Universitat Autònoma de Barcelona

CENTRE FOR RESEARCH IN AGRICULTURAL GENOMICS

Functional characterization of REVEILLE 8 and NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED interaction in the diurnal regulation of anthocyanin biosynthesis

PhD Thesis
Pablo Pérez García

Barcelona, 2015

Universitat Autònoma de Barcelona

CENTRE FOR RESEARCH IN AGRICULTURAL GENOMICS

Facultad de Biociencias

Departamento de Biología Animal, de Biología Vegetal y de Ecología

Programa de Doctorado en Biología y Biotecnología Vegetal

Functional characterization of REVEILLE 8 and NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED interaction in the diurnal regulation of anthocyanin biosynthesis

Memoria presentada por Pablo Pérez García para optar al título de doctor por la Universidad Autónoma de Barcelona

La Directora de Tesis:

Dra. Paloma Más Martínez

El candidato a Doctor:

Pablo Pérez García

La Tutora de Tesis:

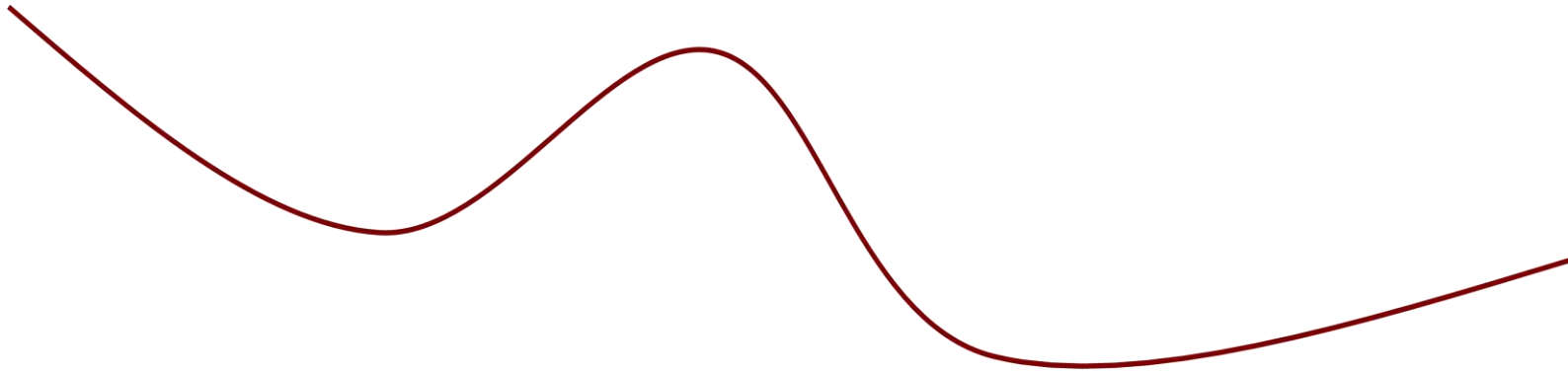
Dra. M. Carmen Martínez Gómez

A mi Pichoncito

A mi Titazo

A mi Tururu

(Si Fung, y a ti también…)



Introduction

Introduction

1. Circadian clocks in plants

Nearly all organisms, from bacteria to humans, have evolved a sophisticated mechanism that allows them to perceive changes in environmental signals and generate rhythms with a period of 24 hours (Bell-Pedersen *et al.* 2005, Jolma *et al.* 2010). The mechanism, known as circadian clock, is able to synchronize physiology and metabolism in anticipation to the predictable daily changes (mostly changes in light and temperature) that occur during the day and night cycle. Despite its tight connection with the environment, the circadian clock is an endogenous oscillator able to sustain rhythms even in the absence of environmental transitions (Costa 2001). However, under these constant conditions (free-running), the internal period of the clock is close to, but not exactly, 24 hours (hence the term circadian, from the Latin *circa*: "approximately" and *dies*: "day"). Several evidences have now conclusively demonstrated that the circadian system includes a resetting mechanism by which it is synchronized every day to the proper time (Sehgal *et al.* 2007, Winfree 1970). Circadian clocks also exhibit a remarkable property known as temperature compensation. By virtue of this property, clocks are able to maintain a relatively constant period over a physiological range of temperatures. This property is essential for the clock to properly "measure time" regardless the unpredictable changes in temperature (although the daily temperature transitions at the day/night are still able to reset the clock) (Somero 2004). It has been proposed that circadian oscillations provide an adaptive advantage by allowing organisms to anticipate the predictable environmental changes during the day/night cycle and to coordinate simultaneous, sequential or temporally incompatible events (Gerhart-Hines and Lazar 2015, Yerushalmi and Green 2009). Thus, the circadian system acts as an endogenous processor of environmental signals to synchronize metabolic and developmental activities.

The three main properties that characterize circadian rhythms include: phase, period and amplitude (Mas 2008). These properties are very useful to compare the circadian function in wild-type and in mutants in which the clock does not run properly. Phase refers to the state of a rhythm relative to another reference rhythm. Usually, the environmental changes during the day/night cycle are used as the reference rhythm. A Zeitgeber (German: "time giver") is any environmental cue able to synchronize the clock. Thus, the circadian phase is often defined by a Zeitgeber Time (ZT) (by convention ZT0 usually correlates with dawn). The period is the time required to complete a rhythmic cycle. Short periods are sometimes coupled with advanced phase and long periods with delayed phase. The amplitude is defined as the half

distance between the peak of an oscillation and the lowest point of the same oscillation. High amplitude and low amplitude phenotypes indicate an increase or a decrease on the circadian output, respectively.

Classical studies have functionally organized the circadian system in three main modules: the Input Pathways, the Central Oscillator and the Output Pathways (de Montaigu *et al.* 2010, Ueda *et al.* 2001). The Input Pathways refer to all the clock components responsible for perceiving the environmental signals and transmit this information to synchronize the Central Oscillator. This Oscillator, considered as the "heart" of the clock, is responsible for the generation of rhythms, while the Output Pathways refer to the biological processes that are rhythmically oscillating. Although this classical view of the circadian system is very valuable to understand the clock, it is now well-accepted that the circadian system is far more complicated, with intricate connections between the different modules, with components acting within the central oscillator and also in the input and output pathways, and with blurred delimitations among the different modules (Devlin and Kay 2001, Wijnen and Young 2006). Thus, the circadian system should be considered as a sophisticated network with reciprocal regulations among components rather than a simple lineal pathway with three main modules (Figure 1).

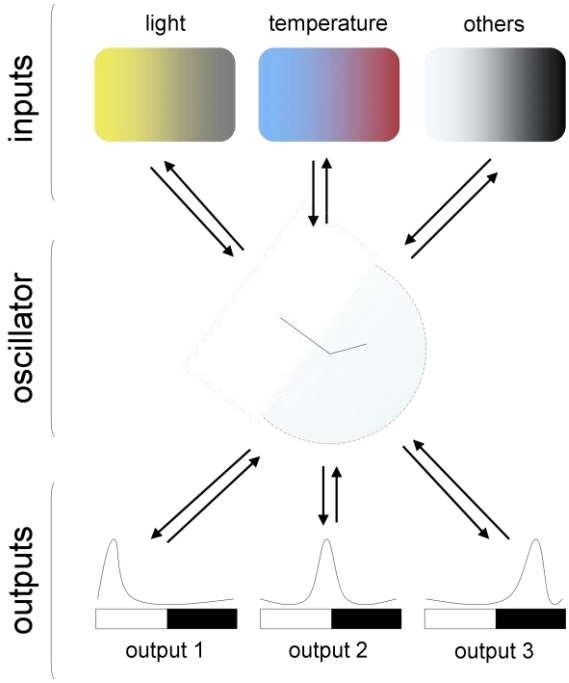


Figure 1. Classical organization of the circadian system. Input Pathways transmit the environmental information encoded in signals such as light and temperature to the Central Oscillator that generates rhythms in multiple biological processes or Clock Outputs. This lineal pathway is an over-simplification as multiple interconnections exist among the different modules and different components can be part of more than one module.

The first reports that recognized diurnal rhythms are dated to the fourth century Before Christ. Androsthenes described the daily leaf movements of *Tamarindus indicus* (the tamarind tree) in the island of Tylos in the Persian Gulf during the marches of Alexander the Great (Bretzl 1903). From these initial observations, the scientific findings on circadian rhythms continue in 1729 when the French astronomer de Marian reported that the daily leaf movements of the sensitive heliotrope plant (probably *Mimosa podia*) persisted under constant darkness (de Mairan 1729). Nearly a century passed before the period length of these leaf movements was accurately measured under constant conditions and found to be close to 24 hours. Together, these analyses confirmed that rhythms were endogenous and not simply responses to environmental cues (de Candolle 1832). Animal circadian rhythms were not rigorously described until much later, with the study of pigment rhythms in arthropods (Kiesel 1894) and daily activity in rats (Richter 1922). The endogenous nature of leaf movement rhythms was a matter of debate until the experiments performed with the fungus *Neurospora crassa* were conducted in the space (Sulzman *et al.* 1984). This work showed that rhythms were truly endogenous and not driven by some subtle and undetected geophysical cue associated with the rotation of the earth on its axis.

In plants, the circadian clock activity also allows the anticipation to the predictable changes in the environmental conditions to be prepared well in advanced these conditions arise. Thus, the circadian clocks coordinate physiology and metabolism to the most appropriate or advantageous time of day or season (Harmer 2009). By modulating the timing of responses, plants are able to save energy and resources and thus, the circadian clock function is of vital importance for plant fitness and survival. Most of what we know about circadian rhythms in plants has been performed using the model species *Arabidopsis thaliana*. However, relevant studies have also provided insightful clues about clock function in crops and plants of agronomical importance (McClung 2013).

In *Arabidopsis*, perception of light environmental changes is achieved through a battery of photoreceptors that act coordinately to synchronize the central oscillator (Franklin *et al.* 2014, McClung and Davis 2010) (section 3). The molecular mechanisms responsible for the generation of rhythms seem to rely on the reciprocal regulation among core clock components (section 2), which follows a sequential regulatory wave at every phase of the diurnal cycle (Carré and Veflingstad 2013, Troncoso-Ponce and Mas 2012) (section 4). Related to the clock outputs, a wide variety of processes are regulated by the clock in *Arabidopsis*, which reflects its importance controlling nearly all stages of plant development and many

essential aspects of growth and metabolism (de Montaigu, et al. 2010, Kinmonth-Schultz *et al.* 2013) (section 5).

2. Main components at the core of the *Arabidopsis* circadian clock

Many different approaches had led to the identification of an impressive amount of clock components that are directly or indirectly connected to the clock. Characterization of mutant and over-expressing plants has provided insightful clues about the role of these components within the circadian signaling pathway and their possible regulatory and functional roles. Below are described some of the main components of the *Arabidopsis* clock (Figure 2) and their circadian phenotypes while their regulatory interactions are described in section 4.

The initial identification of clock components was aided by the use of plants expressing the promoter of the morning-expressed clock output *CHLOROPHYLL A/B-BINDING PROTEIN 2 (CAB2)* fused to the luciferase (LUC). Mutagenesis of *CAB2::LUC* plants and subsequent screening by *in vivo* analysis of the rhythmic luminescence, a number of mutants with altered circadian period, phase or amplitude were discovered (Millar *et al.* 1995). One of the first characterized mutant plants displayed an early circadian phase and a short period phenotype for gene expression as well as for other circadian outputs under a wide range of temperature and light conditions (Makino *et al.* 2002, Millar, et al. 1995, Somers *et al.* 1998b, Strayer *et al.* 2000). Cloning of the gene, denominated *TIMING OF CAB EXPRESSION 1 (TOC1)* or *PSEUDO RESPONSE REGULATOR 1* (*PRR1)* revealed that it encoded a protein containing at its NH_2-terminus a motif similar to the receiver domain characteristic of the response regulators. However, TOC1 lacks the conserved phospho-accepting aspartate residue present in canonical response regulators (Makino, et al. 2002, Strayer, et al. 2000). In addition, TOC1 contains a distinctive COOH-terminal motif similar to that found in the COSTANS (CO) family of transcription factors. In addition to the short period phenotype and early phase of *toc1* mutant plants mentioned above (Millar, et al. 1995, Somers, et al. 1998b), constitutive over-expression of TOC1 (TOC1-ox) results in arrhythmic gene expression while additional copies of rhythmic *TOC1* expression (TOC1 MiniGene, TMG lines) rendered a delayed phase and a long period phenotype (Más *et al.* 2003). Further phenotypic studies of TOC1 mutants and over-expressing plants revealed that TOC1 plays an important role as a molecular link connecting the central oscillator with the light input to the clock (Mas *et al.* 2003). TOC1 is also important for proper photomorphogenesis, floral transition and plant responses to drought, through the Abscisic Acid (ABA) hormone signaling (Ding *et al.* 2007, Legnaioli *et al.* 2009). A number of different regulatory mechanisms contribute to regulation of TOC1 rhythmic gene and protein

expression. The mechanisms include changes in chromatin structure, transcriptional regulation and protein degradation by the proteasome pathway (Mas 2008) (consult section 4).

TOC1 forms part of a protein family including other four members (PRR3, PRR5, PRR7 and PRR9). These members were also shown to be part of the circadian system (Matsushika *et al.* 2000). The expression of the genes is regulated by the clock and displays a sequential peak of expression from dawn (*PRR9*) to dusk (*TOC1).* Furthermore, mutation and over-expression of the PRRs render a range of circadian phenotypes. Plants expressing non-functional transcripts of *PRR7* or *PRR9,* present long period phenotypes while *prr5* mutant plants show a short period phenotype. Notably, single mutations of any PRR render less severe circadian phenotypes than those displayed by *toc1* loss of function plants. The *prr7/prr9* double mutants show a longer period than that of single mutant plants (Farré *et al.* 2005, Nakamichi *et al.* 2005) while plants *prr5/prr7/prr9* triple mutants are arrhythmic under constant light (Nakamichi, et al. 2005), which suggest that they play redundant roles. TOC1 and PRR5 seem to act as transcriptional repressors in the expression of the preceding PRR target genes, most likely by direct binding to their promoters, as inferred by chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) (Huang *et al.* 2012, Nakamichi *et al.* 2012).

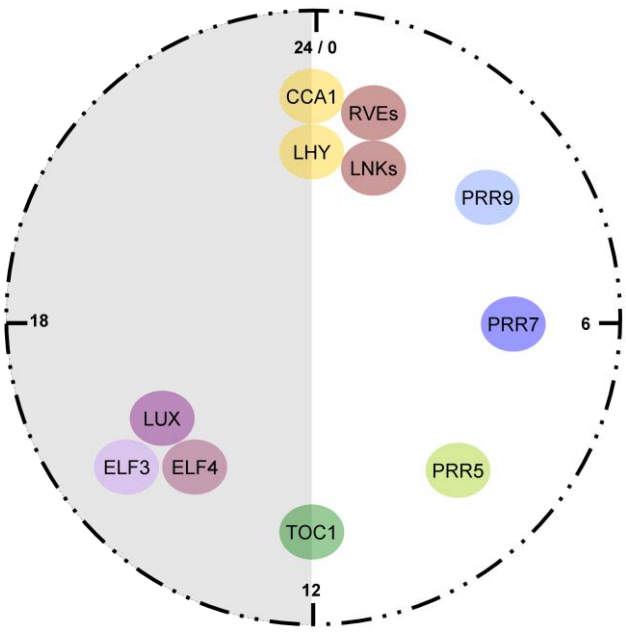


Figure 2. Expression of the main *Arabidopsis* clock components around the clock. In the morning, *RVEs*, *LNKs*, *CCA1*, *LHY*, *PRR9* and *PRR7* are expressed. The PRR genes are sequentially expressed. In the night, the EC components expression gets activated. Expression of *CCA1/LHY* (orange), *RVEs* and *LNKs* (red), *PRR9* (pale blue), *PRR7* (dark blue), *PRR5* (pale green), *TOC1* (dark green), EC (purple) under 12-h light:12-h darkness (LD) conditions. Numbers indicate the Zeitgeber Time in hours. White and grey semicircles represent the light and dark period, respectively.

Two single MYB transcription factors *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1)* and *LATE ELONGATED HYPOCOTYL* (*LHY)* that are expressed in the morning seem to play a key role at the core of the clock (Schaffer *et al.* 1998, Wang and Tobin 1998). LHY and CCA1 have highly similar DNA-binding domains and show strong homology throughout their protein sequences. CCA1 was initially identified as a factor able to bind to the *CAB2* promoter (Wang *et al.* 1997)

while LHY was described as a relevant component for the initiation of flowering (Schaffer 1997). The *cca1* or *lhy* single mutant plants show a clear circadian short period and advanced phase as compared to wild-type plants, while double mutant plants are arrhythmic (Alabadí *et al.* 2002, Mizoguchi *et al.* 2002). Over-expression of either gene also renders arrhythmic clock gene expression as well as arrhythmia in other clock outputs (Wang and Tobin 1998). Other morning-expressed clock components include the REVEILLE/LHY-CCA1-LIKE (RVE/LCL) and the NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED (LNK) protein families. Some members of the RVE family have been shown to be connected with the clock while the LNK protein family has been related with light and circadian function. The studies described in this Thesis deal with the interaction and function of these components, which are described in more detailed in sections 2.1 and 2.2.

In addition to TOC1, other clock components acting during the evening include three interacting factors forming the so-called EVENING COMPLEX (EC) (Nusinow *et al.* 2011). The EC is composed of the single MYB, SHAQYF-type GARP transcription factor LUX ARRHYTHMO (LUX) (also known as PHYTOCLOCK 1 (PCL1) (Hazen *et al.* 2005, Onai and Ishiura 2005), the EARLY FLOWERING 3 (ELF3) (McWatters et al. 2000) and the EARLY FLOWERING 4 (ELF4) (Doyle et al. 2002). *lux* mutants are arrhythmic (Hazen, et al. 2005) and display a long hypocotyl phenotype (Millar, et al. 1995) while ELF3 and ELF4 were both identified as mutants with alterations in flowering time (Hicks *et al.* 1996, Zagotta *et al.* 1992). Similar to *lux* mutants, individual *elf3* and *elf4* mutants lead to arrhythmia, which demonstrate the key role of these proteins at the core of the clock. The EC also regulates the expression of *PRR9* and is essential in the control of hypocotyl growth (see section 5.1).

2.1 RVE protein family

As mentioned above, CCA1 and LHY are both key regulators of the circadian clockwork. These single MYB transcription factors belong to a family of eleven members (Andersson *et al.* 1999, Carré and Kim 2002) sharing a high degree of sequence similarity, particularly evident on their MYB domain (Figure 3). Five of the eleven members of the family can be further clustered into a subfamily. These five members were named as LHY/CCA1-LIKE (LCL) (Schmied and Merkle 2005) or RVE (Andersson, et al. 1999, Carré and Kim 2002) (Figure 3) and share sequence identity not only in the MYB domain but also in a region at the C-terminal end of the proteins denominated LCL domain (Farinas and Mas 2011). The LCL domain is not present in CCA1, LHY or in the other members of the family.

The role of the single MYB transcription factors on circadian function has been studied in detail for some of them. For instance, the late-morning expressed EARLY-PHYTOCROME-RESPONSIVE 1/RVE7 (EPR1/RVE7) was initially discovered in a screening for direct targets of the photoreceptor PHYTOCHROME. Plants over-expressing *EPR1/RVE7* (EPR1/RVE7-ox) showed a reduced circadian expression of the clock output *CAB2* (Kuno *et al.* 2003). This phenotype is not dependent of CCA1 and LHY function, as their expression is not affected in EPR1/RVE7-ox plants. Moreover, EPR1/RVE7 protein represses its own expression, which suggests that its circadian expression is regulated by a negative feedback loop. The authors proposed that EPR1/RVE7 might be part of a slave oscillator (Kuno, et al. 2003).

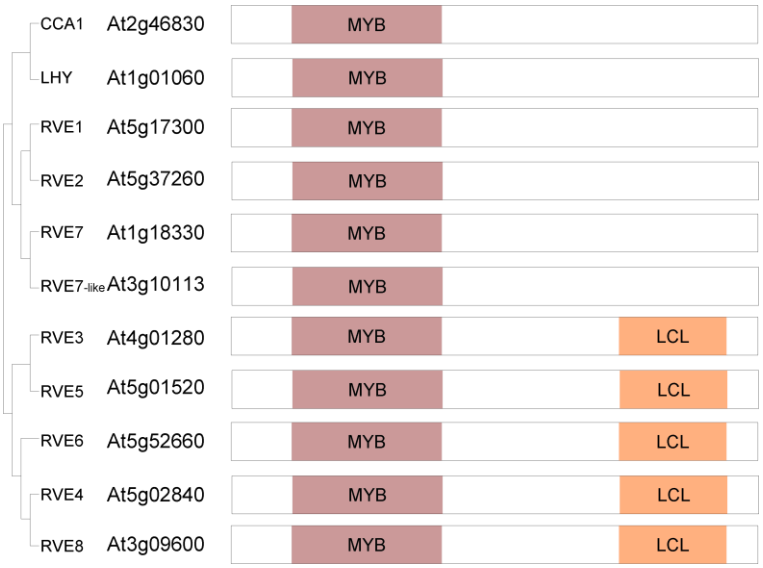


Figure 3. Schematic representation depicting the phylogenetic tree of the single MYB protein family. The phylogenetic tree is based on sequence similarities among members of the family. Pale red boxes represent the MYB domain while orange boxes represent the LCL domain shared by the five RVE subfamily members.

Another RVE member, CIRCADIAN 1/REVEILLE 2 (CIR1/RVE2) was also connected to the clock (Zhang *et al.* 2007). *CIR1/RVE2* expression shows a circadian oscillation peaking before dawn and this rhythmic oscillation requires a functional *CCA1* and *LHY* expression. Constitutive over-expression of *CIR1/RVE2* results in a short period circadian phenotype for *TOC1*, *LUX*, *CCA1* and *LHY* oscillation and a low amplitude specifically for *CCA1* and *LHY*. In addition to its role modulating the expression of core-clock components, CIR1/RVE2-ox plants display delayed flowering, longer hypocotyls and reduced seed germination under continuous dark conditions. These findings suggest that CIR1/RVE2 might be part of a feedback loop important in the control of circadian outputs and in the modulation of the pace of the clock, most likely through CCA1 and LHY function. Another connection between the clock and the RVEs was

identified in studies with RVE1 (Rawat *et al.* 2009). The expression of *RVE1* is clock-regulated, with peak transcript abundance close to dawn. The *rve1* mutants do not affect the circadian expression of core components but RVE1 seems to be important for the circadian regulation of a hormonal clock output such as auxin signaling. Consistently, mutant plants display alterations in the regulation of auxin-dependent hypocotyl growth (See section 5.1).

RVE8, another member of the RVE family, was identified in a screening searching for proteins able to bind to the EVENING ELEMENT (EE) circadian motif (Harmer and Kay 2005). *RVE8* mRNA rhythmically oscillates with a peak of expression around dawn (Farinas and Mas 2011) although the protein seems to peak few hours later after dawn (Hsu et al. 2013). Miss-expression of RVE8 leads to a number of phenotypes in different clock outputs and under different conditions. Over-expression of RVE8 (RVE8-ox) delays flowering, particularly under long day conditions (LgD) while the hypocotyl length of RVE8-ox plants is shorter under a wide range of light fluences. In contrast, *rve8* loss of function plants flower later than WT plants and the length of the hypocotyl is longer (Farinas and Mas 2011, Rawat *et al.* 2011). High temperature might also affect RVE8 activity since circadian phenotypes of RVE8 miss-expressing plants are reduced at lower temperature (Rawat, et al. 2011). Transcriptionally, miss-expression of RVE8 affects the circadian expression of core clock genes. For instance, loss of function *rve8* plants showed a long circadian period and delayed phase in the expression of the core clock genes *TOC1* and *CCA1* as well as for the output gen *CAB2.* Conversely, RVE8-ox plants displayed a short period and advanced phase of circadian gene expression. The phenotypic analysis also revealed that even though CCA1 and RVE8 share sequence similarity in their MYB domains and have a similar phase of expression, they perform opposite roles on the circadian clock. Indeed, while CCA1 represses *TOC1* expression, RVE8 act as a positive regulator (Perales and Más 2007). Similarly, RVE8 also activates the expression of other evening-expressed genes like *PRR5* (Rawat, et al. 2011). The mechanisms behind RVE8 and CCA1 regulation of circadian gene expression seem to involve changes in chromatin remodeling (Farinas and Mas 2011, Perales and Más 2007) (please see details on section 4).

Other members of the RVE family are also connected with the circadian clock (Hsu *et al.* 2013). The analysis of *rve4* and *rve6* single mutant plants did not reveal obvious circadian phenotypes. The pattern of expression of the evening-expressed clock output *COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2 (CCR2)* remained unchanged in the absent of a functional RVE4 or RVE6. However, the *rve4/rve6/rve8* triple mutant showed a more dramatic long period phenotype than the single *rve8* single mutant, suggesting a partial redundancy in the regulation of clock gene expression. Further analysis revealed significant changes on the

waveforms of the evening-expressed clock genes *TOC1* and *PPR5* in the *rve4/rve6/rve8* triple mutant plants (Hsu, et al. 2013). The phase of *TOC1* and *PRR5* expression was markedly delayed while the amplitude of *PRR5* was diminished. These results suggest that RVE4, 6 and 8 might have redundant roles in the control of evening-expressed core genes, although the circadian phenotypes of *rve8* single mutant plants, not observed in single *rve4* or *rve6* mutations, suggest a possible hierarchy in their functions.

2.2 LNK protein family

A screening for genes involved in clock resetting using pulses of light in the middle of the night led to the discovery of a protein family named NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED (LNK) (Rugnone *et al.* 2013). The LNK protein family is composed of four members sharing a sequence similarity of about 30%, with LNK1 and LNK2 being about 35% similar and LNK3 and LNK4 showing a 60% of similarity. The four of them display a rhythmic pattern of gene expression, with a peak in the morning, similar to that of *CCA1*, *LHY* and *RVE8* (Rugnone, et al. 2013). Analysis of *lnk1* and *lnk2* single mutants showed a long hypocotyl phenotype under constant light conditions. The growth phenotypes were more severe in double *lnk1/lnk2* mutant plants compared to single mutants (Rugnone, et al. 2013) suggesting a possible functional redundancy of LNK1 and LNK2 in the control of hypocotyl elongation. Analysis of other clock outputs such as the photoperiodic regulation of flowering time revealed a severe flowering phenotype for the double *lnk1/lnk2* mutants (Rugnone, et al. 2013). Analysis of circadian genes expressed in the morning revealed a longer circadian period in *lnk1/lnk2* mutants compared to the one observed in WT plants (Rugnone, et al. 2013, Xie *et al.* 2014). Notably, analysis of *PRR5* gene expression revealed more severe alterations, including a very long circadian period and clear decreased amplitude. *TOC1* expression was also decreased in *lnk1/lnk2* mutant plants, which suggests that LNK1 and LNK2 might function as specific transcriptional activators of *PRR5* and *TOC1* expression. In turn, TOC1 appears to directly bind to the *LNK* promoters to activate their expression, as inferred by ChIP-seq (Huang, et al. 2012), ChIP-Q-PCR (Rugnone, et al. 2013) and analysis of *toc1-2* mutant plants (Rugnone, et al. 2013). Notably, analysis of *lnk3* and *lnk4* mutant plants showed no obvious circadian phenotype for gene expression (Xie, et al. 2014).

Bimolecular Fluorescent Complementation (BiFC) assays and firefly luciferase complementation imaging revealed some clues about the molecular components involved in LNK function. Indeed, LNK1 and LNK2 were found to rhythmically interact with CCA1, LHY, RVE4 and RVE8 (Xie, et al. 2014). Further studies using LNK1 stradiol inducible lines showed

that following LNK1 induction, the expression of *TOC1* and *PRR5* was highly increased. As RVE8 is also a transcriptional activator of *TOC1* and *PRR5* (Farinas and Mas 2011) (Rawat, et al. 2011) (see section 2.1), the possibility that LNK1 and RVE8 act as transcriptional co-activators of *TOC1* and *PRR5* was examined. Indeed, the increased *TOC1* and *PRR5* expression after RVE8 induction was severely reduced in the *lnk1/lnk2* background, suggesting that RVE8 might require functional LNK1 and LNK2 to fully activate *TOC1* and *PRR5* expression. Similarly, activation by LNK1 required the presence of RVE4 and RVE8 (Xie, et al. 2014).

LNK1 function has been also connected to temperature. Ambient temperature has been shown to be an important synchronizing cue for the clock (McClung and Davis 2010, Wigge 2013). Pulses of temperature during the day resulted in up-regulation of *LNK1* expression, particularly during the night, which suggest a possible gating by the clock (Mizuno *et al.* 2014b). Analysis of *LNK1* induction by temperature in several clock mutants, including the components of the EC, previously shown to be important for temperature signaling (Mizuno, et al. 2014b) revealed that the gating effect of *LNK1* induction at night was abolished in the absence of a functional EC. ChIP experiments showed that this regulation might occur through direct binding, as ELF3 and LUX were able to bind to the *LNK1* promoter (Mizuno, et al. 2014b). Altogether, these results establish a direct connection of LNKs with the light and temperature signaling to the clock in coordination with some essential clock components.

3. Entrainment of the *Arabidopsis* circadian clock

The circadian clock is not only a robust mechanism able to sustain rhythms under constant environmental conditions but also a flexible system that synchronizes every day with the environmental changes that occur during the day-night cycle. As mentioned above, the main Zeitgebers in plants are light and temperature. Over the past years, research studies have been intensively focused on identifying components and mechanisms responsible for clock synchronization by light. Changes in light quality and quantity, particularly around the dawn/dusk transitions, modulate the expression and activity of core clock components. The changes in gene expression modulate the amplitude, period and phase of the clock to perfectly adjust the external environmental time with the internal period of the clock. In diurnal organisms, like plants, and following the Aschoff´s rule (Aschoff 1960), the higher the intensity of light, the shorter the circadian period. Also, light at dusk advances the phase of the clock whereas light pulses at dawn leads to delayed phases. Notably, light in the middle of the day have no effect on the circadian phase (Wijnen and Young 2006). This reflects a very interesting

feature of circadian clocks known as “gating” by which the clock controls its sensibility to light at different times-of-day.

Molecularly, light is perceived in plants by a complex array of photoreceptors. For instance, Red (RL) and Far Red (FRL) light are preferentially sensed by the PHYTOCROME family (PHYA to PHYE in *Arabidopsis thaliana*) of photoreceptors (Clack *et al.* 1994, Rockwell *et al.* 2006, Sharrock and Quail 1989). CRYPTOCROMES (CRY1, 2 and 3) on the other hand are responsible for the UV-A/blue light (BL) perception (Ahmad and Cashmore 1993, Lin *et al.* 1996) together with PHOTOTROPINS (PHOT1 and PHOT2) (Huala *et al.* 1997, Kagawa *et al.* 2001) and members of the ZEITLUPE family (ZEITLUPE, ZTL, FLAVIN-BINDING, KELCH REPEAT, F-BOX 1, FKF1 and LOV KELCH PROTEIN 2, LKP2 (Nelson et al. 2000, Schultz et al. 2001, Somers et al. 2000)). PHOT1 and PHOT2 are important in responses that orientate the plant to the light source and chloroplast movement through the cytoplasm. Recent studies have also shown UV-B RESISTANCE 8 (UVR8) as the photoreceptor of UV-B light (Rizzini *et al.* 2011).

The PHY photoreceptors use phytochromobilin (POB) as a chromofore that is bound to the rest of the protein by a covalent bond. A complex reorganization of the PHY protein structure occurs when light is sensed by the photoreceptors (Rockwell, et al. 2006). Two main conformers are found in PHYs, the Pr and Pfr. Under conditions of high RL versus FRL, the major conformer is the Pr form. Conversely, PHY switch to the Pfr form with light conditions enriched in FRL. It was defined the role of PHYs in clock entrainment by comparing the effects of different light quality and quantity in Wild-Type (WT) and *phy* mutants plants (Yanovsky *et al.* 2000). The studies indicated that PHYA function as a key photoreceptor that entrains the clock mainly at low fluencies of RL and BL light while PHYB is more relevant synchronizing the clock at higher fluencies of RL. Further studies showed that the direct interaction between PHYB and CRY2 was important for transmitting the information of both RL and BL to entrain the clock (Más *et al.* 2000). The interaction between PHYA and CRY1 (Ahmad 1998) demonstrated their joint function in the light input to the clock (Devlin and Kay 2000). It is noteworthy that the quintuple mutant (*phyA/phyB/phyC/phyD/phyE*) still sustained robust circadian rhythms (Hu *et al.* 2013, Strasser *et al.* 2010), which suggest that other photoreceptors are able to compensate the function of PHYs in the quintuple mutant.

The CRY photoreceptor protein family perceives light signals through the flavin adenine dinucleotide (FAD) and pterin chromophores. CRY1 and CRY2 have been shown to participate in the BL input to the clock. Studies with loss of function mutants showed that the circadian period of plants lacking a functional CRY1 rendered a long period phenotype under low and high fluencies of BL but not under intermediate fluencies (Somers *et al.* 1998a). c*ry2* mutant plants on the other hand also showed a long period phenotype just under low BL

fluencies. In double *cry1/cry2* mutant plants, the long period is observed under all BL intensities, suggesting a partial redundancy of CRY1 and CRY2 on clock entrainment by BL. The *cry1/cry2* double mutant still sustained circadian oscillations (Devlin and Kay 2000), which suggests that in contrast to what is described in animal systems (Van Der Horst *et al.* 1999), the plant CRYs are not essential components of the central oscillator.

The ZEITLUPE protein family is composed of three members: ZTL, FKF1, and LKP2 (Baudry *et al.* 2010, Park *et al.* 2010, Yu *et al.* 2008). These proteins contain three specific domains including a BL absorbing PAS domain (Per-ARNT-Sim/LOV), which binds the flavin mononucleotide chromophore (Ito *et al.* 2012), an F-box domain with E3 ligase activity and a Kelch domain responsible for interactions with substrates. ZTL, FKF1 and LKP2 contribute to the ubiquitin-mediated clock protein degradation by conferring substrate specificity to the SCF E3 ubiquitin ligase complexes (Ito, et al. 2012). ZTL was identified in a screening as a mutant that lengthened the circadian period under free running conditions and displayed altered photoperiodic regulation of flowering time (Somers *et al.* 2004, Somers, et al. 2000). The LOV domain (for light, oxygen, or voltage) is a specialized domain with potential blue-light absorbing capacity. *ZTL* transcript is constitutively express throughout the day although the protein follows a circadian regulation with a peak at the end of the light period. GI interacts with ZTL preventing its degradation and this interaction is enhanced by BL and results in the stabilization of ZTL (Kim *et al.* 2007). ZTL regulation of circadian period is accomplished via the precise regulation of TOC1 and PRR5 protein stability (Kiba *et al.* 2007, Más, et al. 2003).

The circadian clock can be entrained by temperature as robustly as by light although the mechanisms behind this synchronization remain to be fully elucidated. The transcription of some clock core genes is regulated in response to changes in ambient temperature (Mizuno *et al.* 2014a). For instance *PRR7, PRR9, GI* and *LUX* are up-regulated in response to a temperature upshift specifically during the dark period. The *PRR7, PRR9, GI* and *LUX* clock genes are common targets of the EC night-time repressor, which suggest that warm temperatures might antagonize EC activity, whereas cold temperatures stimulate it (Mizuno, et al. 2014a). In terms of clock synchronization, high temperatures appear to be equivalent to daylight while low temperatures are interpreted as darkness (McClung 2006). This suggests that light and temperature signals may converge at some shared entrainment mechanisms.

In addition to light and temperature, other factors such as sucrose accumulation, might contribute to clock synchronization (Haydon *et al.* 2013). Photosynthetic production of sugars is a well study process controlled by the clock (Graf *et al.* 2010). Haydon and colleagues demonstrated that photosynthetically derived sugars contribute to entrainment of the *Arabidopsis thaliana* circadian clock. The authors found that following its activation by light at

dawn, the expression of the clock component *PRR7* is directly repressed by sugars. As PRR7 is a transcriptional repressor of the core clock gene *CCA1,* repression of PRR7 by sucrose results in activation of *CCA1* early in the light period, in a process that is gated by the clock. Thus, sugar oscillations define a "metabolic dawn" that contributes to circadian entrainment and maintenance of rhythms in *Arabidopsis*.

The changes in ambient temperature are able to entrain every day the clock. However, the clock is also characterized by a remarkable property, known as temperature compensation (Ruoff 1992, Somero 2004). Essentially, and to be valuable as a timing device, the circadian system should run at the same pace regardless the variations in temperature. This way the clock does not run faster at higher temperatures or slower at lower temperatures, and thus is able to maintain a period close to 24 hours. The capacity of buffering the variations in temperature (within a physiological range) is in clear contrast to what is happening in many biochemical reactions. The components and mechanisms by which the circadian clock is capable of compensating changes in temperature are only starting to emerge. The expression of some clock components is significantly modulated by high or low temperatures (Mizuno, et al. 2014a, Mizuno, et al. 2014b, Nakamichi *et al.* 2010, Salomé *et al.* 2010) and similarly, alternative splicing dependent on high or low temperature renders different functional isoforms that affect clock activity (James *et al.* 2012, Park *et al.* 2012, Seo *et al.* 2012). Recent studies have provided some clues about the mechanism responsible for temperature compensation. The mechanism relies on the perfect balance between two opposing, temperature-dependent activities: phosphorylation by the protein kinase CK2 and the transcriptional activity of CCA1. The balance of these two activities at the different temperatures is essential for the clock to sustain a 24-hour period (Portolés and Mas 2010).

4. Regulatory mechanisms responsible for the generation of rhythms at the core of the *Arabidopsis* central oscillator

A wide range of mechanisms are responsible for the precise generation of the rhythmic oscillations. The mechanisms pervade many regulatory layers including transcriptional and post-transcriptional, translational and post-translational as well as epigenetic. Circadian regulation of alternative splicing (Hong *et al.* 2010, Wang *et al.* 2012), protein phosphorylation (Fujiwara *et al.* 2008, Portolés and Mas 2010) ubiquitination (Cui *et al.* 2013), degradation (Kiba, et al. 2007, Más, et al. 2003) and subcellular localization (Herrero *et al.* 2012, Kim *et al.* 2013) are some of the regulatory processes described to be connected with the *Arabidopsis* circadian clock. Based on their relevance to our studies, here we only briefly describe the

transcriptional, epigenetic and protein-protein interaction mechanisms important for clock function.

4.1 Transcriptional regulatory mechanisms at the core of the *Arabidopsis* circadian clock

The very first indication about a transcriptional reciprocal regulation among clock components came from studies revealing that the morning-expressed MYB transcription factors CCA1 and LHY negatively regulated the expression of the evening-phased clock gene *TOC1* (Alabadí, et al. 2002, Harmer *et al.* 2000) by directly binding to the EE present at the *TOC1* promoter. These initial studies also indicated that TOC1 functioned as an activator of *CCA1* and *LHY* expression. However, recent work has experimentally and mathematically (Gendron *et al.* 2012, Huang, et al. 2012, Pokhilko *et al.* 2012) changed this view and showed that TOC1 is in fact a repressor of *CCA1* and *LHY* expression. The studies revealed that morning- and evening-expressed clock core genes are regulated through the repressing activity of TOC1 (Huang, et al. 2012). ChIP-seq analyses also indicated that this regulation occurs through direct binding of TOC1 to the promoters of its target genes. The global repressing function of TOC1 transcriptionally connects the morning- and evening-expressed oscillator genes (Figure 4). An additional transcriptional loop has been identified between CCA1 and CHE (Pruneda-Paz *et al.* 2009). The clock component CCA1 HIKING EXPEDITION (CHE) is a transcription factor belonging to the class I TCP (TB1, CYC, PCFs) family. CHE is able to bind to the consensus class I TCP-binding site (TBS) (GGNCCCAC) at the *CCA1* promoter to inhibit its expression. CCA1 and LHY in turn repress *CHE* expression by directly binding to its promoter. It was suggested that the protein-protein interaction between CHE and TOC1 might be important for the recruitment of TOC1 to the *CCA1* promoter.

CCA1 and LHY also regulate other members of the PRR family. For instance, CCA1 and LHY are able to activate *PRR7* and *PRR9* expression and in turn, PRR7 and PRR9 bind to the promoters of *CCA1* and *LHY* to inhibit their expression (Farré, et al. 2005, Nakamichi, et al. 2005, Salomé and McClung 2005a). It is noteworthy that CCA1 and LHY act as repressors for most core clock genes except for *PRR7* and *PRR9*. It will be interesting to elucidate the components and mechanisms conferring this differential function. PRR5 also inhibits *CCA1/LHY* expression late during the day (Nakamichi, et al. 2010) while the repression is completed with TOC1 function around dusk. Altogether, these results suggest that the sequential waves of PRR repressing activity negatively regulate *LHY* and *CCA1* transcription throughout the day. Additional factors might be responsible for the repression until the mid-late night, when *CCA1* and *LHY* expression start to rise.

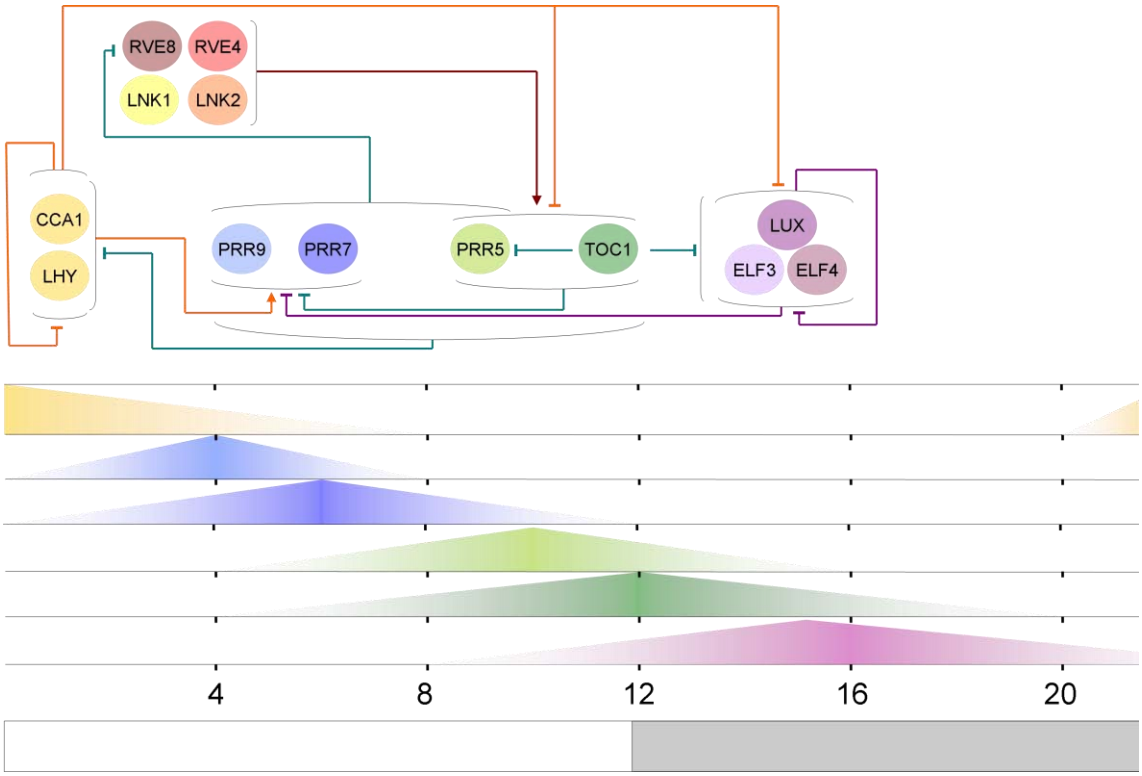


Figure 4. Transcriptional regulatory networks at the core of the *Arabidopsis* circadian clock. *CCA1* and *LHY* are expressed at dawn and repress evening-expressed genes such as *TOC1* and *PRR5* and the EC components. CCA1 and LHY also function as activators of *PRR7* and *PRR9* expression. In turn, *LHY* and *CCA1* expression is inhibited throughout the day by the action of the PRRs. Expression of *PRR7* and *9* is down-regulated during the night by the EC. This enables *LHY/CCA1* transcription to rise again at the following dawn. PRRs act also as repressors of other PRRs expressed at an earlier phase. This allows the temporal separation of expression in consecutive phases. The RVE-LNK complex activates *PRR5* and *TOC1* expression while TOC1 has a regulatory effect on the EC components. The color coded scheme in the lower panel indicates the circadian expression of morning components (orange), PRR9 (pale blue), PRR7 (dark blue), PRR5 (pale green), TOC1 (dark green), EC (purple) under LD conditions. Numbers indicate the Zeitgeber Time in hours. White and grey boxes represent light and dark period, respectively.

Other evening-expressed components also regulate the morning loop. Indeed, the EC represses *PRR9* expression by ELF3 and LUX binding to the *PRR9* promoter (Chow *et al.* 2012, Dixon *et al.* 2011, Helfer *et al.* 2011). As PRR9 represses *CCA1* and *LHY* expression, the EC is indirectly able to positively regulate *CCA1* and *LHY* (by repressing their repressor). In turn, CCA1 and LHY directly interact with the promoters of the EC genes, preventing the expression of its components (Lau *et al.* 2011, Li *et al.* 2011, Portolés and Mas 2010). This regulation forms an indirect feedback loop connecting the morning-expressed genes with the EC. Notably, *LUX* and *ELF4* are themselves transcriptionally repressed by the EC (Chow, et al. 2012, Kikis *et al.* 2005), which establishes a self-regulatory transcriptional mechanism.

4.2 Chromatin-dependent mechanisms at the core of the *Arabidopsis* circadian clock

Changes in chromatin architecture have also emerged as a central mechanism coupled with the rhythmic oscillation of clock gene expression. The degree of chromatin compaction highly varies in a dynamic process that regulates the accessibility of chromatin to the transcriptional machinery and other regulators (Li *et al.* 2007). The N-terminal tails of histones is modified by a number of covalent modifications including among others, acetylation, methylation and ubiquitination. Histone hyper-acetylation has been proposed to result in an open chromatin structure that facilitates transcriptional activation while histone hypo-acetylation leads to repression by chromatin compaction. Histone acetyltransferases (HATs) are the enzymes responsible for the addition of the acetyl groups and conversely, histone deacetylases (HDACs) remove the acetylation. The first report connecting chromatin changes with the circadian clock came from studies describing circadian changes in histone H3 acetylation (H3Ac) at the *TOC1* promoter (Perales and Más 2007). The study suggested that the transcriptional repression of *TOC1* by CCA1 might be due to changes in chromatin remodeling. Indeed, over-expression of CCA1 leads to a hypo-acetylated state of histones at the *TOC1* promoter that correlates with the transcriptional repression (Perales and Más 2007). The rhythmic changes in histone acetylation at the *TOC1* promoter are also regulated by RVE8 (Farinas and Mas 2011). However, the molecular function of CCA1 and RVE8 are quite different as RVE8 favors histone hyper-acetylation. Indeed, over-expression of RVE8 advances the rising phase of *TOC1* and correlates with an increased pattern of acetylation (Farinas and Mas 2011). Conversely, a delayed rising phase and a decreased pattern of histone acetylation are observed in *rve8* loss-of-function mutant. These results indicate that RVE8 favors the rising phase of *TOC1* through modulation of histone acetylation, which counterbalance the repressing activity of CCA1. Following *TOC1* peak of expression, HDAC activities contribute to *TOC1* declining phase specifically at the light to dark transition. The HDAC activities appear to antagonize RVE8 function so that the declining phase of *TOC1* is initiated. The rhythmic oscillation of H3Ac is not exclusive for *TOC1* but also pervades other oscillator promoters including *CCA1, LHY, PRR9, PRR7* and *LUX* (Malapeira *et al.* 2012, Song and Noh 2012).

Members of the plant TOPLESS/TOPLESS RELATED PROTEIN (TPL/TPR) protein family were shown to interact with PRR5, 7 and 9 proteins and this interaction is important for repression of *CCA1* and *LHY* transcription. The mechanism behind this regulation involves the histone deacetylase activities of HISTONE DEACETYLASE6 (HDA6) that forms a complex together with PRR9 and TPL to repress circadian gene expression (Wang *et al.* 2013). Other marks such as histone methylation (Berr *et al.* 2010, Guo *et al.* 2010, Sanchez *et al.* 2010), demethylation (Jones *et al.* 2010, Lu *et al.* 2011) and ubiquitination (Himanen *et al.* 2012)

appear to be also important for circadian clock progression, although the components and mechanisms behind these epigenetic regulations remain to be fully elucidated.

4.3 Functional protein complexes of MYB transcription factors and their relevance for circadian and light signaling pathways

Protein-protein interactions of morning and evening clock proteins have been shown to be essential for circadian function (Seo and Mas 2014). Focusing just on CCA1 and LHY, many different partners have been identified, and the formation of their corresponding protein complexes appears to regulate light, temperature and circadian signaling pathways. CCA1 and LHY form homo and heterodimers in the nucleus (Lu *et al.* 2009, Yakir *et al.* 2009) and the differential ratios might modulate their DNA binding capabilities, protein complex stability or subcellular localization. CCA1 and LHY also interact with the regulatory subunits of the protein kinase CK2 (Sugano *et al.* 1998, Sugano *et al.* 1999). The interaction is important for controlling the circadian period length (Daniel *et al.* 2004, Portolés and Más 2007, Sugano, et al. 1999) and for maintaining a constant period over a physiological range of temperatures (Portolés and Mas 2010). Light signaling is also modulated by the interaction of CCA1 with ELONGATED HYPOCOTYL5 (HY5), FAR RED-IMPAIRED RESPONSE1 (FAR1) and FAR RED-ELONGATED HYPOCOTYL3 (FHY3) (Andronis *et al.* 2008, Li, et al. 2011). Similar to the opposite roles as activator (e.g. of *PRR9* and *7*) or repressor (e.g. of *TOC1*), CCA1 synergistically increases the DNA binding activity of HY5 on the *CAB2* promoter (Andronis, et al. 2008) but disrupts the transcriptional activating function of FHY3, HY5 and FAR1 on the *ELF4* promoter (Li, et al. 2011). Interaction of CCA1 and LHY with other partners appears to aid their transcriptional activity. For instance, the interaction of CCA1 and LHY with DE-ETIOLATED 1 (DET1), a negative regulator of light-regulated gene expression, facilitates the binding to the promoters of their target genes to repress their expression (Lau, et al. 2011). DET1 also forms a complex with LHY and with the E3 ubiquitin ligase SINAT5, an *Arabidopsis* homologue of the Drosophila SINA RING-finger protein (Park, et al. 2010). The complex seems to be important for the initiation of flowering time through the SINAT5-dependent ubiquitination of LHY in a process that is regulated by DET1. By both yeast two-hybrid and direct protein–protein interaction assays, CCA1 and LHY were also shown to interact with different members of the TCP (TEOSINTE BRANCHED1, CYCLOIDEA and Proliferating Cell Nuclear Antigen Factor) plant-specific family of transcription factors. As TCP transcription factors are involved in the regulation of nuclear genes encoding organelle proteins, it was suggested that the connection of TCP with the clock might be important for the time-of-day coordination of organellar functions (Giraud *et al.* 2010). As mentioned in section 2.2, CCA1, LHY, RVE4 and RVE8 directly interact with LNK1 and

LNK2. It is possible that these interactions act as a "Morning Complex" important for the transcriptional modulation of circadian gene expression during day time (Xie, et al. 2014) in a similar fashion to that displayed by the EC (Nusinow, et al. 2011).

5. Biological processes controlled by the clock

The high percentage of genes controlled by the circadian clock is translated into rhythms in a wide variety of biological processes in which these clock-controlled genes are involved. The clock outputs pervade many different signaling pathways that are timely regulated in synchronization with the environment. Nearly all developmental transitions in plants are temporally coordinated at the most appropriate diurnal or seasonal time. Similarly, key metabolic pathways including that of anthocyanin biosynthesis are tightly controlled by the clock. In the following sections, we briefly describe the role of the circadian clock modulating the timing of these processes as miss-expression of *RVE8* and *LNKs* alter some developmental transitions and the diurnal accumulation of anthocyanins.

5.1 Clock outputs: developmental processes

Hypocotyl growth

Following germination, the embryonic hypocotyl elongates in a process that is regulated by multiple factors including light and the circadian clock. Indeed, hypocotyl growth depends on cell expansion, a process that follows a diurnal rhythm with a peak at the end of the night. Consistently, many clock mutants and over-expressing lines, including those of *RVE8* (section 2.1) and *LNK*s (section 2.2) display alterations in hypocotyl length. Molecularly, members of the basic Helix Loop Helix (bHLH) family of plant transcription factors, denominated PIFs (PHYTOCHROME INTERACTING FACTORS) has been shown to be master regulators of growth (Jeong and Choi 2013). The very precise post-translational regulations by light and transcriptional control by the clock seem to define the diurnal waveform of growth. Indeed, through the action of PHYB, light during the day targets PIF protein degradation by the 26S proteasome pathway (Lorrain *et al.* 2008) while the EC represses the promoters of *PIF4* and *PIF5* until the end of the night (Lu *et al.* 2012, Nusinow, et al. 2011). This dual light-clock regulation leads to PIF protein accumulation specifically at the end of the dark period. Similar to the effects of light, sucrose seems also to promote cell elongation, most likely by a post-translational regulatory mechanism that affect the stability of the PIF proteins (Stewart *et al.*

2011). Remarkably, the circadian clock gates the effects of sucrose on hypocotyl elongation (Fankhauser and Staiger 2002, Yamashino 2013).

The circadian clock also regulates hypocotyl growth through the modulation of the GIBBERELLIC ACID (GA) hormone activity (Arana *et al.* 2011, de Lucas *et al.* 2008). The DELLA proteins are key repressors of GA-responsive growth by inhibiting a subset of GA-related genes. Notably, DELLAs repress PIF4 activity and this repression is counteracted by the DELLAs destabilization through GA (de Lucas, et al. 2008). Removing the DELLA proteins allows PIF4 to bind to the G-box motifs to induce the expression of genes involved in hypocotyl growth around dawn. The circadian clock also gates the transcriptional regulation of the GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), which results in higher stability of DELLA proteins during day and higher GA sensitivity at night (Arana, et al. 2011). Other hormones such as auxins (AUX) are also important for hypocotyl elongation. Several pathways act in parallel to mediate the AUX-dependent regulation of hypocotyl growth. The clock itself also gates the time of maximal AUX responsiveness to coincide with the time in which hypocotyl elongation reaches its maximum rate (Covington and Harmer 2007). Here again, PIF4 and PIF5 play a crucial role regulating the transcription of several AUX-related genes (Franklin *et al.* 2011, Hornitschek *et al.* 2012, Nozue *et al.* 2011). Notably, RVE1 has a role modulating hypocotyl growth. This function is independent of PIFs and seems to occur through regulation of AUX biosynthesis (Rawat, et al. 2009). Overall, these studies show how plants can integrate the circadian clock, light and hormone signaling to optimize hypocotyl growth.

Flowering time

The developmental transition from a vegetative to a reproductive stage is regulated in plants by many different pathways including the one controlled by day-length or photoperiod. *Arabidopsis thaliana* is a facultative long-day plant, i.e. flower earlier under long-day than under short-day conditions, and the circadian clock is the mechanism that allows plants to measure day-length. Consistently, clock mutants and over-expressing lines, including those of RVE8 (section 2.1) and LNKs (section 2.2) display alterations in the photoperiodic regulation of flowering time. The photoperiodic flowering pathway regulates the amount of florigen, in *Arabidopsis* the FLOWERING LOCUS T (FT) protein, which determines the flowering time (Kobayashi *et al.* 1999). From leaves, where FT is synthesized, the protein is translocated to the shoot apical meristem to regulate floral development. Under long-day conditions, FT accumulation relies on a very precise regulation of its activator CONSTANS (CO) (Samach *et al.* 2000, Suárez-López *et al.* 2001). To induce *FT* transcription, *CO* expression is precisely

regulated by the circadian clock. In the morning, CYCLING DOF FACTORs (CDFs) (Fornara *et al.* 2009) directly repress *CO* transcription while CCA1 and LHY repress *FKF1* and *GI*, which are negative regulators of CDFs (Imaizumi *et al.* 2005, Schaffer, et al. 1998). In the afternoon, the FKF1 and GI proteins form a complex that targets CDF proteins to degradation under the long-day conditions (Sawa *et al.* 2007). ZTL and LKP2 also interact with GI in a BL dependent manner and contribute to the degradation of CDFs (Kim, et al. 2007). Around this time, *CDF* transcripts are also repressed by PRR9, PRR7 and PRR5 (Nakamichi, et al. 2010). Recently, the FLOWERING BHLH (FBH) factors have been identified as transcriptional activators controlling the amplitude of *CO* expression (Ito, et al. 2012). Post-translational mechanisms of CO protein stabilization are also important for day-length sensing. Light signals perceived by PHY and CRY stabilize CO protein only in long-day afternoons (Valverde *et al.* 2004). In the morning, PHYB and the ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1) contribute to the degradation of the CO protein (Lazaro *et al.* 2012). In the dark, CO protein is actively degraded by the SUPPRESSOR OF PHYA-105 1 (SPA1) and the CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) complex (Zuo *et al.* 2011). However, under blue light, CRY2 binds to SPA1/COP1 complex to suppress their activity. CO degradation is also counteracted in the late afternoon by FKF1, which binds to and stabilizes CO protein in a blue light-dependent manner (Song *et al.* 2012). Thus, the complex interplay of light and the circadian clock modulates *CO* transcriptional and post-translational mechanisms for proper day-length sensing and flowering time.

5.2 Clock outputs: metabolic pathways

A wide variety of metabolic pathways are controlled by the clock including among others, processes such as photosynthesis (Michael *et al.* 2008, Noordally *et al.* 2013), redox homeostasis (Lai *et al.* 2012), starch metabolism (Graf, et al. 2010), nutrient assimilation (Salomé *et al.* 2013) or secondary metabolism (Love *et al.* 2004). The circadian regulation of metabolism seems to ensure optimal growth and physiology in synchronization with the environment. Circadian oscillations also allow the temporal separation of incompatible metabolic processes so that they occur at a biologically beneficial time of day or year. Remarkably, there is increasing evidence that metabolic outputs can in turn influence the circadian timing and might also comprise independent circadian oscillators (Haydon, et al. 2013). In the following subsections, we focus on anthocyanin biosynthesis as this clock output is directly regulated by RVE8 and LNKs.

Phenylpropanoid pathway

Phenylpropanoids are organic compounds found throughout the plant kingdom that influence many key aspects of plant metabolism. The pathway generates a variety of important secondary products including monolignols, flavonoids, phenolic acids and stilbenes (Vogt 2010). They perform important functions such as strengthening cell walls, UV sun screening, symbiotic nitrogen fixation and photo-oxidative damaging protection (Ariizumi and Toriyama 2011, Bennett and Wallsgrove 1994, Cheynier *et al.* 2013, Nakabayashi *et al.* 2014). The diversity of phenylpropanoids is the result of the modification and amplification of a limited set of core structures derived from the shikimate pathway (Ferrer *et al.* 2008, Maeda and Dudareva 2012, Tohge *et al.* 2013, Vogt 2010), which is the pathway responsible for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan). The enzyme phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine into cinnamic acid, which in turn is converted to p-coumaroyl-CoA by the cinnamate 4-hydroxylase (C4L) and 4-coumarate-CoA ligase (4CL). The p-coumaroyl-CoA compound is the precursor of many phenylpropanoid products (Figure 5). In *Arabidopsis*, four genes have been identified to encode isomers of PAL (Hamberger *et al.* 2007). Plants lacking functional PALs have been used to show that the isomers PAL1 and PAL2 have a prevalent role in the flavonoid pathway (Olsen et al. 2008). Analyses of gene expression and enzyme activities suggested that the 4CL3 had a primary role on flavonoid biosynthesis while the 4CL1 and 4CL2 mainly regulate reactions of other branches of the phenylpropanoids pathway (Ehlting et al. 1999). Overall, phenylpropanoids are of high interest because their beneficial functions in human health including anticancer and anti-inflammatory properties.

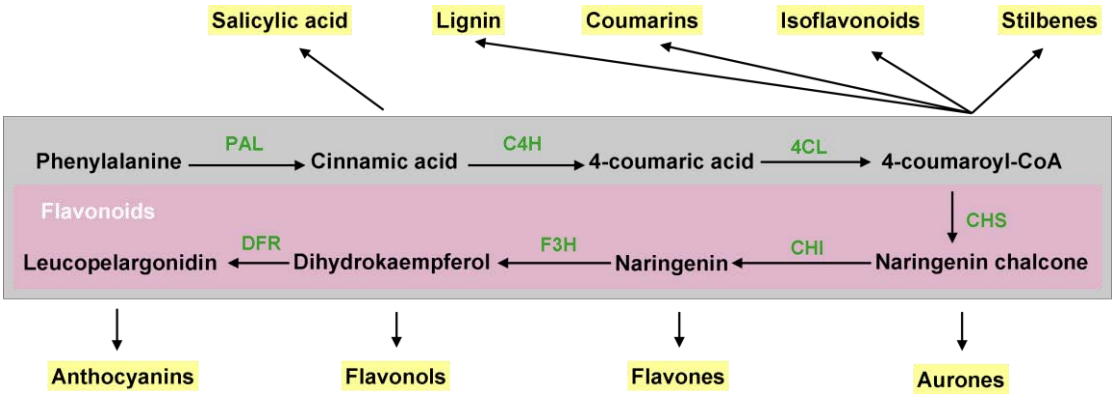


Figure 5. Schematic view of some branches deriving from phenylpropanoid metabolism. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone flavavone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol reductase; Purple box indicate flavonoids pathaway. Yellow boxes are compounds produced from the phenylpropanoids metabolism.

Anthocyanin biosynthesis

Flavonoids are one of the largest groups of secondary metabolites that derive from the phenylpropanoid pathway. The main classes of flavonoids include the colourless to pale yellow flavonols, the red to purple anthocyanins and the colourless proanthocyanidins. Flavonoids contribute to the agronomic, industrial and nutritional value of plant products (Jaakola 2013, Zhang *et al.* 2014). Particularly, anthocyanins are a group of pigments involved in many essential processes such as the attraction of pollinators, seed dispersion, protection against irradiations and defense against microbial agents (Buer *et al.* 2010, Shi and Xie 2014). In the following subsections, we briefly describe the anthocyanin biosynthetic pathway and the main endogenous molecular regulators as well as the exogenous signals and pathways modulating anthocyanin accumulation.

In *Arabidopsis*, the expression of the so-called early biosynthesis genes (EBGs) including *CHALCONE SYNTHASE, CHALCONE FLAVANONE ISOMERASA, FLAVANONE 3-HYDROXYLASE, FLAVONOID 3`-HYDROLASE (CHS, CHI, F3H* and *F3`H* respectively) precedes the late biosynthesis genes (LBGs) such as *DFR, LDOX*, ANTHOCYANIN REDUCTASE (ANR) and UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRASNFERASE (UF3GT). The expression of these genes is regulated by the R2R3-type MYB transcription factors such as PAP1 (Borevitz *et al.* 2000) and PAP2 (Gonzalez *et al.* 2008). The MYBs interacts with a WD40 protein, TRANSPARENT TESTA GLABRA1 (TTG1) and with bHLH proteins such as TRANSPARENT TESTA8 (TT8; bHLH042), GLABRA3 (GL3; bHLH001) and ENHANCER OF GLABRA3 (EGL3; bHLH002) to form a WD40-bHLH-MYB (WBM) complex (Gonzalez, et al. 2008) (Figure 6). Other MYBs such as MYB11, MYB12, and MYB111, MYB113 and MYB114 can also regulate the EBGs and LBGs in a TTG1-independent manner. The positive function of the WBM complex is counteracted by the MYB protein MYB-LIKE 2 (MYBL2) that functions as a negative regulator of anthocyanin biosynthesis (Matsui *et al.* 2008). Transient expression and protein interaction studies have proved that MYBL2 competes with positive regulators as PAP1 for the binding with bHLH proteins causing a reduction in the WBM activity (Matsui, et al. 2008). The regulation might be determined by the quantitative competition of positive and negative components (Song et al. 2011). If this is the case, the switch between activation/repression would be determined by the competition of PAP1 and others positive regulators with the MYBL2 protein. Phenotypic characterization of these factors confirmed their role on the anthocyanin biosynthetic pathway. Indeed, plants over-expressing *PAP1, PAP2, MYB113* or *MYB114* show an increased accumulation of anthocyanins (Gonzalez, et al. 2008) while *pap1* single mutant plants or RNAi plants with down-regulation of *PAP1, PAP2, MYB113* or *MYB114* accumulate less anthocyanins in leaves

and seedlings (Gonzalez, et al. 2008). Over-expression of *PAP1* in other species also leads to anthocyanin accumulation (Li *et al.* 2010, Zuluaga *et al.* 2008), suggesting a conserved function throughout evolution.

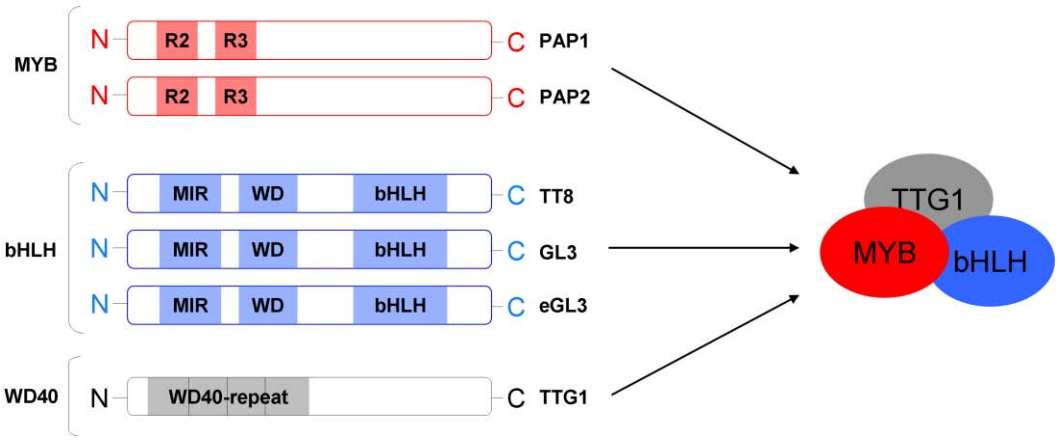


Figure 6. MBW complexes involved in anthocyanin production. Schematic representation of the MYB, bHLH and WD40 transcription factors that form the MBW complexes. The color-coded specific domains are also represented for each factor.

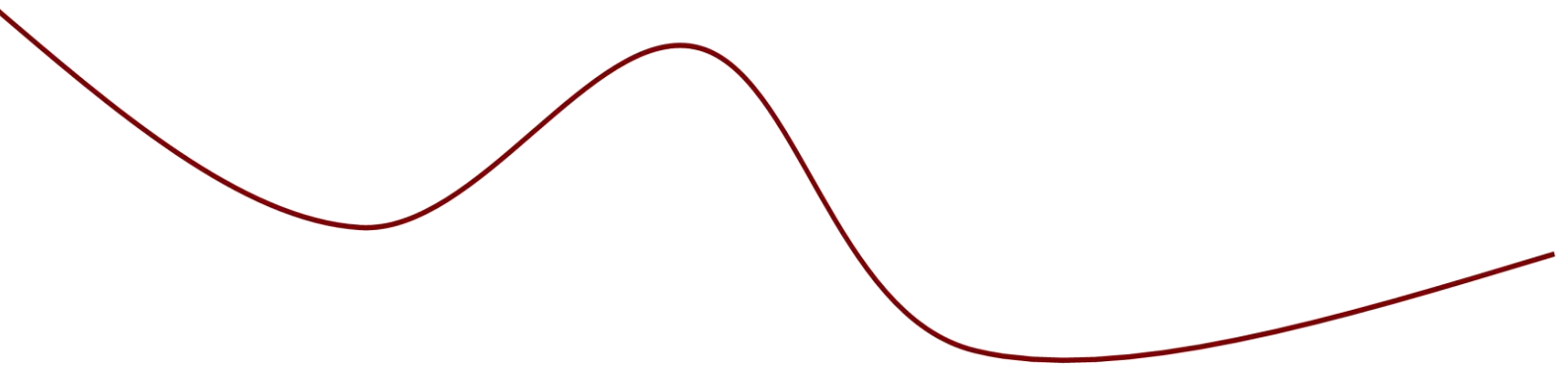
First evidences demonstrating a role for GL3 and EGL3 on anthocyanin production came from transient expression assays in which *GL3* was co-expressed with other MYB transcription factor in *Matthiola incata* (Ramsay *et al.* 2003). Later studies using single and double mutants *gl3/egl3* confirmed the role of these factors on anthocyanin production. TTG1 is the only WD40 protein member currently determined to regulate anthocyanin production. Mutations on *TTG1* locus result in pleiotropic effects on several plant developmental processes including anthocyanin accumulation in vegetative tissues, deficiency of proanthocyanidins in the seed coat, alterations on trichome initiation and seed mucilage production (Walker et al. 1999). In concordance with these results, *TTG1* is found constitutively expressed in all plant tissues across all developmental stages suggesting its role in multiple processes.

Anthocyanin biosynthesis is activated by light (Cominelli et al. 2008) while darkness reduces anthocyanin content (Ang *et al.* 1998). Although the specific mechanism by which light regulates anthocyanin quantity is not fully understood, the light-dependent regulation of the expression of anthocyanin-related genes might play a key role (Cominelli, et al. 2008). For instance, the expression of the *PAP1, PAP2, GL3, EGL3* and *TT8* is induced by light. Furthermore, over-expression of *PAP1* is not able to overcome the inhibition of anthocyanin biosynthesis under dark conditions (Cominelli, et al. 2008). In addition, light signaling components have been demonstrated to control anthocyanin biosynthesis. The bZIP protein HY5, which is a positive regulator of photomorphogenesis (Ang, et al. 1998) has a key role on

anthocyanin production. Under FR light conditions, HY5 is able to interact with PIF3 and bind to specific elements in the promoters of anthocyanin structural genes to activate their expression (Shin et al. 2007).

Other important factor having a positive role on anthocyanin accumulation is sucrose (Teng et al. 2005). Sucrose induces the expression of several anthocyanin-related genes by directly regulating *PAP1* expression. In addition, some hormones have a positive effect on anthocyanin accumulation but this effect disappears in the absence of sucrose (Loreti et al. 2008). Nitrogen can also regulate anthocyanin biosynthesis. Nitrogen and anthocyanin accumulation are inversely correlated (Lea et al. 2007): high nitrogen concentrations correlate with low anthocyanins and conversely high anthocyanin is accompanied by decreased nitrogen accumulation. Gene expression analysis have confirmed that nitrogen depletion induces *PAP1* and *PAP2* expression (Lillo *et al.* 2008) with *PAP2* showing a higher response to nitrogen depletion than *PAP1*. Differential responses were also observed for the bHLH transcription factors, with GL3 but not EGL3 responding to nitrogen limiting conditions (Lea, et al. 2007). Consistently, *gl3* mutant plants did not respond to nitrogen depletion and did not accumulate more anthocyanins than WT plants or *egl3* mutant plants, which responded as the WT. Taken together, these results indicate that specific components of the WBM complexes might have different roles regulating anthocyanin accumulation.

Plant hormones such as AUX, ABA (Hoth *et al.* 2010, Jeong *et al.* 2004), GAs (Weiss *et al.* 1995) or cytokinin (Deikman and Hammer 1995, Morgan and Drew 1997) also regulate anthocyanin biosynthesis. In the case of the hormone Jasmonic Acid (JA), its role on anthocyanin production has been extensively studied. The F-box protein CORONATIVE INSENSITIVE 1 (COI1), main component of the JA signal transduction pathway, has been shown to be necessary for the transcriptional activation of LBGs and the regulatory genes *PAP1, PAP2* and *GL3* by JASMONIC ACID (JA) (Shan et al. 2009). COI1 form part of a complex responsible for the degradation of the JA ZIM-domain (JAZ) proteins (Xu et al. 2002). The JAZ proteins are able to interact with the C-terminal of GL3, EGL3 and TT8 and with PAP1 and GL1 interfering with the formation of a functional WBM complex (Qi et al. 2011). JA induces the degradation of JAZ proteins allowing the formation of active WBM complexes and thus the production of anthocyanin is resumed. Ethylene (ET) suppresses anthocyanin accumulation most likely by repressing the expression of *TT8, GL3* and *PAP1* while concomitantly stimulating the expression of the negative regulator *MYBL2* (Jeong et al. 2010). ET is in part responsible for the suppression of sugar-inducible anthocyanin synthesis in *Arabidopsis* plants growing under light. This regulation seems to be mediated through down-regulation of the expression of the sucrose transporter S*UCROSE-PROTON SYMPORTER 1 (SUC1)* in roots.



Objectives

Objectives

The general aim of this Thesis is the characterization of **the role of REVEILLE8/LHY-CCA1-LIKE5 (RVE8/LCL5) within the *Arabidopsis* circadian clock**. This general aim was conceived through the completion of the following specific objectives:

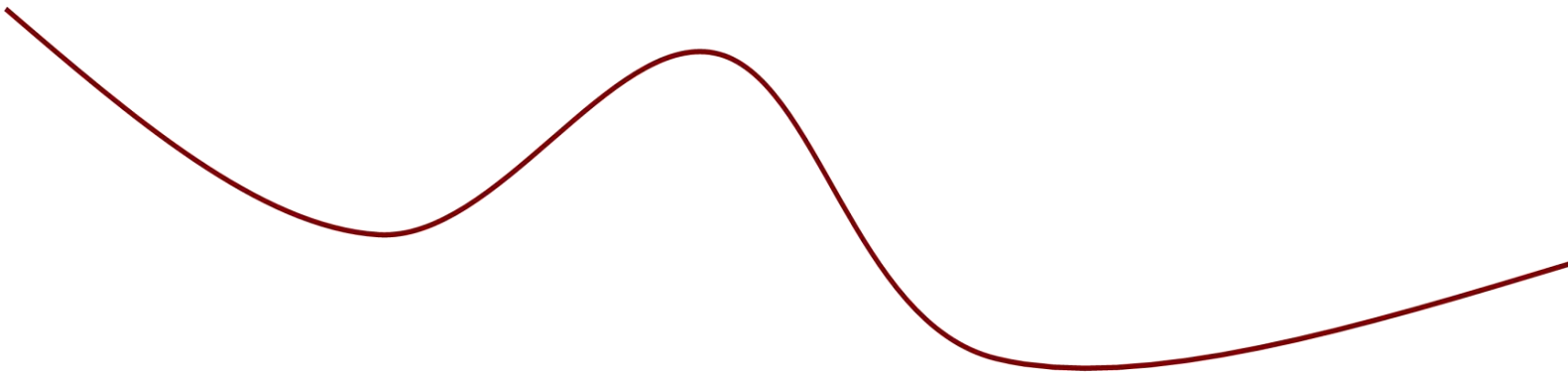
1. To unravel the transcriptional networks directly controlled by RVE8 by using RNA-seq approaches under particular environmental conditions and identifying regulated genes ascribed to specific pathways.

2. To determine the transcriptional oscillatory patterns of genes regulated by RVE8 by performing time course analyses over the diurnal cycle in plants miss-expressing RVE8.

3. To identify the RVE8 interacting proteins that modulate RVE8 function by performing a yeast-two hybrid screening followed by time course analyses through co-immunoprecipitation in plants.

4. To characterize the transcriptional activity of RVE8 and the effects of its interaction with LNKs by examining gene expression and protein binding to target genes through chromatin immunoprecipitation assays.

5. To dissect the physiological relevance of RVE8–LNK interaction by measuring the anthocyanin content in the different genetic backgrounds.



Results and Discussion

Results and Discussion

1. Genome-wide transcriptional profiling of RVE8 over-expressing plants

To identify the transcriptional networks controlled by RVE8, we compared the transcriptomic profiles of WT and RVE8-ox plants by genome-wide RNA sequencing (RNA-seq). Previous studies have reported an increased circadian period length of higher order *rve* mutants compared with single mutants (Hsu, et al. 2013), suggesting a functional redundancy among the RVE family. Therefore, we made used of the RVE8-ox plants for our RNA-seq analysis. To reduce the effects due to changes in the circadian phase by RVE8 over-expression, sampling was performed with plants grown under constant light and temperature conditions (without light or temperature entrainment). Under these conditions, direct RVE8 target genes can be identified, excluding those miss-expressed due to the change of the circadian phase in RVE8-ox plants (Figure 7). Our analysis revealed 1,074 differentially expressed genes, with *RVE8* at the top most significantly different. Functional categorization of the proteins encoded by the miss-regulated target genes revealed a wide variety of biological processes, including among others, signal transduction, response to stress and developmental processes (Figure 8).

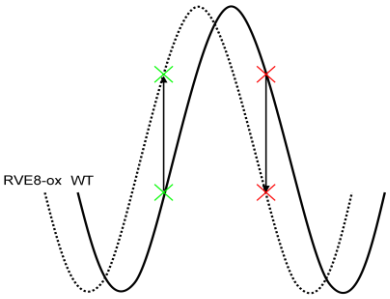


Figure 7. Schematic representation of the phase shift in RVE8-ox plant compared to WT. Depending on the time of sampling (green and red crosses) miss-regulated genes might appear as up-regulated (green crosses) or down-regulated (red crosses) when actually the differences in gene expression are due to the changes in the circadian phase.

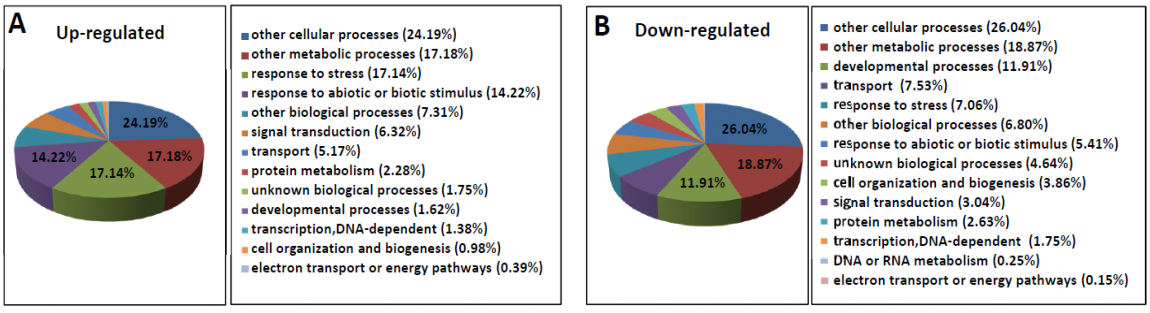


Figure 8. Biological processes miss-regulated in RVE8-ox plants. Functional categorization by annotation (GO Biological Processes) of genes up-regulated (A) and down-regulated (B) in RVE8-ox. Pie charts show the most relevant percentages calculated as the number of annotations to terms in a GO slim category (x100) divided by the number of total annotations to terms in the ontology.

Inspection of the data also revealed that a number of up-regulated genes were highly co-expressed (Figure 9A) and could be ascribed to the flavonoid biosynthetic pathway (Figure 9B-D). Genes situated upstream or downstream the anthocyanin-related genes were not affected by the over-expression of RVE8, suggesting that the up-regulation was specific for the anthocyanin-related genes (Figure 9E).

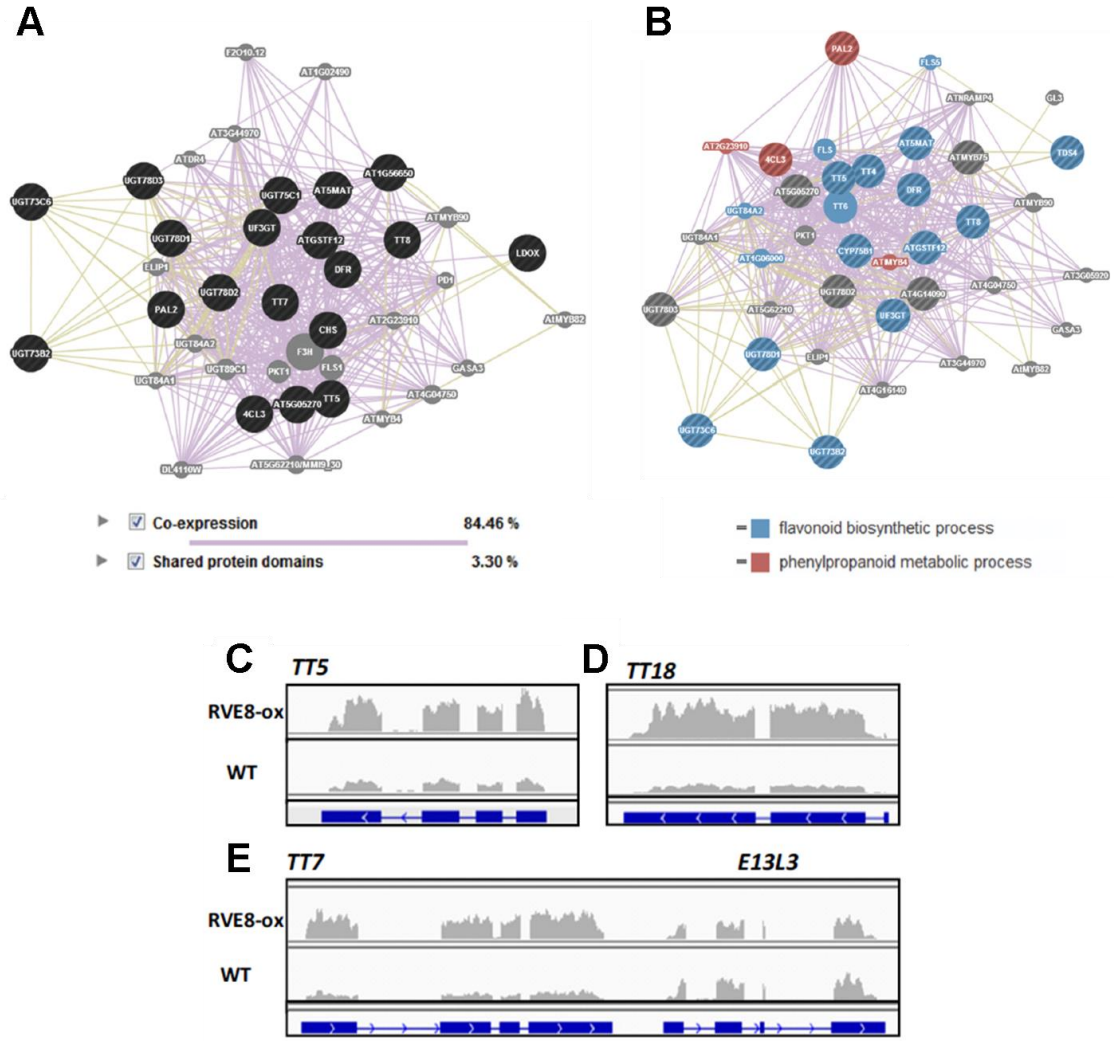


Figure 9. The anthocyanin biosynthetic pathway is miss-regulated in RVE8-ox plants. (A) Co-expression analysis of up-regulated genes in RVE8-ox plants that can be clustered together in the flavonoid biosynthetic pathway (B). (C–E) Visualization of RNA-seq reads by using the Integrative Genomics Viewer browser for the indicated anthocyanin biosynthetic loci. As a control, RNA-seq data from the *E13L3* locus (*GLUCAN ENDO-1, 3-BETAGLUCOSIDASE LIKE PROTEIN 3*) just downstream of *TT7* is shown in (E).

2. Oscillatory waveforms of anthocyanin-related genes over a diurnal cycle

The expression of most of the anthocyanin biosynthetic genes is controlled by the clock, with a rhythmic oscillatory pattern peaking around dawn under LL conditions (Figure 10A) in a similar trend to that observed for *RVE8*. Intriguingly, the peak phase of expression for the anthocyanin-related genes appears to change under LD cycles, and in some instances, the waveforms displayed a double peak around ZT4 and ZT12 with a clear decrease around midday (Figure 10B-C).

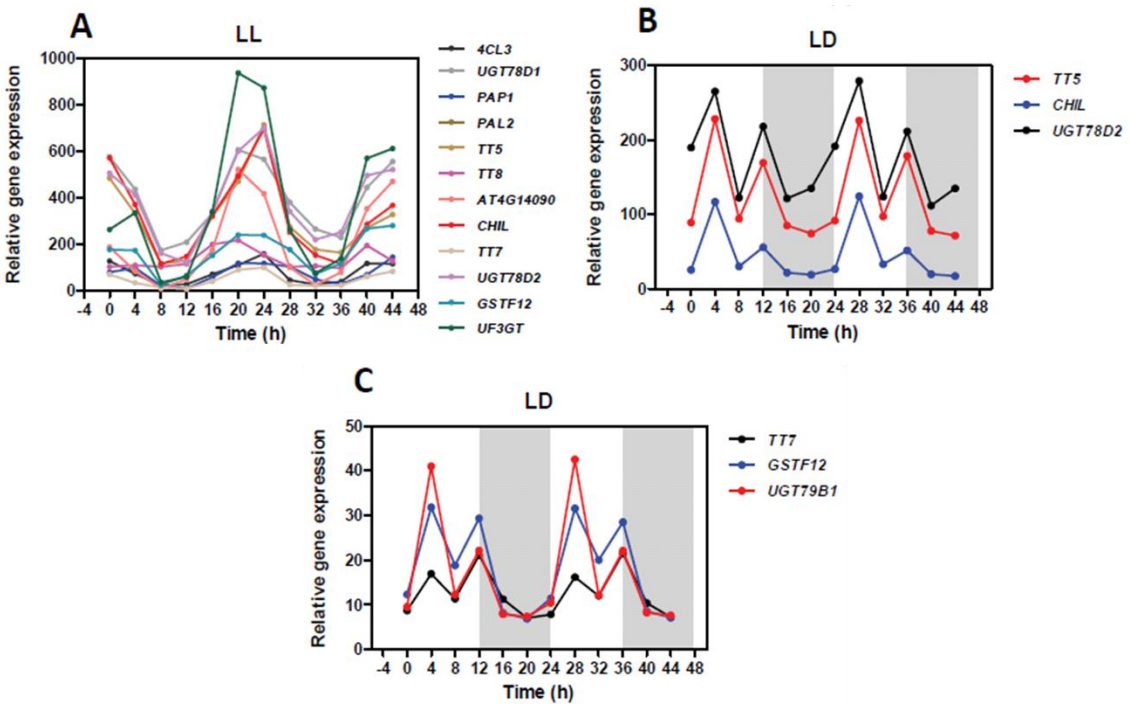


Figure 10. Oscillatory waveforms of anthocyanin-related genes. (A) Time course analysis over two circadian cycles under LL conditions of anthocyanin-related genes. Expression data was obtained from the publicly available web tool *DIURNAL* using the LL12-LDHH dataset with a cutoff value of 0.8. (B-C) Time course analysis over two circadian cycles under LD conditions of anthocyanin-related genes. Expression data was obtained from the publicly available web tool *DIURNAL* using the COL-LDHH dataset with a cutoff value of 0.8.

To verify the RNA-seq data, we performed a time course analysis under LD conditions to analyze the expression of the anthocyanin-related genes in WT and RVE8-ox plants. Our results showed that transcript abundance was significantly increased in RVE8-ox plants, particularly during daytime (Figure 11A-F), whereas no significant differences in gene expression were observed in WT and RVE8-ox during the night period. The decreased expression around ZT7 was quite evident in RVE8-ox plants. The expression of other regulatory

no biosynthetic anthocyanin genes was not significantly affected (Figure 11G-H) with the exception of *PAP1* and *TT8* (Figure 11E-F). The regulation appears to be gated mostly during the day but it is not constant, as at midday, other endogenous factors and/or mechanisms are partially able to overcome the RVE8-mediated activating function of the anthocyanin pathway. External factors like light (Cominelli, et al. 2008), sucrose (Teng, et al. 2005), nitrogen (Lea, et al. 2007) and plant hormones (Jeong, et al. 2010, Qi, et al. 2011) might also contribute to the production of anthocyanin.

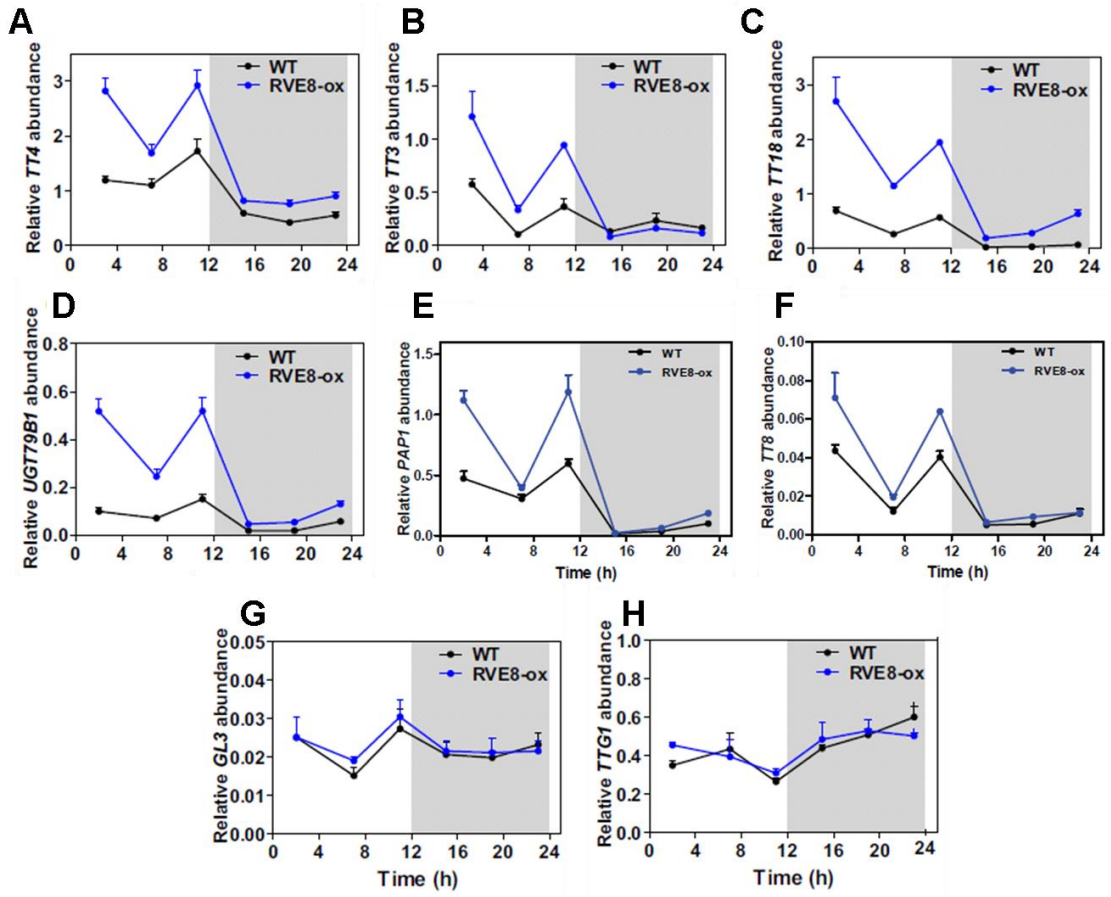


Figure 11. Up-regulation of anthocyanin biosynthetic genes in RVE8-ox plants. Time course analysis by RT-Q-PCR of *TT4* (A), *TT3* (B), *TT18* (C), *UGT79B1* (D), *PAP1* (E), *TT8* (F), *GL3* (G) and *TTG1* (H) in WT and RVE8-ox plants grown under LD cycles. mRNA abundance was normalized to *IPP2* expression. Values represent means + SEM. White, day; gray, night.

Our experimental and *in silico* analyses suggest that in RVE8-ox plants, the diurnal expression of genes involved in the anthocyanin biosynthetic pathway is up-regulated in a phase-dependent manner. Regulation of peak phase of expression is essential for circadian function and its control over metabolic outputs. The circadian oscillation of core clock components is indeed characterized by a specific peak phase of expression occurring at different times during the day-night cycle (e.g. dawn, morning, dusk or late evening). Similar to

CCA1 and *LHY*, *RVE8* mRNA expression follows a circadian regulation reaching a peak close to dawn (Farinas and Mas 2011, Rawat, et al. 2011). The similarities in amino acid sequence and circadian expression suggest that CCA1 and RVE8 might perform also a similar function. Indeed, these factors directly regulate *TOC1* expression through changes in chromatin remodeling. However, their roles are antagonistic: while CCA1 repression of *TOC1* correlates with a hypo-acetylated state of histones at the *TOC1* promoter (Perales and Más 2007), RVE8 activates *TOC1* expression most likely through histone acetylation (Farinas and Mas 2011). Over-expression of RVE8 leads to hyper-acetylation while *rve8* mutation results in hypo-acetylation. Miss-expression of *RVE8* also affects the phase of the clock, with an advanced and delayed phase in RVE8-ox and *rve8* mutants, respectively (Farinas and Mas 2011, Rawat, et al. 2011) (Figure 12).

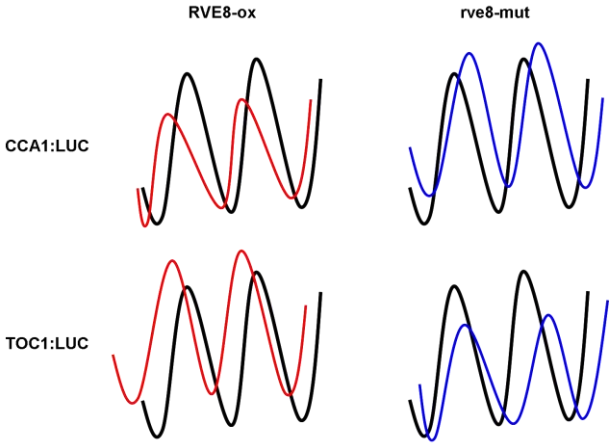


Figure 12. *CCA1* and *TOC1* expression in RVE8 miss-expressing plants. Circadian phenotypes of *CCA1* and *TOC1* expression in RVE8-ox and *rve8*-mut plants. Black line corresponds to WT expression. Red and blue lines correspond to expression in RVE8-ox and *rve8*-mut plants, respectively.

3. Interaction of LNK proteins with RVE8 in yeast-two hybrid assays

To further dissect the molecular mechanism underlying RVE8 function, we performed a yeast two-hybrid screening to identify RVE8 interacting proteins. The full-length coding sequence of RVE8 was used as a bait to screen a random-primed *Arabidopsis thaliana* cDNA library. Using a high confidence score (predicted biological score, PBS) (Formstecher *et al.* 2005) we identified three RVE8 interacting factors belonging to the LNK protein family (Figure 13). The LNK proteins have been shown to follow a circadian regulation and to affect the pace of the clock (Rugnone, et al. 2013).

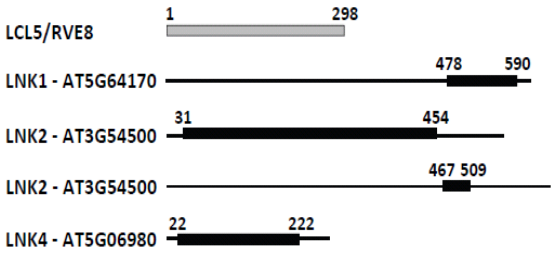


Figure 13. Yeast-two hybrid analysis. Schematic representation of the putative Selected Interaction Domains (SID, black boxes) that were obtained by identifying the domains shared by all prey fragments matching the reference protein.

The yeast two-hybrid screening is thus consistent with a previous report showing the rhythmic interaction of LNK1 and LNK2 with RVE8 and with RVE4 (Xie, et al. 2014). Analysis of the RVE8 co-expressed gene network by the *ATTED-II* and *GENEVESTIGATOR* web tools uncovered the members of the LNK family as highly significant genes co-expressed with RVE8 (Figure 14). Other proteins highly co-expressed with RVE8 are the core-clock components CCA1 and LHY.

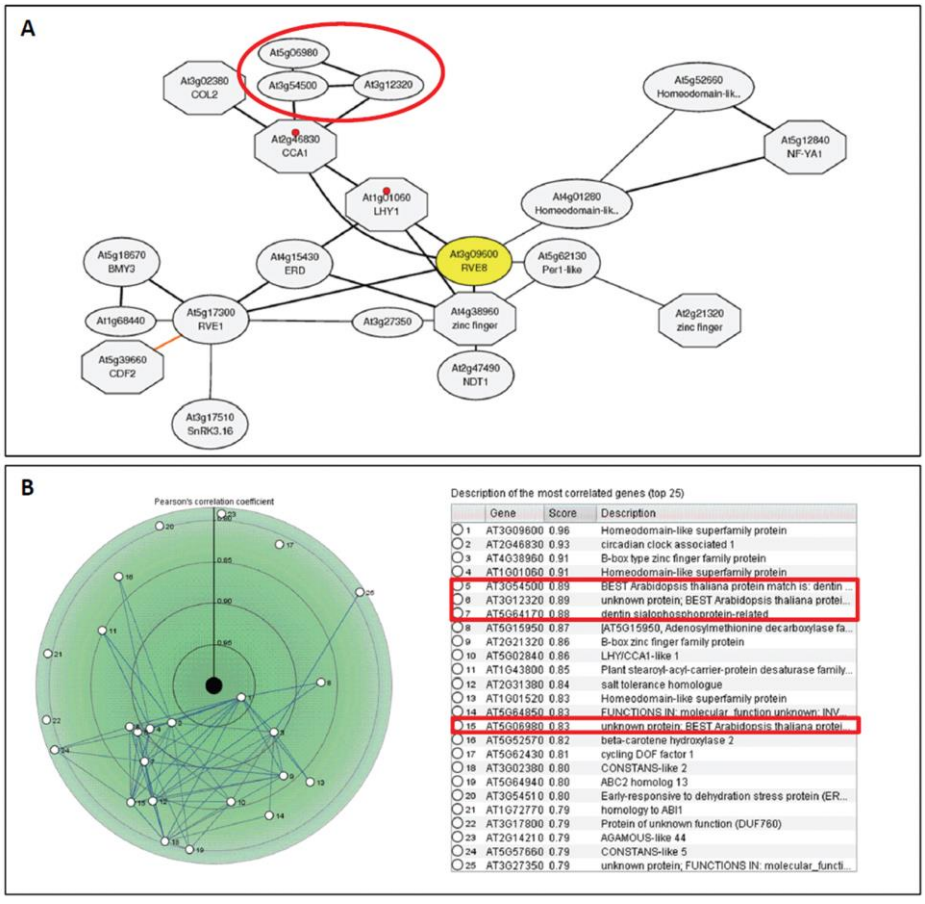


Figure 14. Analysis of *RVE8* co-regulated gene networks. Correlation of *LNKs* with *RVE8* as inferred by co-expression analysis using the *ATTED-II* web tool (A) or the *GENEVESTIGATOR* database (B). In both analyses, the Pearson's correlation coefficient is used to determine the correlation. The *LNK* genes are highlighted by the red oval (A) and red rectangles (B).

4. Generation of different RVE8 and LNKs genetic backgrounds

To verify the biological relevance of LNK interaction with RVE8, transgenic plants over-expressing each of the *LNKs* under the control of the constitutive 35S promoter were generated. Homozygous single insertion lines were chosen for further analyses (see Materials and Methods section). Three independent lines were selected from each genotype, and the level of over-expression was quantified by RT-Q-PCR (Figure 15). Analysis of the genetic interaction and studies of over-expressing *LNK* plants were highly complicated by the fact that plants lost the transgen over generations, resulting in lines with low over-expression (particularly evident for *LNK1* and *LNK2*). Extensive characterization of multiple lines finally allowed us to select a number of independent lines that moderately over-expressed *LNKs*. Similar problems for maintaining over-expression have been found for other clock components (e.g. CCA1-ox or TOC1-ox).

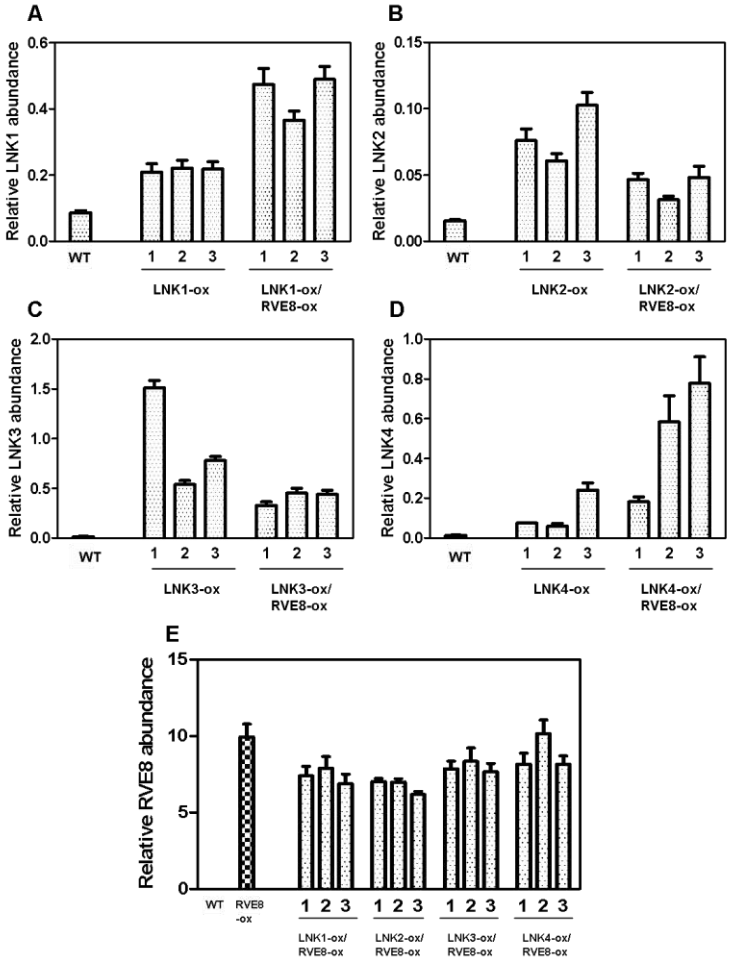


Figure 15. Gene expression analysis in the different transgenic lines used in our studies. Gene expression by RT-Q-PCR of LNK1, 2, 3, 4 in over-expressing WT and RVE8-ox plants (A-D). Gene expression by RT-Q-PCR of *RVE8* in double LNK and RVE8 over-expressing plants (E). Samples were collected at ZT7. mRNA abundance was normalized to *IPP2* expression. Values represent means + SEM.

5. Direct interaction of LNKs with RVE8 by co-immunoprecipitation in plants

To further support the interaction between LNKs and RVE8 and to expand the studies to LNK3 and LNK4, we performed co-immunoprecipitation experiments with plants over-expressing RVE8 and LNK1, LNK3 or LNK4 proteins. Our results revealed a clear interaction at ZT7 and a weaker interaction at ZT11 (Figure 16). No evident immunoprecipitation was observed at other time points examined (ZT2, ZT15, ZT19, and ZT23). No bands with mobility close to that of the LNK proteins were observed when similar procedures were performed with WT plants or with samples similarly processed but without antibody, which confirmed the specificity of the interactions.

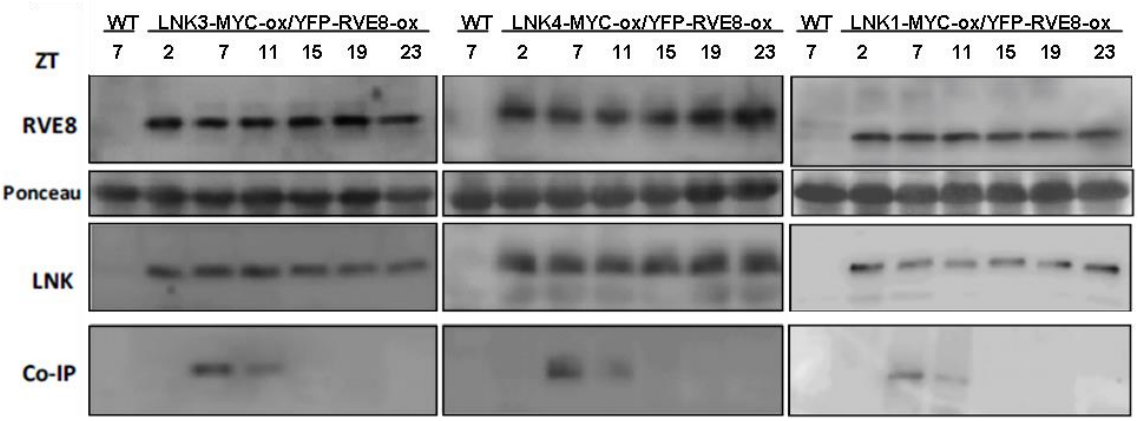


Figure 16. LNK proteins interact with RVE8. Western-blot analysis of LNK3-MYC-ox/YFP-RVE8-ox, LNK4-MYC-ox/YFP-RVE8-ox and LNK1-MYC-ox/YFP-RVE8-ox plants immunoprecipitated (Co-IP) with anti-GFP antibody following detection with anti-MYC antibody. Western-blot analysis of RVE8 and LNK protein accumulation is shown above and below the Ponceau staining. Plants were grown under LD cycles and processed at the indicated ZTs.

We found that the members of the LNK protein family interact with RVE8 mostly around ZT7. These findings suggest that despite the constitutive *LNK* over-expression, the interaction is timely controlled. Competition with endogenous LNK proteins is not likely responsible for the observed pattern of interaction, as no evident immunoprecipitation was observed at time points when the endogenous expression is very low (ZT15, ZT19, and ZT23). RVE8 and LNK protein abundance did not manifestly change at the different time points examined, suggesting that changes in protein stability are not driving the interaction. These results open the interesting possibility that additional factors might be involved in the

interaction or that specific states of the RVE8 and/or LNK proteins are more susceptible to interact. For instance, post-translational modifications can influence the interaction. Some examples are already described in the literature: hyper-phosphorylated TOC1 and PRR5 isoforms are more susceptible to interact with ZTL (Fujiwara, et al. 2008). Further studies will be necessary to ascertain the additional factors and mechanisms influencing the RVE-LNK interaction.

It is noteworthy that the interaction among RVEs, LNKs, CCA1 and LHY has been proposed to form a "morning complex" (Xie, et al. 2014) in line with the previously described "evening complex". Yet, the existence of this complex and its role controlling circadian gene expression and clock outputs has not been fully determined. Interaction between the single MYB transcription factors CCA1 and LHY to form homo and heterodimers is also important for their circadian function (Lu, et al. 2009, Yakir, et al. 2009). They also interact with components of other pathways providing the nodules linking the clock with processes regulated in a circadian manner. For instance, the interaction of CCA1 with regulators of light signaling such as HY5, FAR1, FHY3 (Andronis, et al. 2008, Li, et al. 2011) might serve as an entry point for the numerous processes that are regulated by both light and the clock.

6. The anthocyanin related targets of RVE8 are also regulated by LNKs

We next interrogated previously published RNA-seq datasets of *lnk1/lnk2* double mutant plants (dm) (Rugnone, et al. 2013). Comparisons of RVE8-ox and dm RNA-seq experiments under constant light and temperature conditions revealed that among the overlapping genes in both datasets (154) (Figure 17A-B), about 72% of the up-regulated genes in RVE8-ox plants were down-regulated in dm plants (Figure 17C; 17G) whereas only about 9% of the overlapping genes up-regulated in RVE8-ox plants were also up-regulated in the dm (Figure 17D). Similar low percentages were obtained when down-regulated genes in RVE8-ox RNA-seq dataset were compared with up- or down-regulated genes in dm plants (Figure 17E-F). These results suggest that without light or temperature entrainment, RVE8 and LNKs might co-activate a subset of their target genes, as previously suggested (Xie, et al. 2014).

When we focused on the up-regulated anthocyanin genes in the RVE8 RNA-seq dataset, we found that nearly all of them were significantly down-regulated in dm plants (Figure 18). Intriguingly, RNA-seq analysis with plants grown under LgD conditions (Rugnone, et al. 2013) showed that many of the anthocyanin-related genes were not down-regulated but highly up-regulated in the dm plants (figure 19A-B). RT-Q-PCR analysis of dm plants grown under LD cycles confirmed a clear up-regulation particularly during the day (figure 19C-D).

These intriguing results are consistent with the observed different waveforms of the anthocyanin genes under LD and LL cycles (Figure 10) and suggest that timing by the clock and/or the external environmental conditions are important for LNK function in the anthocyanin pathway.

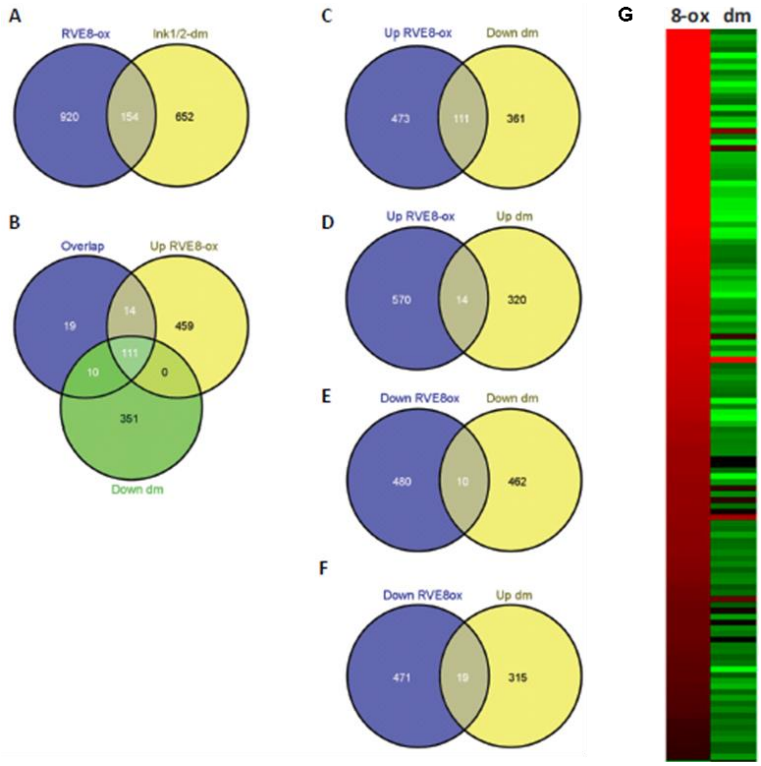


Figure 17. Overlapping target genes in RVE8-ox and dm plants. Venn diagrams depicting the number of overlapping genes from the RVE8-ox RNA-Seq assays (this study) and the dm RNA-Seq dataset (3). (A) Analysis of all miss-regulated genes. Analysis of the 154 overlapping genes (B) and the 111 genes up-regulated in RVE8-ox and down-regulated in dm (C). Comparisons of up-regulated datasets (D), down-regulated (E) and genes down-regulated in RVE8-ox and up-regulated in dm (F). The RNA-Seq assays were performed in both cases in plants grown under constant light and temperature without entrainment to ensure that the identified targets are not resulting from changes in circadian phases. (G) Heatmap comparing the up-regulated transcripts in RVE8-ox (8-ox) and the corresponding expression in dm plants. Red indicates high expression and green, low expression.

RVE8-ox	
Gene ID	Gene Symbol
AT1G30530	UDP-GLUCOSYL TRANSFERASE 78D1 (UGT78D1)
AT1G56650	PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1)
AT1G65060	4-COUMARATE:COA LIGASE 3 (4CL3)
AT2G36790	UDP-GLUCOSYL TRANSFERASE 73C6 (UGT73C6)
AT3G29590	(AT5MAT)
AT3G53260	PHENYLALANINE AMMONIA-LYASE 2 (PAL2)
AT3G55120	TRANSPARENT TESTA 5 (TT5)
AT4G09820	TRANSPARENT TESTA 8 (TT8)
AT4G14090	
AT4G22880	LEUCOANTHOCYANIDIN DIOXYGENASE (LDOX)
AT4G34135	UDP-GLUCOSYLTRANSFERASE 73B2 (UGT73B2)
AT5G05270	CHALCONE ISOMERASE LIKE (CHIL)
AT5G07990	TRANSPARENT TESTA 7 (TT7)
AT5G13930	TRANSPARENT TESTA 4 (TT4)
AT5G17030	UDP-GLUCOSYL TRANSFERASE 78D3 (UGT78D3)
AT5G17050	UDP-GLUCOSYL TRANSFERASE 78D2 (UGT78D2)
AT5G17220	GLUTATHIONE S-TRANSFERASE PHI 12 (GSTF12)
AT5G42800	DIHYDROFLAVONOL 4-REDUCTASE (DFR)
AT5G54060	UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRANSFERASE (UF3GT)

dm	
Gene ID	Gene Symbol
AT1G30530	UDP-GLUCOSYL TRANSFERASE 78D1 (UGT78D1)
AT1G56650	PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1)
AT1G65060	4-COUMARATE:COA LIGASE 3 (4CL3)
AT3G29590	(AT5MAT)
AT3G53260	PHENYLALANINE AMMONIA-LYASE 2 (PAL2)
AT3G55120	TRANSPARENT TESTA 5 (TT5)
AT4G09820	TRANSPARENT TESTA 8 (TT8)
AT4G14090	
AT4G22880	LEUCOANTHOCYANIDIN DIOXYGENASE (LDOX)
AT5G05270	CHALCONE ISOMERASE LIKE (CHIL)
AT5G07990	TRANSPARENT TESTA 7 (TT7)
AT5G13930	TRANSPARENT TESTA 4 (TT4)
AT5G17050	UDP-GLUCOSYL TRANSFERASE 78D2 (UGT78D2)
AT5G17220	GLUTATHIONE S-TRANSFERASE PHI 12 (GSTF12)
AT5G42800	DIHYDROFLAVONOL 4-REDUCTASE (DFR)
AT5G54060	UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRANSFERASE (UF3GT)

Figure 18. The anthocyanin pathway is miss-regulated in both RVE8-ox and dm plants. List of relevant components of the anthocyanin pathway that are mis-regulated in the RVE8-ox RNA-Seq assays (this study) and the dm RNA-Seq dataset (Rugnone, et al. 2013). The RNA-Seq were performed in both cases with plants grown under constant light and temperature without entrainment.

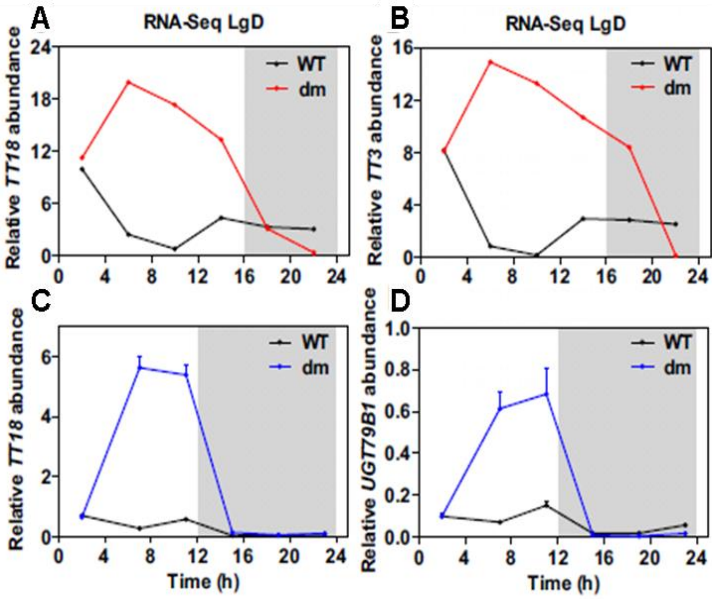


Figure 19. LNK proteins regulate anthocyanin gene expression. Time course analysis of *TT18* (A) and *TT3* (B) transcriptional profiles from RNA-seq data of dm plants under LgD (Rugnone, et al. 2013). Time course analysis by RT-Q-PCR of *TT18* (C) and *UGT79B1* (D) in WT and dm plants grown under LD cycles. mRNA abundance was normalized to *IPP2* expression. Values represent means + SEM. White, day; gray, night.

Based on our results, it would be interesting to examine the role of other members of the RVE family in the regulation of anthocyanin gene expression. Likewise, the fact that CCA1 and LHY are very similar in sequence to RVE8 (Carré and Kim 2002) and due to the physical interaction between LNK1 and LNK2 with CCA1 and LHY (Xie, et al. 2014), it is plausible that CCA1 and LHY might play also a role regulating anthocyanin gene expression. However, based on the differences between CCA1 and RVE8 function, it is also possible that the CCA1/LHY interaction with LNKs is only relevant for core circadian gene expression and that anthocyanin regulation is more exclusive of LNKs and RVEs. It would also very interesting to characterize

lnk3, lnk4 single mutants and *lnk3/lnk4* double mutant plants, to check similarities and differences with *lnk1*, *lnk2* single mutants and *lnk1/lnk2* double mutants phenotypes. The generation of the quadruple mutant *lnk1/lnk2/lnk3/lnk4* would also allow us to decipher the processes in which the LNKs are indispensable.

7. The phase-specific binding of RVE8 to the promoters of anthocyanin biosynthetic genes is antagonized by LNKs

RVE8 is able to directly bind to the promoters of its target circadian genes (Farinas and Mas 2011, Rawat, et al. 2011, Xie, et al. 2014). Therefore, we next investigated whether RVE8 binds *in vivo* to the promoters of the anthocyanin biosynthetic genes. First, we performed ChIP assays with RVE8-ox plants and examined by QPCR the promoter amplification. We found a significant enrichment of the *TT18, UGT79B1* and *TT4* promoters and a lower amplification of other anthocyanin-related gene promoters (Figure 20). The binding appeared to be specific as we obtained lower amplification when samples were similarly processed but without antibody or when a promoter of an unrelated gene was used as a negative control (Figure 20).

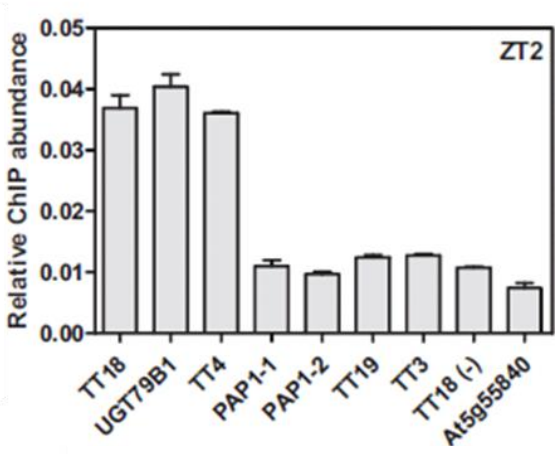


Figure 20. Binding of RVE8 to the promoters of the anthocyanin-related genes. ChIP assays using RVE8-ox plants grown under LD cycles and sampled at ZT2. The promoters of the indicated genes were amplified by QPCR. Samples similarly processed but in the absence of antibody [TT18(−)] were used as control. The promoter of an unrelated gene (At5g55840) was used as a negative control.

Our results also revealed that the declining mRNA accumulation from ZT2 to ZT7 (Figure 11) was accompanied by a concomitant decrease in RVE8 binding to the promoters of the *TT18*, *UGT79B1*, and *TT4* genes (Figure 21A-C). Remarkably, the decreased binding at ZT7 was specific for the anthocyanin-related genes and not for other previously described RVE8

circadian targets such as *TOC1* (Figure 21A) or *PRR5* (Figure 21C). ChIP analysis at ZT11 also revealed the absence or reduced RVE8 binding to the anthocyanin-related gene promoters but not to the *TOC1* promoter (Figure 21D). These results suggest a different mechanism in the regulation of anthocyanin-related genes and the evening-expressed clock genes.

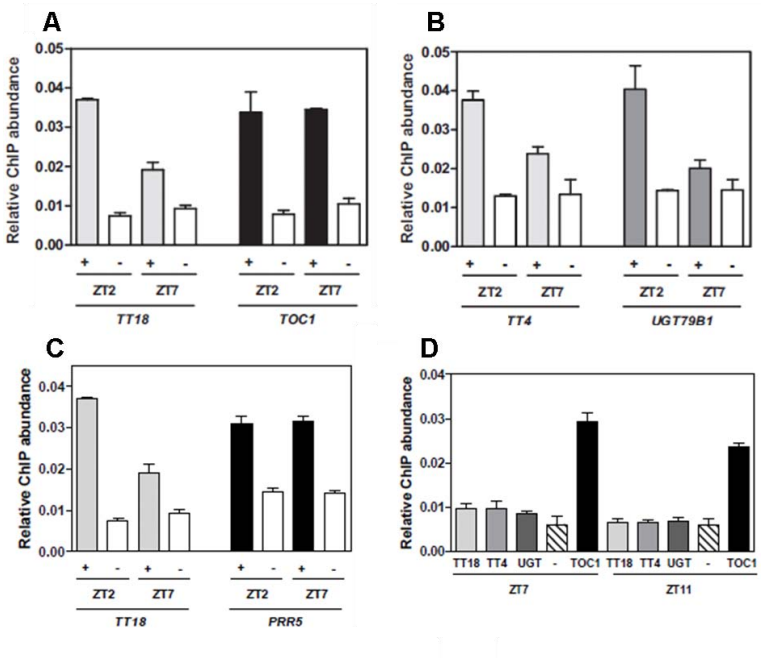


Figure 21. Differential binding of RVE8 to the promoters of its anthocyanin-related target genes depending of the Zeitgeber Time. ChIP-QPCR comparing RVE8 binding at ZT2 and ZT7 to the promoters of (A) *TT18* and *TOC1* and (B) *TT4* and *UGT79B1*. (C) ChIP-QPCR analysis comparing RVE8 binding at ZT2 and ZT7 to the promoters of *TT18* and *PRR5.* (D) ChIP-QPCR analysis comparing RVE8 binding at ZT7 and ZT11 to the promoters of *TT18, TT4, UGT79B1* (*UGT)* and *TOC1*. Values are represented as means + SEM.

Our results show that RVE8 not only regulate core evening-expressed genes but also anthocyanin-related genes expressed in the morning. Binding to morning and evening gene promoters might occur at different motifs, as some of the anthocyanin-related genes lack the EE motif. It would be interesting to identify the cis-acting elements responsible for RVE8 binding to the promoters of the anthocyanin biosynthetic genes. Similarly to RVE8, CCA1 and LHY also bind to morning- and evening-expressed genes. However, they act as activators of the morning genes (e.g. *PRR7* and *PRR9*) and repressors of evening genes (e.g. *TOC1*). In contrast, RVE8 activates both the morning and evening-expressed genes.

We next examined whether RVE8 binding was altered in plants miss-expressing LNKs. First, we compared binding in RVE8-ox and in RVE8-ox/dm plants using sets of lines that expressed comparable amounts of RVE8 (Figure 22A). We found that RVE8 binding to the anthocyanin gene promoters was significantly enriched in the absence of functional LNK1 and

LNK2 (Figure 22B-C), whereas the opposite effect was observed for binding to the *TOC1* promoter (Figure 22B). These results are in agreement with data showing that RVE8, LNK1, and LNK2 act together as co-transcriptional activators of *PRR5* and *TOC1* expression. The results are also in line with the notion that anthocyanin and circadian gene expression are oppositely modulated by the RVE8–LNK interaction. Notably, the increased RVE8 binding in RVE8-ox/dm plants was phase specific, as no significant differences in binding were observed when the ChIP assays were performed at ZT2 (Figure 22D).

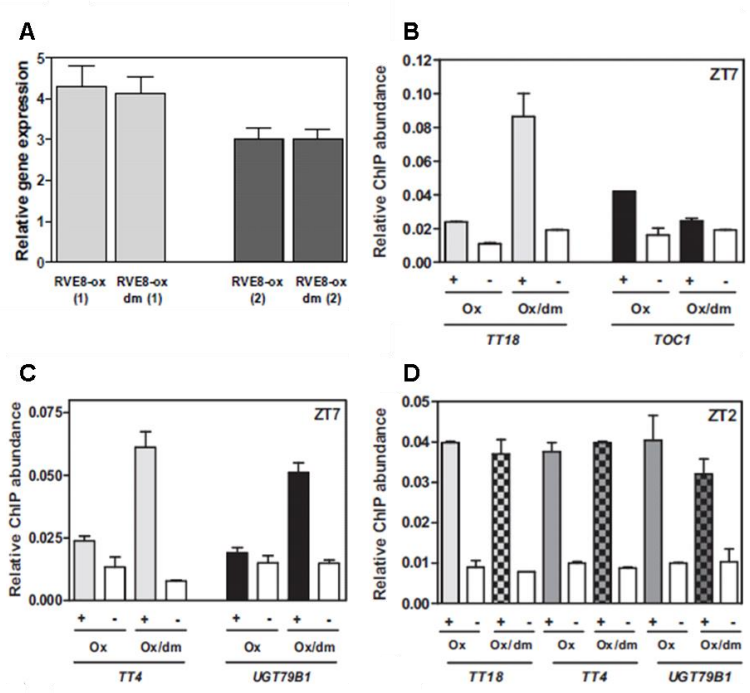


Figure 22. Increased binding of RVE8 to the promoters of its anthocyanin-related target genes in the absent of functional LNK1 and LNK2. (A) Two sets (1 and 2) of RVE8- ox and RVE8-ox/dm lines (analyzed at ZT7) expressing similar amounts of *RVE8* were used for ChIP assays. Comparison of RVE8 binding by ChIP-QPCR using RVE8-ox (Ox) and RVE8-ox/dm (Ox/dm) plants sampled at ZT7. The promoters of (B) *TT18* and *TOC1* and (C) TT4 and *UGT79B1* were amplified. (D) ChIP-QPCR using RVE8-ox (Ox) and RVE8-ox/dm (Ox/dm) plants sampled at ZT2. Values are represented as means + SEM.

Our results suggest that the phase-specific interference of LNKs on RVE8 binding might be responsible for the decreased anthocyanin gene expression around midday. If our conclusions are correct, then RVE8 binding should be affected by LNK over-expression. Indeed, ChIP analysis with RVE8-ox and double RVE8-ox/LNK1-ox plants showed that RVE8 binding was abolished in the double over-expressing lines, specifically at ZT7 but not at ZT2 (Figure 23A-B). However, again, the effect was not observed at the *TOC1* and *PRR5* promoters (Figure 23A; 23D). ChIP analysis of RVE8-ox/LNK3-ox plants rendered similar results (Figure 23C-D).

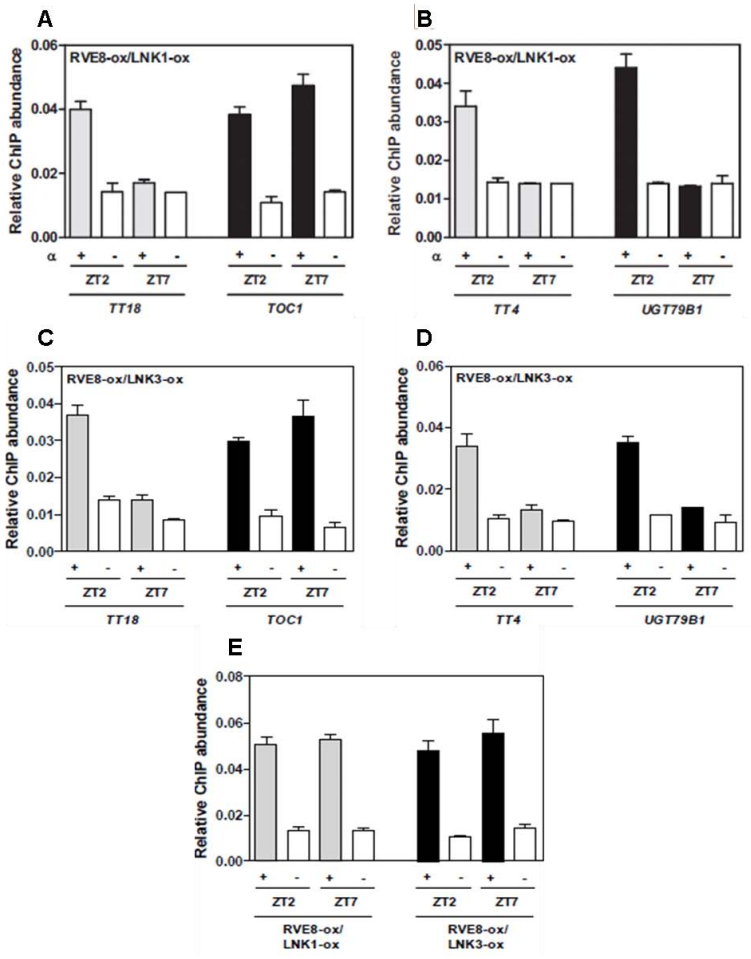


Figure 23. Decreased binding of RVE8 to the promoters of its anthocyanin-related target genes by over-expression of LNKs. (A and B) Comparison of RVE8 binding by ChIP-Q-PCR assays using RVE8-ox/LNK1-ox plants sampled at ZT2 and ZT7. The promoters of (A) *TT18* and *TOC1*, and (B) *TT4* and *UGT79B1* were amplified. ChIP-QPCR analysis of RVE8-ox/LNK3-ox plants sampled at ZT2 and ZT7. Specific regions at the promoters of (C) *TT18* and *TOC1,* and (D) *TT4* and *UGT79B1* were amplified. (E) Comparison of RVE8 binding to the *PRR5* promoter by ChIP-QPCR assays using RVE8-ox/LNK1-ox and RVE8-ox/LNK3-ox plants sampled at ZT2 and ZT7. Values are represented as means + SEM.

Activation of *TOC1* and *PRR5* expression by RVE8 is reduced in the absence of a functional LNK1 and LNK2 and conversely, the activating function of LNK1 and LNK2 is decreased in *rve4* or *rve8* mutant backgrounds. These and other findings demonstrate that RVE8 and LNKs act as co-transcriptional activators in the regulation of *PRR5* and *TOC1* expression (Xie, et al. 2014). Notably, our results indicate that the time-specific interaction of LNKs with RVE8 antagonizes RVE8 positive effect on anthocyanin gene expression in the afternoon. Around dawn, RVE8 binds to the anthocyanin biosynthetic gene promoters

regardless the presence of LNKs. However, in the afternoon, there is a reduction of RVE8 binding that is concomitant with its interaction with LNKs. Studies with the different *LNK* and *RVE8* over-expressing and mutant plants further support this notion, demonstrating that that LNKs interfere with the activating function of RVE8 in the control of anthocyanin gene expression. It is possible that LNK interaction with RVE8 and possibly with other factors increases the affinity of the complex for the EE motifs and thus favoring the binding of the complex to the promoters of the core clock genes. The results showing that over-expression of LNK1 and LNK2 does not render a circadian phenotype (Xie, et al. 2014) but leads to anthocyanin phenotypes also highlight fundamental differences in their regulatory functions. It would be very interesting to fully investigate the mechanism behind the RVE8-LNK pivotal regulation of anthocyanin biosynthesis and core-clock gene expression. The switch between activation to repression is not exclusive for LNKs-RVE8 but it is found in other components such as CCA1 and LHY (Salomé, et al. 2010).

8. RVE8-LNK function in the regulation of anthocyanin accumulation

To dissect the physiological relevance of RVE8–LNK interaction, we measured anthocyanin content in the different genetic backgrounds. As shown in Figure 24A, increased anthocyanin accumulation was observed in RVE8-ox plants, whereas the anthocyanin content was even higher in dm plants. These results are consistent with the transcriptional changes observed in these plants and with the positive role for RVE8 and the negative function of LNK1 and LNK2 in the control of the anthocyanin pathway. Our studies also showed an increased accumulation of anthocyanin in RVE8-ox/dm compared with RVE8-ox (Figure 24A). The anthocyanin content correlated with the up-regulation of the anthocyanin-related genes, particularly around the mid-to-late day (Figure 24B-D). The RVE8-ox phenotypes were not due to decreased *LNK* gene expression in RVE8-ox plants (Figure 25).

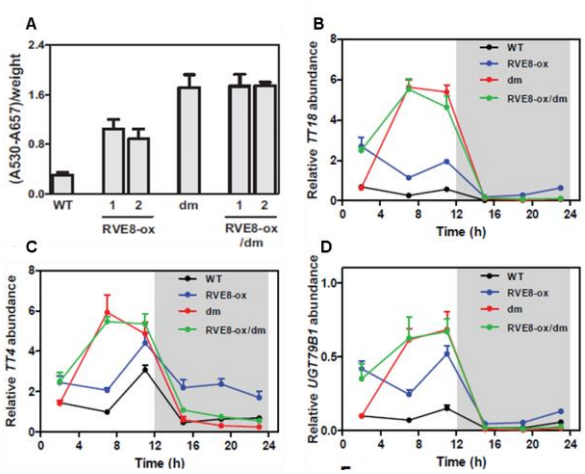


Figure 24. Anthocyanin content in RVE8-ox and RVE8-ox/dm plants correlates with the transcriptional regulation of anthocyanin genes. (A) Anthocyanin content in WT, dm, in two different lines of RVE8-ox and RVE8-ox/dm. Time course analysis by RT-QPCR of *TT18* (B), *TT4* (C) and *UGT79B1* (D) in WT, RVE8-ox, dm and RVE8-ox/dm. Plants were grown under LD cycles. mRNA abundance was normalized to *IPP2* expression. Values represent means + SEM. White shading: day; gray shading: night.

We next reasoned that anthocyanin content in double over-expressing plants should revert the RVE8-ox phenotype. Indeed, single LNK and double RVE8–LNK over-expression led to a significant reduction in anthocyanin content (Figure 26). Consistently, analysis of LNK-ox/RVE8-ox plants revealed a down-regulation of anthocyanin gene expression, particularly evident around ZT7 (Figure 27). Comparisons of anthocyanin gene expression in LNK-ox plants in the presence or absence of RVE8-ox showed that over-expression of RVE8 in LNK-ox plants led to increased expression particularly around ZT2, although the overall expression was still lower than in WT plants (Figure 28).

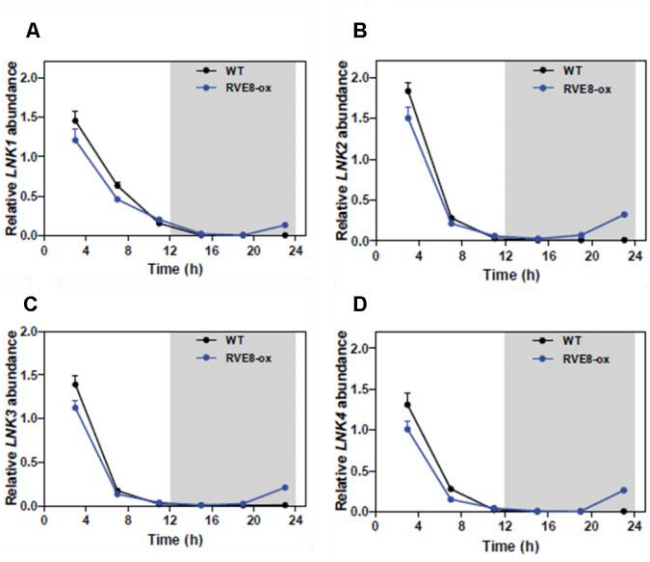


Figure 25. Expression pattern of *LNK* genes in WT and RVE8-ox backgrounds. Time course analysis by RT-QPCR of *LNK1* (B), *LNK2* (C), *LNK3* (D) and *LNK4* (E) in WT plants and in RVE8-ox plants under LD cycles. Plants were grown under LD cycles. mRNA abundance was normalized to *IPP2* expression. Values represent means + SEM. White shading: day; gray shading: night.

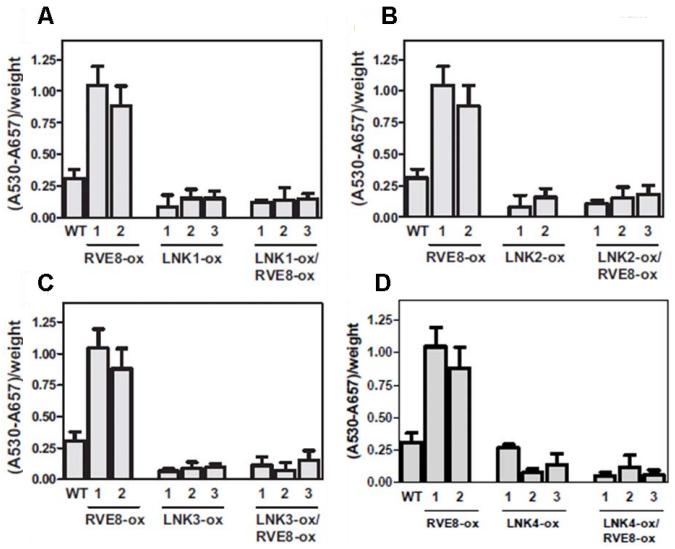


Figure 26. Analysis of anthocyanin content in single and double over-expressors of *RVE8* and *LNK*. Anthocyanin content in (A) single LNK1-ox and double over-expressing (RVE8-ox/LNK1-ox) plants, (B) single LNK2- ox and double over-expressing (RVE8-ox/LNK2-ox) plants, (C) single LNK3-ox and double over-expressing (RVE8-ox/LNK3-ox) plants and (D) single LNK4-ox and double over-expressing (RVE8-ox/LNK4-ox) plants.

Altogether, our results are consistent with an activating function of RVE8 by direct binding to the promoters on anthocyanin genes that is antagonized by the repressing activity of LNKs on anthocyanin accumulation. Different factors and mechanisms might be involved in the regulation of a clock output such as anthocyanin accumulation versus regulation of core

clock gene expression. The different factors might influence the regulatory activity of RVE8 and LNKs. The fact that activation of *TOC1* by RVE8 occurs later during the day compared with the earlier activation of anthocyanin gene expression by RVE8 might be responsible for a timely regulated set of different activities. The results showing that over-expression of LNK1 and LNK2 does not render a circadian phenotype (Xie, et al. 2014) but leads to anthocyanin phenotypes also highlight fundamental differences in the regulatory functions.

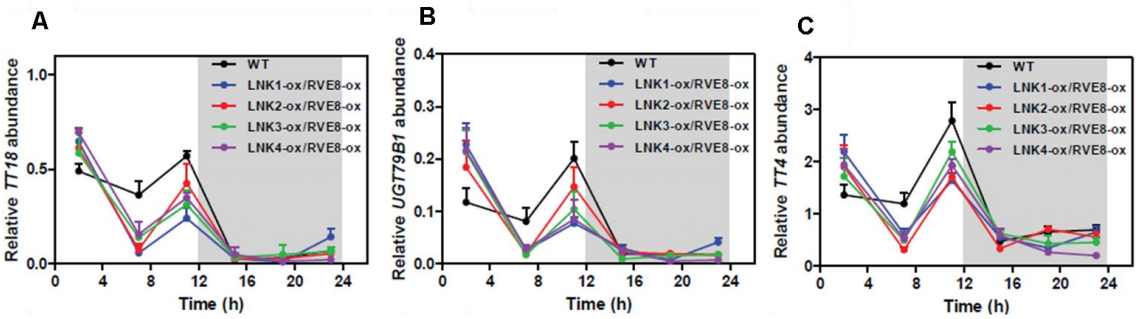


Figure 27. Analysis of transcriptional regulation of anthocyanin genes in double over-expressors of RVE8 and LNK. Time course analysis by RT-QPCR of *TT18* (A), UGT79B1 (B) and *TT4* (C) in LNK-ox/ RVE8-ox. Plants were grown under LD cycles. mRNA abundance was normalized to *IPP2* expression. Values represent means + SEM. White shading: day; gray shading: night.

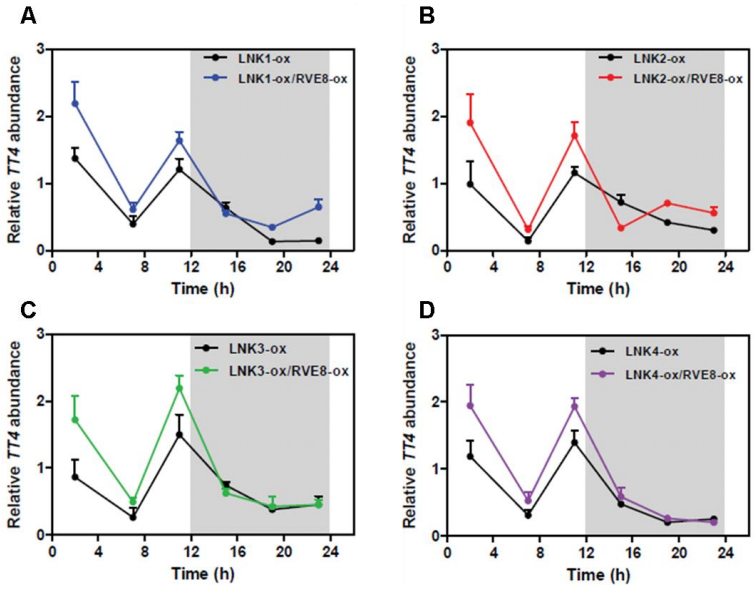


Figure 28. Up-regulation of anthocyanin genes expression in RVE8-ox plants is more relevant at ZT2. Comparison of *TT4* gene expression in single LNK-ox versus double LNK-ox/ RVE8-ox plants (A-D). Plants were grown under LD cycles. mRNA abundance was normalized to *IPP2* expression. Values represent means + SEM. White shading: day; gray shading: night.

Alteration of anthocyanin accumulation in plants miss-expressing RVE8 and/or LNKs might influence downstream plant responses. For instance, anthocyanins act as sunscreen that reduces light impact on photosynthetic tissues and dissipate energy excess that is harmful for the cell (Smillie and Hetherington 1999, Weger *et al.* 1993). Energy capture occurs at a much faster rate than electron transport and dissipation, hence over-excitation of the photosynthetic apparatus is constant. Over-excitation manifests itself as a repression of photosynthesis, a phenomenon called photoinhibition (Long *et al.* 1994). It would be interesting to check whether RVE8-ox plants that accumulate more anthocyanins also have increased protection against high irradiance light. Similarly, CCA1 and LHY have been shown to be related to defense mechanisms in plants. The high accumulation of anthocyanins in RVE8-ox plants might also provide increase resistance against pathogen infection. Lastly, ecological studies with RVE8-ox plants monitoring the behavior of pollinators could lead to interesting findings.

9. RVE8-LNK interaction shapes the diurnal oscillation of anthocyanin gene expression under different photoperiodic conditions

Gene expression analysis under different photoperiodic conditions provided some clues about the physiological relevance of RVE8–LNK interaction (Figure 29). Indeed, a recurrent pattern was observed consisting of (1) a clear up-regulation and a peak of expression about 4 h after dawn that is facilitated by RVE8 activating function; (2) a down-regulation around midday, favored by LNK repressing activity that is followed by a second peak of expression under longer photoperiods; and (3) a subsequent declining phase that coincides in all cases with the dark period. Notably, the down-regulation was completely abolished under LL conditions, which demonstrates the inductive role of light during the night period (Figure 29).

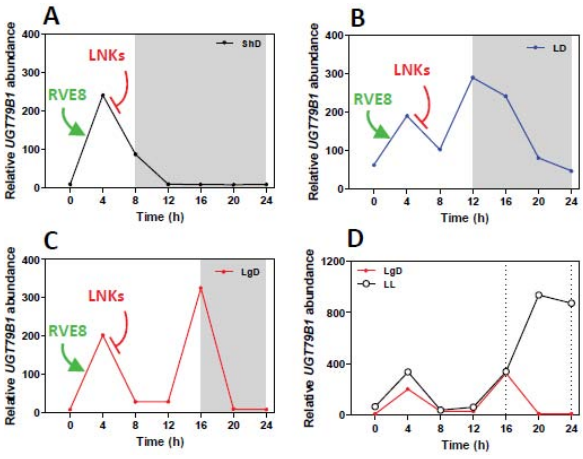


Figure 29. Photoperiodic regulation of *UGT79B1* expression. Time course analysis of *UGT79B1* expression under (A) Short-days (Shd), (B) LD (12h light:12h dark) cycles and (C) Long-days (LgD). (D) Comparison of the oscillatory waveforms under LgD and constant light (LL). Expression data was obtained from the publicly available web tool *DIURNAL*. The activating function by RVE8 (green arrow) and the repressing activity by LNKs (red line ending in a perpendicular line) are also depicted. White shading: day; gray shading: night. The dotted lines in (D) delimit the beginning and end of the dark period under LgD.

10. A model of RVE8-LNK function in the control of anthocyanin and clock gene expression

Our results suggest that around dawn, RVE8 up-regulates anthocyanin gene expression by directly associating to the promoters of a subset of anthocyanin biosynthetic genes. The up-regulation is overcome at midday by the repressing activity of LNK proteins, as inferred by the increased anthocyanin gene expression in *lnk1/lnk2* double mutant plants. Chromatin immunoprecipitation assays using LNK and RVE8 miss-expressing plants show that RVE8 binding to target promoters is precluded in LNK over-expressing plants and conversely, binding is enhanced in the absence of functional LNKs, which provides a mechanism by which LNKs antagonize RVE8 function in the regulation of anthocyanin accumulation. Based on their previously described transcriptional co-activating function, our study defines a switch in the regulatory activity of RVE8-LNK interaction, from a synergic co-activating role of evening-expressed clock genes to a repressive antagonistic function modulating anthocyanin biosynthesis around midday (Figure 30).

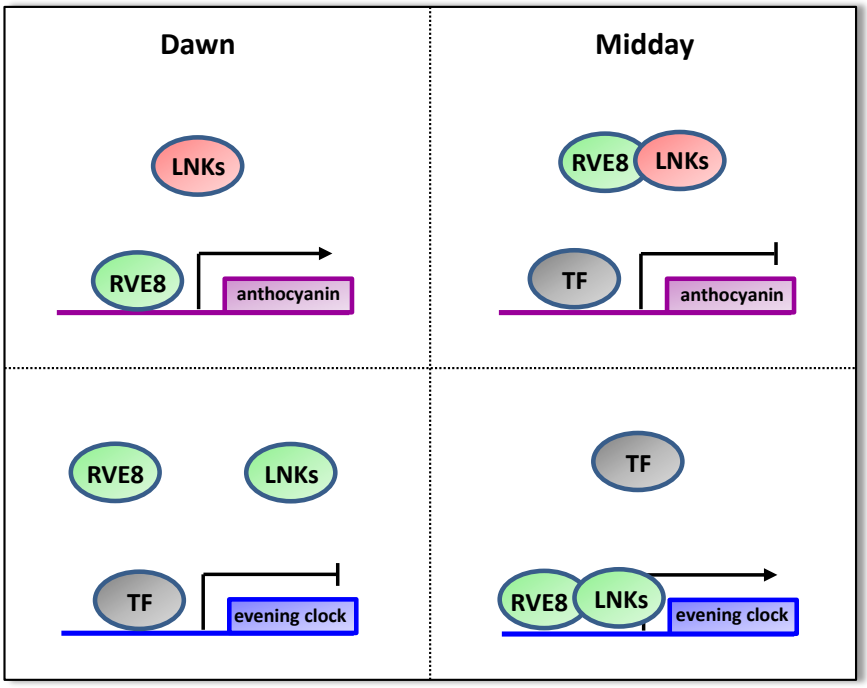


Figure 30. . Graphical representation of the functional relevance of the time-dependent sequestration of RVE8 by LNK proteins. The upper panels represent regulation of anthocyanin-related gene expression (purple line and rectangle) at dawn and midday (left and right, respectively). At midday, unknown transcription factors (TF) (grey oval) might account for the repression. Lower left panel, an evening-expressed clock gene (blue line and rectangle) (e.g. *TOC1*) is repressed at dawn by a TF (e.g. CCA1) while the interaction of RVE8 with LNKs at midday allows its activation. Green color denotes proteins with activating function while red color represents proteins with repressing functions.

Conclusions

Conclusions

1. Nearly all the genes involved in the anthocyanin biosynthetic pathway are up-regulated in RVE8-ox, which suggests **a major role for RVE8 in the control of the anthocyanin biosynthesis**. The expression of most of these genes is controlled by the clock, with a rhythmic oscillatory pattern peaking around dawn. The peak phase of expression for the anthocyanin-related genes changes under light/dark cycles, with waveforms showing a double peak with a clear decrease around midday.

2. Transcript abundance of the genes involved in **anthocyanin biosynthesis is significantly increased in RVE8-ox plants particularly during daytime**, whereas no significant differences in gene expression are observed during the night period. The decreased expression around midday is still evident in RVE8-ox plants, suggesting **a complex mechanism of regulation that is able to overcome the activating function of RVE8 over-expression.**

3. The **LNK proteins directly interact with RVE8** as inferred by yeast-two hybrid assays and co-immunoprecipitation analyses in plants. Despite the over-expression, the interaction is not constant throughout the day/night cycle but **it is gated by the clock to specifically occur around midday**.

4. The **LNKs and RVE8 function in the control of anthocyanin regulation is opposed to their role exerted on circadian core gene expression**. Although RVE8 and LNKs co-activate a subset of their clock-related target genes, RVE8 acts as an activator, whereas LNKs are repressors of the anthocyanin biosynthetic pathway.

5. RVE8 binds *in vivo* to the promoters of the anthocyanin biosynthetic genes but the interaction with LNKs at midday sequesters RVE8 from these promoters, which results in down-regulation of the anthocyanin-related target genes. These conclusions are evident when comparing anthocyanin gene expression and RVE8 binding in the absence of functional LNKs or in the presence of over-expressing LNKs.

6. The activating function of RVE8 by direct binding to the promoters of the anthocyanin genes is antagonized by the repressing activity of LNKs, and **this complex pattern of regulation perfectly fits with the anthocyanin accumulation on the different genetic backgrounds examined**.

7. A recurrent pattern in anthocyanin gene expression is observed and consists of (*1*) a clear **up-regulation and a peak of expression about 4-hours after dawn that is facilitated by RVE8** activating function; (*2*) a **down-regulation around midday, favored by LNK repressing activity** that is followed by **a second peak of expression under longer photoperiods**; and (*3*) a subsequent **declining phase that coincides in all cases with the dark period**. The down-regulation is completely abolished under LL conditions, which demonstrates the inductive role of light during the night period.

8. We conclude that **anthocyanin content is modulated by the phase-dependent interaction of RVE8** (and most likely other RVEs) **with LNKs.** The interaction defines **the timing of RVE8 binding to the promoters of the anthocyanin structural genes and thus in consonance with the photoperiodic conditions, plants might precisely control anthocyanin accumulation.**

Resumen en Castellano

Resumen en Castellano

El reloj circadiano es un mecanismo celular endógeno presente en prácticamente todos los organismos. Una función clave del reloj es la sincronización del metabolismo, fisiología y desarrollo con los cambios medioambientales diurnos y estacionales generados por la rotación de la tierra sobre su propio eje. Se ha propuesto que las oscilaciones circadianas proporcionan una ventaja adaptativa al permitir que los organismos anticipen las transiciones durante el ciclo diurno/nocturno y coordinen procesos simultáneos, secuenciales o temporalmente incompatibles. En los últimos años, numerosos estudios bioquímicos, moleculares y genéticos han proporcionado una visión cada vez más completa de la función y organización circadiana en plantas. Los ritmos circadianos se generan en primera instancia mediante las regulaciones recíprocas entre componentes centrales del reloj que producen una ritmicidad en expresión génica, procesamiento de mRNA, abundancia de proteína y actividad. A pesar de estos avances, aún se dispone de muy poca información sobre los componentes y mecanismos que conectan las señales medioambientales con las rutas de salida del reloj, y en concreto, con los ritmos del metabolismo celular en plantas. El trabajo realizado durante esta Tesis Doctoral se ha centrado en el estudio del papel de los componentes circadianos REVEILLE 8 (RVE8) y NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED (LNKs) en la regulación de la oscilación rítmica de la biosíntesis de antocianinas a lo largo del día. Al amanecer, RVE8 activa la expresión de genes de la ruta de síntesis de antocianinas mediante la unión directa a los promotores de algunos de los genes de esta ruta metabólica. La regulación positiva de RVE8 es antagonizada hacia la mitad del día por la acción represora de las proteínas LNK, tal y como se deduce del dramático incremento en la expresión de genes de antocianinas en plantas dobles mutantes *lnk1/lnk2*. Mediante técnicas de inmunoprecipitación de cromatina usando plantas con la expresión de RVE8 y LNKs alterada, se observa que la unión de RVE8 a los promotores disminuye en presencia de los LNK y aumenta en su ausencia, lo que pone de manifiesto un mecanismo mediante el cual las proteínas LNK antagonizan la función activadora de RVE8 sobre los genes implicados en la biosíntesis de antocianinas. Dado que RVE8 y LNKs han sido descritos como co-activadores transcripcionales de genes del reloj, nuestro estudio define un cambio en la actividad reguladora de la interacción RVE8-LNK, desde una función sinérgica activadora de genes de reloj que se expresan por la tarde, a una función represora que modula la expresión de genes implicados en la biosíntesis de antocianinas a mitad del día.

Materials and Methods

Materials and Methods

1. Plant material, sterilization and transformation

Arabidopsis thaliana (Columbia, Col-0) plants were used in our studies. Seeds were vapor-phase sterilized using 35ml of bleach and 1,5ml of concentrated HCl. Sterilization by the chlorine fumes was performed for 6-12 hours. Seeds were plated on Murashige and Skoog (MS) medium supplemented with 30g/l of sucrose, 5mM MES/KOH, pH 5.7 and the appropriate antibiotics for selection. Seeds were vernalized at 4ºC for 72 hours and subsequently transferred to chambers with environmentally controlled conditions (INKOA S.L.). Unless otherwise indicated, seedlings were grown under LD conditions (12h light:12h dark) with 60-100 µmol $m^{-2}s^{-1}$ of cool white fluorescent light at 22°C.

Generation of transgenic plants was performed following the floral dip protocol (Clought, 1998). The *CCA1::LUC* (Salomé and McClung 2005b) and *TOC1::LUC* (Perales and Más 2007) reporter lines were used for most studies. The RVE8-ox, *rve8* mutant lines and the *lnk1/lnk2* double mutant plants were previously described (Farinas and Mas 2011, Rugnone, et al. 2013). These plants were transformed with the LNKs-ox constructs to generate single LNK-ox plants (LNK1-ox, LNK2-ox, LNK3-ox and LNK4-ox) and double LNK-ox/RVE8-ox plants (LNK1-ox/RVE8-ox, LNK2-ox/RVE8-ox, LNK3-ox/RVE8-ox, LNK4-ox/RVE8-ox) in the *CCA1::LUC* and *TOC1::LUC* background (Table 1). *Agrobacterium tumefaciens* (strain GV2660) transformed with the constructs of interest was used for plant transformation (Logemann *et al.* 2006). Bacteria were plated for 48 hours at 28ºC (until OD_{600} was about 2.0) in 30 ml of sterile YEB medium (5 g/L beef extrac, 1 g/L yeast extract, 5 g/L pepton, 5 g/L sucrose, 0.5 g/L MgCl2) supplemented with antibiotics (ampicillin 100 µg/ml, rifampicin 100 µg/ml and spectinomycin 100 µg/ml). The bacteria solution was added to 5% sucrose solution containing 0.03% of Silwet L-77. *Arabidopsis* inflorescences were dipped into the Agrobacterium solution for 10-30 seconds under gentle agitation. Plants were placed under a lid cover for 24 hours to ensure high humidity.

Table 1. Plants used in this study

Name	Reporter	Background	Resistance reporter	Resistance construct
WT	*TOC1::LUC*	Col0	GENT	
WT	*CCA1::LUC*	Col0	PPT	
35S:YFP-RVE8	*CCA1:LUC*	Col0	PPT	KAN
35S:YFP-RVE8	*TOC1:LUC*	Col0	GENT	KAN
lnk1/lnk2	-	Col0	-	KAN
35S:YFP-RVE8/*lnk1/lnk2*	*CCA1:LUC*	Col0	PPT	KAN
35S:YFP-RVE8/lnk1/lnk2	*TOC1:LUC*	Col0	GENT	KAN
35S:LNK1.1-MYC	*TOC1:LUC*	Col0	GENT	HYGR
35S:LNK1.1-MYC / YFP-RVE8	*TOC1:LUC*	Col0	GENT	HYGR, KAN
35S:LNK1.1-MYC	*CCA1:LUC*	Col0	PPT	HYGR
35S:LNK1.1-MYC / YFP-RVE8	*CCA1:LUC*	Col0	PPT	HYGR, KAN
35S:LNK2.1-MYC	*TOC1:LUC*	Col0	GENT	HYGR
35S:LNK2.1-MYC / YFP-RVE8	*TOC1:LUC*	Col0	GENT	HYGR, KAN
35S:LNK2.1-MYC	*CCA1:LUC*	Col0	PPT	HYGR
35S:LNK2.1-MYC / YFP-RVE8	*CCA1:LUC*	Col0	PPT	HYGR
35S:LNK3-MYC	*TOC1:LUC*	Col0	GENT	HYGR
35S:LNK3-MYC / YFP-RVE8	*TOC1:LUC*	Col0	GENT	HYGR, KAN
35S:LNK3-MYC	*CCA1:LUC*	Col0	PPT	HYGR
35S:LNK3-MYC / YFP-RVE8	*CCA1:LUC*	Col0	PPT	HYGR, KAN
35S:LNK4.1-MYC	*TOC1:LUC*	Col0	GENT	HYGR
35S:LNK4.1-MYC / YFP-RVE8	*TOC1:LUC*	Col0	GENT	HYGR, KAN
35S:LNK4.1-MYC	*CCA1:LUC*	Col0	PPT	HYGR
35S:LNK4.1-MYC / YFP-RVE8	*CCA1:LUC*	Col0	PPT	HYGR, KAN

2. Molecular cloning

The over-expressing constructs of LNK1, 2 3 and 4 were generated by PCR amplification of each of the *LNK* coding sequences (CDS). The PCR fragments were cloned into the pENTR/TOPO using the TOPO reaction (Gateway®) and following the manufacturer recommendations. For optimal results, 0.5:1–2:1 molar ratio of PCR products were added to the reaction. The resulting vector containing each of the *LNK* CDS was used to transform chemically competent *E. coli* (one shot TOP10 cells, Gateway®). The transformed bacteria were spread on selective plates containing 50 µg /ml kanamycin and incubated overnight at 37°C. Approximately 4 resistant colonies were selected for amplification and plasmid purification using the Plant Mini-Prep Kit (Qiagen). The absence of mutations was confirmed by sequencing using the M13 and F13 primers (Gateway®). The *LNK* CDS were introduced in the destiny vector pGWB517 (P_{35S}-*att*R1-Cm^r-*ccd*B-*att*R2-4xMyc-T_{NOS}; Hygromycin resistance) (Nakagawa *et al.* 2007a, Nakagawa *et al.* 2007b) by homologous recombination using the LR reaction (Gateway®) and following the manufacturer recommendations. For optimal results, 150 ng of entry clone and 150 ng of destination vector were added to the reaction. The resulting

expression vectors (containing the *LNK* CDS fused to 4X MYC in the C-terminal under the control of the 35S promoter) were used to transform chemically competent *E. coli* (one shot TOP10, Gateway®). Bacteria were spread on selective plates containing 100 µg /ml spectinomycin and incubated overnight at 37°C. Approximately 4 resistant colonies were selected for amplification and plasmid purification using the Plant Mini-Prep Kit (Quiagen). The expression vectors carrying the *LNK* CDS were introduced by electroporation into the *Agrobacterium tumefaciens* strain GV2660 as described above.

3. RNA-seq analysis

For the RNA-Seq experiments, plants were directly grown under LL conditions at 22ºC for fourteen days. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's recommendations. RNA sequencing was performed by BaseClear (Leiden, Netherlands). The FASTQ sequence reads were generated using the Illumina Casava pipeline version 1.8.3. Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing adapters and/or PhiX control signal were removed using an in-house filtering protocol. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0. Sequence analysis was performed by Sequentia Biotech (Barcelona, Spain). High-quality reads were aligned against the *Arabidopsis thaliana* reference genome sequence (TAIR10 Genome Release) with TopHat (version 2.0.9). The resulting alignment files were used as input for HTSeq (doi: 10.1101/002824) and the TAIR10 annotation file to calculate transcript expression values. All the statistical analyses were performed with R using the libraries: SERE (doi:10.1186/1471-2164-13-524), ArrayQualityMetrics (10.1093/bioinformatics/btn647), HTSFilter (10.1093/bioinformatics/btt350) and TCC (10.1186/1471-2105-14-219). The overall quality of the experiment was evaluated on the basis of the similarity between replicates by using several approaches. The algorithm SERE calculates similarity scores among samples assuming a binomial distribution of the read counts. The library ArrayQualityMetrics was used to perform a clustering analysis and a PCA analysis on the basis of the read counts in the different samples. HTSFilter was used to identify the minimum normalized read count to remove transcripts with very low and excessively variable expression across the samples. TMM normalization was used to normalize counts across the experiments, and exact test was used with an FDR threshold of 0.05. Transcripts with TMM normalized read counts < 64.126 in all the samples were removed. The final dataset was analysed with TCC to identify differentially expressed (DE) transcripts between the over-expressing line and the WT. The Integrative

Genomics Viewer (IGV) was used to visualize the data (Thorvaldsdóttir H, 2012; Robinson JT, 2011). Heatmaps were produced after standardization of the expression values by using MeV (http://www.tm4.org/mev.html). Genes were classified into broad functional categories (GO annotations) using the web-based tool of *The Arabidopsis Information Resource* (TAIR) (http://www.arabidopsis.org/index.jsp), which renders over-represented and significant functional terms as compared to the frequency in the whole genome. RVE8 co-expressed gene network was obtained using various publicly available resources (*GENEVESTIGATOR*; https://www.genevestigator.com/gv/), (*GENEMANIA*; http://www.genemania.org/) and *ATTED-II* (http://atted.jp/data/locus/820117.shtml). The waveforms of circadian expression under the different environmental conditions were analyzed using the web-based tool available in the *DIURNAL* database (http://diurnal.mocklerlab.org/) (Michael, 2008, Mocker 2007).

4. Gene expression analyses by RT-QPCR

Seedlings were synchronized under LD cycles for fourteen days and samples were taken every four hours over a diurnal cycle. RNA was purified using the Maxwell 16 LEV simply RNA Tissue kit (Promega). RNA was incubated with RNase-free TURBO DNase (Ambion) to reduce genomic DNA contamination. Single strand cDNA was synthesized using iScript™ Reverse Transcription Supermix for RT-Q-PCR (BioRad) following manufacturer recommendations. For QPCR analysis, cDNAs were diluted 5-fold with nuclease-free water and QPCR was performed with the iTaq Universal SYBR Green Supermix (BioRad) in a 96-well CFX96 Touch Real-Time PCR Detection System (BioRad). The *IPP2* gene (*ISOPENTENYL PYROPHOSPHATE: DIMETHYL-ALLYL PYROPHOSPHATE ISOMERASE*) was used as control (Huang, 2012). The amplification data were analyzed using the second derivative maximum method. Resulting Cp values were converted into relative expression values using the comparative Ct method. The list of primers used in this study is shown below in Table 2.

5. Yeast-two hybrid assays

For the yeast two-hybrid screening (Hybrigenics Services, S.A.S. Paris, France), the full-length coding sequence of *RVE8* was PCR-amplified and cloned into the pB27 vector as a C-terminal fusion to LexA (N-LexA-RVE8-C). The construct was checked by sequencing and used as a bait to screen a random-primed *Arabidopsis thaliana* seedlings cDNA library constructed into the pP6 vector. The pB27 and pP6 vectors derive from the original pBTM116 (Vojtek A, 1995) and

pGADGH plasmids, respectively. About 62 million clones (6-fold the complexity of the library) were screened using a mating approach with YHGX13 (Y187 ade2-101::loxP-kanMX-loxP, matα) and L40ΔGal4 (matα) yeast strains as previously described (Fromont, 1997). Around 370 His+ colonies were selected on a medium lacking tryptophan, leucine and histidine, and supplemented with 50 mM 3-aminotriazole to handle bait autoactivation. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher, 2005). The PBS relies on two different analyses. First, a local score takes into account the redundancy and independency of prey fragments as well as the distribution of the reading frames and stop codons in overlapping fragments. Second, a global score takes into account the interactions found in all the screens performed using the same library. This global score represents the probability of an interaction being nonspecific. For practical use, the scores were divided into four categories, from A (highest confidence) to D (lowest confidence). A fifth category (E) specifically flags interactions involving highly connected prey domains previously found several times in screens performed on libraries derived from the same organism. The PBS scores have been shown to positively correlate with the biological significance of interactions (Rain, 1997; Wojcik, 2002).

6. Protein extraction and co-immunoprecipitation by Western-blot

Fourteen day-old seedlings were grounded in liquid nitrogen and proteins extracted in 1 ml of co-immunoprecipitation buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl; 0.5% NP-40, 1 mM EDTA, 3 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 µg ml-1 leupeptin, 1 µg ml-1 aprotinin, 5 µg ml-1 antipain, 1 µg ml-1 pepstatin, 5 µg ml-1 chymostatin and 50 µM MG132). Protein extracts were centrifuged twice for 20 minutes at 4ºC and the supernatant was recovered. Protein concentration was calculated using the Bradford method (Bradford, 1976) and proteins were denatured in loading buffer (80 mM Tris-HCL pH 6.8; SDS 1,6%; DTT 0,1 M; Glycerol 5%; Bromophenol blue) at 100ºC for 10 min and immediately subjected to SDS-PAGE electrophoresis. To that end, 10% acrylamide (amresco(R)) gels were prepared for the detection of RVE8, LNK3 and LNK4 while 8% acrylamide gels were used for LNK1. Approximately 100 µg of total proteins were loaded per lane. Protein samples were run at 80mV for 20 minutes and 100mV for 60 to 90 minutes. Proteins were transferred for 60 minutes at 100mV to Polyvinylidene Fifluoride (PVDF) membranes and stained with Red

Ponceau following standard protocols. The membranes were blocked with 7,5% powdered milk dissolved in PBS-T (PO_4H_2K 1,8 mM; PO_4HNa_2 10 mM; NaCl 137 mM; KCl 2,7 mM; 0,05% Tween) for 2 hours at room temperature. The membranes were incubated overnight at 4ºC with Anti-MYC (Sigma) antibody for LNK1-MYC, LNK3-MYC and LNK4-MYC detection or Anti-GFP (Invitrogen) antibody for YFP-RVE8 detection. The antibodies were diluted to 1:1000 in TBS-T. The membranes were washed three times with TBS-T for 10 minutes each time. Membranes were incubated for 60-90 minutes with anti-mouse (for MYC detection) or anti-rabbit (for GFP detection) antibodies diluted to 1:500 in TBS-T. The membranes were subsequently washed three times with TBS-T for 10 minutes each time. Proteins were detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo scientific) with the LAS-4000 luminescence detector (Fuji).

For co-immunoprecipitation assays, four samples of protein extracts (approximately 1 mg) were incubated for 2 h at 4ºC with the GFP antibody coupled to magnetic particles (GFP-Trap®_M, Chromotek). Immunocomplexes were washed 5 times followed by additional washing with PBS (composición del PBS). Immunoprecipitated proteins from the four samples were pulled together and eluted by adding Laemmli buffer (80 mM Tris-HCL pH 6.8; SDS 1,6%; DTT 0,1 M; Glycerol 5%; Bromophenol blue). Proteins were denatured at 95ºC for 10 min and analyzed by Western-blot as described above.

7. Analysis of anthocyanin content

Roots from 4 week-old plants were removed with scissors and rosette leaves were weighted and placed in 1.5 ml microfuge tubes before rapid immersion in liquid N_2. Relative anthocyanin accumulation was determined with at least six groups of eight plants from each genotype. Grounded tissues were incubated overnight in the dark with 300 µl of methanol acidified with 1% HCl. Following the addition of 200 µl of Milli-Q H_2O, anthocyanins were separated from chlorophylls by adding 500 µl of chloroform. Samples were centrifuged for 3 minutes and 400 µl of a solution containing 60% Methanol, 1% HCl, 40% Milli-Q H_2O was added to the supernatants. Total anthocyanin content was quantified by measuring the absorbance at 530 nm and 657 nm. Subtraction of the A657 from the A530 gives the relative accumulation of anthocyanin (Rabino, 1986). Values were graphically depicted relative to the weight of triplicate samples.

8. ChIP assays

ChIP assays were performed essentially as previously described. Approximately 1 g of fourteen (Perales and Más 2007) day-old seedlings were fixed at the indicated ZT in 30 ml of ice-cold fixation buffer (0.4 M Sucrose, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM PMSF, 1% Formaldehyde, 0.05% Triton X-100) for 15 min under vacuum. Fixation was stopped by addition of ice-cold glycine 0.125 M and vacuum incubation for 5 min. Seedlings were then washed 3 times with ice-cold water and dried. The resulting seedlings were grounded in liquid nitrogen and the powder was filtered twice with miracloth. Extraction was performed with extraction buffer I (0.4 M Sucrose, 10 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol, 1 mM PMSF, 5 µg/ml Leupeptin, 1 µg/ml Aprotinin, 5 µg/ml Antipain, 1 µg /ml Pepstatin, 5 µg/ml Chymostatin and 50 µm MG132). Nuclei were then purified by centrifugation at 4ºC for 20 minutes at 1000g. Nuclei were washed four times by centrifugation at 4ºC for 20 minutes at 1000g with 2ml of extraction buffer II (0.25 M Sucrose, 10 mM Tris-HCl pH 8.0, 10 mM MgCl2, 1% Triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF, 5 µg/ml Leupeptin, 1 µg/ml Aprotinin, 5 µg/ml Antipain, 1 µg /ml Pepstatin, 5 µg/ml Chymostatin and 50 µm MG132). Nuclei were resuspended in 1 ml of nuclei lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, 5 µg/ml Leupeptin, 1 µg/ml Aprotinin, 5 µg/ml Antipain, 1 µg /ml Pepstatin, 5 µg/ml Chymostatin and 50 µm MG132). 300 µl of chromatin was sonicated to approximately 500–1000 bp fragments with a sonicator (Bioruptor Next Generation, Diagenode). Following centrifugation at 12.000 x g for 10 minutes at 4ºC, 100 µl of soluble chromatin (the supernatant) was diluted in 400 µl of ChIP dilution buffer (15 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA, 1 mM PMSF, 5 µg/ml Leupeptin, 1 µg/ml Aprotinin, 5 µg/ml Antipain, 1 µg /ml Pepstatin, 5 µg/ml Chymostatin and 50 µm MG132) and incubated overnight at 4ºC with 50µl of Magnetic beads (Dynabeads protein G, Invitrogen) and with 1:1000 Anti-GFP antibody (Invitrogen). Immunocomplexes were washed twice with 900 µl of low salt buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA), twice with 900µl of high salt buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA), twice with 900 µl of LiCl wash buffer (10 mM Tris-HCl pH 8.0, 0.25 M LiCl, 1% NP-40, 1% Sodium Deoxycholate, 1 mM EDTA) and twice with 900 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Immunocomplexes were eluted 300 µl with 1% SDS and 0.1 M NaHCO3 followed by 1 hour at 65°C to break the bonds between the antibodies and the proteins. Next, 220 mM NaCl were added to precipitate the DNA, following incubation overnight at 65°C for reverse cross-linking. Immunoprecipitated DNA was isolated using the QIAquick kit (Qiagen) following the manufacturer instructions. ChIP samples were quantified

by QPCR with the iTaq Universal SYBR Green Supermix (BioRad) in a 96-well CFX96 Touch Real-Time PCR Detection System (BioRad). ChIP values were calculated relative to the input values. Samples similarly processed but omitting the antibody in the incubation were used as negative control (-). Amplification of the promoter of an unrelated gene (At5g55840) was also used as a negative control. The list of primers used for promoter amplification is shown in Table 2.

Table 2. List of primers used in this study

Name	Sequence	Experiment	Position	
LNK1_TOPO_F	CACCATGGGTAGTGGAACAAACCA	Molecular Clonning		
LNK1_TOPO_R	ATTGTTGTCACTTGTTACAACTTCTG	Molecular Clonning		
LNK2_TOPO_F	CACCATGTTTGATTGGGAAGAAGAA	Molecular Clonning		
LNK2_TOPO_R	TCACAATTTTCTTTTGTTTCCTTGGGATGC	Molecular Clonning		
LNK3_TOPO_F	CACCATGGATTGTTATGCTGAAGAGCT	Molecular Clonning		
LNK3_TOPO_R	CTACTGTACTCTTTCCGACAGAGG	Molecular Clonning		
LNK4_TOPO_F	CACCATGGATCGTTATTCGAGGAGGA	Molecular Clonning		
LNK4_TOPO_R	CCAAATATGATGAAACTCTCTTATCC	Molecular Clonning		
RVE8_TOPO_F	TTATGCTGATTTGTCGCTTGTTGAGTTC	Molecular Clonning		
RVE8_TOPO_R	TGCTGATTTGTCGCTTGTTGAGTTCTTG	Molecular Clonning		
LINK1_TOPO_STOP_Rev	TTAATTGTTGTCACTTGTTACAACTTCTG	Molecular Clonning		
LINK2_TOPO_STOP_Rev	TCACAATTTTCTTTTGTTTCCTTGGGATGC	Molecular Clonning		
LINK3_NcoI_F	CATGCCATGGGCATGGATTGTTATGCTGAAGA	Molecular Clonning		
LINK3_XhoI_R	CCGCTCGAGCTACTGTACTCTTTCCGACAGA	Molecular Clonning		
LINK4_NcoI_F	CATGCCATGGGCATGGATCGTTATTCGAGGA	Molecular Clonning		
LINK4_XhoI_R	CCGCTCGAGTTACCAAATATGATGAAACTCTC	Molecular Clonning		
TT18_ChIP_F	TCTCCTAATTTCTTCCCTCCAA	ChIP Assays	-352	-373
TT18_ChIP_R	TGAGCTTCTCAGGTTTCTTCTG	ChIP Assays	-291	-312
UF3GT_ChIP_F	TGCTGCAAGGCTTTTACAGA	ChIP Assays	-324	-343
UF3GT_ChIP_R	GAATTGCGGTGTCTTGTGTG	ChIP Assays	-206	-225
TT4_ChIP_F	CAAAGCCCTTTGTTGGTGTA	ChIP Assays	-447	-466
TT4_ChIP_R	TTTGCAACAACAACAACTCATATT	ChIP Assays	-279	-302
PAP1_ChIP_F (promoter)	GTAAAAGAATATCTAATTTTAAGAAAAGACTTCAAA	ChIP Assays	-446	-481
PAP1_ChIP_R (promoter)	TACCAGTTTTTAGAATGTGCATCTTCAC	ChIP Assays	-499	-526
PAP1_ChIP_F (gene)	CACCAAGTTCCTGTAAGAGCTGGTATG	ChIP Assays	100	126
PAP1_ChIP_R (gene)	TCTATTCAGAAAATTGATTAATACCCGGTAT	ChIP Assays	217	247
TT19_ChIP_F	TCTAATAAAATGCCAACACATCTACTCTCAT	ChIP Assays	-99	-129
TT19_ChIP_R	TTGTTACGAATAAGAAAGATTTTACTATATGCAC	ChIP Assays	-21	-54
TT3_ChIP_F	TTTCCAGTTTTCGCAAAGAAA	ChIP Assays	-302	-322
TT3_ChIP_R	GACCTCTTCTCTGACGTCTTACG	ChIP Assays	-147	-169
TOC1_ChIP_F2	TAATATGAGCCAATCGGTAATACGA	ChIP Assays	-1482	-1457
TOC1_ChIP_R2	GGTTGGGAAACAAATAATCAAGTTG	ChIP Assays	-1382	-1357
PRR5_ChIP_EE_F	TGCAAACCTATGTACCAAACAGA	ChIP Assays	-1096	-1066
PRR5_ChIP_EE_R	AAATCCCACTCGTGACTTTTG	ChIP Assays	-1016	-995
At5g55840_ChIP_F	GATTCTGCTTCTCACCAA	ChIP Assays	133	150
At5g55840_ChIP_R	ATTCAGCAATAGCCACAA	ChIP Assays	306	323
IPP2_EXP_F	CATGCGACACACCAACACCA	Expression Analysis		
IPP2_EXP_R	TGAGGCGAATCAATGGGAGA	Expression Analysis		
TT18_EXP_F	AACGCGAGTGGACAATTGGAATG	Expression Analysis		
TT18_EXP_R	GCGTACTCACTCGTTGCTTCTATG	Expression Analysis		
UF3GT_EXP_F	TGGAGGTTGGATTCAGCAACCG	Expression Analysis		
UF3GT_EXP_R	ACCCAAACCCGCAATGGCTAAC	Expression Analysis		
CHS_EXP_F	TTCCGCATCACCAACAGTGAAC	Expression Analysis		
CHS_EXP_R	CGCACATGCGCTTGAACTTCTC	Expression Analysis		
TT3_EXP_F	AGGAAGGAAGCTACGATGATGCC	Expression Analysis		
TT3_EXP_R	TGTCGGCTTTATCACTTCGTTCTC	Expression Analysis		
GL3_EXP_F	ACCGTCAATTGCAAGCACAAGG	Expression Analysis		
GL3_EXP_R	GCAACCCTTTGAAGTGCTTCTTTG	Expression Analysis		
TTG1_EXP_F	GCAGCCTGATTGGATTGGTATTGC	Expression Analysis		
TTG1_EXP_R	TTGATCACTTCACATCTGCACCTC	Expression Analysis		
PAP1_EXP_F	CTGTAAGAGCTGGGCTAAACCG	Expression Analysis		
PAP1_EXP_R	AGACCACCTATTCCCTAGAAGCC	Expression Analysis		
LINK1_EXP_F	TGGAAACAGACCGGAGAAAGGC	Expression Analysis		
LINK1_EXP_R	TCCAGCATACTTGTCTGCTTCACC	Expression Analysis		
LINK2_EXP_F	CTCAGTTGAGGACCAGCCATATC	Expression Analysis		
LINK2_EXP_R	TCCTCTGACCGTACAGCTCTT	Expression Analysis		
LNK3_EXP_F	GCGCGACCAGTAGCAATAACAG	Expression Analysis		
LNK3_EXP_R	AATGGAGCTTCCTCCTTGAAAGAC	Expression Analysis		
LNK4_EXP_F	GGCTACAGAAATGTTGACTG	Expression Analysis		
LNK4_EXP_R	CTGTTGTGAGTTCTTTGCAAG	Expression Analysis		

References

Ahmad, M. and Cashmore, A.R. (1993) HY4 gene of A. thaliana encodes a protein with characteristics of a blue-light photoreceptor.

Alabadí, D., Yanovsky, M.J., Más, P., Harmer, S.L. and Kay, S.A. (2002) Critical role for CCA1 and LHY in maintaining circadian rhythmicity in Arabidopsis. *Current Biology*, **12**, 757-761.

Andersson, C., Harmer, S., Schultz, T. and Kay, S. (1999) The Reveille (REV) family of DNA binding proteins and the circadian clock. In *10th international conference on Arabidopsis research, Melbourne*, pp. 4-8.

Andronis, C., Barak, S., Knowles, S.M., Sugano, S. and Tobin, E.M. (2008) The clock protein CCA1 and the bZIP transcription factor HY5 physically interact to regulate gene expression in Arabidopsis. *Molecular plant*, **1**, 58-67.

Ang, L.-H., Chattopadhyay, S., Wei, N., Oyama, T., Okada, K., Batschauer, A. and Deng, X.-W. (1998) Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of Arabidopsis development. *Molecular cell*, **1**, 213-222.

Arana, M.V., Marín-de la Rosa, N., Maloof, J.N., Blázquez, M.A. and Alabadí, D. (2011) Circadian oscillation of gibberellin signaling in Arabidopsis. *Proceedings of the National Academy of Sciences*, **108**, 9292-9297.

Ariizumi, T. and Toriyama, K. (2011) Genetic regulation of sporopollenin synthesis and pollen exine development. *Annual review of plant biology*, **62**, 437-460.

Aschoff, J. (1960) Exogenous and endogenous components in circadian rhythms. In *Cold Spring Harbor symposia on quantitative biology*: Cold Spring Harbor Laboratory Press, pp. 11-28.

Baudry, A., Ito, S., Song, Y.H., Strait, A.A., Kiba, T., Lu, S., Henriques, R., Pruneda-Paz, J.L., Chua, N.-H. and Tobin, E.M. (2010) F-box proteins FKF1 and LKP2 act in concert with ZEITLUPE to control Arabidopsis clock progression. *The Plant Cell Online*, **22**, 606-622.

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L. and Zoran, M.J. (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nature Reviews Genetics*, **6**, 544-556.

Bennett, R.N. and Wallsgrove, R.M. (1994) Secondary metabolites in plant defence mechanisms. *New Phytologist*, **127**, 617-633.

Berr, A., McCallum, E.J., Ménard, R., Meyer, D., Fuchs, J., Dong, A. and Shen, W.-H. (2010) Arabidopsis SET DOMAIN GROUP2 is required for H3K4 trimethylation and is crucial for both sporophyte and gametophyte development. *The Plant Cell Online*, **22**, 3232-3248.

Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A. and Lamb, C. (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *The Plant Cell Online*, **12**, 2383-2393.

Bretzl, H. (1903) *Botanische Forschungen des Alexanderzuges*: BG Teubner.

Buer, C.S., Imin, N. and Djordjevic, M.A. (2010) Flavonoids: new roles for old molecules. *Journal of Integrative Plant Biology*, **52**, 98-111.

Carré, I. and Veflingstad, S.R. (2013) Emerging design principles in the Arabidopsis circadian clock. In *Seminars in cell & developmental biology*: Elsevier, pp. 393-398.

Carré, I.A. and Kim, J.Y. (2002) MYB transcription factors in the Arabidopsis circadian clock. *Journal of experimental botany*, **53**, 1551-1557.

Clack, T., Mathews, S. and Sharrock, R.A. (1994) The phytochrome apoprotein family inArabidopsis is encoded by five genes: the sequences and expression ofPHYD andPHYE. *Plant molecular biology*, **25**, 413-427.

Cominelli, E., Gusmaroli, G., Allegra, D., Galbiati, M., Wade, H.K., Jenkins, G.I. and Tonelli, C. (2008) Expression analysis

of anthocyanin regulatory genes in response to different light qualities in Arabidopsis thaliana. *Journal of plant physiology*, **165**, 886-894.

Costa, R. (2001) Introduction: taking stock of circadian clock complexity. In *Seminars in Cell & Developmental Biology*: Elsevier, pp. 267-269.

Covington, M.F. and Harmer, S.L. (2007) The circadian clock regulates auxin signaling and responses in Arabidopsis. *PLoS biology*, **5**, e222.

Cui, X., Lu, F., Li, Y., Xue, Y., Kang, Y., Zhang, S., Qiu, Q., Cui, X., Zheng, S. and Liu, B. (2013) Ubiquitin-specific proteases UBP12 and UBP13 act in circadian clock and photoperiodic flowering regulation in Arabidopsis. *Plant Physiology*, **162**, 897-906.

Cheynier, V., Comte, G., Davies, K.M., Lattanzio, V. and Martens, S. (2013) Plant phenolics: recent advances on their biosynthesis, genetics, and ecophysiology. *Plant Physiology and Biochemistry*, **72**, 1-20.

Chow, B.Y., Helfer, A., Nusinow, D.A. and Kay, S.A. (2012) ELF3 recruitment to the PRR9 promoter requires other Evening Complex members in the Arabidopsis circadian clock. *Plant signaling & behavior*, **7**, 170-173.

Daniel, X., Sugano, S. and Tobin, E.M. (2004) CK2 phosphorylation of CCA1 is necessary for its circadian oscillator function in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 3292-3297.

de Candolle, A.P. (1832) *Phisiologie végétale*.

de Lucas, M., Davière, J.-M., Rodríguez-Falcón, M., Pontin, M., Iglesias-Pedraz, J.M., Lorrain, S., Fankhauser, C., Blázquez, M.A., Titarenko, E. and Prat, S. (2008) A molecular framework for light and gibberellin control of cell elongation. *Nature*, **451**, 480-484.

de Mairan, J. (1729) Observation botanique. *Hist. Acad. Roy. Sci*, **35**, 36.

de Montaigu, A., Tóth, R. and Coupland, G. (2010) Plant development goes like clockwork. *Trends in Genetics*, **26**, 296-306.

Deikman, J. and Hammer, P.E. (1995) Induction of anthocyanin accumulation by cytokinins in Arabidopsis thaliana. *Plant Physiology*, **108**, 47-57.

Devlin, P.F. and Kay, S.A. (2000) Cryptochromes are required for phytochrome signaling to the circadian clock but not for rhythmicity. *The Plant Cell Online*, **12**, 2499-2509.

Devlin, P.F. and Kay, S.A. (2001) Circadian photoperception. *Annual review of physiology*, **63**, 677-694.

Ding, Z., Doyle, M.R., Amasino, R.M. and Davis, S.J. (2007) A complex genetic interaction between Arabidopsis thaliana TOC1 and CCA1/LHY in driving the circadian clock and in output regulation. *Genetics*, **176**, 1501-1510.

Dixon, L.E., Knox, K., Kozma-Bognar, L., Southern, M.M., Pokhilko, A. and Millar, A.J. (2011) Temporal repression of core circadian genes is mediated through EARLY FLOWERING 3 in Arabidopsis. *Current Biology*, **21**, 120-125.

Doyle, M.R., Davis, S.J., Bastow, R.M., McWatters, H.G., Kozma-Bognár, L., Nagy, F., Millar, A.J. and Amasino, R.M. (2002) The ELF4 gene controls circadian rhythms and flowering time in Arabidopsis thaliana. *Nature*, **419**, 74-77.

Ehlting, J., Büttner, D., Wang, Q., Douglas, C.J., Somssich, I.E. and Kombrink, E. (1999) Three 4-coumarate: coenzyme A ligases in Arabidopsis thaliana represent two evolutionarily divergent classes in angiosperms. *The Plant Journal*, **19**, 9-20.

Fankhauser, C. and Staiger, D. (2002) Photoreceptors in Arabidopsis thaliana: light perception, signal transduction and entrainment of the endogenous clock. *Planta*, **216**, 1-16.

Farinas, B. and Mas, P. (2011) Functional implication of the MYB transcription factor RVE8/LCL5 in the circadian control of histone acetylation. *The Plant Journal*, **66**, 318-329.

Farré, E.M., Harmer, S.L., Harmon, F.G., Yanovsky, M.J. and Kay, S.A. (2005) Overlapping and distinct roles of PRR7 and PRR9 in the Arabidopsis circadian clock. *Current Biology*, **15**, 47-54.

Ferrer, J.-L., Austin, M., Stewart, C. and Noel, J. (2008) Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiology and Biochemistry*, **46**, 356-370.

Formstecher, E., Aresta, S., Collura, V., Hamburger, A., Meil, A., Trehin, A., Reverdy, C., Betin, V., Maire, S. and Brun, C. (2005) Protein interaction mapping: a Drosophila case study. *Genome research*, **15**, 376-384.

Fornara, F., Panigrahi, K.C., Gissot, L., Sauerbrunn, N., Rühl, M., Jarillo, J.A. and Coupland, G. (2009) Arabidopsis DOF transcription factors act redundantly to reduce CONSTANS expression and are essential for a photoperiodic flowering response. *Developmental cell*, **17**, 75-86.

Franklin, K.A., Lee, S.H., Patel, D., Kumar, S.V., Spartz, A.K., Gu, C., Ye, S., Yu, P., Breen, G. and Cohen, J.D. (2011) Phytochrome-interacting factor 4 (PIF4) regulates auxin biosynthesis at high temperature. *Proceedings of the National Academy of Sciences*, **108**, 20231-20235.

Franklin, K.A., Toledo-Ortiz, G., Pyott, D.E. and Halliday, K.J. (2014) Interaction of light and temperature signalling. *Journal of experimental botany*, **65**, 2859-2871.

Fujiwara, S., Wang, L., Han, L., Suh, S.-S., Salomé, P.A., McClung, C.R. and Somers, D.E. (2008) Post-translational regulation of the Arabidopsis circadian clock through selective proteolysis and phosphorylation of pseudo-response regulator proteins. *Journal of Biological Chemistry*, **283**, 23073-23083.

Gendron, J.M., Pruneda-Paz, J.L., Doherty, C.J., Gross, A.M., Kang, S.E. and Kay, S.A. (2012) Arabidopsis circadian clock protein, TOC1, is a DNA-binding transcription factor. *Proceedings of the National Academy of Sciences*, **109**, 3167-3172.

Gerhart-Hines, Z. and Lazar, M.A. (2015) Circadian Metabolism in the Light of Evolution. *Endocrine Reviews*, er. 2015-1007.

Giraud, E., Ng, S., Carrie, C., Duncan, O., Low, J., Lee, C.P., Van Aken, O., Millar, A.H., Murcha, M. and Whelan, J. (2010) TCP transcription factors link the regulation of genes encoding mitochondrial proteins with the circadian clock in Arabidopsis thaliana. *The Plant Cell Online*, **22**, 3921-3934.

Gonzalez, A., Zhao, M., Leavitt, J.M. and Lloyd, A.M. (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. *The Plant Journal*, **53**, 814-827.

Graf, A., Schlereth, A., Stitt, M. and Smith, A.M. (2010) Circadian control of carbohydrate availability for growth in Arabidopsis plants at night. *Proceedings of the National Academy of Sciences*, **107**, 9458-9463.

Guo, L., Yu, Y., Law, J.A. and Zhang, X. (2010) SET DOMAIN GROUP2 is the major histone H3 lysine 4 trimethyltransferase in Arabidopsis. *Proceedings of the National Academy of Sciences*, **107**, 18557-18562.

Hamberger, B., Ellis, M., Friedmann, M., de Azevedo Souza, C., Barbazuk, B. and Douglas, C.J. (2007) Genome-wide analyses of phenylpropanoid-related genes in Populus trichocarpa, Arabidopsis thaliana, and Oryza sativa: the Populus lignin toolbox and conservation and diversification of angiosperm gene families This article is one of a selection of papers published in the Special Issue on Poplar Research in Canada. *Botany*, **85**, 1182-1201.

Harmer, S.L. (2009) The circadian system in higher plants. *Annual review of plant biology*, **60**, 357-377.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.-S., Han, B., Zhu, T., Wang, X., Kreps, J.A. and Kay, S.A. (2000) Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. *Science*, **290**, 2110-2113.

Harmer, S.L. and Kay, S.A. (2005) Positive and negative factors confer phase-specific circadian regulation of transcription in Arabidopsis. *The Plant Cell Online*, **17**, 1926-1940.

Haydon, M.J., Mielczarek, O., Robertson, F.C., Hubbard, K.E. and Webb, A.A. (2013) Photosynthetic entrainment of the

Arabidopsis thaliana circadian clock. *Nature*, **502**, 689-692.

Hazen, S.P., Schultz, T.F., Pruneda-Paz, J.L., Borevitz, J.O., Ecker, J.R. and Kay, S.A. (2005) LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 10387-10392.

Helfer, A., Nusinow, D.A., Chow, B.Y., Gehrke, A.R., Bulyk, M.L. and Kay, S.A. (2011) LUX ARRHYTHMO encodes a nighttime repressor of circadian gene expression in the Arabidopsis core clock. *Current Biology*, **21**, 126-133.

Herrero, E., Kolmos, E., Bujdoso, N., Yuan, Y., Wang, M., Berns, M.C., Uhlworm, H., Coupland, G., Saini, R. and Jaskolski, M. (2012) EARLY FLOWERING4 recruitment of EARLY FLOWERING3 in the nucleus sustains the Arabidopsis circadian clock. *The Plant Cell Online*, **24**, 428-443.

Hicks, K.A., Millar, A.J., Carré, I.A., Somers, D.E., Straume, M., Meeks-Wagner, D.R. and Kay, S.A. (1996) Conditional circadian dysfunction of the Arabidopsis early-flowering 3 mutant. *Science*, **274**, 790-792.

Himanen, K., Woloszynska, M., Boccardi, T.M., De Groeve, S., Nelissen, H., Bruno, L., Vuylsteke, M. and Van Lijsebettens, M. (2012) Histone H2B monoubiquitination is required to reach maximal transcript levels of circadian clock genes in Arabidopsis. *The Plant Journal*, **72**, 249-260.

Hong, S., Song, H.-R., Lutz, K., Kerstetter, R.A., Michael, T.P. and McClung, C.R. (2010) Type II protein arginine methyltransferase 5 (PRMT5) is required for circadian period determination in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, **107**, 21211-21216.

Hornitschek, P., Kohnen, M.V., Lorrain, S., Rougemont, J., Ljung, K., López-Vidriero, I., Franco-Zorrilla, J.M., Solano, R., Trevisan, M. and Pradervand, S. (2012) Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. *The Plant Journal*, **71**, 699-711.

Hoth, S., Niedermeier, M., Feuerstein, A., Hornig, J. and Sauer, N. (2010) An ABA-responsive element in the AtSUC1 promoter is involved in the regulation of AtSUC1 expression. *Planta*, **232**, 911-923.

Hsu, P.Y., Devisetty, U.K. and Harmer, S.L. (2013) Accurate timekeeping is controlled by a cycling activator in Arabidopsis. *Elife*, **2**, e00473.

Hu, W., Franklin, K.A., Sharrock, R.A., Jones, M.A., Harmer, S.L. and Lagarias, J.C. (2013) Unanticipated regulatory roles for Arabidopsis phytochromes revealed by null mutant analysis. *Proceedings of the National Academy of Sciences*, **110**, 1542-1547.

Huala, E., Oeller, P.W., Liscum, E., Han, I.-S., Larsen, E. and Briggs, W.R. (1997) Arabidopsis NPH1: a protein kinase with a putative redox-sensing domain. *Science*, **278**, 2120-2123.

Huang, W., Perez-Garcia, P., Pokhilko, A., Millar, A., Antoshechkin, I., Riechmann, J.L. and Mas, P. (2012) Mapping the core of the Arabidopsis circadian clock defines the network structure of the oscillator. *Science*, **336**, 75-79.

Imaizumi, T., Schultz, T.F., Harmon, F.G., Ho, L.A. and Kay, S.A. (2005) FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in Arabidopsis. *Science*, **309**, 293-297.

Ito, S., Song, Y.H. and Imaizumi, T. (2012) LOV domain-containing F-box proteins: light-dependent protein degradation modules in Arabidopsis. *Molecular plant*, sss013.

Jaakola, L. (2013) New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends in plant science*, **18**, 477-483.

James, A.B., Syed, N.H., Bordage, S., Marshall, J., Nimmo, G.A., Jenkins, G.I., Herzyk, P., Brown, J.W. and Nimmo, H.G. (2012) Alternative splicing mediates responses of the Arabidopsis circadian clock to temperature changes. *The Plant Cell Online*, **24**, 961-981.

Jeong, J. and Choi, G. (2013) Phytochrome-interacting factors have both shared and distinct biological roles. *Molecules and cells*, **35**, 371-380.

Jeong, S.-W., Das, P.K., Jeoung, S.C., Song, J.-Y., Lee, H.K., Kim, Y.-K., Kim, W.J., Park, Y.I., Yoo, S.-D. and Choi, S.-B. (2010) Ethylene suppression of sugar-induced anthocyanin pigmentation in Arabidopsis. *Plant Physiology*, **154**, 1514-1531.

Jeong, S.T., Goto-Yamamoto, N., Kobayashi, S. and Esaka, M. (2004) Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. *Plant Science*, **167**, 247-252.

Jolma, I.W., Laerum, O.D., Lillo, C. and Ruoff, P. (2010) Circadian oscillators in eukaryotes. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, **2**, 533-549.

Jones, M.A., Covington, M.F., DiTacchio, L., Vollmers, C., Panda, S. and Harmer, S.L. (2010) Jumonji domain protein JMJD5 functions in both the plant and human circadian systems. *Proceedings of the National Academy of Sciences*, **107**, 21623-21628.

Kagawa, T., Sakai, T., Suetsugu, N., Oikawa, K., Ishiguro, S., Kato, T., Tabata, S., Okada, K. and Wada, M. (2001) Arabidopsis NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. *Science*, **291**, 2138-2141.

Kiba, T., Henriques, R., Sakakibara, H. and Chua, N.-H. (2007) Targeted degradation of PSEUDO-RESPONSE REGULATOR5 by an SCFZTL complex regulates clock function and photomorphogenesis in Arabidopsis thaliana. *The Plant Cell Online*, **19**, 2516-2530.

Kiesel, A. (1894) Untersuchungen zur physiologie des facettierten auges.

Kikis, E.A., Khanna, R. and Quail, P.H. (2005) ELF4 is a phytochrome-regulated component of a negative-feedback loop involving the central oscillator components CCA1 and LHY. *The Plant Journal*, **44**, 300-313.

Kim, W.-Y., Fujiwara, S., Suh, S.-S., Kim, J., Kim, Y., Han, L., David, K., Putterill, J., Nam, H.G. and Somers, D.E. (2007) ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. *Nature*, **449**, 356-360.

Kim, Y., Lim, J., Yeom, M., Kim, H., Kim, J., Wang, L., Kim, W.Y., Somers, D.E. and Nam, H.G. (2013) ELF4 regulates GIGANTEA chromatin access through subnuclear sequestration. *Cell reports*, **3**, 671-677.

Kinmonth-Schultz, H.A., Golembeski, G.S. and Imaizumi, T. (2013) Circadian clock-regulated physiological outputs: dynamic responses in nature. In *Seminars in cell & developmental biology*: Elsevier, pp. 407-413.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999) A pair of related genes with antagonistic roles in mediating flowering signals. *Science*, **286**, 1960-1962.

Kuno, N., Møller, S.G., Shinomura, T., Xu, X., Chua, N.-H. and Furuya, M. (2003) The novel MYB protein EARLY-PHYTOCHROME-RESPONSIVE1 is a component of a slave circadian oscillator in Arabidopsis. *The Plant Cell Online*, **15**, 2476-2488.

Lai, A.G., Doherty, C.J., Mueller-Roeber, B., Kay, S.A., Schippers, J.H. and Dijkwel, P.P. (2012) CIRCADIAN CLOCK-ASSOCIATED 1 regulates ROS homeostasis and oxidative stress responses. *Proceedings of the National Academy of Sciences*, **109**, 17129-17134.

Lau, O.S., Huang, X., Charron, J.-B., Lee, J.-H., Li, G. and Deng, X.W. (2011) Interaction of Arabidopsis DET1 with CCA1 and LHY in mediating transcriptional repression in the plant circadian clock. *Molecular cell*, **43**, 703-712.

Lazaro, A., Valverde, F., Piñeiro, M. and Jarillo, J.A. (2012) The Arabidopsis E3 ubiquitin ligase HOS1 negatively regulates CONSTANS abundance in the photoperiodic control of flowering. *The Plant Cell Online*, **24**, 982-999.

Lea, U.S., Slimestad, R., Smedvig, P. and Lillo, C. (2007) Nitrogen deficiency enhances expression of specific MYB and bHLH transcription factors and accumulation of end products in the flavonoid pathway. *Planta*, **225**, 1245-1253.

Legnaioli, T., Cuevas, J. and Mas, P. (2009) TOC1 functions as a molecular switch connecting the circadian clock with plant responses to drought. *The EMBO journal*, **28**, 3745-3757.

Li, B., Carey, M. and Workman, J.L. (2007) The role of chromatin during transcription. *Cell*, **128**, 707-719.

Li, G., Siddiqui, H., Teng, Y., Lin, R., Wan, X.-y., Li, J., Lau, O.-S., Ouyang, X., Dai, M. and Wan, J. (2011) Coordinated transcriptional regulation underlying the circadian clock in Arabidopsis. *Nature cell biology*, **13**, 616-622.

Li, X., Gao, M.-J., Pan, H.-Y., Cui, D.-J. and Gruber, M.Y. (2010) Purple canola: Arabidopsis PAP1 increases antioxidants and phenolics in Brassica napus leaves. *Journal of agricultural and food chemistry*, **58**, 1639-1645.

Lillo, C., Lea, U.S. and Ruoff, P. (2008) Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. *Plant, cell & environment*, **31**, 587-601.

Lin, C., Ahmad, M. and Cashmore, A.R. (1996) Arabidopsis cryptochrome 1 is a soluble protein mediating blue light-dependent regulation of plant growth and development. *The Plant Journal*, **10**, 893-902.

Logemann, E., Birkenbihl, R.P., Ülker, B. and Somssich, I.E. (2006) An improved method for preparing Agrobacterium cells that simplifies the Arabidopsis transformation protocol. *Plant Methods*, **2**, 16.

Long, S., Humphries, S. and Falkowski, P.G. (1994) Photoinhibition of photosynthesis in nature. *Annual review of plant biology*, **45**, 633-662.

Loreti, E., Povero, G., Novi, G., Solfanelli, C., Alpi, A. and Perata, P. (2008) Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in Arabidopsis. *New Phytologist*, **179**, 1004-1016.

Lorrain, S., Allen, T., Duek, P.D., Whitelam, G.C. and Fankhauser, C. (2008) Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. *The Plant Journal*, **53**, 312-323.

Love, J., Dodd, A.N. and Webb, A.A. (2004) Circadian and diurnal calcium oscillations encode photoperiodic information in Arabidopsis. *The Plant Cell Online*, **16**, 956-966.

Lu, S.X., Knowles, S.M., Andronis, C., Ong, M.S. and Tobin, E.M. (2009) CIRCADIAN CLOCK ASSOCIATED1 and LATE ELONGATED HYPOCOTYL function synergistically in the circadian clock of Arabidopsis. *Plant Physiology*, **150**, 834-843.

Lu, S.X., Knowles, S.M., Webb, C.J., Celaya, R.B., Cha, C., Siu, J.P. and Tobin, E.M. (2011) The Jumonji C domain-containing protein JMJ30 regulates period length in the Arabidopsis circadian clock. *Plant Physiology*, **155**, 906-915.

Lu, S.X., Webb, C.J., Knowles, S.M., Kim, S.H., Wang, Z. and Tobin, E.M. (2012) CCA1 and ELF3 interact in the control of hypocotyl length and flowering time in Arabidopsis. *Plant Physiology*, **158**, 1079-1088.

Maeda, H. and Dudareva, N. (2012) The shikimate pathway and aromatic amino acid biosynthesis in plants. *Annual review of plant biology*, **63**, 73-105.

Makino, S., Matsushika, A., Kojima, M., Yamashino, T. and Mizuno, T. (2002) The APRR1/TOC1 quintet implicated in circadian rhythms of Arabidopsis thaliana: I. Characterization with APRR1-overexpressing plants. *Plant and Cell Physiology*, **43**, 58-69.

Malapeira, J., Khaitova, L.C. and Mas, P. (2012) Ordered changes in histone modifications at the core of the Arabidopsis circadian clock. *Proceedings of the National Academy of Sciences*, **109**, 21540-21545.

Mas, P. (2008) Circadian clock function in Arabidopsis thaliana: time beyond transcription. *Trends in cell biology*, **18**, 273-281.

Mas, P., Alabadi, D., Yanovsky, M.J., Oyama, T. and Kay, S.A. (2003) Dual role of TOC1 in the control of circadian and

photomorphogenic responses in Arabidopsis. *The Plant cell*, **15**, 223-236.

Más, P., Devlin, P.F., Panda, S. and Kay, S.A. (2000) Functional interaction of phytochrome B and cryptochrome 2. *Nature*, **408**, 207-211.

Más, P., Kim, W.-Y., Somers, D.E. and Kay, S.A. (2003) Targeted degradation of TOC1 by ZTL modulates circadian function in Arabidopsis thaliana. *Nature*, **426**, 567-570.

Matsui, K., Umemura, Y. and Ohme-Takagi, M. (2008) AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis. *The Plant Journal*, **55**, 954-967.

Matsushika, A., Makino, S., Kojima, M. and Mizuno, T. (2000) Circadian waves of expression of the APRR1/TOC1 family of pseudo-response regulators in Arabidopsis thaliana: insight into the plant circadian clock. *Plant and Cell Physiology*, **41**, 1002-1012.

McClung, C.R. (2006) Two-component signaling provides the major output from the cyanobacterial circadian clock. *Proceedings of the National Academy of Sciences*, **103**, 11819-11820.

McClung, C.R. (2013) Beyond Arabidopsis: the circadian clock in non-model plant species. In *Seminars in cell & developmental biology*: Elsevier, pp. 430-436.

McClung, C.R. and Davis, S.J. (2010) Ambient thermometers in plants: from physiological outputs towards mechanisms of thermal sensing. *Current Biology*, **20**, R1086-R1092.

McWatters, H.G., Bastow, R.M., Hall, A. and Millar, A.J. (2000) The ELF3 zeitnehmer regulates light signalling to the circadian clock. *Nature*, **408**, 716-720.

Michael, T.P., Mockler, T.C., Breton, G., McEntee, C., Byer, A., Trout, J.D., Hazen, S.P., Shen, R., Priest, H.D. and Sullivan, C.M. (2008) Network discovery pipeline elucidates conserved time-of-day–specific cis-regulatory modules. *PLoS genetics*, **4**, e14.

Millar, A.J., Carre, I.A., Strayer, C.A., Chua, N.-H. and Kay, S.A. (1995) Circadian clock mutants in Arabidopsis identified by luciferase imaging. *Science*, **267**, 1161-1163.

Mizoguchi, T., Wheatley, K., Hanzawa, Y., Wright, L., Mizoguchi, M., Song, H.-R., Carré, I.A. and Coupland, G. (2002) LHY and CCA1 are partially redundant genes required to maintain circadian rhythms in Arabidopsis. *Developmental cell*, **2**, 629-641.

Mizuno, T., Nomoto, Y., Oka, H., Kitayama, M., Takeuchi, A., Tsubouchi, M. and Yamashino, T. (2014a) Ambient temperature signal feeds into the circadian clock transcriptional circuitry through the EC night-time repressor in Arabidopsis thaliana. *Plant and Cell Physiology*, **55**, 958-976.

Mizuno, T., Takeuchi, A., Nomoto, Y., Nakamichi, N. and Yamashino, T. (2014b) The LNK1 night light-inducible and clock-regulated gene is induced also in response to warm-night through the circadian clock nighttime repressor in Arabidopsis thaliana. *Plant signaling & behavior*, **9**.

Morgan, P.W. and Drew, M.C. (1997) Ethylene and plant responses to stress. *Physiologia Plantarum*, **100**, 620-630.

Nakabayashi, R., Yonekura-Sakakibara, K., Urano, K., Suzuki, M., Yamada, Y., Nishizawa, T., Matsuda, F., Kojima, M., Sakakibara, H. and Shinozaki, K. (2014) Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. *The Plant Journal*, **77**, 367-379.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T. and Kimura, T. (2007a) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *Journal of bioscience and bioengineering*, **104**, 34-41.

Nakagawa, T., Suzuki, T., Murata, S., Nakamura, S., Hino, T., Maeo, K., Tabata, R., Kawai, T., Tanaka, K. and Niwa, Y. (2007b) Improved Gateway binary vectors: high-performance vectors for

creation of fusion constructs in transgenic analysis of plants. *Bioscience, biotechnology, and biochemistry*, **71**, 2095-2100.

Nakamichi, N., Kiba, T., Henriques, R., Mizuno, T., Chua, N.-H. and Sakakibara, H. (2010) PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the Arabidopsis circadian clock. *The Plant Cell Online*, **22**, 594-605.

Nakamichi, N., Kiba, T., Kamioka, M., Suzuki, T., Yamashino, T., Higashiyama, T., Sakakibara, H. and Mizuno, T. (2012) Transcriptional repressor PRR5 directly regulates clock-output pathways. *Proceedings of the National Academy of Sciences*, **109**, 17123-17128.

Nakamichi, N., Kita, M., Ito, S., Yamashino, T. and Mizuno, T. (2005) PSEUDO-RESPONSE REGULATORS, PRR9, PRR7 and PRR5, together play essential roles close to the circadian clock of Arabidopsis thaliana. *Plant and Cell Physiology*, **46**, 686-698.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A. and Bartel, B. (2000) FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. *Cell*, **101**, 331-340.

Noordally, Z.B., Ishii, K., Atkins, K.A., Wetherill, S.J., Kusakina, J., Walton, E.J., Kato, M., Azuma, M., Tanaka, K. and Hanaoka, M. (2013) Circadian control of chloroplast transcription by a nuclear-encoded timing signal. *Science*, **339**, 1316-1319.

Nozue, K., Harmer, S.L. and Maloof, J.N. (2011) Genomic analysis of circadian clock-, light-, and growth-correlated genes reveals PHYTOCHROME-INTERACTING FACTOR5 as a modulator of auxin signaling in Arabidopsis. *Plant Physiology*, **156**, 357-372.

Nusinow, D.A., Helfer, A., Hamilton, E.E., King, J.J., Imaizumi, T., Schultz, T.F., Farré, E.M. and Kay, S.A. (2011) The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. *Nature*, **475**, 398-402.

Olsen, K.M., Lea, U.S., Slimestad, R., Verheul, M. and Lillo, C. (2008) Differential expression of four Arabidopsis PAL genes; PAL1 and PAL2 have functional specialization in abiotic environmental-triggered flavonoid synthesis. *Journal of plant physiology*, **165**, 1491-1499.

Onai, K. and Ishiura, M. (2005) PHYTOCLOCK 1 encoding a novel GARP protein essential for the Arabidopsis circadian clock. *Genes to Cells*, **10**, 963-972.

Park, B.S., Eo, H.J., Jang, I.-C., Kang, H.-G., Song, J.T. and Seo, H.S. (2010) Ubiquitination of LHY by SINAT5 regulates flowering time and is inhibited by DET1. *Biochemical and biophysical research communications*, **398**, 242-246.

Park, M.-J., Seo, P.J. and Park, C.-M. (2012) CCA1 alternative splicing as a way of linking the circadian clock to temperature response in Arabidopsis. *Plant signaling & behavior*, **7**, 1194-1196.

Perales, M. and Más, P. (2007) A functional link between rhythmic changes in chromatin structure and the Arabidopsis biological clock. *The Plant Cell Online*, **19**, 2111-2123.

Pokhilko, A., Fernández, A.P., Edwards, K.D., Southern, M.M., Halliday, K.J. and Millar, A.J. (2012) The clock gene circuit in Arabidopsis includes a repressilator with additional feedback loops. *Molecular systems biology*, **8**.

Portolés, S. and Mas, P. (2010) The functional interplay between protein kinase CK2 and CCA1 transcriptional activity is essential for clock temperature compensation in Arabidopsis. *PLoS genetics*, **6**, e1001201.

Portolés, S. and Más, P. (2007) Altered oscillator function affects clock resonance and is responsible for the reduced day-length sensitivity of CKB4 overexpressing plants. *The Plant Journal*, **51**, 966-977.

Pruneda-Paz, J.L., Breton, G., Para, A. and Kay, S.A. (2009) A functional genomics approach reveals CHE as a component of the Arabidopsis circadian clock. *Science*, **323**, 1481-1485.

Qi, T., Song, S., Ren, Q., Wu, D., Huang, H., Chen, Y., Fan, M., Peng, W., Ren, C. and Xie, D. (2011) The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate

Jasmonate-mediated anthocyanin accumulation and trichome initiation in Arabidopsis thaliana. *The Plant Cell Online*, **23**, 1795-1814.

Ramsay, N.A., Walker, A.R., Mooney, M. and Gray, J.C. (2003) Two basic-helix-loop-helix genes (MYC-146 and GL3) from Arabidopsis can activate anthocyanin biosynthesis in a white-flowered Matthiola incana mutant. *Plant molecular biology*, **52**, 679-688.

Rawat, R., Schwartz, J., Jones, M.A., Sairanen, I., Cheng, Y., Andersson, C.R., Zhao, Y., Ljung, K. and Harmer, S.L. (2009) REVEILLE1, a Myb-like transcription factor, integrates the circadian clock and auxin pathways. *Proceedings of the National Academy of Sciences*, **106**, 16883-16888.

Rawat, R., Takahashi, N., Hsu, P.Y., Jones, M.A., Schwartz, J., Salemi, M.R., Phinney, B.S. and Harmer, S.L. (2011) REVEILLE8 and PSEUDO-REPONSE REGULATOR5 form a negative feedback loop within the Arabidopsis circadian clock. *PLoS genetics*, **7**, e1001350.

Richter, C.P. (1922) A behavioristic study of the activity of the rat. *Comparative Psychology Monographs*.

Rizzini, L., Favory, J.-J., Cloix, C., Faggionato, D., O'Hara, A., Kaiserli, E., Baumeister, R., Schäfer, E., Nagy, F. and Jenkins, G.I. (2011) Perception of UV-B by the Arabidopsis UVR8 protein. *Science*, **332**, 103-106.

Rockwell, N.C., Su, Y.-S. and Lagarias, J.C. (2006) Phytochome structure and signaling mechanisms. *Annual review of plant biology*, **57**, 837.

Rugnone, M.L., Soverna, A.F., Sanchez, S.E., Schlaen, R.G., Hernando, C.E., Seymour, D.K., Mancini, E., Chernomoretz, A., Weigel, D. and Más, P. (2013) LNK genes integrate light and clock signaling networks at the core of the Arabidopsis oscillator. *Proceedings of the National Academy of Sciences*, **110**, 12120-12125.

Ruoff, P. (1992) Introducing temperature-compensation in any reaction kinetic oscillator model.

Salomé, P.A. and McClung, C.R. (2005a) PSEUDO-RESPONSE REGULATOR 7 and 9 are partially redundant genes essential for the temperature responsiveness of the Arabidopsis circadian clock. *The Plant Cell Online*, **17**, 791-803.

Salomé, P.A. and McClung, C.R. (2005b) PSEUDO-RESPONSE REGULATOR 7 and 9 are partially redundant genes essential for the temperature responsiveness of the *Arabidopsis* circadian clock. *The Plant cell*, **17**, 791-803.

Salomé, P.A., Oliva, M., Weigel, D. and Krämer, U. (2013) Circadian clock adjustment to plant iron status depends on chloroplast and phytochrome function. *The EMBO journal*, **32**, 511-523.

Salomé, P.A., Weigel, D. and McClung, C.R. (2010) The role of the Arabidopsis morning loop components CCA1, LHY, PRR7, and PRR9 in temperature compensation. *The Plant Cell Online*, **22**, 3650-3661.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. *Science*, **288**, 1613-1616.

Sanchez, S.E., Petrillo, E., Beckwith, E.J., Zhang, X., Rugnone, M.L., Hernando, C.E., Cuevas, J.C., Herz, M.A.G., Depetris-Chauvin, A. and Simpson, C.G. (2010) A methyl transferase links the circadian clock to the regulation of alternative splicing. *Nature*, **468**, 112-116.

Sawa, M., Nusinow, D.A., Kay, S.A. and Imaizumi, T. (2007) FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. *Science*, **318**, 261-265.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carré, I.A. and Coupland, G. (1998) The late elongated hypocotyl mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. *Cell*, **93**, 1219-1229.

Schaffer, R.J. (1997) LHY, a gene that regulates flowering and hypocotyl elongation of Arabidopsis: University of East Anglia.

Schmied, K. and Merkle, T. (2005) A small family of LHY-CCA1-like (LCL) MYB1R transcription factors: potential co-

regulators of the circadian oscillator. *EMBL/GenBank/DDBJ databases*.

Schultz, T.F., Kiyosue, T., Yanovsky, M., Wada, M. and Kay, S.A. (2001) A role for LKP2 in the circadian clock of Arabidopsis. *The Plant Cell Online*, **13**, 2659-2670.

Sehgal, A., Joiner, W., Crocker, A., Koh, K., Sathyanarayanan, S., Fang, Y., Wu, M., Williams, J. and Zheng, X. (2007) Molecular analysis of sleep: wake cycles in Drosophila. In *Cold Spring Harbor symposia on quantitative biology*: Cold Spring Harbor Laboratory Press, pp. 557-564.

Seo, P.J. and Mas, P. (2014) Multiple Layers of posttranslational regulation refine circadian clock activity in Arabidopsis. *The Plant Cell Online*, **26**, 79-87.

Seo, P.J., Park, M.-J., Lim, M.-H., Kim, S.-G., Lee, M., Baldwin, I.T. and Park, C.-M. (2012) A self-regulatory circuit of CIRCADIAN CLOCK-ASSOCIATED1 underlies the circadian clock regulation of temperature responses in Arabidopsis. *The Plant Cell Online*, **24**, 2427-2442.

Shan, X., Zhang, Y., Peng, W., Wang, Z. and Xie, D. (2009) Molecular mechanism for jasmonate-induction of anthocyanin accumulation in Arabidopsis. *Journal of experimental botany*, **60**, 3849-3860.

Sharrock, R.A. and Quail, P.H. (1989) Novel phytochrome sequences in Arabidopsis thaliana: structure, evolution, and differential expression of a plant regulatory photoreceptor family. *Genes & Development*, **3**, 1745-1757.

Shi, M.-Z. and Xie, D.-Y. (2014) Biosynthesis and Metabolic Engineering of Anthocyanins in Arabidopsis thaliana. *Recent patents on biotechnology*, **8**, 47.

Shin, J., Park, E. and Choi, G. (2007) PIF3 regulates anthocyanin biosynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in Arabidopsis. *The Plant Journal*, **49**, 981-994.

Smillie, R.M. and Hetherington, S.E. (1999) Photoabatement by anthocyanin shields photosynthetic systems from light stress. *Photosynthetica*, **36**, 451-463.

Somero, G.N. (2004) Adaptation of enzymes to temperature: searching for basic "strategies". *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **139**, 321-333.

Somers, D.E., Devlin, P.F. and Kay, S.A. (1998a) Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. *Science*, **282**, 1488-1490.

Somers, D.E., Kim, W.-Y. and Geng, R. (2004) The F-box protein ZEITLUPE confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. *The Plant Cell Online*, **16**, 769-782.

Somers, D.E., Schultz, T.F., Milnamow, M. and Kay, S.A. (2000) ZEITLUPE encodes a novel clock-associated PAS protein from Arabidopsis. *Cell*, **101**, 319-329.

Somers, D.E., Webb, A., Pearson, M. and Kay, S.A. (1998b) The short-period mutant, toc1-1, alters circadian clock regulation of multiple outputs throughout development in Arabidopsis thaliana. *Development*, **125**, 485-494.

Song, H.-R. and Noh, Y.-S. (2012) Rhythmic oscillation of histone acetylation and methylation at the Arabidopsis central clock loci. *Molecules and cells*, **34**, 279-287.

Song, S.-K., Ryu, K.H., Kang, Y.H., Song, J.H., Cho, Y.-H., Yoo, S.-D., Schiefelbein, J. and Lee, M.M. (2011) Cell fate in the Arabidopsis root epidermis is determined by competition between WEREWOLF and CAPRICE. *Plant Physiology*, **157**, 1196-1208.

Song, Y.H., Smith, R.W., To, B.J., Millar, A.J. and Imaizumi, T. (2012) FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. *Science*, **336**, 1045-1049.

Stewart, J.L., Maloof, J.N. and Nemhauser, J.L. (2011) PIF genes mediate the effect of sucrose on seedling growth dynamics. *PLoS One*, **6**, e19894.

Strasser, B., Sánchez-Lamas, M., Yanovsky, M.J., Casal, J.J. and Cerdán, P.D. (2010) Arabidopsis thaliana life without phytochromes. *Proceedings of the National Academy of Sciences*, **107**, 4776-4781.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R., Somers, D.E., Mas, P., Panda, S., Kreps, J.A. and Kay, S.A. (2000) Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog. *Science*, **289**, 768-771.

Suárez-López, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F. and Coupland, G. (2001) CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. *Nature*, **410**, 1116-1120.

Sugano, S., Andronis, C., Green, R.M., Wang, Z.-Y. and Tobin, E.M. (1998) Protein kinase CK2 interacts with and phosphorylates the Arabidopsis circadian clock-associated 1 protein. *Proceedings of the National Academy of Sciences*, **95**, 11020-11025.

Sugano, S., Andronis, C., Ong, M.S., Green, R.M. and Tobin, E.M. (1999) The protein kinase CK2 is involved in regulation of circadian rhythms in Arabidopsis. *Proceedings of the National Academy of Sciences*, **96**, 12362-12366.

Sulzman, F.M., Ellman, D., Fuller, C.A., Moore-Ede, M.C. and Wassmer, G. (1984) Neurospora circadian rhythms in space: a reexamination of the endogenous-exogenous question. *Science*, **225**, 232-234.

Teng, S., Keurentjes, J., Bentsink, L., Koornneef, M. and Smeekens, S. (2005) Sucrose-specific induction of anthocyanin biosynthesis in Arabidopsis requires the MYB75/PAP1 gene. *Plant Physiology*, **139**, 1840-1852.

Tohge, T., Watanabe, M., Hoefgen, R. and Fernie, A.R. (2013) The evolution of phenylpropanoid metabolism in the green lineage. *Critical reviews in biochemistry and molecular biology*, **48**, 123-152.

Troncoso-Ponce, M.A. and Mas, P. (2012) Newly described components and regulatory mechanisms of circadian clock function in Arabidopsis thaliana. *Molecular plant*, **5**, 545-553.

Ueda, H.R., Hagiwara, M. and Kitano, H. (2001) Robust oscillations within the interlocked feedback model of Drosophila circadian rhythm. *Journal of Theoretical Biology*, **210**, 401-406.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A. and Coupland, G. (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science*, **303**, 1003-1006.

Van Der Horst, G.T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S.-i., Takao, M., de Wit, J., Verkerk, A., Eker, A.P. and van Leenen, D. (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature*, **398**, 627-630.

Vogt, T. (2010) Phenylpropanoid biosynthesis. *Molecular plant*, **3**, 2-20.

Walker, A.R., Davison, P.A., Bolognesi-Winfield, A.C., James, C.M., Srinivasan, N., Blundell, T.L., Esch, J.J., Marks, M.D. and Gray, J.C. (1999) The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. *The Plant Cell Online*, **11**, 1337-1349.

Wang, L., Kim, J. and Somers, D.E. (2013) Transcriptional corepressor TOPLESS complexes with pseudoresponse regulator proteins and histone deacetylases to regulate circadian transcription. *Proceedings of the National Academy of Sciences*, **110**, 761-766.

Wang, X., Wu, F., Xie, Q., Wang, H., Wang, Y., Yue, Y., Gahura, O., Ma, S., Liu, L. and Cao, Y. (2012) SKIP is a component of the spliceosome linking alternative splicing and the circadian clock in Arabidopsis. *The Plant Cell Online*, **24**, 3278-3295.

Wang, Z.-Y., Kenigsbuch, D., Sun, L., Harel, E., Ong, M.S. and Tobin, E.M. (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an Arabidopsis Lhcb gene. *The Plant Cell Online*, **9**, 491-507.

Wang, Z.-Y. and Tobin, E.M. (1998) Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell*, **93**, 1207-1217.

Weger, H., Silim, S. and Guy, R. (1993) Photosynthetic acclimation to low temperature by western red cedar

seedlings. *Plant, cell & environment*, **16**, 711-717.

Weiss, D., van der Luit, A., Knegt, E., Vermeer, E., Mol, J.N. and Kooter, J.M. (1995) Identification of endogenous gibberellins in petunia flowers (induction of anthocyanin biosynthetic gene expression and the antagonistic effect of abscisic acid). *Plant Physiology*, **107**, 695-702.

Wigge, P.A. (2013) Ambient temperature signalling in plants. *Current opinion in plant biology*, **16**, 661-666.

Wijnen, H. and Young, M.W. (2006) Interplay of circadian clocks and metabolic rhythms. *Annu. Rev. Genet.*, **40**, 409-448.

Winfree, A.T. (1970) Integrated view of resetting a circadian clock. *Journal of Theoretical Biology*, **28**, 327-374.

Xie, Q., Wang, P., Liu, X., Yuan, L., Wang, L., Zhang, C., Li, Y., Xing, H., Zhi, L. and Yue, Z. (2014) LNK1 and LNK2 are transcriptional coactivators in the Arabidopsis circadian oscillator. *The Plant Cell Online*, **26**, 2843-2857.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D. and Xie, D. (2002) The SCFCOI1 ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. *The Plant Cell Online*, **14**, 1919-1935.

Yakir, E., Hilman, D., Kron, I., Hassidim, M., Melamed-Book, N. and Green, R.M. (2009) Posttranslational regulation of CIRCADIAN CLOCK ASSOCIATED1 in the circadian oscillator of Arabidopsis. *Plant Physiology*, **150**, 844-857.

Yamashino, T. (2013) From a repressilator-based circadian clock mechanism to an external coincidence model responsible for photoperiod and temperature control of plant architecture in Arabodopsis thaliana. *Bioscience, biotechnology, and biochemistry*, **77**, 10-16.

Yanovsky, M.J., Mazzella, M.A. and Casal, J.J. (2000) A quadruple photoreceptor mutant still keeps track of time. *Current Biology*, **10**, 1013-1015.

Yerushalmi, S. and Green, R.M. (2009) Evidence for the adaptive significance of circadian rhythms. *Ecology letters*, **12**, 970-981.

Yu, J.-W., Rubio, V., Lee, N.-Y., Bai, S., Lee, S.-Y., Kim, S.-S., Liu, L., Zhang, Y., Irigoyen, M.L. and Sullivan, J.A. (2008) COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability. *Molecular cell*, **32**, 617-630.

Zagotta, M., Shannon, S., Jacobs, C. and Meeks-Wagner, D.R. (1992) Early-flowering mutants of Arabidopsis thaliana. *Functional Plant Biology*, **19**, 411-418.

Zhang, X., Chen, Y., Wang, Z.Y., Chen, Z., Gu, H. and Qu, L.J. (2007) Constitutive expression of CIR1 (RVE2) affects several circadian-regulated processes and seed germination in Arabidopsis. *The Plant Journal*, **51**, 512-525.

Zhang, Y., Butelli, E. and Martin, C. (2014) Engineering anthocyanin biosynthesis in plants. *Current opinion in plant biology*, **19**, 81-90.

Zuluaga, D.L., Gonzali, S., Loreti, E., Pucciariello, C., Degl'Innocenti, E., Guidi, L., Alpi, A. and Perata, P. (2008) Arabidopsis thaliana MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants. *Functional Plant Biology*, **35**, 606-618.

Zuo, Z., Liu, H., Liu, B., Liu, X. and Lin, C. (2011) Blue light-dependent interaction of CRY2 with SPA1 regulates COP1 activity and floral initiation in Arabidopsis. *Current Biology*, **21**, 841-847.