sensitive to light and show shorter hypocotyl compared to WT (Más et al., 2003a). Furthermore, although Arabidopsis is facultative long day flowering plant, the *toc1* mutant is not able to perceive day-length difference between long day and short day conditions (Más et al., 2003a).

Other members of PRR family have been identified as important clock oscillator components (Adams et al., 2015; Kamioka et al., 2016; Matsushika et al., 2000). *PRR5 (PSEUDO RESPONSE REGULATOR 5)* reaches a peak of expression in the afternoon, approximately 8 hours after dawn (Matsushika et al. 2000). The *prr5* mutant shows a short circadian period, phenotype similar to the one observed in *toc1* plants (Yamamoto et al. 2003). In contrast, plants over-expressing PRR5 have a longer circadian period. The miss-expression of PRR5 also affects clock outputs, with *prr5* mutants having considerably longer

hypocotyls compared to WT when grown under constant red light conditions (Yamamoto et al. 2003). PRR5-ox plants, on the other hand, show very short hypocotyl, i.e. they are hyposensitive to red light (Sato et al. 2002). Miss-expression of PRR5 also affects photoperiodic flowering time, with PRR5-ox plants flowering earlier and *prr5* mutants flowering later than WT under long day conditions (Yamamoto et al. 2003; Sato et al. 2002).

PRR7 (PSEUDO RESPONSE REGULATOR 7) is another member of the PRR family with a peak of expression early during the day (Matsushika et al. 2000). The *prr7* mutant and PRR7-ox plants both show a long period circadian phenotype (Yamamoto et al. 2003; Matsushika et al. 2000). *prr7* mutant plants showed the same output phenotypes as *prr5* mutants, with considerably longer hypocotyls when grown under constant red light and late flowering under long days (Yamamoto et al. 2003).

The *PRR9 (PSEUDO RESPONSE REGULATOR 9)* gene shows a peak of expression very early in the morning. Similar to *prr7* plants, the *prr9* mutants have a slightly longer circadian period than WT plants (Farré et al. 2005). Experiments with PRR9-ox plants show a short period circadian phenotype (Matsushika et al. 2002). Flowering time in *prr9* mutant is only mildly affected with this single mutant flowering slightly later than WT (Ito et al. 2003; Eriksson et al. 2003). The output phenotypes of aberrant *PRR9* expression are more pronounced in higher order mutants (Nakamichi et al., 2010). The evening-expressed components of the circadian system include proteins forming a complex known as the EVENING COMPLEX (EC). The EC is composed of EARLY FLOWERING 3 (ELF3), EARLY FLOWERING 4 (ELF4) and LUX ARRHYTHMO (LUX or PHYTOCLOCK1/PCL1) (Herrero et al., 2012; Nusinow et al., 2011).

ELF3 reaches a peak of expression about 16 hours after dawn. The mutant plants of *elf3* are arrhythmic under constant light conditions, while plants over-expressing ELF3 show an long circadian period (Covington et al., 2001; Hicks

et al., 1996; Reed et al., 2000). The *elf3* mutant was first identified for its early flowering phenotype (Hicks et al., 1996; Zagotta et al., 1992). Additionally, *elf3* mutant plants show a highly elongated hypocotyl (Nieto et al. 2015). Conversely, ELF3-ox plants flower later than WT and have short hypocotyls (Covington et al. 2001; Nieto et al. 2015). ELF3 is a thermosensor protein that regulates temperature entrainment of the clock (Thines and Harmon 2010). Indeed, a recent study showed that ELF3 forms reversible aggregates in the cell in response to temperature changes (Jung et al. 2020). Plants miss-expressing ELF4 showed very similar phenotypes to those reported for ELF3 (Doyle et al., 2002; McWatters et al., 2007). Moreover, a recent study has shown that ELF4 is a mobile element of the clock (Chen et al. 2020). ELF4 is able to move from shoot to root in a temperature-dependent manner and regulate the pace of the root tissue clock (Chen et al. 2020). The *lux* mutants are also arrhythmic and display long hypocotyls and early flowering under both, long day and short day conditions (Hazen et al. 2005; Onai and Ishiura 2005). EC is important in the control of hypocotyl elongation through the regulation of PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and PHYTOCHROME INTERACTING FACTOR 5 (PIF5).

2.1.2. Regulatory network at the *Arabidopsis* central oscillator

Genetic and molecular analyses of the different clock components have provided a view of the complex regulatory network at the core of the Arabidopsis circadian oscillator (**Figure 4**). Overall, the morning-expressed CCA1 and LHY proteins repress the expression of the *PRR* gene family throughout the day (Adams et al., 2015; Alabadi et al., 2001; Kamioka et al., 2016). Repression occurs through the direct binding of CCA1 and LHY to the target promoter genes (Adams et al. 2015; Nagel et al. 2015). In turn, the members of the PRR protein family including TOC1 sequentially repress the expression of *CCA1* and *LHY* (Huang et al. 2012; Nakamichi et al. 2010; Alabadi et al. 2001). PRR proteins bind to promoters of *CCA1* and *LHY in vivo* and act as transcriptional repressors (Nakamichi et al. 2010; Gendron et al.

2012; Huang et al. 2012) The regulation by the PRR protein family restricts the expression of *CCA1* and *LHY* to morning hours (Huang et al., 2012; Nakamichi et al., 2010). TOC1 also represses the other members of the PRR family (Huang et al. 2012).

The expression of the evening genes *ELF3, ELF4* and *LUX* is also repressed by CCA1, LHY and TOC1. CCA1 and LHY repress the *EC* gene expression in the morning, whereas TOC1 represses their expression close to dusk (Li et al., 2011; Lu et al., 2012; Portolés & Más, 2010). This tight control of EC expression allows it to exert its function only during the evening. Conversely, by directly binding to the promoters of *PRR9* and *PRR7*, the EC functions as a repressor of these core clock genes, thereby indirectly promoting *CCA1* expression (Dixon et al., 2011; Helfer et al., 2011; Herrero et al., 2012; McWatters et al., 2007; Zhang et al., 2019). Additionally, EC represses the expression of evening-expressed *GIGANTEA* (*GI*), involved in circadian clock regulation (Nagel et al. 2014; Gould et al. 2006).

Although repressive factors dominate the plant central oscillator network, recent studies have identified activator components of the clock (McClung, 2019). For example, LWD1 (LIGHT-REGULATED WD1) and LWD2 (LIGHT-REGULATED WD2) activate the expression of *CCA1*, *PRR9*, *PRR5* and *TOC1* by direct binding to the promoters of these genes (Wang et al., 2011; Wu et al., 2016). Another recently characterized activator component of the clock is REVEILLE 8 (RVE8) and its co-activator LNK (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED) proteins. Since RVE8 and LNKs directly regulate *BET9* expression and are necessary for BET9 function, they will be discussed in detail in the following sections.

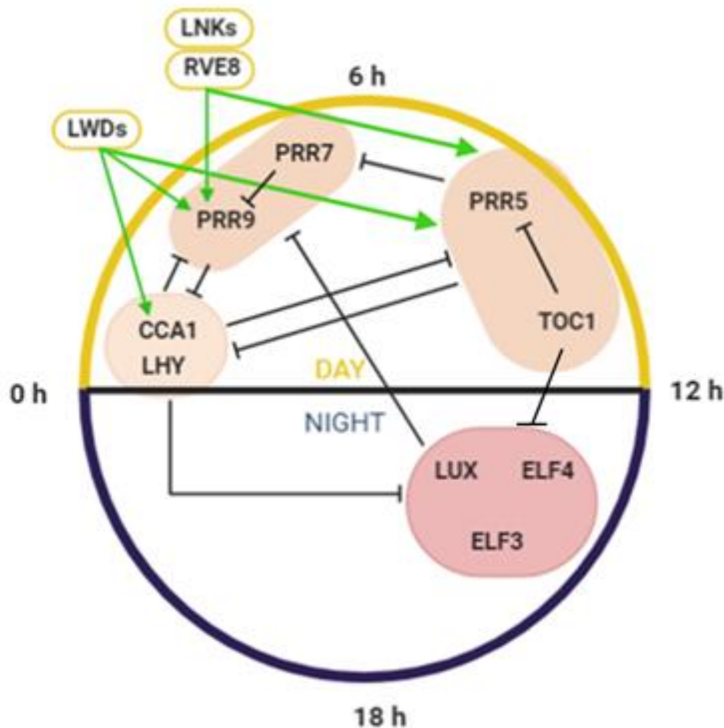


Figure 4. Schematic representation of the main regulatory transcriptional network at the core of the Arabidopsis central oscillator. Members of the core clock oscillator are depicted according to their peak expression from dawn (0h) to dusk (12h). Interactions that promote transcription are represented in green arrows. Repressive interactions are represented in black lines. Clock components belonging to the same functional group are enclosed in pink circles. Modified from Karapetyan & Dong, 2018; Nohales & Kay, 2016.

2.2. Input pathways to the Arabidopsis circadian clock

The *Arabidopsis thaliana* circadian clock is entrained by rhythmic environmental cues, most notably light and temperature (Oakenfull and Davis 2017; Gil and Park 2019). Entrainment of the clock synchronizes the phase of the oscillator with the day and night cycle. This allows the clock to resonate with the daily and seasonal environmental changes. Two main mechanisms synchronize the pace of the central oscillator with the environmental cues: parametric and non-parametric entrainment (Oakenfull and Davis 2017).

Parametric entrainment is a gradual shift in the pace of the clock. For example, increasing light intensity accelerates the pace of the clock, while decreasing light intensity slows down the clock pace, as described by the Aschoff's rule (Aschoff 1979). Consequently, increasing light intensity results in shorter clock period (Somers et al., 1998). Non-parametric entrainment entails quick re-adjustment of the clock at dawn (Millar & Kay, 1996). Besides light and temperature, the plant circadian oscillator is entrained by other signals like humidity (Mwimba et al. 2018), sugar availability (Philippou et al. 2019; Haydon et al. 2013) and hormone accumulation (Singh and Mas 2018).

2.2.1. Clock entrainment by light

Light entrainment is the major resetting factor by which the clock is synchronized to the daily and seasonal changes. Light affects the clock through regulation of chromatin changes, transcription, translation and protein stability (Barneche et al. 2014; Oakenfull and Davis 2017; Sanchez et al. 2020). Circadian entrainment by light initially relies on the function of specific photoreceptors that initiate a complex signaling cascade to reset the oscillator. The central oscillator components in turn are able to modify the sensitivity of plants to the light signal by the diurnal regulation of the expression of these photoreceptors (Fankhauser and Staiger 2002; Oakenfull and Davis 2017). The main photoreceptors in Arabidopsis are divided into four main groups: phytochromes (PHY), cryptochromes (CRY), the ZEITLUPE (ZTL) protein family, and ULTRAVIOLET RESISTANT LOCUS 8 (UVR8) (Sanchez et al. 2020).

Phytochromes are a protein family of five members (PHYA, PHYB, PHYC, PHYD and PHYE) that perceive red and far-red light (Quail et al. 1995). Cryptochromes (CRY1 and CRY2) are photoreceptors of blue light (Cashmore et al. 1999). In addition to cryptochromes, plants perceive blue light through the ZTL protein family also including FLAVIN-BINDING KELCH REPEAT FBOX

(FKF1) and LOV KELCH PROTEIN 2 (LKP2) (Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001).

Of the five members of the phytochrome family, PHYA and PHYB play the most important role in clock perception of light. The *phyA* and *phyB* mutants show a long period phenotype under continuous red light (Somers et al., 1998). This phenotype depends on the light fluence rate; while *phyA* prolonged the clock period only under low-fluence rate red light, *phyB* mutant is affected in the response to high fluence red light (Somers et al., 1998). Additionally, *phyA* mutant shows long period under continuous low-fluence blue light. Double mutant of *phyAphyB* shows additive phenotypes with longer period than WT under different red light fluence rates (Devlin and Kay 2000).

Single *phyA* and *cry1* mutants showed a similar long period phenotype under constant blue light, suggesting that they might participate in the same signaling pathway (Somers et al., 1998). The experiments with *phyA* and *cry1* mutants under different fluence rates of white light showed that CRY1 acts downstream of PHY1 (Devlin and Kay 2000). Additional studies indeed showed that CRY1 acts as a substrate for phosphorylation by PHYA (Ahmad et al. 1998). Additionally, physiological studies, co-immunoprecipitation experiments and Fluorescent Resonance Energy Transfer (FRET) assays demonstrated the direct interaction of CRY2 with PHYB in the control of circadian period length under white light (Más et al., 2000).

The molecular connectors between photoreceptors and the oscillator are beginning to be elucidated. For instance, studies of PHYA function under constant far-red light have identified the EC component ELF4 as a potential target of far-red light signaling (Wenden et al. 2011). PHYA was shown to activate ELF4 expression under far red light conditions, with *phyA* mutant almost abolishing the expression of *ELF4* (Wenden et al. 2011). Other proteins involved in PHYA signaling pathway such as FAR-RED ELONGATED HYPOCOTYL 3 (FHY3), FAR-RED IMPAIRED RESPONSE 1 (FAR1) and

LONG HYPOCOTYL 5 (HY5) directly induce the expression of *ELF4* by binding to cis-element of its promoter (Li et al. 2011). CCA1 and LHY repress the DNA-binding activity of FHY3/FAR1/HY5 complex, suppressing the *ELF4* expression (Li et al. 2011). Another element of the EC, ELF3, was found to interact *in vivo* with PHYB mediating light entrainment of the central oscillator (Huang et al., 2016).

2.2.2. Clock entrainment by temperature

In addition to light, daily temperature changes entrain the plant circadian clock. However, due to pervasive influence of temperature on all biochemical events in the plant, the mechanisms of clock entrainment by thermocycles remain to be fully explored (Gil and Park, 2019).

Although temperature entrains the clock, physiological changes in temperature do not affect the circadian period based on a conserved property of circadian clocks known as temperature compensation (Gil and Park 2019). *CCA1, LHY, TOC1, LUX* and *GI* transcript accumulation shows temperature-sensitive regulation so that at higher temperature *GI*, *TOC1* expression levels increase and *LHY* levels decrease. Conversely temperature decrease from 17°C to 12°C leads to increase in the mRNA levels of *CCA1* and *LHY,* while at 12°C the role of CCA1 becomes more important than LHY, indicating possible different roles for these closely related proteins (Gould et al. 2006). Additionally, alternative splicing of the clock genes *CCA1*, *LHY*, *PRR7*, *TOC1*, *ELF3* and *ZTL* is temperature-dependent (Filichkin et al., 2012; James et al., 2012; Kwon et al., 2014; Yang et al., 2020).

The temperature compensation mechanism in plants has been explained by the interaction of CCA1 and CK2 (CASEIN KINASE 2) (Portolés & Más, 2010). The binding of CCA1 to the promoters of clock genes increased with higher temperature. In turn, CK2 phosphorylates CCA1, and the phosphorylated CCA1 showed less affinity for the target gene promoters. As CK2

phosphorylation also increases with temperature, the study uncovered a novel mechanism whereby two opposing and temperature-dependent activities (CCA1 binding and CK2 phosphorylation) control clock temperature compensation (Portolés & Más, 2010). Using a combination of assays performed at different temperatures, the authors showed that CK2 interferes with the promoter binding of CCA1. Furthermore, the phCK2 counterbalances the higher binding activity of CCA1 at increased temperatures, therefore regulating its transcriptional activity within the oscillator and contributing to temperature compensation (Portolés and Más 2010).

The core clock proteins CCA1 and LHY have different roles in temperature compensation. At lower temperatures, the dynamic balance between CCA1 and GI regulates temperature compensation, while at higher temperatures, the role of CCA1 is taken over by LHY (Gould et al. 2006). PRR7 and PRR9 regulate plant temperature compensation by controlling the expression of *CCA1* and *LHY* upon increased ambient temperature (Salomé et al. 2010).
Light and temperature pathways converge at different points to control circadian system. For example, clock controlled PHYB has a dual role as a light and temperature sensitive photoreceptor (Halliday et al., 2003; Jung et al., 2016; Legris et al., 2016). Recent study revealed that PHYB and EC bind to the same target loci, providing a pathway of integration between the clock and environmental cues (Ezer et al. 2017).

2.3. Output pathways of the Arabidopsis circadian clock

The circadian clock perceives the environmental signals and translates them into precisely timed rhythmic biological responses. A myriad of processes are under the control of the clock including among others, growth, development, and responses to biotic and abiotic stress (Adams and Carré 2011; Greenham and McClung 2015).

2.3.1. Circadian regulation of hypocotyl length

Hypocotyl growth follows a rhythmic pattern under constant light with peak growth at subjective dusk (Dowson-Day & Millar, 1999; Nozue et al., 2007). The molecular mechanism of rhythmic hypocotyl growth is based on the regulation of the light- and clock-controlled bHLH (basic Helix-Loop-Helix) transcription factors PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PHYTOCHROME-INTERACTING FACTOR 5 (PIF5) (Nozue et al. 2007). PIF4 and PIF5 are promoters of hypocotyl growth. The circadian clock EC controls the expression of *PIF4* and *PIF5* genes by binding to their promoters (Nusinow et al. 2011). Additionally, the PRR proteins antagonize the activity of PIFs by repressing their transcription during the day (Martin et al., 2018; Soy et al., 2016; Zhu et al., 2016). Consequentially, PIF4 and PIF5 activity contributing to hypocotyl growth is gated to dawn (Inoue et al., 2018). Moreover, PRRs and PIFs all bind at the promoter of *CDF5* (*CYCLING DOF FACTOR 5*), which encodes a protein that controls hypocotyl elongation (Martin et al., 2018).

2.3.2. Circadian regulation of photoperiodic flowering

Reproductive success of plants depends on proper flowering (Shim et al., 2017). Flowering is connected with many signaling pathways including the circadian clock (Inoue et al., 2017). *Arabidopsis* is a long day flowering plant, i.e. its flowering is promoted under long days and it is delayed under short day conditions (Corbesier et al. 1996). First reports on the connection of the clock and flowering came from the studies of flowering mutants. A number of molecular mechanisms explaining the connection between the circadian clock and the photoperiodic regulation of flowering time have been documented. One example relies on GI (Sawa and Kay 2011; Mizoguchi et al. 2005). GI controls the expression of the key flowering transcription activation factor *CONSTANS (CO)* through the formation of the GI-FKF1 complex (Hwang et al., 2019; Sawa et al., 2007; SB et al., 2010; YH et al., 2012 PROPER FORMAT). *CO* transcription is repressed by the clock-controlled CYCLING DOF FACTOR

(CDF). The GI-FKF1 complex targets the CDF1 protein for proteasome degradation (Sawa et al., 2007; Imaizumi et al., 2005). This allows accumulation of CO in the afternoon. CO protein activates transcription of *FLOWERING LOCUS T (FT),* a florigen that moves from leaves to shoot apical meristem to trigger flowering (Tiwari et al., 2010).

3. REVEILLES and NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED CLOCK COMPONENTS

Our results show a connection of BET9, a bromodomain-containing protein (see below section 5), with the clock components RVEs (REVEILLE) and LNKs (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED). Therefore, we briefly describe here the main findings related to the expression and function of these clock components.

RVE8 (REVEILLE8 or *LHY-CCA1-LIKE5/LCL5)* is a rhythmically expressed oscillator gene with a morning peak of expression and peak protein accumulation at noon (Farinas and Mas 2011a; Rawat et al. 2011). Miss-expression of RVE8 results in altered clock phenotypes, with *rve8* mutant showing delayed phase and longer period of clock gene expression, and RVE8 over-expressing plants resulting in advanced phase and a short period phenotype (Farinas and Mas 2011a; Rawat et al. 2011). Over-expression of RVE8 results in delayed flowering, while *rve8* mutant flowers earlier compared to WT (Farinas and Mas 2011a; Rawat et al. 2011). Besides flowering, hypocotyl elongation is an output pathway affected by RVE8 miss-expression.

RVE8 belongs to the same family of MYB transcription factors as CCA1 and LHY (Andersson et al. 1999; Carré and Kim 2002). The members of this family share high sequence similarity in their MYB domain (Andersson et al. 1999; Carré and Kim 2002). In addition to similarities within MYB domain, a subfamily of five RVE (RVE3, RVE4, RVE5, RVE6 and RVE8) proteins share high

sequence identity in the C-terminal domain LCL (LHY/CCA1-LIKE) (Schmied and Merkle 2005; Farinas and Mas 2011). RVE8 and its closest homologs RVE4 and RVE6 show genetic redundancy as the triple mutant *rve468* showed much longer circadian period than any single *rve* mutant (Hsu et al. 2013; Gray et al. 2017).

Although CCA1/LHY and RVE8 belong to the same protein family, the analysis of their function indicates that they have antagonistic activities within the circadian oscillator. While CCA1 and LHY act as repressors, RVE8 activates the transcription of evening-phased clock genes including *PRR5* and *TOC1, ELF4* and *LUX* (Farinas & Mas, 2011; Hsu et al., 2013; Rawat et al., 2011). RVE8 directly associates to the EE in the promoters of *TOC1* and *PRR5* to regulate their expression.

The RVE8-regulated activation of *TOC1* gene expression correlates with the changes in the acetylation state at the *TOC1* promoter (Farinas and Mas 2011). Chromatin immunoprecipitation (ChIP) assays showed that over-expression of RVE8 favors hyper-acetylation of the H3 at the *TOC1* promoter, leading to the advanced expression phase of *TOC1* expression (Farinas and Mas 2011). This activation antagonizes the repressive function of CCA1 (Farinas and Mas 2011). Conversely, *rve8* mutant has an impaired H3 acetylation and delayed expression of *TOC1* gene (Farinas and Mas 2011). The authors proposed that the repression of *TOC1* expression in the morning occurs because CCA1 prevents binding of RVE8. As the CCA1 abundance decreases, RVE8 is free to bind the *TOC1* promoter and initiate its transcription (Farinas and Mas 2011).

Other activating clock components closely related to RVEs are LNKs (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED). LNKs belong to a protein family of transcription factors composed of 4 members (LNK1, LNK 2, LNK 3, and LNK 4) (Rugnone et al. 2013). The expression of *LNKs* is controlled by the clock, with a peak of expression in the morning (Rugnone et al. 2013). The

lnk1/lnk2 double mutant shows a lengthening of circadian period, particularly relevant for *PRR5* and *TOC1* (Rugnone et al. 2013). The *lnk1/lnk2* mutant plants showed longer hypocotyl length than WT plants or the single mutants, indicating a partially redundant function (Xie et al. 2014). LNK1 and LNK2 interact with the morning-expressed clock components CCA1, LHY, RVE4 and RVE8 (Xie et al. 2014). The authors suggest that RVE8-LNK1 and RVE8-LNK2 form a MORNING COMPLEX (MC) as a novel mechanism within the clock (Xing et al., 2015).

A recent study has also identified the molecular mechanism responsible for the activation of clock gene expression. The mechanism involves the interaction of RVE8 and LNKs with the transcriptional machinery (Ma et al. 2018b). The MYB domain of RVE8 provides the DNA binding specificity to the promoters of *TOC1* and *PRR5*, while the LCL domain serves as an interface for the interaction with LNKs. The LNKs in turn recruit the RNA Polymerase II and the FACT (FACILITATES CHROMATIN TRANSCRIPTION) complex, thus controlling the rhythms of nascent RNAs (Ma et al. 2018b). RVE8 and LNKs are also important in the control of anthocyanin gene expression (Pérez-García et al., 2015), although further studies are necessary to unravel the mechanistic insights behind this regulation.

4. Epigenetic regulation of gene expression

An important regulatory layer of gene expression is chromatin conformation (Pfluger and Wagner 2007). The basic unit of chromatin structure is a nucleosome that consists of 146 bp DNA fragment that is compactly wrapped around a histone octamer (one H3-H4 tetramer and two H2A-H2B dimers) (Richmond et al. 1988). Histones are evolutionarily conserved chromatin-related proteins made of two basic domains: (i) a domain that regulates binding to other histones or DNA and (ii) a N-terminal tail that is a primary site of post-translational modifications (Ronald & Davis, 2017). The covalent modifications

at the histone tail can regulate the chromatin state and control the transcription of genes. Relaxed chromatin favors gene transcription while compacted chromatin structure impedes the binding of the transcriptional machinery, thus suppressing gene transcription (Venkatesh and Workman 2015). Histone modifications favoring a relaxed chromatin state include acetylation and phosphorylation (Jiang and Berger 2017). Depending on the residue modified, repressive histone tail modifications include methylation and ubiquitination (Yelagandula et al. 2014; Nakayama et al. 2001). The histone modifications can regulate gene expression either by changing local electrostatic forces, thus changing DNA conformation or by acting as a recruitment site for transcriptional machinery (Bannister and Kouzarides 2011). Covalent modifications can stand alone or form combinations thereby forming the so-called 'histone code', recognized by the effector proteins and controlling the chromatin architecture (Maksimovic and David 2021). The proteins responsible for deposition and removal of histone marks at the amino acid residues are often referred to as 'writers' and 'erasers', respectively. Proteins recognizing and binding the chromatin marks are termed 'readers'.

4.1. Epigenetic regulation of clock gene expression

Changes on histone acetylation at the *TOC1* promoter were reported to closely match *TOC1* rhythmic expression (Perales and Más 2007). Consistent with the repressing function of CCA1, a hypo-acetylation state of H3 was found in CCA1 over-expressing plants (CCA1-ox), which coincided with the down-regulation of *TOC1* expression in CCA1-ox plants (Perales and Más 2007).

From this initial study, a search was launched to identify the chromatin-related components responsible for the rhythmic histone acetylation. The Histone Acetyl Transferase (HAT) TAF1 (HISTONE ACETYLTRANSFERASE OF THE TAFII250 FAMILY 1) was shown to regulate acetylation at the *TOC1* promoter Hemmes et al., 2012). *PRR5* expression is also rhythmically associated with

changes in histone acetylation (Baerenfaller et al. 2016). The HISTONE ACETYLTRANSFERASE OF THE TAFII250 FAMILY 2 (HAF2) protein reaches peak abundance in the noon leading to acetylation of H3 at the promoter region of *PRR5* enabling the raising expression of these genes (Lee & Seo, 2018).

Not only histone acetylation, but other activating marks such as H3K4me3 – (histone 3 lysine 4 trimethylation) rhythmically oscillate not only at the *TOC1* promoter but at the promoters of all main oscillator genes (Malapeira et al. 2012; Baerenfaller et al. 2016). SET DOMAIN GROUP 2/ARABIDOPSIS TRITHORAX RELATED 3 (SDG2/ATXR3), a histone methyltransferase could be regulating the accumulation of H4K4me3 at the promoters of the clock genes, since miss-expression of SDG2/ATXR3 led to altered H4Kme3 oscillation (Malapeiraet al. 2012). Additionally, functional studies showed that the accumulation of H3K4me3 prevents binding of clock repressors, thus regulating the timing of the declining phase and ensuring a precise circadian oscillation (Malapeira et al. 2012).

Recent studies reported on additional factors regulating the chromatin conformation state at the *TOC1* promoter. For instance, a recent study has shown that CCA1 and LHY recruit the histone deacetylases LDL1/2 (LSD1-LIKE 1/2) and HDA6 (HISTONE DEACETYLASE 6) to control deacetylation at the *TOC1* promoter (Hung et al. 2018). The EC component ELF3 also facilitates the direct binding of the HISTONE DEACETYLASE 9 (HDA9) to the *TOC1* promoter (Lee et al., 2019). Binding of the EC-HDA9 complex leads to histone deacetylation, closed chromatin conformation and consequently low expression of *TOC1* during the night (Lee et al., 2019). Another clock-related component CHE (CCA1 HIKING EXPEDITION) was shown to repress *CCA1* expression by interacting with HD1 (HISTONE DEACETYLASE 1). Consequently, the double mutant of *che/ad1* relieves the repression of *CCA1* (Ng et al. 2017). PRR9 was also proposed to repress *CCA1* and *LHY* gene

expression through its interaction with HDA6 and TOPLESS (TPL) (Wang et al., 2013).

5. BROMODOMAIN AND EXTRA-TERMINAL DOMAIN protein family

BROMODOMAIN AND EXTRA-TERMINAL DOMAIN (BET) proteins are an ancient protein family present in different life kingdoms, from plants to animals and yeast (Florence & Faller, 2001). The BET protein family of Arabidopsis encompasses 12 members whose functions remain to be fully understood. Most plant BET proteins are defined by four domains plant amphipatic domain (PAD), bromodomain (BRD), extra-terminal domain (ET) and transcriptional activation domain (TAD). The defining characteristic of BET proteins is the presence of N-terminal BRD, able to recognize the ε-amino group at the lysine residues of histone tails (Florence & Faller, 2001; Kanno et al., 2004). While BET proteins in animals and yeast have two bromodomains, plant BETs retain only one BRD. Sequence analysis suggests that plant BRD is more similar to the second BRD in the humans (Florence & Faller, 2001). In plants, the first BRD of the animal BET proteins is substituted by the PAD.

Since the affinity for acetylated lysine residues of a single BRD in plant BETs is weaker compared to human and yeast, it has been hypothesized that this can be compensated by dimerization of BET proteins through the PAD, thereby bringing two BRDs together (Florence & Faller, 2001). In plant BETs, PAD and BRD domains are followed by another defining characteristic, the ET domain (Pandey et al. 2002). The ET domain is present in all BET proteins, independent of the organism. It was shown to function as a protein binding domain and as a serine kinase of the BRD4 (BROMODOMAIN 4) protein of the human BET family (Matangkasombut et al. 2000; Z Lygerou et al. 1994; Platt et al. 1999; Devaiah et al. 2012).

The TAD is located at the C-terminal of plant BET proteins (Misra 2011). Studies of the mammalian transcription mechanism indicate that TAD domain is required for recruitment of general transcription factors to initiate transcription or facilitate elongation (Blau et al. 1996). A recent study in plants suggests that the TAD could recruit the BT2 (BTB AND TAZ DOMAIN PROTEIN 2) protein to the chromatin (Misra 2011).

Previous studies have reported a range of functions for BET proteins. For instance, the human BRD4 protein allows transcriptional memory upon cell division (Dey et al. 2003). Human BRD2 (BROMODOMAIN 2) and BRD3 (BROMODOMAIN 3) proteins enable transcription elongation by RNA Polymerase II (LeRoy et al. 2008). Similarly, BRD4 positively regulates the RNA Polymerase II-mediated gene transcription of the human immunodeficiency virus type 1 (Jang et al., 2005; Yang et al., 2005). Since they recognize histone acetylation and relate with RNA Polymerase II, BET proteins have been a target of human cancer research (Shu and Polyak 2016; Spriano et al. 2020).

In plants, initial studies focused on BET4 (GLOBAL TRANSCRIPTION FACTOR GROUP E1/GTE1 or IMBIBITION-INDUCIBLE 1/IMB1). IMB1 was reported to be important in the control of the seed germination (Duque and Chua 2003). *IMB1* is expressed during seed imbibition and studies with the *imb1* mutant showed that IMB1 is involved in promoting seed germination by not only negatively regulating the hormone ABA (abscisic acid) signalling pathway but also positively regulating the PHYA pathway (Duque and Chua 2003).

Another study showed that BET3 (GLOBAL TRANSCRIPTION FACTOR GROUP E4/GTE4) controls the onset of cell cycle and maintenance of cell proliferation (Airoldi et al. 2010). The *gte4* mutants seem to be unable to hold a pool of undifferentiated cells, as seen by partial loss of the root quiescent center (QC) and aberrant root morphology. Additional experiments with *gte4*

mutant showed a range of defective phenotypes, from impaired seed germination to defects in size and shape of aerial organs like leaves and flowers (Airoldi et al. 2010). The regulation of cell cycle by bromodomain-containing proteins has been identified in other organisms (Dey et al. 2003; Maruyama et al. 2002).

The BET12 (GLOBAL TRANSCRIPTION FACTOR GROUP E6/GTE6) regulates the development of juvenile and mature leaves in Arabidopsis (Chua et al. 2005). Importantly, the authors identified the molecular mechanism behind this phenotype. ChIP (chromatin immunoprecipitation) experiments showed that GTE6 binds to the promoter of *ASYMMETRIC LEAVES1* (*AS1*) to initiate the transcription of this gene (Chua et al. 2005). Experiments with GTE6 over-expressing plants indicate that GTE6 protein regulates the acetylation and chromatin remodeling at the promoter of *AS1*, thereby regulating its expression (Chua et al. 2005).

The first study reporting on BET9 (GLOBAL TRANSCRIPTION FACTOR GROUP E9/GTE9) identified BET9 as an interactor of the BT1 (BTB and TAZ DOMAIN 1) transcriptional regulator protein in a yeast two-hybrid screening (Du and Poovaiah 2004). BET9 was also shown to interact with another member of the BT family, BT2 (Misra et al. 2018). *In vivo* experiments showed that BT2 co-immunoprecipitated with BET9, confirming their interaction in plants (Misra et al. 2018). The loss-of-function mutant of *bet9* mimicked the phenotype of *bt2,* with hypersensitive phenotypes to glucose and ABA during seed germination (Misra et al. 2018). When BT2 over-expressing plants were crossed with *bet9* mutants, the resistance was not regained confirming that BET9 was required for BT2-mediated response to sugars and ABA (Misra et al. 2018). The transcription levels of *BET9* changed after different abiotic stresses (Baxter et al., 2007; Lee et al., 2005; Rizhsky et al., 2004). Upon 2 hours of oxidative stress, the transcript abundance of *BET9* increased more than four-fold (Baxter et al. 2007). Experiments under low temperature also indicate that *BET9* expression is cold-regulated (Lee et al., 2005). Additionally, *BET9*

transcript abundance was elevated in leaves under a combination of drought and heat stress (Rizhsky et al. 2004).

The BET10 (GLOBAL TRANSCRIPTION FACTOR GROUP E11/GTE11) shares a high degree of identity with BET9. Like BET9, BET10 protein interacts with BT1 (Du and Poovaiah 2004). More specifically, a GST pull-down assay confirmed that BET10 interacts with BTB (Broad-Complex, Tramtrack, and Bric-a-Brac) domain of BT1 (Du and Poovaiah 2004). Additionally, the same study reported that BET10 interacts with additional members of the family such as BT2 and BT4 (Du and Poovaiah 2004). The studies also demonstrated that BET10 was able to activate transcription from promoters of four different tested genes (Du and Poovaiah 2004). These results reinforced the role of BET10 protein regulating transcription. Similar to *bet9*, loss-of-function *bet10* mutant mimicked the phenotype of *bt2* mutant and germination was hypersensitive to glucose and ABA (Misra et al. 2018). The authors suggested that the BET proteins and BT2 could form a transcription-regulating mechanism with BET proteins providing chromatin recognition component and BT2 protein providing DNA sequence specificity (Misra et al. 2018). It is also noteworthy that while the transcriptional changes of *BET9* were detected upon abiotic stress, the changes in *BET10* transcript occurred upon pathogen infections (Ascencio-Ibáñez et al. 2008; Truman et al. 2006), which suggest that BET9 and BET10 could also have independent functions.

OBJECTIVES

The research goal of this Doctoral Thesis is to elucidate the functional connection of the bromodomain-containing protein BET9 (BROMODOMAIN AND EXTRA-TERMINAL DOMAIN 9) within the *Arabidopsis thaliana* circadian system. This main goal was accomplished through several specific goals:

1. **Elucidation of *BET9* rhythmic oscillation and identification of clock components contributing to *BET9* circadian regulation.** We performed time course analyses of *BET9* expression in plants miss-expressing the clock components RVE8 (REVEILLE 8) and LNKs (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED).

2. **Generation and characterization of plants miss-expressing BET9.** We generated plants over-expressing BET9 and characterized T-DNA insertion mutants of *BET9* and *BET10*. We also generated *bet9/bet10* double mutant plants.

3. **Characterization of the effects on oscillator gene expression and histone acetylation of plants miss-expressing BET9.** We performed time course analyses of oscillator gene expression in WT, BET9 over-expressing plants and *bet9/bet10* double mutants. We also analyzed the pattern of histone acetylation at the promoters of oscillator genes.

4. **Generation and characterization of plants miss-expressing BET9 and RVE8.** We generated plants over-expressing BET9 in a *rve8* mutant background, and plants over-expressing RVE8 in a *bet9* mutant.

5. **Identification of the functional connection of BET9 and RVE8 in the control of oscillator gene expression.** We performed time course analyses of oscillator gene expression and analyzed BET9 and RVE8 binding to target gene promoters in plants over-expressing BET9 in a *rve8* mutant background, and in plants over-expressing RVE8 in a *bet9* mutant background.

RESULTS

1. Arabidopsis BET9 genomic structure and protein domains

The *BET9* gene (AT5G14270) (also known as *GLOBAL TRANSCRIPTION FACTOR GROUP E 9, GTE9*) is located at the sense strand of chromosome five of the *Arabidopsis thaliana* genome. The genomic structure of *BET9* consists of 10 exons (**Figure 5A**) and the gene encodes a protein containing four functional domains: i) a plant amphipatic domain (PAD) that might be involved in protein dimerization; ii) a bromodomain (BRD), highly conserved domain shown to recognize acetyl lysine residues in many species; iii) an extra-terminal domain (ET), unique to proteins from the BET family, potentially involved in protein-protein interactions; and iv) a transcription activation domain (TAD) possibly involved in the regulation of gene transcription; (Du and Poovaiah 2004; Brian Florence and Faller 2001) (**Figure 5B**).

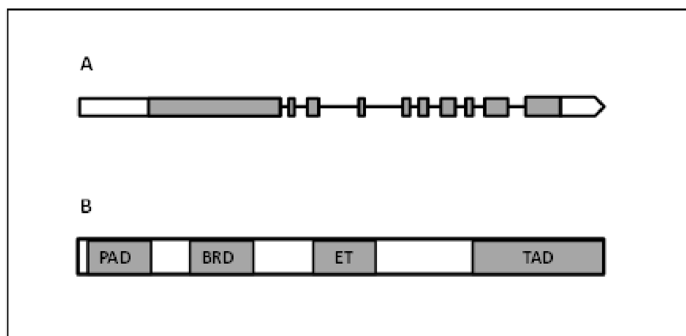


Figure 5. *BET9* gene structure and protein domains. (A) Genomic structure of *BET9*. Grey box: exon, grey line: intron; white box: 5'UTR and white arrow: 3'UTR. (B) Schematic diagram of BET9 protein. PAD: Plant Amphipatic Domain. BRD: bromodomain. ET: Extra-Terminal. TAD: Transcription Activation Domain.

BET proteins are found across kingdoms and show an ancient evolutionary history (Florence & Faller, 2001). Arabidopsis BET protein family contains 12 members that belong to the also called GTE protein family. Their phylogenetic analyses indicated that BET10 is the closest homolog to BET9 (**Figure 6**)

(Misra et al. 2018). The overview of the plant BET protein family is given in Table 2.

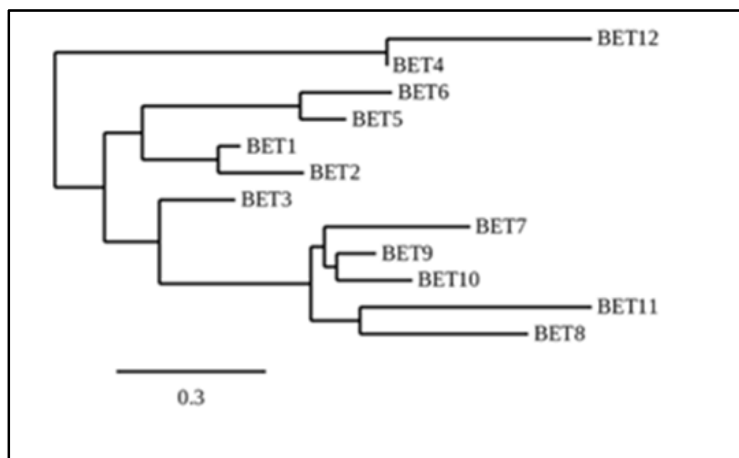


Figure 6. Phylogram of plant BET protein family. Protein sequences of 12 homologues were aligned using MUSCLE (multiple sequence comparison by log-expectation) (Edgar 2004). The phylogram shows the evolutionary relationship among the Arabidopsis BET proteins.

Table 2 *Arabidopsis thaliana* GTE protein family (Modified from Misra 2011).

Locus	BET member	GTE member	Gene name	Protein length (aa)
AT1G73150	1	3		461
AT1G17790	2	5		487
AT1G06230	3	4		766
AT2G34900	4	1	IMB1	386
AT5G65630	5	7		590
AT5G10550	6	2		581
AT3G27260	7	8		813
AT5G63320	8	10	NPX1	1061
AT5G14270	9	9		689
AT3G01770	10	11		620
AT5G46550	11	12		494
AT3G52280	12	6		386

IMB1: IMBIBITION-INDUCIBLE 1; NPX1: NUCLEAR PROTEIN

2. Bromodomain structure prediction

In order to get further insights of BET9 protein, we used the Protein Data Bank (PDB) (www.rcsb.org) (Dhalluin et al. 1999) to visualize in PyMol (www.pymol.org/2), the already defined structure of the bromodomain of the human p300/CBP-associated factor (P/CAF) (**Figure 7**). It consists of 4 left-handed and antiparallel helices (Dhalluin et al. 1999), with loops between helices (loop ZA and loop BC) lined with conserved residues, and forming a hydrophobic pocket able to bind to acetylated lysine (Owen et al. 2000).

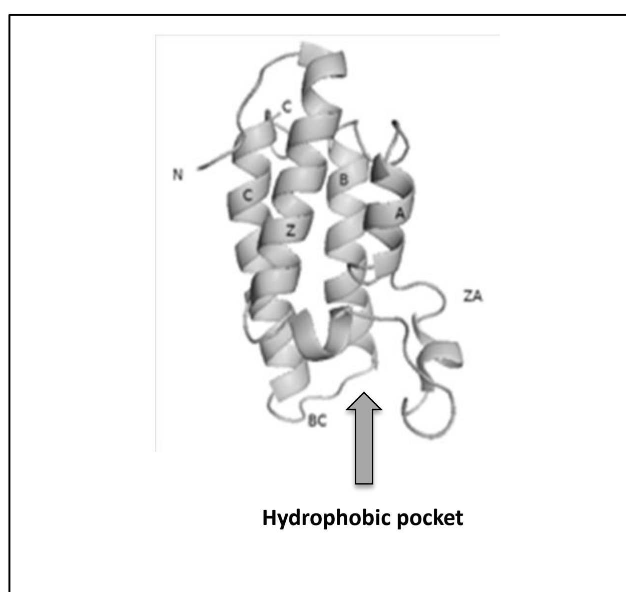


Figure 7. Three-dimensional structure of the P/CAF bromodomain. The structure was downloaded from the Protein Data Bank (PDB) (code 1N72) (Dhalluin et al. 1999).

3. BET9 gene expression and protein localization

We next used publicly available datasets to obtain information about *BET9* gene expression and protein subcellular localization (Klepikova et al. 2016; Hooper et al. 2017). Our analyses revealed that *BET9* is pervasively expressed in *Arabidopsis thaliana* (Klepikova et al. 2016) including developing seeds, hypocotyls, cotyledons, roots, leaves and flowers (**Figure 8**).

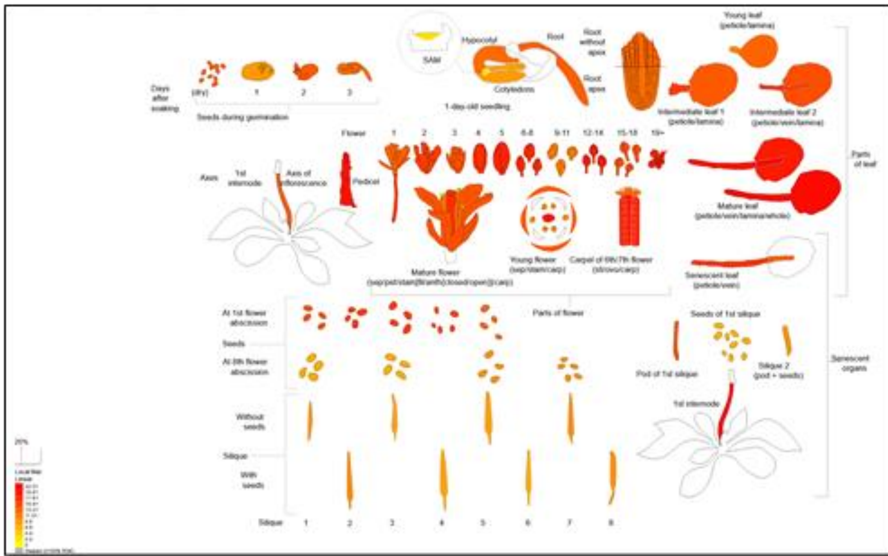


Figure 8. Schematic drawing of *BET9* expression pattern across Arabidopsis organs. RPKM values from zero to local maximum across organs (Klepikova et al. 2016). Image from the ePlant (bar.utoronto.ca/eplant) online tool (Waese et al. 2017).

Our *in silico* analyses (Wilkins et al. 2010) also indicated an oscillatory pattern of *BET9* expression with a peak of expression close to dusk and a trough close to dawn (**Figure 9**). The results suggest that the circadian clock might regulate the expression and possibly the function of BET9.

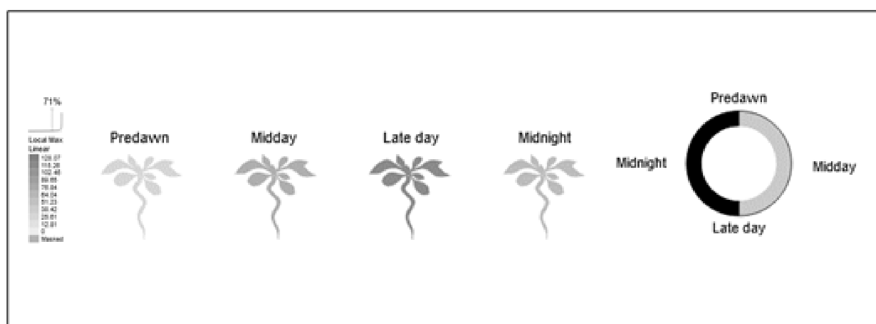


Figure 9. Schematic drawing of diurnal *BET9* expression. RPKM values from zero to local maximum during the day (Wilkins, Bräutigam, and Campbell 2010). Image obtained from the ePlant (bar.utoronto.ca/eplant) online tool (Waese et al. 2017).

Predictions of BET9 protein subcellular localization also estimate its nuclear localization (**Figure 10**), which is consistent with the presence of the chromatin-related bromodomain, and BET9 plausible nuclear-localized function.

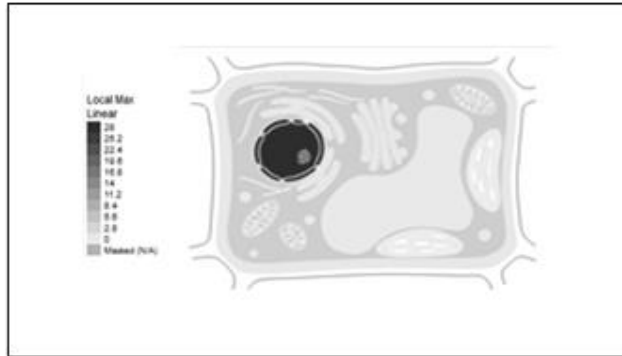


Figure 10. Prediction of BET9 subcellular localization of BET9. RPKM values from zero to local maximum across the cell (Hooper et al. 2017). Image obtained from the ePlant (bar.utoronto.ca/eplant) online tool (Waese et al. 2017).

4. Rhythmic oscillation of *BET9* and *BET10* gene expression

To further investigate the diel and circadian oscillation of *BET9* expression throughout the day, we used the *DIURNAL* database (Mockler et al. 2007) (diurnal.mocklerlab.org), which is a publicly available web-based repository of diurnal and circadian gene expression datasets. The analyses confirmed that *BET9* oscillates with a peak of expression close to or anticipating dusk under short day (ShD) and long day (LgD) conditions (**Figure 11A-B**). A similar pattern of expression was observed under constant light (LL) conditions, albeit with slightly reduced amplitude (**Figure 11C**). Time course analyses by RT-QPCR (Reverse Transcription-Quantitative Polymerase Chain Reaction) of plants grown under the different entraining and free-running conditions confirmed the oscillatory pattern of expression (**Figure 11D-F**). Together, the *in silico* analyses and the time course RT-QPCR assays indicate that *BET9*

expression is controlled by the clock with a peak phase close to dusk, similar to other oscillator genes like *TOC1*.

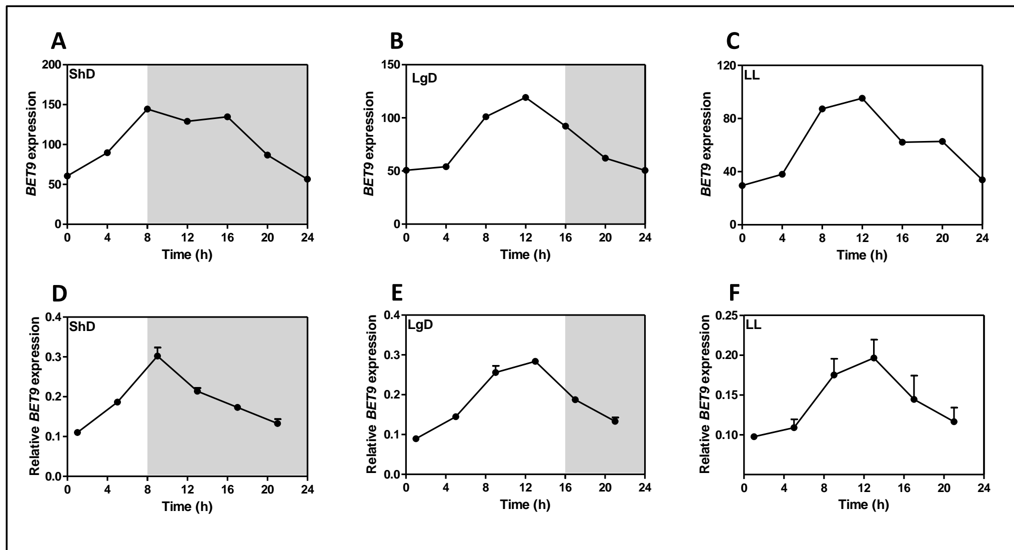


Figure 11. Diurnal and circadian expression of *BET9*. Analyses of *BET9* expression using the *DIURNAL* database (diurnal.mocklerlab.org). The analyses were performed under different environmental conditions: (A) ShD (8h light:16h dark), (B) LgD (16h light:8h dark) and (C) LL (constant light). Cut-off value: 0.8. Time course analyses by RT-QPCR of plants growing under (D) ShD (8h light:16h dark), (E) LgD (16h light:8h dark) and (F) LL (constant light). Seedlings were grown for 10 days under ShD and LgD conditions and samples were collected every 4h over a 24h cycle. For analyses under LL, plants were grown for 8 days under LgD, and then transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* (*ISOPENTENYL PYROPHOSPHATE:DIMETHYLALLYL PYROPHOSPHATE ISOMERASE 2*) expression values. The data represent mean + SEM of two biological replicates. White box in A-B and D-E: light; grey box in A-B and D-E: darkness.

We also examined the expression of *BET10*, the closest homologue of *BET9*. The *in silico* analyses and our time course assays by RT-QPCR revealed an oscillation of *BET10* expression under LL conditions (**Figure 12A-B)** with a peak phase comparable to the one observed for *BET9.* Our results thus show

that the expression of both *BET9* and *BET10* rhythmically oscillates and is controlled by the circadian clock.

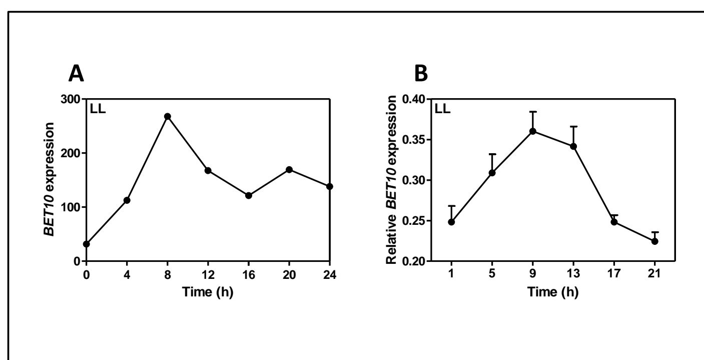


Figure 12. Diurnal and circadian expression of *BET10.* (A) Analysis of *BET10* expression using the *DIURNAL* database (diurnal.mocklerlab.org). The analysis was performed under LL conditions. Cut-off value: 0.6. (B) Time course analyses by RT-QPCR of plants growing under LL. Plants were grown for 8 days under LgD (16h light:8h dark) and then transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* expression values. The data represent mean + SEM of two biological replicates.

5. *BET9* expression is regulated by the REVEILLE protein family

Based on the circadian regulation of *BET9* expression, we next aimed to identify the clock factors controlling *BET9* circadian oscillation. To that end, we analyzed *BET9* expression in clock mutant and over-expression lines. Previously published data showed that the transcription of *PRR5* and *TOC1* is controlled by RVE8 (Farinas and Mas 2011a; Xie et al. 2014; Ma et al. 2018a; Rawat et al. 2011). As *BET9* showed a peak phase of expression similar to *PRR5* and *TOC1*, we used RVE8 miss-expression plants (Farinas and Mas 2011a) to examine *BET9* oscillation.

Time course analyses by RT-QPCR showed that *BET9* expression was up-regulated in RVE8-ox plants, with an advanced peak of expression compared

to WT (**Figure 13A**). Contrarily, *BET9* expression was phase-delayed in *rve8* mutant plants resulting in a down-regulation close to subjective dusk, reaching a peak in the middle of the subjective night (**Figure 13B**).

Analyses of *rve* triple mutant plants (*rve468* composed of *rve8* mutant and its closest homologs *rve4* and *rve6*) (Hsu, Devisetty, and Harmer 2013)*,* showed a clear down-regulation of *BET9* expression from CT9 (Circadian Time 9, 9 hours after subjective dawn) until CT17 (**Figure 13C**). Notably, the *BET9* gene expression phenotypes were remarkably similar to those observed for *TOC1* (**Figure 13D-F**). Together, these results suggest that RVEs contribute to the circadian oscillation of *BET9* expression in a similar way to that observed to *TOC1*.

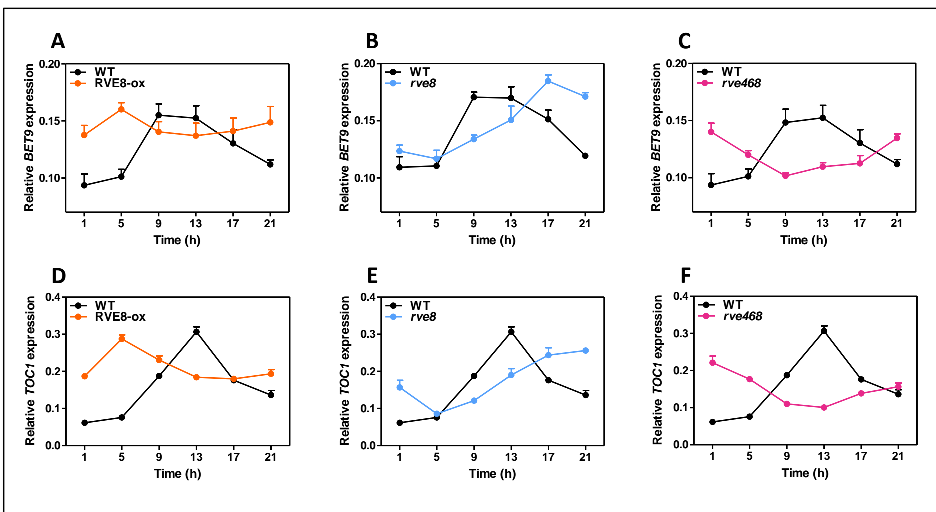


Figure 13. *BET9* and *TOC1* expression in *rve* mutant plants. Time course analysis by RT-QPCR of *BET9* expression in (A) RVE8-ox; (B) *rve8* single mutant, and (C) *rve468* triple mutant compared to WT. Time course analysis by RT-QPCR of *TOC1* expression in (D) RVE8-ox; (E) *rve8* single mutant, and (F) *rve468* triple mutant compared to WT. Seedlings were grown for 8 days under LgD, and transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* expression values. The data represent mean + SEM of two biological replicates.

6. *BET9* expression is altered in *lnk1/lnk2* double mutant plants

RVE8 forms a protein complex with LNKs (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED) (Ma et al. 2018a; Xie et al. 2014) and the protein complex recruits the transcriptional machinery to control the transcript initiation and elongation of *TOC1* and *PRR5* expression (Ma et al. 2018a). As our results indicate that RVE8 regulates *BET9* expression following a similar trend to that observed for *TOC1*, we explored whether LNKs also regulated *BET9* expression. To that end, we performed a time course analysis by RT-QPCR in *lnk1/lnk2* double mutant plants (Rugnone et al. 2013). Our results showed that the expression of *BET9* was severely down-regulated in *lnk1/lnk2* mutant compared to WT plants (**Figure 14A**) following a similar trend to that observed for *TOC1* and *PRR5* gene expression (**Figure 14B-C**). The results indicate that LNKs are important for the activation of *BET9* expression. The RVE-LNK protein complex likely regulates *BET9* expression following the same molecular mechanisms as for *TOC1* and *PRR5* (Ma et al. 2018a) (i.e. by recruiting the transcriptional machinery).

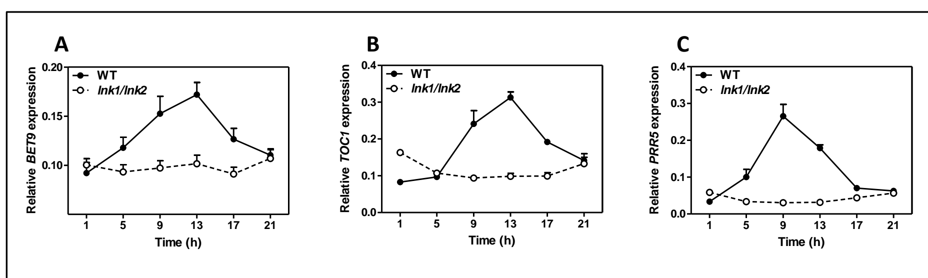


Figure 14. *BET9*, *TOC1* and *PRR5* expression is affected in *lnk1/lnk2* double mutant plants. Time course analysis by RT-QPCR of (A) *BET9*; (B) *TOC1*, and (C) *PRR5* expression in *lnk1/lnk2* double mutant compared to WT. Seedlings were grown for 8 days under LgD, and transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* expression values. The data represent mean + SEM of two biological replicates.

7. RVE8 binds to the *BET9* promoter

Due to the similar patterns of *BET9* and *TOC1* in RVE miss-expressing plants, and based on previous studies showing that RVE8 directly associates to the *TOC1* promoter (Rawat et al. 2011; Farinas and Mas 2011a), we examined the possible direct binding of RVE8 to the *BET9* locus. To that end, we performed Chromatin Immunoprecipitation (ChIP) assays with an anti-GFP antibody to immunoprecipitate RVE8-GFP followed by Q-PCR. We found specific amplification of the *BET9* promoter in a region containing the previously described Evening Element (EE) motif important for evening-phased rhythmic oscillation (Alabadí et al. 2001) (set of primers #1, **Figure 15A**), whereas reduced enrichment was observed in a region far from the EE (set of primers #2) or from the negative controls *TA3 (TRANSCRIPTIONALLY INACTIVE RETROTRANSPOSON 3)* and *CCA1* (**Figure 15B**). As expected, we found a significant enrichment of RVE8 at the *TOC1* and *PRR5* promoters (**Figure 15C**). Altogether, our results show that RVE8 regulation of *BET9* expression most likely occurs through direct binding of RVE8 to the *BET9* promoter.

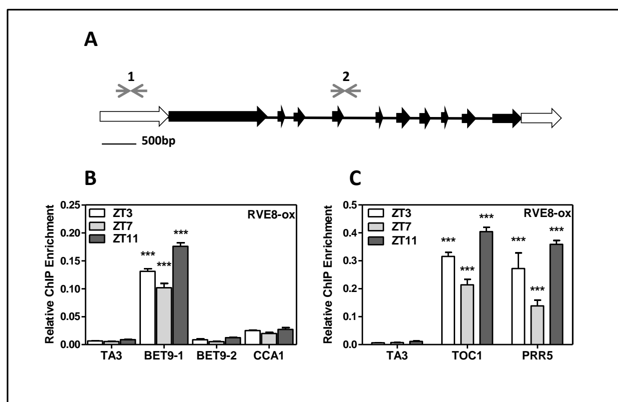


Figure 15. RVE8 binding to the *BET9* promoter. (A) Schematic diagram of *BET9* locus indicating the position of the two sets of primers used for ChIP (grey arrows). Set #1 amplifies a region containing the EE motif whereas set #2 amplifies a region +2318 bp downstream of the transcription start site. White arrows indicate UTRs and black arrows delimit exons. (B) ChIP-Q-PCR analyses of RVE8-ox plants showing RVE8 binding to EE in *BET9* promoter (BET9-1) during the day. *TA3*, downstream *BET9* position (BET9-2) and *CCA1* were used as negative controls. (C) ChIP-Q-PCR

analysis showing RVE8 binding to *TOC1* and *PRR5* promoters. Seedlings were grown for 10 days under LgD conditions and samples were collected at ZT3 (Zeitgeber time 3, 3 hours after dawn), ZT7 and ZT11. Enrichment was calculated relative to the input values. The data represent mean +SEM of three biological replicates. Unpaired Student's *t*-test was used to determine the significant difference in enrichment at *BET9-1, TOC1 and PRR5* loci compared to *TA3* (*** $p < 0.001$).

8. Generation and characterization of *BET9* mis-expressing plants

In order to study the function of BET9, we characterized two T-DNA insertion mutant lines (Alonso et al., 2003) (GK_826H06 and SALK_119044). The reduced expression of *BET9* in both lines was confirmed by RT-QPCR (**Figure 16A**). To investigate the effect of over-expressing the *BET9* gene, we also generated constructs expressing the *BET9* CDS under the cauliflower mosaic virus (CaMV) 35S promoter and used the Green Fluorescent Protein (GFP) as a tag fused to the N-terminal and C-terminal positions. We obtained independent, one-insertion, homozygous BET9-ox lines with different degrees of over-expression, as confirmed by RT-QPCR (**Figure 16B**).

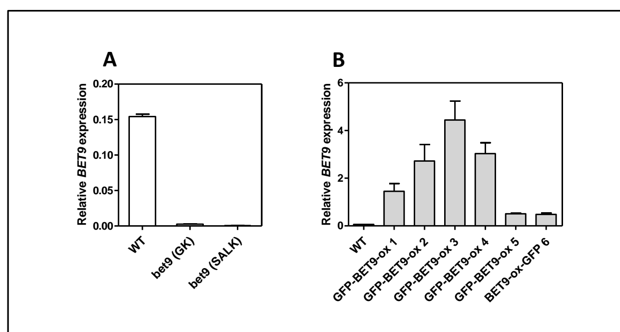
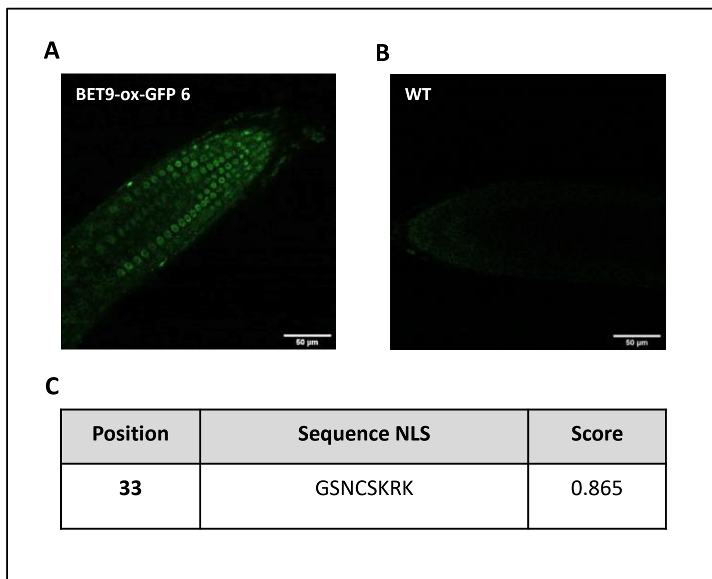


Figure 16. Characterization of BET9 mis-expressing lines. (A) RT-QPCR analysis of *BET9* expression in *bet9* T-DNA insertion lines compared to WT. (B) *BET9* expression analyzed by RT-QPCR in different BET9-ox lines compared to WT. Seedlings were grown for 10 days under LgD and samples were collected at ZT1. Values were normalized to *IPP2*. The data represent mean +SEM of two biological replicates.

9. Analysis of BET9 subcellular localization

Subcellular localization studies by confocal microscopy of root tip cells using the plants expressing BET9 fused to GFP showed clear fluorescent signals localized in the nucleus whereas no fluorescence was observed in WT plants imaged with the same conditions (**Figure 17A-B**). The nuclear localization is consistent with the subcellular localization predictions (**Figure 10**), and with the bioinformatics analysis of BET9 amino acid sequence (Lin and Hu 2013), which predicts a nuclear localization signal (NLS) in positions 33 to 40 (**Figure 17C**).



Position	Sequence NLS	Score
33	GSNCSKRK	0.865

Figure 17. Subcellular localization of BET9. (A) Root tip cells of BET9-ox-GFP 6 plants and (B) Root tip cells of WT plants were visualized under confocal microscope. Scale bar: 50µm. Seedlings were grown for 7 days under LgD conditions. (C) NLS of BET9 protein as identified by using the SeqNLS algorithm (mleg.cse.sc.edu/seqNLS) (Lin and Hu 2013).

10. Over-expression of *BET9* affects the expression of circadian clock genes

In order to investigate the role of BET9 regulating the circadian clock, we examined the expression of core clock genes in plants miss-expressing *BET9*. Time course analyses by RT-QPCR of plants entrained under LgD conditions for 8 days followed by two days under LL showed that the raising phase of *PRR5* and *TOC1* expression was advanced in GFP-BET9-ox 5 (**Figure 18A-B**) The expression of both genes was clearly up-regulated compared to WT plants. Up-regulation was also observed for other evening-expressed gene such as *ELF4* (**Figure 18F**) as well as morning-expressed genes such as *CCA1*, *PRR9* and *PRR7* (**Figure 18C-E**), Similar results were obtained when a second over-expressing line (line 6) was analyzed (**Figure 19**).

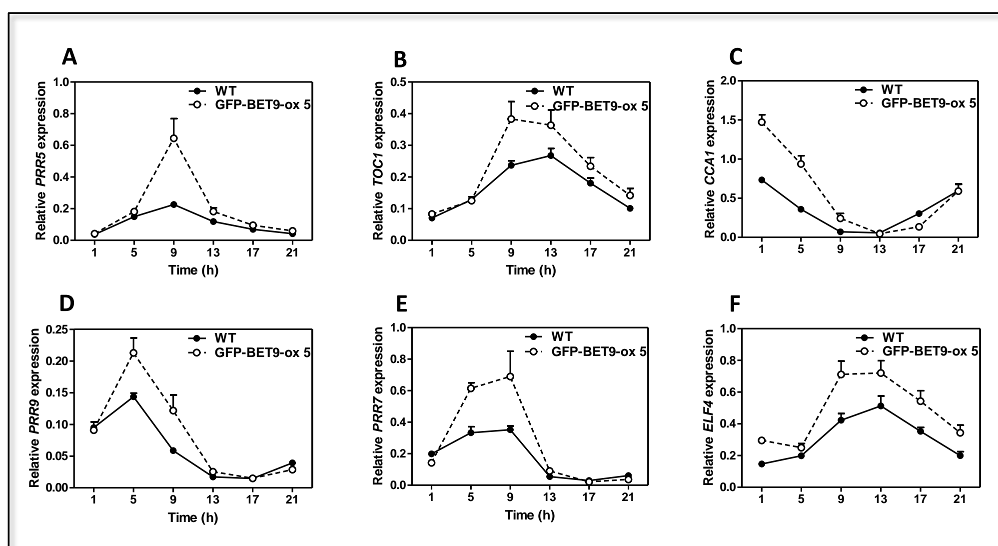


Figure 18. Alteration of clock gene expression in BET9-ox compared to WT plants. Time course analysis by RT-QPCR of (A) *PRR5*; (B) *TOC1*; (C) *CCA1*; (D) *PRR9;* (E) *PRR7*, and (F) *ELF4* expression in GFP-BET9-ox 5 compared to WT. Seedlings were grown for 8 days under LgD, and transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* expression values. The data represent mean + SEM of two biological replicates.

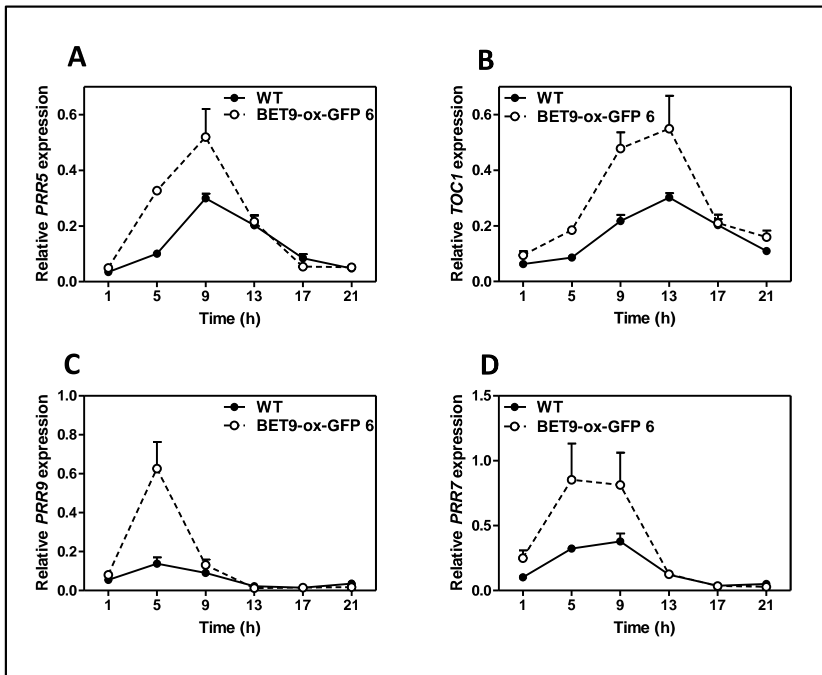


Figure 19. Verification of BET9-ox molecular phenotypes in a second over-expressing line. Time course analysis by RT-QPCR of (A) *PRR5*; (B) *TOC1*; (C) *CCA1*; (D) *PRR9;* (E) *PRR7*, and (F) *ELF4* expression in BET9-ox-GFP 6 compared to WT. Seedlings were grown for 8 days under LgD, and transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* expression values. The data represent mean + SEM of two biological replicates.

The two over-expressing lines have the GFP tag in different terminals of the BET9 protein. The similar phenotypes of these lines indicate that the GFP tag does not interfere with the BET9 functionality. Altogether, our results indicate that proper expression of *BET9* is important in the control of clock gene expression.

11. Over- expression of *BET9* affects clock promoter activity

To verify BET9 function within the clock, we used the GFP-BET9-ox construct to transform plants expressing the promoter of *PRR5* fused to the

LUCIFERASE (*PRR5::LUC*). Plants grown under LD (12h light:12h dark) cycles were transferred to a luminometer, and promoter activity was examined *in vivo* under LL. Our results showed an advanced phase and up-regulation of *PRR5::LUC* activity in GFP-BET9-ox 3 plants compared to WT (**Figure 20A**). These results are consistent with the changes in gene expression observed in BET9-ox plants. Analyses of the circadian period using BioDare (biodare2.ed.ac.uk) (Zielinski et al. 2014) also showed that GFP-BET9-ox 3 plants have significantly shorter circadian period compared to WT (**Figure 20B**). The results suggest that proper expression of *BET9* is important for regulating clock promoter activity.

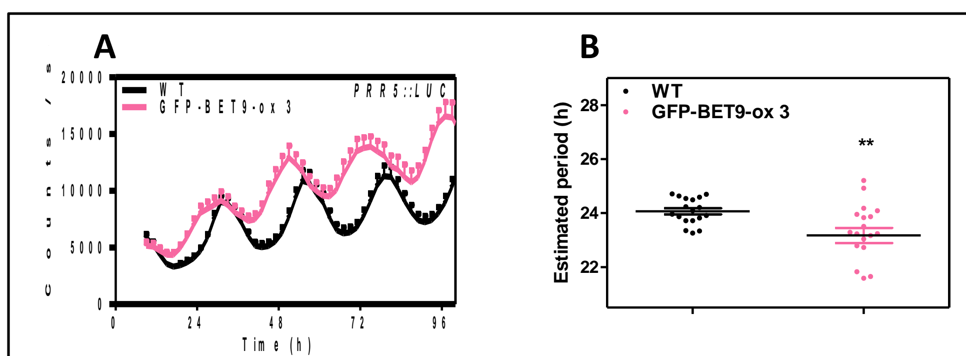


Figure 20. BET9 over-expression affects *PRR5* promoter activity. (A) *In vivo* luminescence analysis of *PRR5::LUC* activity in WT and GFP-BET9-ox 3 plants. (B) Estimated period of *PRR5::LUC* bioluminescence calculated by BioDare (biodare2.ed.ac.uk). Plants were grown for 7 days under LD (12h light:12h dark), and transferred to LL for 7 days where luminescence was measured every two hours. Data represent mean + SEM of two biological replicates. Unpaired Student's *t*-test was used to determine statistical significance of differences in period length of BET9-ox compared to WT (*p < 0.05; ** p < 0.01).

12. Circadian clock gene expression is altered in *bet9/bet10* double mutant plants

Time course analyses by RT-QPCR of the *bet9* mutant plants did not render any relevant changes of clock gene expression compared to WT (**Figure 21A-D**).

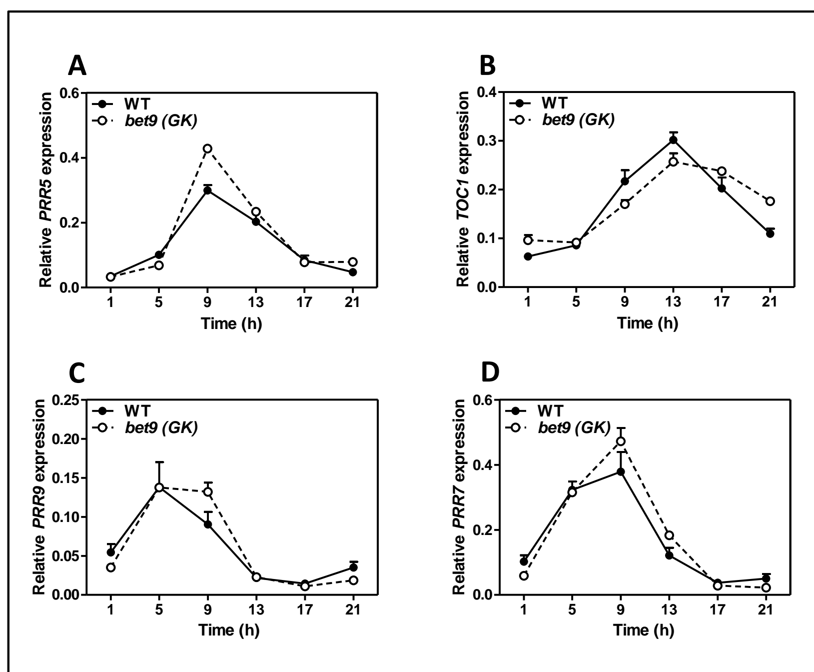


Figure 21. Analyses of clock gene expression in *bet9* mutant compared to WT plants. Time course analysis by RT-QPCR of (A) *PRR5*; (B) *TOC1*; (C)*PRR9*, and (D) *PRR7* expression in *bet9* mutant compared to WT. Seedlings were grown for 8 days under LgD, and transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* expression values. The data represent mean + SEM of two biological replicates.

We reasoned that a possible functional redundancy of BET9 and BET10 could be responsible for the lack of clock gene expression phenotypes. To explore this possibility, we characterized a T-DNA insertion mutant line of *BET10* (**Figure 22A**), and generated double *bet9/bet10* mutant plants by crossing the single mutants. The reduced expression of *BET9* and *BET10* was confirmed by RT-QPCR (**Figure 22B**).

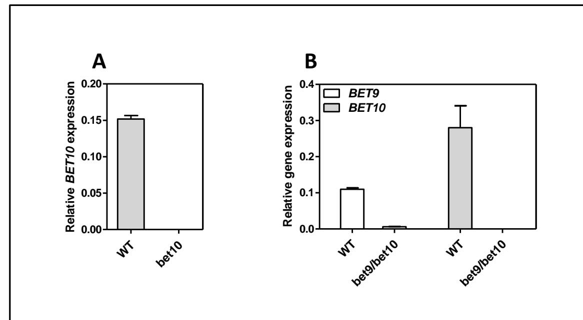


Figure 22. Characterization of *bet10* and *bet9/bet10* mutant lines. (A) RT-QPCR analysis of *BET10* expression in a *bet10* T-DNA insertion line compared to WT. (B) RT-QPCR analysis of *BET9* and *BET10* expression in a *bet9*/*bet10* line compared to WT. Seedlings were grown for 8 days under LgD and samples were collected at ZT5. Values were normalized to *IPP*2. The data represent mean +SEM of two biological replicates.

Time course analyses by RT-QPCR of *bet9/bet10* double mutant plants entrained under LgD conditions followed by two days under LL showed a decreased amplitude for all clock genes examined (**Figure 23A-F**). These results suggest that BET9 and BET10 might be redundant proteins in the regulation of clock gene expression. We are currently generating the *bet9/bet10* double mutant plants in the *TOC1::LUC* and *PRR5::LUC* background to examine whether the absence of functional BET9 and BET10 affects the promoter activity of these clock genes.

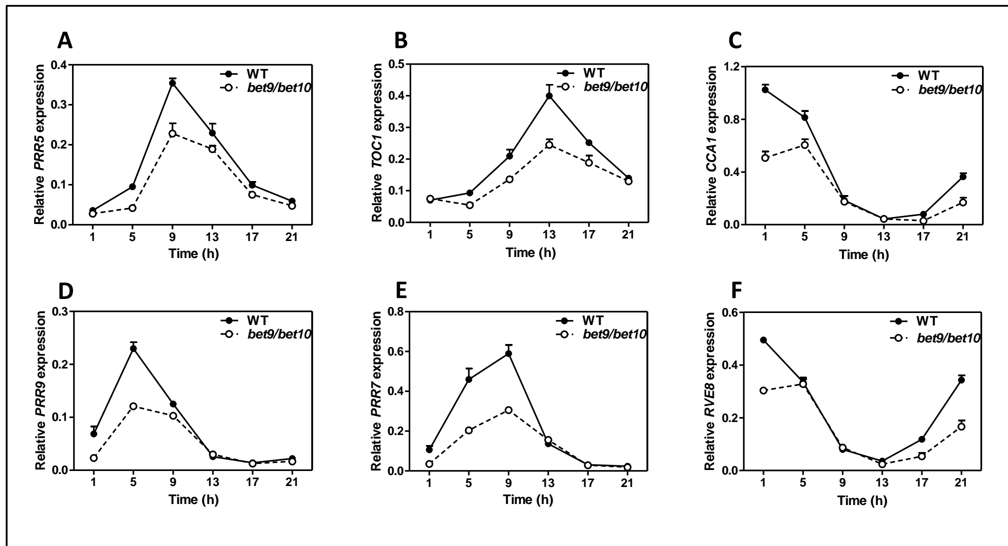


Figure 23. Analyses of clock gene expression in *bet9/bet10* double mutant compared to WT plants. Time course analysis by RT-QPCR of (A) *PRR5*; (B) *TOC1*; (C) CCA1; (D) *PRR9*; (E) *PRR7*, and (F) *RVE8* expression in *bet9/bet10* double mutant compared to WT. Seedlings were grown for 8 days under LgD, and transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* expression values. The data represent mean + SEM of two biological replicates.

13. BET9 binds to *TOC1* and *PRR5* promoters

As the expression of *TOC1* and *PRR5* is affected by *BET9* over-expression and in *bet9/bet10* double mutant plants, we analyzed if BET9 directly binds to the promoters of these clock genes. ChIP analyses followed by Q-PCR was performed with BET9-ox-GFP 6 plants grown under long day conditions (16h light: 8h darkness) and transferred to LL for two days before sampling at CT7 (7 hours after subjective dawn). Immunoprecipitation of BET9 with an anti-GFP antibody followed by amplification of the promoter regions of *TOC1* and *PRR5* (**Figure 24A**) showed a clear enrichment in the regions containing the EE motif, important for their rhythmic oscillation (Alabadí et al. 2001) (**Figure 24B**). Therefore, BET9 seems to regulate *TOC1* and *PRR5* expression by direct binding to their promoters.

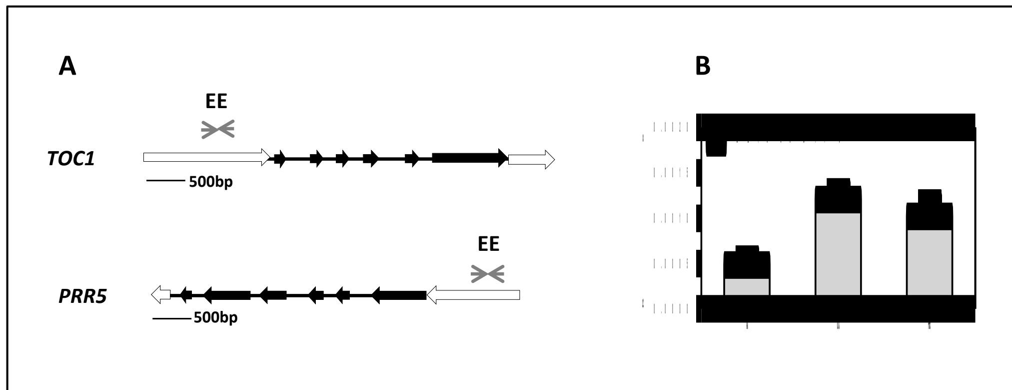


Figure 24. BET9 binds to the promoters of *TOC1* and *PRR5*. (A) Schematic diagram of *TOC1* and *PRR5* loci. White arrows indicate UTRs and black arrows delimit exons. Grey arrows represent primer pairs at the EE motif of clock gene promoters. (B) ChIP-Q-PCR assay of BET9-ox-GFP 6 plants showing BET9 binding to EE of *TOC1* and *PRR5* promoters. *TA3* was used as negative control. Seedlings were grown for 8 days under LgD, and then transferred to LL for 2 days before sampling at CT7. Enrichment was calculated relative to the input values. The data represent mean + SEM of two biological replicates.

14. BET9 regulates the acetylation state of the *TOC1* promoter

Changes in histone acetylation at the *TOC1* promoter contribute to the rhythmic oscillation of *TOC1* expression (Perales and Mas, 2007). Furthermore, RVE8 modulates the acetylation at *TOC1* promoter (Farinas and Mas, 2011). Interestingly, bromodomains are able to interact with acetylated lysine residues (Dhalluin et al. 1999). As both BET9 and RVE8 affect the expression of *TOC1*, we compared the acetylation state of *TOC1* locus in WT, BET9-ox and *bet9/bet10* plants. ChIP-Q-PCR analyses were performed with plants grown under long day conditions and transferred to LL for two days before sampling at CT7. Acetylated sites were immunoprecipitated using an anti-acetylation H3 lysine 9 antibody (αAcH3K9). Our results showed that BET9-ox-GFP 6 plants had higher acetylation at the EE motif of *TOC1* promoter, whereas *bet9/bet10* double mutant plants showed a hypo-acetylation

state (**Figure 25**). As histone acetylation is an activating mark (Allfrey et al. 1964), the ChIP results are consistent with the changes in *TOC1* expression in BET9-ox and *bet9/bet10* compared to WT. Therefore, proper expression of *BET9* is important, directly or indirectly, in the control of histone acetylation at the *TOC1* promoter.

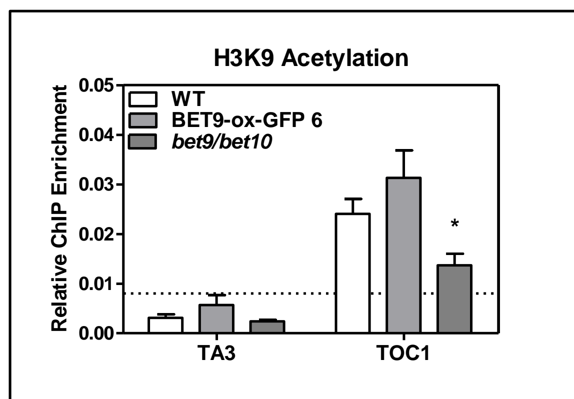


Figure 25. BET9 partially regulates the H3K9 acetylation at the *TOC1* promoter. ChIP-Q-PCR assay performed with WT, BET9-ox and *bet9/bet10* lines showing acetylation state of *TOC1* promoter. Seedlings were grown for 8 days under LgD, and then transferred to LL for 2 days before sampling at CT7. Enrichment was calculated relative to the input values. The data represent mean ±SEM of two biological replicates. Unpaired Student's t-test was used to determine statistical significance of different H3K9 acetylation levels of WT compared to *bet9/bet10* (*p < 0.05).

15. RVE8 binding to clock gene promoters in *bet9* mutant plants

Since BET9 and RVE8 are both activators of clock gene expression, we explored their genetic interaction, and generated transgenic lines over-expressing *RVE8* in the *bet9* mutant background (**Figure 26A**). Comparative ChIP analyses showed that the binding of RVE8 to the target promoters was similar in plants over-expressing RVE8 in a WT background than in the *bet9* mutant background (**Figure 26B**). As both lines showed similar over-expression of RVE8 (**Figure 26A**), the results suggest that BET9 is not required for RVE8 binding to the promoters of *TOC1* and *PRR5* (**Figure 26B**).

However, and due to the functional redundancy of BET9 and BET10, it would be necessary to investigate RVE8 binding in the *bet9/bet10* double mutant background. The results would confirm whether BET9 and BET10 influence binding of RVE8 to its target gene promoters. We are currently generating RVE8-ox plants in the *bet9/bet10* double mutant background to investigate this idea.

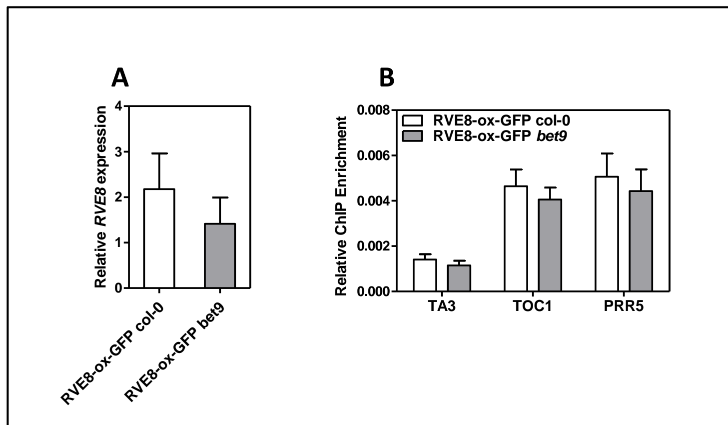


Figure 26. RVE8 binding to clock gene promoters in a WT and *bet9* mutant backgrounds. (A) RT-QPCR analysis of *RVE8* expression in a WT and a *bet9* mutant backgrounds. All the values are normalized to *IPP2* expression values. (B) Comparative ChIP analysis of RVE8 binding to clock promoters using RVE8-ox plants in a WT and in a *bet9* mutant backgrounds. Seedlings were grown for 8 days under LgD, and then transferred to LL for 2 days before sampling at CT7. *TA3* was used as negative control. Enrichment was calculated relative to the input values. The data represent mean + SEM of two biological replicates.

16. Clock gene expression of RVE8-ox in a *bet9* mutant background

To further characterize the genetic interaction of RVE8 and BET9, we performed time course analysis by RT-QPCR comparing clock gene expression in plants over-expressing RVE8 in a WT and in a *bet9* mutant

background. Time course analysis by RT-QPCR showed the up-regulation of *TOC1* and *PRR5* expression in RVE8-ox plants in the WT background (**Figure 27A**). Analyses of RVE8-ox plants in the *bet9* mutant background showed similar patterns of expression (**Figure 27A**), suggesting that the absence of a functional BET9 does not affect RVE8 regulatory function. As for the ChIP assays, the analyses of plants over-expressing RVE8 in the *bet9/bet10* double mutant background is essential to confirm whether BET9 and BET10 indeed contribute to RVE8 function.

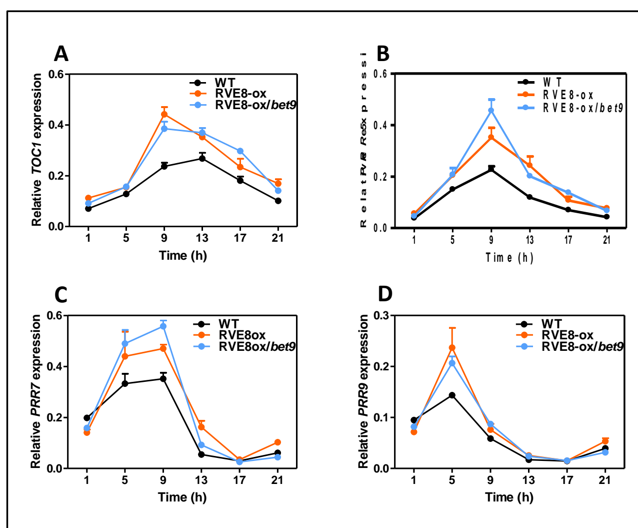


Figure 27. Analysis of clock gene expression in plants over-expressing RVE8 in WT and *bet9* mutant backgrounds. Time course analysis by RT-QPCR of (A) *TOC1*; (B) *PRR5*; (C) *PRR7*, and (D) *PRR9* expression in plants over-expressing RVE8 in a WT and a *bet9* mutant background. Seedlings were grown for 8 days under LgD, and transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* expression values. The data represent mean + SEM of two biological replicates.

17. RVE8 is required for proper BET9 binding to clock gene promoters

To further study the genetic interaction of RVE8 and BET9, we also generated lines over-expressing *BET9* in the *rve8* mutant background and selected the ones with similar (or slightly reduced) over-expression compared to that of

BET9-ox in a WT background (**Figure 28A**). Comparative ChIP analyses showed that the binding of BET9 to the target promoters was reduced (or abolished) in the *rve8* mutant background compared to WT (**Figure 28B**). The results suggest that RVE8 is required for BET9 binding to the promoters of *TOC1* and *PRR5* (**Figure 28**).

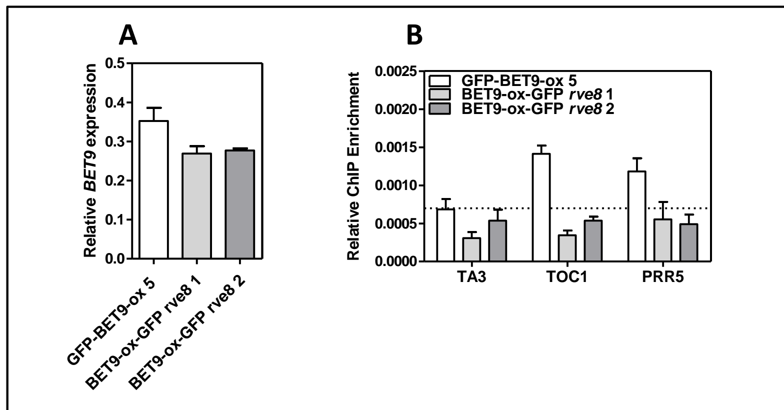


Figure 28. RVE8 is required for proper BET9 binding to clock gene promoters. (A) RT-QPCR analysis of GFP-BET9-ox 5 in WT, *rve8* 1 and *rve8* 2. All the values are normalized to *IPP2* expression values. (B) Comparative analyses of BET9 binding to clock gene promoters by ChIP assays using BET9-ox plants in a WT and a *rve8* mutant backgrounds. Seedlings were grown for 8 days under LgD, and then transferred to LL for 2 days before sampling at CT7. *TA3* was used as negative control. Enrichment was calculated relative to the input values. Data represent mean + SEM of two biological replicates.

18. RVE8 is required for proper regulation of BET9 clock gene expression

We next analyzed clock gene expression in plants over-expressing BET9 in the WT and *rve8* mutant backgrounds. Time course analyses by RT-QPCR of plants synchronized under LgD followed by two days under LL showed that the up-regulation of clock gene expression in BET9-ox plants was abolished in the *rve8* mutant background (**Figure 29**). The absence of a functional RVE8 led to

a delayed phase suggesting that BET9 requires RVE8 for exerting its regulatory function. These results are consistent with the reduced binding of BET9 to the target promoters in the *rve8* background (**Figure 28B**).

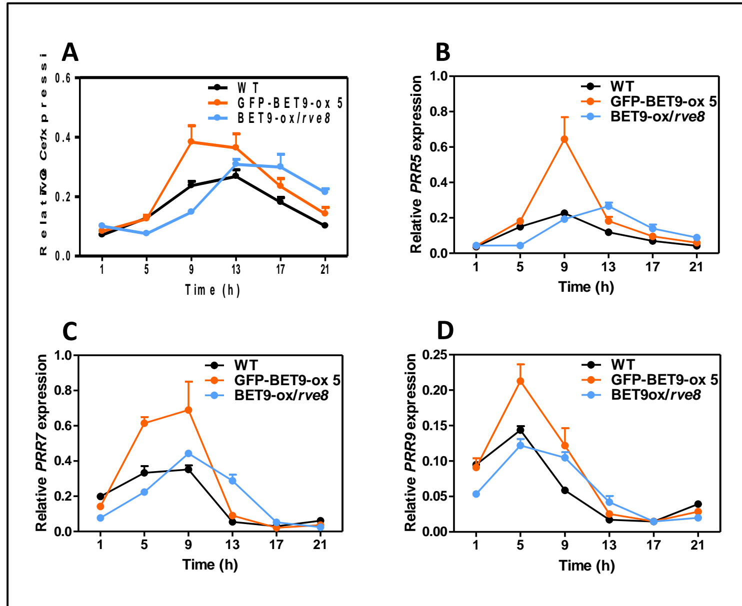


Figure 29. Analysis of clock gene expression in plants over-expressing BET9 in WT and *rve8* mutant backgrounds. Time course analysis by RT-QPCR of (A) *TOC1*; (B) *PRR5*; (C) *PRR7*, and (D) *PRR9* expression in plants over-expressing BET9 in a WT and a *bet9* mutant background. Seedlings were grown for 8 days under LgD, and transferred to LL for 2 days before sampling every 4h over a 24h cycle. Values were normalized to *IPP2* expression values. The data represent mean + SEM of two biological replicates.

DISCUSSION

In this Doctoral Thesis, we have investigated the function of BET9, a bromodomain-containing protein, within the Arabidopsis circadian system. Our studies show that the rhythmic oscillation of *BET9* expression is controlled by direct binding of the clock activator RVE8 to the *BET9* promoter. Furthermore, we found a role for BET9 regulating histone acetylation at the *TOC1* promoter, and show the genetic interaction data of BET9 and RVE8 in the control of clock gene expression. The circadian oscillator network is comprised by a wide number of repressors. In our studies, we have identified BET9 as an activator of clock gene expression.

Arabidopsis BET proteins contain a single bromodomain (Brian Florence and Faller 2001) in contrast to the bromodomains in tandem present in mammalian BET proteins (Kanno et al. 2004). The BRD of Arabidopsis BET proteins shares homology with the second BRD in mammals (Brian Florence and Faller 2001), whereas plants lack the first BRD of mammalian BET proteins and instead contain a plant-specific amphipathic domain (PAD), which may allow dimerization with other BET proteins (Brian Florence and Faller 2001). The ET domain in plant BET proteins present sequence similarity to the N-terminal portion of the ET domain in mammals and it is predicted to function as a protein-protein interaction domain (Zoi Lygerou et al. 1994; Brian Florence and Faller 2001; Rahman et al. 2011).

The *in silico* studies indicate that *BET9* is expressed in virtually all organs of the plant. Diurnal and circadian analyses also showed that *BET9* expression oscillates with a peak phase close to dusk (and subjective dusk). This pattern of expression resembles that of *PRR5* and *TOC1* (Strayer et al. 2000; Matsushika et al. 2000). The oscillation is likely to occur at different organs since RNA-Seq analyses at the shoot apex also revealed the oscillatory pattern of *BET9* with a similar peak phase of expression (N. Takahashi et al. 2015). Photoperiod and light might affect *BET9* expression as its amplitude seems to decrease with longer photoperiods and constant light conditions. It is likely that BET9 protein accumulation and function also oscillate, which provide a link

between the circadian clock, BET9 function and the targets that BET9 regulates.

BET10, the closest homolog of BET9, might complement the regulatory function of BET9. BET9 and BET10 share about 71.5% of amino acid identity (Edgar 2004) and cluster together in a clade within the phylogenetic tree of the Arabidopsis BET proteins. The similar peak phase of *BET9* and *BET10* expression and the phenotypes of *bet9/bet10* double mutant plants (see below) indeed suggest that BET9 and BET10 could perform redundant or partially redundant functions. Plant BET proteins contain a PAD domain, which is predicted to be important for dimerization between BET proteins (Florence and Faller, 2001). It would be interesting to examine the possible protein-protein interaction of BET9 and BET10, and the relevance of such interaction for their function.

The ET domain might also be important for the interaction with other proteins. Indeed, previous studies have identified that BET9 and BET10 interact with BT2, a BTB-domain (Broad-Complex, Tramtrack, and Bric-a-Brac) protein involved in plant responses to hormones and stress conditions (Misra, McKnight, and Mandadi 2018). The study proposed that BET9 and BET10 contribute to BT2-dependent responses to sugar and the hormone ABA (abscisic acid) (Misra, McKnight, and Mandadi 2018). The authors also proposed that BET9 and BET10 increase the affinity of the BT2-CULLIN3 ubiquitin ligase complex for the acetylated chromatin at the 35S enhancers (Misra 2011).

The study also shows that BET9-BET10-BT2 complex controls the 35S enhancer-mediated transcription and that mutation in any member of this complex leads to impaired transcription of 35S-regulated genes (Misra 2011). However, we have obtained lines over-expressing RVE8 under 35S promoter in the *bet9* mutant background. It is possible that BT2 and BET10 could aid in the activation of the 35S enhancer even in the absence of a functional BET9. In this regard, analyses of lines over-expressing RVE8 in the *bet9/bet10*

double mutant background will not only clarify whether RVE8 requires BET9 and BET10 proteins to exert its regulatory function, but also if BET9 and BET10 are both required to regulate 35S enhancer-mediated transcription.

Our results show that *BET9* expression is altered in *rve468* triple mutant plants indicating that these members of the RVE family are important for controlling *BET9* expression. However, we observed clear phenotypes also in the single *rve8* mutant. These results suggest that although RVE4, RVE6 and RVE8 might play a redundant regulatory function, as previously described (Hsu, Devisetty, and Harmer 2013), the absence of a functional RVE8 is sufficient to alter *BET9* expression. The contrasting phenotypes of RVE8 over-expression and mutation in the control of *BET9* also suggest a direct mechanism of control. This notion is supported by the results showing that changes of *BET9* gene expression in *rve8* mutant and RVE8-ox plants are similar to those observed for *TOC1*, which is directly regulated by RVE8. Our ChIP assays indeed confirmed the direct binding of RVE8 to the *BET9* promoter.

We propose that RVE8 is important for the activation of *BET9* following a similar mechanism that activates *PRR5* and *TOC1* (Farinas and Mas 2011b; Ma et al. 2018a; Rawat et al. 2011; Xie et al. 2014). This mechanism involves the interaction with the LNK proteins and the recruitment of the transcriptional machinery to the target genes (Ma et al. 2018a). Consistently, the expression of *BET9* is severely down-regulated in *lnk1/lnk2* double mutant plants, following a similar trend to that observed for *PRR5* and *TOC1* (Xie et al. 2014; Ma et al. 2018a). Regulation of *BET9* expression also relies on binding of RVE8 to the *BET9* promoter at the EE, a motif that is important for evening gene expression of *TOC1* (Alabadí et al. 2001) and most likely for *BET9*.

Our results also showed that the expression of morning and evening-phased oscillator genes was up-regulated in BET9-ox and down-regulated at their peak of expression in *bet9/bet10* double mutant plants. These results indicate that proper expression of *BET9* and *BET10* is important for the activation of clock gene expression, thus aiding the clock genes to reach their full amplitude.

Although the circadian network is comprised of an ample battery of repressors, new studies are increasingly identifying clock activators. Our results indicate an activating function of BET9 of clock gene expression. It is noteworthy that miss-expression of BET9 is able to overcome the complex regulatory network at the core of the clock. Indeed, the peak phase of expression of all oscillator genes examined are affected following the same trend, i.e. up-regulated in BET9-ox and down-regulated in *bet9/bet10*. This is not consistent with the regulatory network at the core of the clock. For instance, as TOC1 represses the morning-expressed clock genes, having less TOC1 in *bet9/bet10* should result in the up-regulation of morning-expressed genes. However, this is not the case, which suggests that BET9 regulation overcomes the oscillator network in plants miss-expressing BET9.

BET9 might bind to acetylated histones as shown for other BRD-containing proteins. The human genome encodes for 61 bromodomains, which bind to different acetylated lysines, depending on the structure of the region flanking the residue (Filippakopoulos et al. 2012). For example, a member of human BET family, BRD2 (BROMODOMAIN-CONTAINING PROTEIN 2), specifically interacts with acetylated H4K12 lysine residues (Kanno et al. 2004), whereas another member, BRD4 (BROMODOMAIN-CONTAINING PROTEIN 4) binds to acetylated tails of histone 3 (H3) and histone 4 (H4) (Dey et al. 2003). BET proteins have been also shown to bind to acetylated lysine residues in other organisms (de la Cruz et al., 2005; Kanno et al., 2004; Yang, 2004).

Few studies have conclusively reported the binding of BET proteins to acetylated residues in plants. An example includes BET3 (BROMODOMAIN AND EXTRA-TERMINAL DOMAIN 3, also known as GENERAL TRANSCRIPTION FACTOR GROUP E4/GTE4), which was proposed to regulate cell cycle genes by interacting with acetylated marks (Airoldi et al. 2010). The BET12 protein (BROMODOMAIN AND EXTRA-TERMINAL DOMAIN 12, also known as GENERAL TRANSCRIPTION FACTOR GROUP E6/GTE6) was proposed to regulate the ASYMMETRIC LEAVES1 (AS1) gene transcription by modulating acetylation of H3 and H4 tails (Chua et al., 2005).

The similarities in both sequence and predicted structure of BET9 BRD with the BET proteins suggest that BET9 could bind to acetylated histone residues through its BRD. If that is the case, and based on our results showing increased acetylation at the target promoters in BET9-ox plants, our results may also suggest that BET9 might recruit Histone acetyltransferases (HATs) or other factors that facilitate histone acetylation. The activation of clock gene expression in BET9-ox plants is consistent with the fact that increased histone acetylation has been associated with a relaxed chromatin conformation that favors gene transcription (Hernández-Rosas, López-Rosas, and Saavedra-Vélez 2020).

It is also possible that the increased acetylation at the promoters of clock genes in BET9-ox plants could occur by directly preventing the binding of histone deacetylases (HDACs) to the targeted loci. Indeed, BDF1 (BROMODOMAIN FACTOR 1), a yeast homolog of the plant BET proteins, physically prevents binding of the SIR2 (SIRTUIN 2) deacetylase to genes located at the boundary between heterochromatin and euchromatin (Ladurner et al. 2003). This mechanism effectively maintains a high acetylated state of H4 that enables active transcription of the targeted loci (Ladurner et al. 2003). A similar regulatory mechanism might prevail in the BET9-ox plants. However, the expression of clock genes still decline following their peak of expression in BET9-ox, whereas a raising phase is still present in *bet9/bet10* mutant plants. These results suggest that if BET9 and BET10 prevent HDACs binding to the clock target loci, this prevention mostly occurs at the peak phase of their expression.

Previous studies have shown that circadian rhythms of oscillator gene expression associate with the rhythmic accumulation of H3 acetylation and H3K4 trimethylation (H3K4me3) (Malapeira, Khaitova, and Mas 2012). Functional analyses showed that H3K4me3 contributed to the transition from activation to repression by delineating the timing of clock repressor binding (Malapeira, Khaitova, and Mas 2012). In a similar trend, BET9 and the associated histone acetylation at the promoters of oscillator genes might

prevent binding of clock repressors, thereby facilitating the proper amplitude of the oscillator gene expression. Further studies will be necessary to prove this hypothesis.

Another mechanism explaining BET9 function could be centered on RVE8, which provides the DNA binding sequence specificity and increased acetylation through a still unknown mechanism. In humans, the MYB DNA binding domain of the c-MYB protein also enables histone tail acetylation (Mo et al. 2005). The single MYB domain of RVE8 shares sequence similarity with c-MYB, and consistently, RVE8 miss-expressing plants modulate the pattern of histone acetylation at the *TOC1* promoter (Farinas and Mas 2011b). Thus, the DNA binding specificity of RVE8 and the subsequent increased acetylation might permit binding of BET9 to the acetylated residues. The genetic interaction studies showing that BET9 requires RVE8 for its full regulatory function support this notion. This regulatory function might rely on the direct protein-protein interaction of RVE8 with BET9. However, our preliminary analyses failed to show this interaction.

BET9 binding to histone acetylated residues might further facilitate an open conformation of chromatin that favors binding of clock activators such as RVE8. As mentioned above, BET9 and BET10 increase the affinity of the BT2-CULLIN3 ubiquitin ligase complex for the acetylated chromatin at the 35S enhancers (Misra 2011). The human BET protein, BRD2 functions as an adaptor for the E2F transcription factor and chromatin remodeling complexes to regulate *cyclin A* gene expression (Sinha, Faller, and Denis 2005). Another example includes the CREB Binding Protein (CBP) acting as a bridge between transcriptional co-activators (CREB, Ca^{+2}/CYCLIC AMP-RESPONSIVE ELEMENT BINDING) and RNA Polymerase II. Our genetic interaction analyses do not support for now a model in which BET9 facilitate the activating function of RVE8, although studies using RVE8-ox in the *bet9/bet10* double mutant background are essential to fully prove this idea.

One possible output of BET9 function could be ABA signaling pathway. As mentioned above, BET9 contributes to plant responses to ABA (Misra, McKnight, and Mandadi 2018). The *bet9* and *bet10* single mutants showed a hypersensitive response to ABA, resulting in impaired germination (Misra, McKnight, and Mandadi 2018) TOC1 also directly regulates ABA signaling and plant responses to drought (Legnaioli, Cuevas, and Mas 2009). As BET9 regulates *TOC1* expression, it is possible that the connection of BET9 with the ABA signaling pathway might rely, at least in part, on TOC1. In this regard, it would be interesting to conduct genetic interaction studies between BET9 and TOC1, and check ABA-regulated processes, including plant responses to drought.

The current network of clock gene expression in *Arabidopsis thaliana* is full of repressors. Our studies have identified an activator of oscillator gene expression. BET9 function might ensure that the oscillator gene expression reaches full amplitude so that the clock components can exert their circadian function at the proper time during the day or night. BET9 regulatory function also paves new ways to manipulate clock activity by modulating chromatin conformation and accessibility of clock activators. Manipulation of the clock function in synchrony with the diurnal and seasonal environmental changes is relevant for its possible biotechnological application to crops of agronomical interest.

CONCLUSIONS

In this Doctoral Thesis, we have examined the functional connection of the bromodomain-containing protein BET9 (BROMODOMAIN AND EXTRA-TERMINAL DOMAIN 9) with the *Arabidopsis thaliana* circadian clock. The main conclusions of our studies can be summarized as follows:

1. ***BET9* and *BET10* expression is controlled by the circadian clock.** *BET9* and *BET10* rhythmically oscillate under entraining and free-running conditions with a peak of expression around dusk.

2. ***BET9* expression is directly regulated by the clock component RVE8.** RVE8 binds to the *BET9* gene promoter and activates its expression. The clock components LNKs are also essential for *BET9* activation.

3. **BET9 contributes to the up-regulation of clock gene expression.** Over-expression of BET9 up-regulates morning- and evening-expressed clock genes.

4. **BET9 and BET10 have a redundant role in the control of clock gene expression.** The expression of morning- and evening-expressed clock genes is down-regulated in *bet9/bet10* double mutant plants, whereas gene expression is only marginally altered in *bet9* single mutant plants.

5. **BET9 and BET10 favor histone 3 acetylation at the *TOC1* promoter.** Over-expression of BET9 results in a hyperacetylated state of HISTONE 3 at the *TOC1* promoter, whereas HISTONE 3 acetylation is reduced in *bet9bet10* double mutant plants.

6. **BET9 requires RVE8 to exert its function.** Activation of gene expression and BET9 binding to its target gene promoters is reduced in the absence of a functional RVE8.

SUMMARY

The circadian clock is an endogenously generated timekeeping mechanism that generates 24-hour rhythms in multiple biological processes. The rhythmic oscillations provide an adaptive advantage, allowing organisms to anticipate and adjust to the environmental changes that occur during the day and night cycle. The generation of the rhythms rely on the oscillations in gene expression and protein function at the core of the oscillator. Over the last years, changes in chromatin marks have been identified as an important mechanism contributing to the rhythmic oscillations. However, we are still far from the identification of the chromatin-related components that are responsible for the rhythmic regulation of these chromatin changes. In this Doctoral Thesis, we have characterized the function of the BROMODOMAIN AND EXTRA-TERMINAL DOMAIN 9 (BET9) protein within the *Arabidopsis thaliana* circadian clock. We have identified the rhythmic oscillation of *BET9* expression, which is controlled by the binding of the clock activator known as REVEILLE 8 (RVE8) to the *BET9* promoter. Characterization of *bet9* mutant plants and lines over-expressing BET9 (BET9-ox) showed that BET9 functions as an activator of clock gene expression. Chromatin immunoprecipitation assays (ChIP) also showed that BET9 directly binds to the promoters of essential clock genes such as *TIMING OF CAB2 EXPRESSION 1* (*TOC1*) and *PSEUDO RESPONSE REGULATOR 5* (*PRR5*). Increased binding of BET9 to these promoters in BET9-ox plants correlated with increased HISTONE 3 acetylation at the *TOC1* promoter, and conversely, with hypoacetylation in the *bet9* mutant. Genetic interaction studies showed that BET9 requires a functional RVE8, as the BET9-ox gene expression phenotypes and BET9 binding to the gene target promoters were abolished in the *rve8* mutant background. Our studies have thus uncovered a chromatin-related protein that together with RVE8 contributes to the activation of clock gene expression.

RESUMEN

El reloj circadiano es un mecanismo endógeno que genera ritmos de 24 horas en múltiples procesos biológicos. Las oscilaciones rítmicas proporcionan una ventaja adaptativa, permitiendo a los organismos anticipar y ajustarse a los cambios ambientales que ocurren en el ciclo de día-noche. La generación de los ritmos se basa en las oscilaciones rítmicas de expresión génica y función de proteínas del oscilador circadiano. En los últimos años, los cambios en marcas de cromatina se han identificado como un mecanismo importante que contribuye a las oscilaciones rítmicas. Sin embargo, estamos aún lejos de la identificación de todos los componentes relacionados con la cromatina que son responsables de la regulación rítmica de estos cambios de cromatina. En la presente Tesis Doctoral, se describe la caracterización de la función de la proteína BROMODOMAIN AND EXTRA-TERMINAL DOMAIN 9 (BET9) en el reloj circadiano de *Arabidopsis thaliana*. Hemos identificado la oscilación rítmica de la expresión *BET9*, que está controlada por la unión del activador de reloj denominado REVEILLE 8 (RVE8) al promotor de *BET9*. La caracterización de plantas mutantes de *bet9* y líneas que sobre-expresan BET9 (BET9-ox) mostró que BET9 funciona como un activador de la expresión génica del reloj. Los ensayos de inmunoprecipitación de cromatina (ChIP) también mostraron que BET9 se une directamente a los promotores de genes de reloj esenciales como *TIMING OF CAB2 EXPRESSION 1* (*TOC1*) y *PSEUDO RESPONSE REGULATOR 5* (*PRR5*). Observamos que el aumento de la unión de BET9 a estos promotores en plantas BET9-ox se correlacionaba con el aumento de la acetilación de la HISTONA 3 en el promotor de *TOC1* y, a la inversa, con la hipoacetilación en el mutante *bet9*. Los estudios de interacción genética mostraron que BET9 requiere un RVE8 funcional, ya que los fenotipos de expresión génica de plantas BET9-ox y la unión de BET9 a los promotores diana quedaban abolidos en el fondo mutante *rve8*. Por tanto, nuestros estudios han descubierto una proteína relacionada con la cromatina que junto con RVE8 contribuyen a la activación de la expresión génica del reloj.

MATERIALS AND METHODS

1. Plant material and growth conditions

Columbia (Col-0) ecotype of *Arabidopsis thaliana* was used as background for all experiments performed in this study. All the T-DNA insertion lines of *bet9* (GK_826H06 and SALK_119044) and *bet10* (GK_856G10) were obtained from Nottingham Arabidopsis Stock Centre (NASC). RVE8-ox (Farinas and Mas 2011a), *rve8* (SALK_016333C) (Farinas and Mas 2011a), *rve468* (SALK_137617, SALK_069978, SALK_016333C) (Hsu et al., 2013), *lnk1/lnk2* (SALK_024353, GK_484F07) (Rugnone et al. 2013) have been described previously. *bet9* (GK_826H06) and *bet10* (GK_856G10) single mutants were crossed to generate double *bet9/bet10* mutant. Homozygous mutant plants were identified using locus-specific and T-DNA insertion-specific primer pairs. Primers used for genotyping are listed in Table 3. Transgenic lines used in this study are listed in Table 4.

Sterilization of *Arabidopsis* seeds was performed using liquid-phase sterilization with sodium hypochlorite (NaClO, i.e. bleach). 1.43 mL of bleach was diluted with 3.57 mL of water containing 0.02 mL of Tween 20 (Merck) non-ionic detergent. This solution was mixed with absolute ethanol (99% EtOH) for a final ethanol concentration of 70%. Seeds were surface sterilized in this solution for 12 minutes, washed three times with EtOH and left to dry in laminar flow hood.

Following sterilization, seeds were sown on Murashige and Skoog (MS) medium without sucrose and kept in darkness at 4^0C for 2 to 4 days. Upon stratification, seeds were transferred to chambers for plant growth (Inkoa Sistemas). All the experiments were performed at 22^0C under 60-100 µmol $m^{-2}s^{-1}$ of cool white fluorescent light and different photoperiod (short day, long day or constant light) conditions, as indicated for each experiment.

2. Plasmid construction and generation of transgenic plants

The coding sequence (CDS) of *BET9* gene was amplified by PCR (Polymerase Chain Reaction) and cloned into pENTR/D-TOPO vector (Invitrogen, Gateway®) following the instructions of the manufacturer. The resulting entry vector was introduced into chemically competent E.coli (One Shot® TOP10, Gateway®). Resistant bacterial colonies were obtained by overnight incubation at 37^{0}C on 50 µg/ml kanamycin selection plates. 10 resistant colonies were selected and amplified overnight in Yeast Extract Beef (YEB) liquid medium containing 50 µg/ml kanamycin antibiotic. Liquid colony PCR was used to detect colonies containing the *BET9* CDS. The plasmids were purified from 5 positive liquid colonies containing the construct using Plant Mini-Prep Kit (Qiagen). The correct sequence of *BET9* CDS was confirmed by capillary sequencing with M13 and F13 primers (Gateway®). Entry vector was used to deliver *BET9* CDS without mutations into plant destination vectors pGWB505 (35S pro, C-sGFP) and pGWB506 (35S pro, N-sGFP) using LR reaction (Gateway®). The obtained expression vectors were amplified in chemically competent E.coli (One Shot® TOP10, Gateway®) and further introduced to *Agrobacterium tumefaciens* strain GV2660. Transformed Agrobacteria were grown for 48 hours at 28^{0}C on solid YEB containing selection antibiotics (ampicillin 100 µg/ml, rifampicin 100 µg/ml and spectinomycin 100 µg/ml). The resulting colonies were moved to liquid YEB media containing selection antibiotics (ampicillin 100 µg/ml, rifampicin 100 µg/ml and spectinomycin 100 µg/ml) and grown at 28^{0}C with agitation until OD600 ≈ 2.0. 30 mL of liquid medium were added to 120 ml of sucrose and Silwet L-77 detergent solution. The WT plants were transformed through *Agrobacterium tumefaciens* (GV2260)-mediated DNA transfer (Clough and Bent 1998) by dipping the inflorescences for 20-30 seconds in the solution containing the Agrobacterium, sucrose and detergent. Subsequently, plants were kept in dark and humid place for 48h and then moved to growth chamber. One-insert, homozygous

lines in T3 generation were obtained by antibiotic selection plates (kanamycine 50 µg/mL or hygromycine 40 µg/mL).

3. RNA extraction and real-time quantitative PCR analysis

For the gene expression time course analysis, 10-day-old seedlings were collected every four hours over a 24 hour period under diurnal or circadian conditions. Total RNA was extracted using Maxwell® RSC Plant RNA Kit (Promega). 1µl of extracted RNA was used to synthetize single strand complementary cDNA with iScript™ Reverse Transcription Supermix for RT-Q-PCR (Bio-rad). Obtained cDNA was diluted with nuclease-free water for a final cDNA concentration of 10 ng/µl. Q-PCR was performed using Brilliant III Ultra-Fast SYBR Green Q-PCR Master Mix (Agilent) with a 96-well CFX96 Touch Real-Time PCR detection system (Bio-Rad) following the protocol from manufacturer. All the samples used for Q-PCR experiments were run in three technical replicates, using *IPP2* (*ISOPENTENYL PYROPHOSPHATE: DIMETHYL-ALLYL PYROPHSPHATE ISOMERASE*) gene levels as reference. Relative transcript levels were calculated using the 2(-Delta Delta C(T)) method (Livak and Schmittgen 2001). The primers used in this study are shown in Table 3.

4. Chromatin immunoprecipitation

Upon stratification, plants were grown under LgD conditions at 22^{0}C for 8 days, then moved to constant light for 2 days and sampled on the third day. The samples were collected and processed following previously published protocol (Yamaguchi et al. 2014) with small modifications. 350-400 mg of fresh plant tissue was fixed using vacuum infiltration with solution of PBS (Phosphate-buffered saline) and 1% formaldehyde (16% formaldehyde solution (w/v) methanol-free, Thermo Fisher Scientific). Fixation process did not last longer than 15 minutes and was terminated with 0.125M glycine solution as quencher. Samples were rinsed twice with ice-cold PBS, quickly dried on paper and

frozen in liquid nitrogen. Fixed samples were processed by grinding and homogenizing in nuclei extraction buffer (100mM MOPS pH 7.6, 10mM $MgCl_2$, 0.25M sucrose, 5% Dextran T-40, 2.5% Ficoll 400, 40mM β-Mercaptoethanol, 1 x Protease Inhibitor Cocktail), followed by nuclei lysis in filter sterilized buffer (50mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 1% SDS). Extracted chromatin was shredded in ChIP dilution buffer (16.7mM Tris-HCl pH 8.0, 167 mM NaCl, 1.2mM EDTA, 0.01% SDS) using sonication device (Bioruptor® NextGen, Diagenode) (treatment: 30s on, 30s off for 8 cycles at low intensity) and pre-cleaned by incubation with Protein G-Dynabeads magnetic beads (Invitrogen by Thermo Fisher Scientific). Cleaned solution was incubated overnight with Anti-GFP (αGFP) (Invitrogen by Thermo Fisher Scientific) or Anti-acetyl-Histone H3 (Lys9) (αAcH3K9) (Merck) antibody. Overnight immunoprecipitation with antibody was followed by 4 hours of incubation with Protein G-Dynabeads magnetic beads. Beads were washed two times with each ice-cold: low salt buffer (0.1% SDS, 1% Triton-X, 2mM EDTA, 20mM Tris-HCl pH 8.0, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton-X, 2mM EDTA, 20mM Tris-HCl pH 8.0, 500mM NaCl), LiCl buffer (0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.0) and 0.5 TE buffer. The samples were separated from the beads by incubation at 65^0C. DNA was reverse-crosslinked by incubation at 95^0C for 15 minutes. DNA was purified using QIAquick kit (Qiagen) and enrichment levels were quantified using Brilliant III Ultra-Fast SYBR Green Q-PCR Master Mix (Agilent) with a 96-well CFX96 Touch Real-Time PCR detection system (Bio-Rad) following the protocol from manufacturer. All the ChIP samples were run in three technical replicates. *TA3* retrotransposon was used as a negative control. Enrichment values for each primer pair were calculated relative to 2% input (total chromatin) values. The list of ChIP-Q-PCR primers is provided in Table 3.

5. *In vivo* luminescence assay

In vivo luminescence assay was done as previously described (Ma et al. 2018a). Plants were grown under 12:12 photoperiod for 7 days at 22^{0}C and subsequently transferred to 96-well white microplates (Berthold). Each well was filled with a mix of 160 µl of MS medium without sucrose and 40 µl of 1,44mM D-Luciferin (Sigma). Plates were synchronized under 12:12 photoperiod for one more day and subsequently moved to constant light at 22^{0}C. Luminescence was measured using LB960 luminometer (Berthold Technologies) by Microwin 2010 software (Mikrotek Laborsysteme). Luminescence rhythms were analysed using BioDare2 software (biodare2.ed.ac.uk) repository for circadian data (Zielinski et al. 2014). Period, phase and amplitude were calculated using Fast Fourier Transform-Non-Linear Least Squares suite. At least two biological replicates were done for each experiment. Each experiment included 8-12 seedlings per genotype.

6. Confocal imaging

Plants were grown for seven days in MS medium without sucrose under long day photoperiod at 22^{0}C. The root tip cells were observed using FV3000 Confocal Laser Scanning Microscope (Olympus) (excitation, 488nm; emission, 509 nm) with 20x and 60x objectives to detect subcellular localization of the GFP signal. WT plants grown in same conditions were used as negative control.

7. Statistical analysis

All the statistical analysis and representation of data has been performed in Prism 5 (GraphPad). Statistical significance was calculated using two-tailed *t* test with 95% confidence interval (*p < 0.05; ** p < 0.01; *** p < 0.001). All the experiments have at least two biological replicates performed on different days and analysed separately.

Table 3: List of primers used in this study

Name	Sequence	Experiment
BET9_T-DNA_L	TGTTATGGCTGATACACTTCGTAA	Genotyping
BET9_T-DNA_R	TCAGTAGTACAACCCAGGGGATAG	Genotyping
GabiKat_RBo3144	GTGGATTGATGTGATATCTCC	Genotyping
BET10_T-DNA_L	CATCTTTTGCACCAAAAGAGG	Genotyping
BET10_T-DNA_R	AAGCCCTTCAGAGTTTTCTGC	Genotyping
SALK_LBb1.3	ATTTTGCCGATTTCGGAAC	Genotyping
BET9_TOPO_F	CACCATGACAGAGAGAAACGGTGGTTT	Cloning
BET9_TOPO_R	ATCAATCTCTCCCTCTTCTATATCTATTTC	Cloning
BET9_TOPO_STOP_R	ATAGATATAGAAGAGGGAGAGATTGATTGA	Cloning
IPP2_EXP_F	CCAGGACAATGCACTAGGTGTG	Expression
IPP2_EXP_R	AGGGAGTGAACTCATCGACTGG	Expression
BET9_EXP_F	AGTTTACCGAGGGCATCAAAGGG	Expression
BET9_EXP_R	AGCGCTTGTAGCTCCATCCAAC	Expression
BET10_EXP_F	AAGCACGGTTACAAGCAGAA	Expression
BET10_EXP_R	CTCAAGCAATGCCTGCCG	Expression
TOC1_EXP_F	TCTTCGCAGAATCCCTGTGAT	Expression
TOC1_EXP_R	GCTGCACCTAGCTTCAAGCA	Expression
PRR5_EXP_F	AATGGTGGTGATGCCCAGAG	Expression
PRR5_EXP_R	GCACTCCATCTGTACTGCGT	Expression
PRR7_EXP_F	AAGTAGTGATGGGAGTGGCG	Expression
PRR7_EXP_R	GAGATACCGCTCGTGGACTG	Expression
PRR9_EXP_F	ACCAATGAGGGGATTGCTGG	Expression
PRR9_EXP_R	TGCAGCTTCTCTCTGGCTTC	Expression
RVE8_EXP_F	ACTCTTCGTGGAGCAGAAGCTG	Expression
RVE8_EXP_R	TGAAGCACTGGAGGCTGTTTAGC	Expression
LNK1_EXP_F	TGGAAACAGACCGGAGAAAGGC	Expression
LNK1_EXP_R	TCCAGCATACTTGTCTGCTTCACC	Expression
LNK2_EXP_F	CTCAGTTGAGGACCAGCCATATC	Expression
LNK2_EXP_R	TCCTCTGACCGTACAGCTCTT	Expression
CCA1_EXP_F	TCGAAAGACGGGAAGTGGAACG	Expression
CCA1_EXP_R	GTCGATCTTCATTGGCCATCTCAG	Expression
TA3_ChIP_F	CTGCGTGGAAGTCTGTCAAA	ChIP assay
TA3_ChIP_R	CTATGCCACAGGGCAGTTTT	ChIP assay
BET9_1_ChIP_F	ACCGTTAAGTGTGGAATCAGACA	ChIP assay
BET9_1_ChIP_R	GCAGAATGACGAATACGACCG	ChIP assay
BET9_2_ChIP_F	GTAATAGCTTGGGAAGTGTGTCC	ChIP assay

BET9_2_ChIP_R	TTTGCATGTTATTCCAGACCAC	ChIP assay
TOC1_ChIP_F	ATAAACGAAACGAAGCCGAATC	ChIP assay
TOC1_ChIP_R	CAAACATATCAAAAGGTCGACAGAA	ChIP assay
PRR5_ChIP_F	TGCAAACCTATGTACCAAACAGA	ChIP assay
PRR5_ChIP_R	AAATCCCACTCGTGACTTTTG	ChIP assay
CCA1_ChIP_F	CACGTGTCGACAAACTGGTG	ChIP assay
CCA1_ChIP_R	GTTCCGGGACTACCTGAAAGG	ChIP assay

Table 4: List of *Arabidopsis thaliana* lines used in this study

Line	Gene	Reporter	Tag	Reference
Col-0	-	-	-	N/A
Col-0	-	*TOC1::LUC*	-	Perales and Más 2007
Col-0	-	*PRR5::LUC*	-	Kamioka et al. 2016
bet9	At5g14270	-	-	Alonso et al. 2003
bet10	At3g01770	-	-	Alonso et al. 2003
bet9/bet10	At5g14270 At3g01770	-	-	In this study
rve8	At3g09600	*TOC1::LUC*	-	Farinas and Mas 2011
rve4/rve6/rve 8	At5g02840 At5g52660 At3g09600	*CCR2::LUC*	-	Hsu et al. 2013
lnk1/lnk2	At5g64170 At3g54500	-	-	Rugnone et al. 2013
BET9-ox	At5g14270	-	C-sGFP	In this study
BET9-ox	At5g14270	*TOC1::LUC*	N-sGFP	In this study
BET9-ox	At5g14270	*PRR5::LUC*	N-sGFP	In this study
RVE8-ox	At3g09600	*TOC1::LUC*	C-sGFP	Farinas and Mas 2011
BET9-ox/*rve8*	At5g14270/ At3g09600	*TOC1::LUC*	N-sGFP	In this study
RVE8-ox/*bet9*	At3g09600/ At5g14270	*TOC1::LUC*	C-sGFP	In this study

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Review

Chromatin Dynamics and Transcriptional Control of Circadian Rhythms in *Arabidopsis*

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Abstract: Circadian rhythms pervade nearly all aspects of plant growth, physiology, and development. Generation of the rhythms relies on an endogenous timing system or circadian clock that generates 24-h oscillations in multiple rhythmic outputs. At its bases, the plant circadian function relies on dynamic interactive networks of clock components that regulate each other to generate rhythms at specific phases during the day and night. From the initial discovery more than 13 years ago of a parallelism between the oscillations in chromatin status and the transcriptional rhythms of an *Arabidopsis* clock gene, a number of studies have later expanded considerably our view on the circadian epigenome and transcriptome landscapes. Here, we describe the most recent identification of chromatin-related factors that are able to directly interact with *Arabidopsis* clock proteins to shape the transcriptional waveforms of circadian gene expression and clock outputs. We discuss how changes in chromatin marks associate with transcript initiation, elongation, and the rhythms of nascent RNAs, and speculate on future interesting research directions in the field.

Keywords: Arabidopsis; circadian clock; chromatin; transcriptional rhythms

1. The Plant Circadian Clock

The circadian clock is an endogenous timing mechanism able to generate biological rhythms with a period of 24 h. Although the circadian system is daily synchronized by changes in light and temperature, it is also capable of sustaining the circadian oscillations under constant environmental conditions [1]. The circadian clock regulates an ample range of physiological, developmental and metabolic processes ensuring that they are appropriately phased in concordance with the cellular demands [2]. The mechanisms responsible for the generation of the rhythms are quite complex and involve the orchestrated expression and function of key essential components [3,4]. This molecular oscillator is exquisitely connected with synchronizing cues to timely drive the rhythms of the biological processes or rhythmic outputs controlled by the clock [5].

The circadian function is particularly important in plants, possibly due to their sessile nature and the need for constantly monitoring the environment for proper adaptation and survival. Indeed, the anticipatory function of the plant circadian system has been proposed to provide an adaptive advantage and improve fitness [6–9]. At its basis, a common mechanism responsible for the generation of rhythms in eukaryotic cells relies on circadian negative feedback loops of activators that drive the expression of negative components, which feedback to inhibit their own expression [10]. This basic transcriptional regulatory mechanism is complemented by additional layers of circadian control including among others chromatin regulation, RNA metabolism and changes in cellular and subcellular localization [11–14].

The molecular components of the plant circadian system have been extensively identified and characterized in the model plant *Arabidopsis thaliana*. Circadian studies on crops have also started to uncover the divergences but also the similarities of clock components in plant model systems and other crops [15,16]. The firstly identified *Arabidopsis* clock component, *TIMING OF CAB EXPRESSION (TOC1)* or *PSEUDO RESPONSE REGULATOR1 (PRR1)*, was initially isolated nearly 25 years ago [17], and characterized as an evening-expressed gene, important in the regulation of circadian rhythms and flowering time [18–23]. Two additional clock components, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), were later identified and characterized [24,25]. CCA1 and LHY are two morning-expressed and partially redundant single MYB-containing proteins that form heterodimers to fulfill their circadian function [26,27]. TOC1, CCA1, and LHY were proposed to form a transcriptional feedback loop essential for circadian rhythmicity in *Arabidopsis* [20].

Research over the last years has contributed to extend our knowledge on the molecular circadian network, adding numerous new components and regulatory mechanisms [28]. For instance, other members of the *PSEUDO RESPONSE REGULATOR* (*PRR*) gene family including *PRR3*, *PRR5*, *PRR7*, and *PRR9* [29,30] were found to be closely associated with the *Arabidopsis* clock [31,32]. The *PRR* gene family members are sequentially expressed, starting with *PRR9* with a peak-phase closed to dawn, followed by *PRR7* and *PRR5* at midday and by *TOC1* with a peak-phase of expression at dusk [19,29]. Other evening-expressed components include the *EARLY FLOWERING 3* (*ELF3*) and *ELF4* genes coding for two plant-specific proteins without recognizable domains [33,34] and *LUX ARRHYTMO* (*LUX*) coding for a single MYB-like GARP transcription factor [35,36]. These three clock proteins were first identified by genetic screens for components involved in flowering and hypocotyl regulation [33,37–39]. The proteins were found to interact forming a multi-protein complex called the EVENING COMPLEX (EC) [40,41]. Recent studies have indicated that the EC components may also have independent functions from the EC [42–44].

The main clock components engage in highly complex regulatory networks that ensure the specific phase of oscillator gene expression during the day and night (Figure 1). For instance, CCA1 and LHY repress the *PRR*s [45], which in turn suppress *CCA1* and *LHY* transcription [31]. TOC1 not only represses *CCA1* and *LHY* expression [46–48] but nearly all the oscillator genes by binding to their promoters [48]. The *EC* components are repressed by CCA1 and LHY in the morning [49–51] and by TOC1 in the evening [48]. In turn, the EC acts as a transcriptional repressor directly binding to the *PRR9*, *PRR7*, and *LUX* promoters and repressing their expression [52–55]. By repressing the repressors of *CCA1* and *LHY*, the EC indirectly promotes *CCA1* and *LHY* expression.

The identification of the regulatory function of the clock components uncovered a prevalent number of repressors, opening the question about the mechanisms of circadian transcriptional activation. Over the past recent years, a number of new components have been proposed to function as activators of clock gene expression (Figure 1). One example includes the *LIGHT-REGULATED WD1* (*LWD1*) and *LWD2* genes, encoding WD (Trp and Asp)-containing proteins [56]. The LWDs directly bind to the promoters of *CCA1*, *PRR9*, *PRR5*, and *TOC1* to activate their expression [57,58]. Another example includes REVEILLE 8 (RVE8 also known as LHY-CCA1-LIKE5 or LCL5), a protein that belongs to the CCA1 and LHY single-MYB protein family [59,60]. Despite being members of the same family of plant transcription factors, RVE8 activates the expression of *TOC1* and *PRR5*, thus, in an opposite way to the repressing function of CCA1 and LHY [59,61]. RVE8 also directly activates the expression of *PRR9*, *ELF4* and *LUX* [62]. Other members of the RVE protein family appear to be functionally redundant with RVE8, as the *rve4rve6rve8* triple mutant accentuates the long period phenotype of *rve8* single mutant [62].

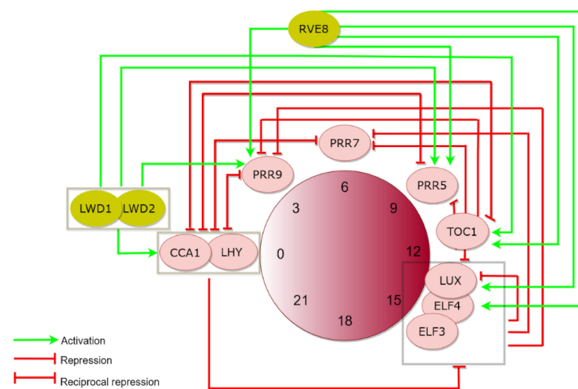

Figure 1. Schematic drawing depicting the basic regulatory network at the core of the *Arabidopsis* circadian oscillator. Oscillator genes are expressed at different phases during the day and night, from morning hours on the left on the central circle (clock) to evening hours on the right. Repression is depicted as red lines ending in small perpendicular dashes whereas activation is indicated by the green arrows. Clock components that interact to perform their regulatory function are encircled in grey line boxes. CCA1 and LHY are repressors of *PRR* genes, including *TOC1.* PRRs in turn repress the expression of *CCA1* and *LHY*. Expression of *Evening Complex (EC)* components (*LUX*, *ELF4*, *ELF3*) is repressed by both CCA1/LHY and TOC1. The EC represses expression of *PRR9*, *PRR7*, and *LUX.* The regulatory network is dominated by repressive interactions, although recent studies have uncovered a number of activating factors such as LWD1/2 and RVE8, which activate the expression of multiple morning- and evening-expressed oscillator genes. Please consult the main text for further details.

Overall, it is well established that generation of 24-h rhythms requires the accurate coordination of the expression and activities of numerous clock components. These components regulate each other through multiple regulatory mechanisms to ultimately control plant physiology and development in synchronization with the environment [12]. In this review, we focus on one of these regulatory mechanisms: chromatin changes and its connection with circadian transcriptional regulation. We do not attempt to provide an exhaustive description of all what is known related to the topic but rather to provide an update on the most recent and relevant discoveries functionally linking chromatin status and the plant circadian clock. Readers are encouraged to consult recent reviews that have in-depth descriptions on this and other specific topics [14,63,64].

2. Transcriptional Dynamics and Chromatin Status

Transcriptional regulation is intimately connected with chromatin status, which can be modified by changes in DNA methylation [65], histone covalent modifications [66,67], nucleosome remodeling and replacement of core histone with histone variants [68] and higher-order chromatin location and organization [69]. The four core histones (H2A, H2B, H3, and H4) can be modified at different amino acid residues by a repertoire of modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, glycosylation, ADP ribosylation, biotinylation, and carbonylation [67]. These modifications are able to alter the accessibility of chromatin to the transcription machinery, thus influencing the transcriptional outcome [66].

Acetylation of histones is controlled by histone acetyltransferases (HATs) and has been mostly associated with gene activation [70]. Plants have multiple HATs [71], which have been functionally characterized to a different degree. One major class of plant HATs present homology with the yeast and Tetrahymena GCN5 (GENERAL CONTROL NONDEREPRESSIBLE 5) family [72]. GCN5 appears to be important in the regulation of many processes including cell differentiation, organogenesis, and responses to light and cold [73]. The acetylation of histones can be reverted by histone deacetylases such as RPD3 (REDUCED POTASSIUM DEPENDENCY PROTEIN 3)-like and SIR2 (SILENT INFORMATION REGULATOR PROTEIN 2)-like, which are conserved across all eukaryotes [70,71].

Histones can be also methylated by histone methyltransferases (HMTs) including a group of SET (SU(VAR)/E(Z)/TRX) domain proteins. Histone methylation is associated either with gene activation or with repression depending on the amino acid residue of the modification [74]. For instance, histone H3 methylation of lysine 4 (H3K4) or lysine 36 (H3K36) is generally associated with activation of gene expression, whereas methylation of lysine 9 (H3K9) or lysine 27 (H3K27) is usually related to heterochromatin and gene repression [74]. Histone methylation is also reversible through the action of histone demethylases such as lysine-specific demethylase 1 (LSD1) and Jumonji C (JmjC) domain-containing proteins, which play important roles in the regulation of plant growth and development [75].

3. Functional Link between Chromatin and Circadian Transcription

The connection between chromatin changes and the *Arabidopsis* circadian clock was first reported about thirteen years ago [76]. The study showed that the rhythmic changes in *TOC1* mRNA expression were associated with parallel oscillations in histone acetylation [76]. The trough of *TOC1* expression coincided with histone deacetylation and with maximal CCA1 repressor binding [76]. Later studies reported that other histone marks also associate with the chromatin state at the *TOC1* promoter [77,78]. The accumulation of some of these histone marks such as histone 3 lysine 4 trimethylation (H3K4me3) was found to antagonize the binding of clock repressors, ensuring that repression occurred at the proper time during the day and night cycle [78]. The rhythms in histone marks were found not only at the *TOC1* promoter but in many oscillator loci [63]. From that point on, a number of chromatin-related factors were identified as "writers" and "erasers" of the histone marks important for the circadian oscillation. Here we describe the most relevant studies over the last couple of years (Table 1).

Table 1. List of the most recent findings connecting chromatin changes and circadian oscillator genes in *Arabidopsis*.

Histone Mark	Clock/Chromatin-Related Factor	Regulated Clock Component	Reference
Acetylation	HAF2	*PRR5, LUX*	[79]
Deacetylation	Sin3-HDAC	*CCA1, PRR9*	[80]
	ELF3-HDA9	*TOC1*	[81]
	EC-HDA9-HOS15	*GI*	[82]
	HDA6-CCA1/LHY	*TOC1*	[83]
	HDA6-TOC1	*CCA1, LHY*, other clock genes	[84]
Methylation	SDG2 (ATXR3)	*CCA1, LHY*	[78,85]
	RVE8/LNKs	*PRR5, TOC1 nascent RNAs*	[86]
Demethylation	JMJ14	*CCA1, LHY*	[85]
	CCA1/LHY-LDL1/2	*TOC1*	[83]
	TOC1-LDL1/2	*CCA1, LHY, other clock genes*	[84]
	JMJ13	*CCA1, LHY*	[87]
Monoubiquitination	HUB1/HUB2	*CCA1*	[88]
Histone Variant H2A.Z	ELF3-SWR1	*PRR7, PRR9*	[89]

It is well established that increasing patterns of histone acetylation at the promoters of clock genes correlate with their rising phase of expression [63]. A recent study has provided some clues about chromatin-related factors contributing to this histone acetylation [79]. The study has shown that the expression of *HAF2*, a histone acetyltransferase of the TAFII250 family 2, is activated at midday, and this activation promotes histone acetylation at the *PRR5* and *LUX* loci, coincident with their raising phase of expression [79]. If histone acetylation associates with activation, what are the chromatin-related components that facilitate the histone deacetylation during the declining phase? A number of histone deacetylases had been already identified [71]. However, a recent report has provided evidence that the evolutionarily conserved Sin3-histone deacetylase complex (HDAC) is connected with the plant clock [80]. The study showed that components of the Sin3-HDAC

complex, SAP30 FUNCTION-RELATED 1 (AFR1), and AFR2, are circadianly-regulated. Moreover, the evening-expressed AFR proteins contribute to the repression of *CCA1* and *PRR9* during the night, facilitating histone deacetylation by directly binding to their promoters. Thus, rhythmic histone deacetylation by the Sin3-HDAC complex contributes to shape the appropriate circadian waveforms of morning-expressed circadian genes [80].

Other histone deacetylases have been identified in studies of evening-expressed genes such as *TOC1,* which is also regulated by changing histone deacetylation patterns [81]. Indeed, HISTONE DEACETYLASE 9 (HDA9) and ELF3 directly interact and regulate the declining phase of *TOC1* after dusk. This regulation relies on the direct binding of HDA9 to the *TOC1* promoter through the interaction with ELF3. The EC-HDA9 complex facilitates histone deacetylation and represses *TOC1* expression during the night. The components of the EC also interact with HDA9 and with HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 15 (HOS15), a WD40 repeat protein at the promoter of the clock- and flowering-related gene *GIGANTEA* (*GI*), leading to histone deacetylation and transcriptional repression of *GI* [82]. PRR9 also interacts with a member of the plant Groucho/Tup1 corepressor family, TOPLESS/TOPLESS-RELATED (TPL/TPR) and with HDA6, defining together a repressive complex at the promoters of *CCA1* and *LHY* [90]. The studies thus provide examples of the direct interaction between clock components and chromatin-related factors, which underscores the importance of chromatin status and regulation of circadian clock gene expression.

In addition to histone acetylation/deacetylation, other histone marks are also associated with circadian gene expression. For instance, the circadian accumulation of H3K4me3 at clock loci was proposed to be mediated by the histone methyltransferase SDG2/ATXR3 (SET DOMAIN GROUP 2/ARABIDOPSIS TRITHORAX RELATED 3) [78]. In a more recent study, Song et al. have not only verified the role of SDG2/ATXR3 controlling circadian histone methylation but also identified a role for the Jumonji C domain–containing histone demethylase (JMJ14) as regulator of circadian oscillations [85]. Notably, the study has reported a feedback between histone modifications and the diurnal regulation of circadian clock genes [85]. On one hand, the histone methyltransferase SDG2 (as a "writer") and the histone demethylase JMJ14 (as an "eraser") regulate the expression of circadian oscillator genes by modulating H3K4me3 accumulation. In turn, CCA1 and LHY were shown to regulate directly the diurnal expression of *JMJ14* and indirectly that of *SDG2*, which leads to the rhythmic patterns of H3K4me3 accumulation in the target loci. Furthermore, a genome-wide analysis showed a limited overlap between H3K4me3 and H3K9ac marks in morning-phased and evening-phased genes, suggesting specific roles of different histone modifications controlling diurnal gene expression in *Arabidopsis*. Another study has recently shown that the expression of *TOC1* is also repressed by histone demethylation [83]. In this case, the repression requires the coordinated interaction of CCA1/LHY with the Lysine-Specific Demethylase 1 (LSD1)-like histone demethylases, LDL1 and LDL2 [75]. LDL1 and LDL2 also interact with the histone deacetylase HDA6 providing a double mechanism for repression of *TOC1* expression by both histone demethylation and deacetylation [83]. Notably, the authors have also recently shown that HDA6 and LDL1/2 can in turn interact with TOC1, and the complex contributes to the repression of *CCA1*, *LHY*, and other circadian related genes [84]. Therefore, the LDL1/2-HDA6 complex seems to play a relevant role controlling the expression of a subset of clock-related genes.

ELF3 also represses target gene expression at the end of the day by directly interacting with a protein from the chromatin-related SWI2/SNF2-RELATED (SWR1) complex [89]. The SWR1 complex associates with chromatin and catalyzes the histone variant H2A.Z exchange at genomic sites. H2A.Z is a well-conserved histone variant [91] that influences transcriptional activities of associated genes [92]. Consistently, the EC-SWR1 complex is able to bind to the *PRR7* and *PRR9* loci to control both the deposition of H2A.Z and the repression of these genes at dusk. The study thus provides a mechanism by which repressive chromatin domains are temporally defined by the circadian clock [89].

A majority of studies on the transcriptional circadian regulation has mainly focused on steady-state mRNA expression. A recent report however, has provided evidence on the rhythms in transcriptional synthesis, circadian nascent RNAs and chromatin modifications [86]. The study showed a modular

function of RVE8, with its MYB domain responsible for the DNA binding, and its LCL domain providing the platform for the interaction with the clock components known as NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED proteins (LNKs) [61,93–95]. LNKs rhythmically recruit the RNA Polymerase II and the transcript elongation FACT complex to co-occupy the promoters of the clock genes *TOC1* and *PRR5* [86]. The RVE8-LNKs interaction and the recruitment of the transcriptional machinery ultimately define not only transcript initiation and elongation but also the chromatin status including changes in histone marks such as H3K4me3 accumulation. Analyses of nascent RNAs by nuclear run-on transcription by bromouridine immunocapture indeed showed that the rhythmic occupancy of the transcriptional machinery results in oscillatory nascent RNAs [86].

Another recent study has provided further information on transcript elongation and pre-mRNA processing of *CCA1*. The study focused on histone H2B monoubiquitination (H2Bub), which in *Arabidopsis* is controlled by HISTONE MONOUBIQUITINATION1 (HUB1) and HUB2 E3 ubiquitin ligases together with the UBIQUITIN-CONJUGATING ENZYME 1 (UBC1) and UBC2 E2-conjugating enzymes [88]. HUB proteins interact with the previously uncharacterized RNA-binding motif-containing proteins, SPEN3 and KHD1 [88]. In the *spen3-1* and *hub1-4* mutants, H2Bub accumulation was reduced and *CCA1α* and *CCA1β* splice isoforms were altered. The mutant plants showed short circadian period length phenotypes in agreement with the reduced expression of *CCA1*. Overall, the study showed that H2Bub deposition associated with *CCA1* transcript elongation and pre-mRNA processing are two processes that are facilitated by the HUB1/HUB2 complex [88].

The circadian clock controls many outputs or rhythmic biological processes that occur at the most appropriate diurnal or seasonal time. Chromatin marks have been recently associated with seasonal regulation [96]. Indeed, genome-wide analyses in a natural population of perennial *Arabidopsis halleri* have uncovered a close connection of histone H3 lysine 27 trimethylation (H3K27me3) deposition with the control of seasonal gene regulation. The seasonal accumulation of H3K27me3 is phase-delayed in comparison with the H3K4me3 oscillation, most prevalently for genes associated with environmental memory. The authors thus proposed that H3K27me3 marks can control seasonal responses by monitoring past transcriptional activity for long-term regulation of expression in a subset of genes in plants grown under natural environmental conditions [96].

One fundamental clock output is the photoperiodic regulation of flowering time [97,98]. Many studies have previously shown the importance of chromatin remodeling at flowering-related loci [99]. A recent study has proposed a hierarchical graphical model inferring genome-wide gene regulatory networks connecting flower development and circadian signaling [100]. The study identified two major connecting hubs: HFR1 (LONG HYPOCOTYL IN FAR-RED) and LHY. Indeed, the network analyses showed that LHY controls a number of transcription factors directly related with flower development [100]. Notably, during the transition to flowering, *LHY* shows in turn a significant change in H3K4me3 at the shoot apical meristem [101]. HFR1 directly interacts with the histone acetyltransferase HAC1 (HISTONE ACETYLTRANSFERASE 1) and bind to *AG* (AGAMOUS, a floral development factor) to activate its expression via histone acetylation. Consistently, the authors found a flower-specific peak of H3K27ac at the *AG* gene body closely coinciding with a HFR1 binding motif. HFR1 plays a key role in the transducing signals from light and temperature to influence circadian signaling and flowering development. It would be interesting to apply this kind of approaches with time series to further infer dynamics and new connections between chromatin changes at the core of the oscillator and in clock related outputs.

Another example connecting chromatin changes with the regulation of flowering time was recently provided by a study on the florigen gene *FLOWERING LOCUS T (FT)*. *FT* shows a 24-h oscillation under long-day (LD) conditions with a peak of expression during the day. At dusk, the HISTONE DEACETYLASE 2C (HD2C) is recruited to the *FT* locus and deacetylates histones to repress *FT* transcription. HD2C competes with CONSTANS (CO), the activator of *FT*, for the binding of the *MORF-RELATED GENE 2* (*MRG2*) [102]. Thus, the study involves a histone deacetylase and histone methylation readers to shape the photoperiodic-dependent waveform of *FT* expression. H2B

monoubiquitination and SPEN3 function are not only important for *CCA1* transcript elongation and pre-mRNA processing as mentioned above [88], but are also important in the regulation of the flowering [88]. Indeed, the *spen3-1* mutant plants showed a delay in flowering time that correlated with an enhanced expression of the flowering-related gene *FLOWERING LOCUS C* (*FLC*), most likely due to an increased distal versus proximal ratio of its antisense *COOLAIR* transcript [88].

FT is regulated by the precisely coordinated action of several players. For instance, CO forms a protein complex together with the B and C subunits of Nuclear Factor Y (NF-Y) to activate *FT* expression close to dusk. In contrast, the Polycomb repressive complex 1 (PRC1) and PRC2 proteins silence *FT* expression. PRC proteins show H3K27 methyltransferase activity that generates H3K27 trimethylation (H3K27me3) and maintain this mark, also facilitating other repressive marks [103]. A recent study has shown that the NF-CO complex favors a reconfiguration of the chromosomal conformation at *FT* resulting in reduced binding of Polycomb proteins to the *FT* promoter [104]. This chromatin looping and reduced binding of Polycomb proteins relieve the Polycomb-mediated silencing, resulting in *FT* de-repression near dusk.

Another example includes the role of histone demethylation in the regulation of flowering time. The study shows that JMJ13, which possesses H3K27me3 site-specific demethylase activity, acts as a flowering repressor, and modulates flowering time in a photoperiod- and temperature-dependent manner [87]. The study also shows that the expression of main clock genes such as *LHY* and *CCA1* and flowering-related genes such as *CO* was up-regulated in *jmj13* mutant plants [87]. These results open the question of whether JMJ13 directly regulates clock through changes in histone demethylation at their loci. JMJ5/JMJ30 has been also connected with circadian regulation and in particular with temperature compensation [105]. However, this function appears not to involve changes in H3K36 methylation at the circadian clock loci [105].

A recent study has shown that the circadian clock regulates other outputs such as seed dormancy through the concerted action of the ATP-dependent chromatin-remodeling factor PICKLE (PKL) [106] and the EC component LUX [107]. The two proteins interact and bind to the locus of the *DELAY OF GERMINATION1* (*DOG1*) gene, which encodes a protein involved in seed dormancy [108]. The H3K27me3 accumulation at the *DOG1* locus was reduced in *pkl* or *lux* mutants. The authors conclude that the circadian clock, through LUX and its interaction with PKL, modulates seed dormancy during seed development by controlling the expression of *DOG1*. This regulation might be important to prevent seeds from becoming overly dormant [107].

4. Future Perspectives

Circadian studies are rapidly expanding our view on how the circadian system works in different parts of the plant, and how mobile signals are able to synchronize clocks in distal organs [109–113]. Over the past recent years, it has become increasingly clear that circadian information is shared through short- and long-distance communication. The strength of circadian cell-to-cell coupling differs among cells and tissues [114,115]. For example, coupling is minimum among cotyledon cells [116], variable in leaves [117–119], high in roots [120] and between the vasculature and neighbor mesophyll cells [121], and very high within cells at the shoot apex [122]. Long-distance circadian synchronization on the other hand, seems to occur through shoot-to-root photosynthetic signaling [123], light piping down the root [124] and by the movement of ELF4 from shoots to regulate the period of the root clock in a temperature-dependent manner [113]. The studies highlight specific and autonomous circadian function, which urgently calls for studies on changes of the chromatin status not only with a temporal resolution (circadian or seasonal) but also with spatial definition in order to identify cell, tissue-, and organ-specific circadian chromatin landscapes.

Likewise, over recent years, chromatin conformation capture approaches have provided an unprecedented three-dimensional view of chromatin organization [125]. Studies with animal cells have uncovered a hierarchical system with compartment, domains and loops, playing important roles in the control of transcription [126]. Similar studies in plants have now shown that plant cells contain

comparable high-order structures [69] with the notable exception of the TAD-like loop domains found in mammals or the lack of a plant CTCF-like insulator protein [126]. It would be then interesting to fully understand the functional divergences of the high-order chromatin formation and organization in plants compared to animals. Responses of plant chromatin conformation to different environmental and cellular signals would be also interesting to elucidate, focusing on the functional connection between their formation and their specific biological functions. Circadian changes on chromatin conformation and nuclear localization in different tissues and organs would be also worth exploring. We surely have ahead many interesting discoveries within the plant circadian field.

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