Molecular characterization of the circadian function and the transcriptional regulatory network at the core of the pistil circadian clock

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INTRODUCTION

Introduction

1. Overview of the circadian clock function

The circadian clock is cellular mechanism found in almost all organisms. It confers 24h rhythms on processes ranging from gene expression to behavior, by synchronizing with the environmental changes caused by the rotation of earth. Environmental signals act as a zeitgeber time (ZT) (from the German for "time givers") to reset the clock every day. This synchronization is an active process called entrainment (Roenneberg et al., 2007). Circadian clocks are autonomous, producing robust 24h circadian rhythms even in the absence of daily environmental transitions (i.e., under constant light and/or temperature conditions). Circadian rhythms in the absence of the entraining signals are called free-running rhythms. A diurnal rhythm is not circadian if it does not persist under constant environmental conditions (Vitaterna et al., 2001). The circadian clock control of biological processes ensure that these processes take place at appropriate times of the day (Vitaterna et al., 2001). Disruption of the biological rhythms can impair fitness, health, and well-being of organisms (Finger and Kramer, 2021).

Conceptually, the circadian clock system consists of three functional modules: input pathways, central oscillator, and output pathways (Figure 1). The input pathways perceive and transmit environmental cues to entrain the central oscillator. Synchronizing cues include variation in light quality, light quantity, temperature and even humidity. All the external environmental cues that convey the time information to the central oscillator can be considered as input pathways. The central oscillator is composed of core clock components that reciprocally regulate each other through interlocked feedback loops (Bell-Pedersen et al., 2005). Output pathways refer to the biological processes whose rhythms are controlled by the central oscillator (Yakir et al., 2007). Thus, environmental signals perceived and transduced via input pathways are responsible for entraining the central oscillator, which generates rhythmic output pathways enabling a vast of biological processes to occur at appropriate times during the day and night cycle (Greenham and McClung, 2015).

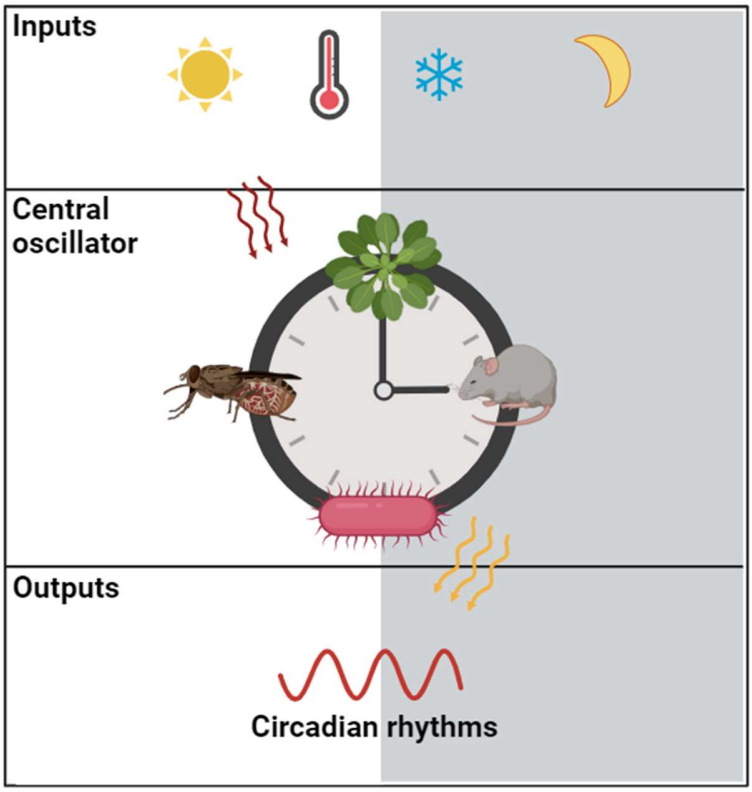


Figure 1. A simplified schematic view of the circadian clock system. Inputs (top), Central oscillator (center), and Outputs (bottom). Created by using BioRender.

Circadian rhythms are described with mathematical terms including period, phase, and amplitude (Harmer, 2009). Circadian period refers to the duration of one full cycle. Under free-running conditions, the period of circadian rhythms sustain similar (albeit not exactly) period as the entraining condition. Amplitude refers to half of the difference between the peak and trough of a given rhythm while phase refers to the state of a rhythm oscillation relative to the state of the entraining time (Parsons et al., 2020).

Plasticity of the circadian period appears to be a conserved property from mammals to plants (Azzi et al., 2017; Hotta et al., 2007; Scheer et al., 2007). The increased intensity of the light input to the oscillator shortens the period of the circadian clock under continuous light (LL) conditions (Aschoff, 1979; Devlin and Kay, 2000). In addition, higher temperature and sucrose also shorten the circadian period (Frank et al., 2018; Gould et al., 2006; Haydon et al., 2013b; Mehra et al., 2009; Salomé et al., 2010a; Shin et al., 2017).

Conversely, the circadian period is longer with lower light intensities or lower temperature (Aschoff, 1979; Devlin and Kay, 2000; Salomé et al., 2010a). Another remarkable property of the circadian system is a phenomenon known as "gating", whereby the same-strength stimuli applied at different times of the day can result in different responses. Circadian gating may enable plants to respond better to the wide range and intensities of environmental signals that they are constantly exposed to (Hotta et al., 2007). The circadian clock can also sustain robust rhythms with a period close to 24h over a broad range of physiological temperatures, which is termed temperature compensation (Gould et al., 2006). Nutritional compensation is also documented as the circadian period remains stable over different nutritional applications (Iwasaki and Kondo, 2000).

In many multicellular organisms, circadian clocks generate cell-autonomous oscillations (Doherty and Kay, 2010; Jolma et al., 2010). These cellular circadian rhythms are integrated into the tissue or organismal level to achieve coordinated physiological responses. In mammals, this has been documented as hierarchical and tissue-specific function of networked circadian clocks. The circadian clock in the suprachiasmatic nucleus (SCN) in the brain is known as a master clock, whereas clocks in peripheral tissues, such as liver, are termed peripheral oscillators. Similarly, a hierarchical multi-oscillator network orchestrates the *Arabidopsis* circadian system (Takahashi et al., 2015). The circadian clock at the shoot apex functions as a master oscillator influencing the circadian activity in roots. Studies on specific plant organs and tissues are uncovering both the circadian autonomy of some organs (Bordage et al., 2016; Endo et al., 2014; James et al., 2008; Thain et al., 2000; Thain et al., 2002; Wenden et al., 2012; Yakir et al., 2011b) as well as the coupling and coordination of rhythms within the plant (Chen et al., 2020; Endo et al., 2014; Gould et al., 2018; Greenwood et al., 2019; Takahashi et al., 2015). Therefore, the plant circadian system comprises autonomous tissue-specific rhythms complemented with cell-to-cell coupling and long-distance coordination (Nakamichi, 2020; Sorkin and Nusinow, 2021).

2. The plant circadian system

The plant circadian clock regulates a significant fraction of the transcriptome and participates in the regulation of many processes including among others, physiology,

development, and metabolism (Caldeira et al., 2014; Footitt et al., 2017; Haydon et al., 2013a; Mwimba et al., 2018a). Having an appropriate functional circadian clock confers a higher fitness and adaptive advantages to plants (Green et al., 2002). Indeed, plants matching their internal clock period with the 24h period of the environment contain more chlorophyll, fix more carbon, grow faster, and survive better than plants with clock periods differing to that of the environment (Dodd, 2005). Rather than being a single perfectly synchronized timer, the clock can be sensitive to different cues, run at different speeds, and drive distinct processes in different cell types and tissues (Greenwood and Locke, 2020). In *Arabidopsis thaliana*, two different circadian clocks are distinguished having different sensitivities to external temperature (Michael et al., 2003). Stomatal guard cells have a different period from the surrounding epidermal and mesophyll leaf cells (Yakir et al., 2011a). By comparing transcript levels in guard cells with whole plants, differences in the expression of some oscillator genes are identified (Yakir et al., 2011b), which underlies cell-specific differences in clock properties. This flexibility of the plant clock might help plants regulate such a wide range of developmental and physiological processes.

Arabidopsis thaliana, as the model plant, has been extensively used to explore the molecular mechanisms of the circadian clock function. About 30% of the *Arabidopsis* transcriptome is regulated by the circadian clock (Covington et al., 2008; Harmer et al., 2000). Circadian clocks also control a similar proportion of the transcriptome in rice (*Oryza sativa*) (Filichkin et al., 2011), papaya (*Carica papaya*) (Zdepski et al., 2008), maize (*Zea mays*) (Hayes et al., 2010; Khan et al., 2010), soybean (*Glycine max*) (Marcolino-Gomes et al., 2014), and poplar (*Populus trichocarpa*) (Filichkin et al., 2011; Hoffman et al., 2010). Currently, circadian-related studies are increasingly conducted with crops, highlighting the potential of applying the circadian clock gene variation into marker-assisted breeding programs in crops (Campoli et al., 2012; Lee et al., 2022; Li et al., 2020; Lou et al., 2011; Yang et al., 2013). The studies suggest that circadian alleles can be crucial for global adaptation of crops to a broad range of latitudes. Consistent variation in circadian period along a latitudinal gradient in annual populations of the wild plants and the selectively bred crop, provide novel evidence of natural and artificial selection for circadian performance (Greenham et al., 2017).

2.1. The *Arabidopsis* central oscillator

The *Arabidopsis* central oscillator is composed of three main interacting transcription–translation regulatory modules: the morning, the central, and the evening loops (Figure 2). The PSEUDO-RESPONSE REGULATOR known as the TIMING OF CAB EXPRESSION1 (TOC1/PRR1) and two single MYB transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), play pivotal roles at the central oscillator. CCA1 and LHY repress their own expression as well as the expression of their repressor TOC1 (Alabadí et al., 2001). TOC1 directly binds to promoters of *CCA1* and *LHY* through the CCT domain of TOC1 (Gendron et al., 2012). This core loop is further connected with other PSEUDO-RESPONSE REGULATOR family members (i.e., PRR5, PRR7 and PRR9) comprising the morning loop, as well as with the evening loop including LUX ARRHYTHMO (LUX), EARLY FLOWRING 3 (ELF3), and EARLY FLOWERING 4 (ELF4) and GIGANTEA (GI) to create the complex architecture of the plant circadian clock (Adams et al., 2015).

The PRR9, PRR7, and PRR5 proteins are expressed throughout the day, and physically associate with the *CCA1* and *LHY* promoters and repress their transcription (Nakamichi et al., 2010b). All of these loops are connected with the Evening Complex (EC), formed by ELF3, ELF4, and LUX, that directly represses *PRR9, PRR7, GI*, and *LUX* expression (Herrero et al., 2012b). In addition to CCA1 and LHY, TOC1 also represses the expression of the morning-expressed (*CCA1*, *LHY* and *PRRs*) and the evening-expressed (*LUX*, *ELF4* and *GI*) genes (Gendron et al., 2012; Huang et al., 2012). Additional key clock components are expressed at other times during the day or night ensuring smooth shapes of the oscillatory waves and fine-tuning the precision of the clock (Nohales and Kay, 2016). Altogether, the complex regulatory circadian network at the core of the clock ensures that the morning and evening clock transcripts precisely peak at their corresponding phases during the day and night cycle.

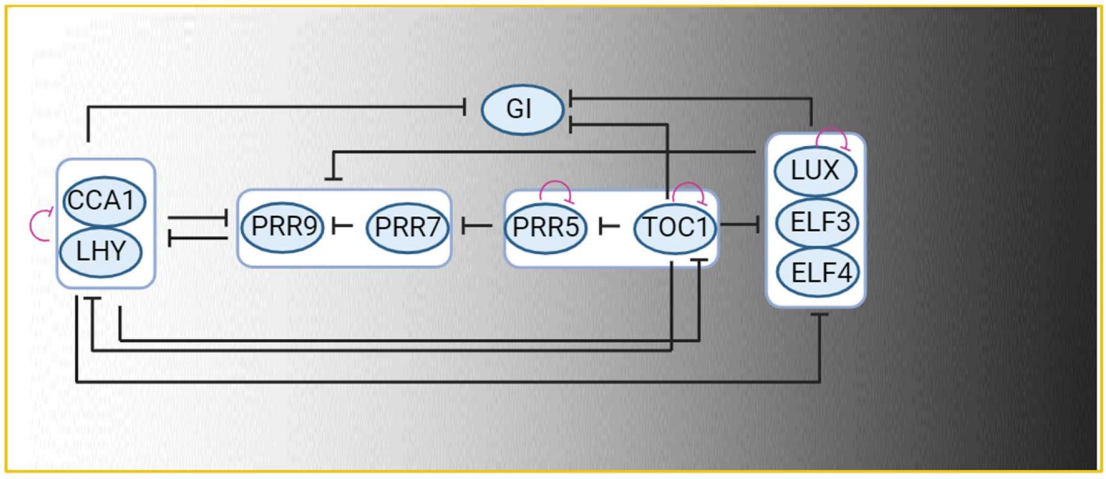


Figure 2. A schematic diagram showing the transcriptional regulatory network at the core of the clock in *Arabidopsis thaliana*. The sequential expression of each clock main component during a light-dark 24h cycle is shown from left to right. The black and red lines ending in vertical dashes represent repression of transcription. Modified from (Nohales and Kay, 2010) using BioRender.

Over-expression or mutation of the main core clock components affect the circadian rhythms altering the circadian period by the clock (Table 1). For instance, constitutive over-expression of CCA1 (CCA1-ox) abolishes circadian rhythmicity of genes expressed at different phases (Matsushika et al., 2007; Wang and Tobin, 1998). *cca1-1* mutant plants show a short-period phenotype (Alabadí et al., 2002; Green and Tobin, 1999) while the double mutants *cca1-1lhy-R* plants are unable to sustain oscillations (Alabadí et al., 2002). However, *cca1-1lhy-R* plants sustain circadian oscillations under entraining cycles, suggesting that the *Arabidopsis* circadian clock is also dependent on other clock components (Alabadí et al., 2002). Both *prr7* and *prr9* mutant plants show longer period than WT under LL conditions, whereas their circadian periods are very similar to WT under constant darkness (DD) (Farré et al., 2005). PRR7 and PRR9 play a partially redundant role in sustaining the *Arabidopsis* circadian clock as circadian rhythms in *prr79* double mutant plants display longer period than single mutants (Farré et al., 2005; Nakamichi et al., 2007). The rhythms are also severely perturbed in the *prr57* double mutant plants showing a very short circadian period with markedly reduced amplitude (Nakamichi et al., 2005) while the *prr579* triple mutant plants are arrhythmic (Nakamichi *et al.*, 2005).

Analyses of evening-expressed clock mutant plants such as the *toc1* mutant show a shorter period length under LL (Más et al., 2003; Millar et al., 1995) while over-expression of TOC1 (TOC1-ox) leads to arrhythmia (Más et al., 2003). The mutation of *elf3* also shows arrhythmia. Mutant plants lacking the other components of the EC are also arrhythmic (Doyle et al., 2002; Hazen et al., 2005) suggesting an indispensable role of these clock components sustaining circadian oscillations.

Table 1. Circadian period of *Arabidopsis* clock mutants

Genotype	Period under free-running conditions	References
elf3	No change	(Hicks et al., 1996)
prr7	No change	(Nakamichi et al., 2005)
prr59	No change	(Nakamichi et al., 2005)
cca1	Short	(Alabadí et al., 2002)
toc1	Short	(Más et al., 2003; Millar et al., 1995)
prr5	Short	(Nakamichi et al., 2005)
prr57	Short	(Nakamichi et al., 2005)
PRR9-ox	Short	(Matsushika et al., 2007b)
prr9	Long	(Nakamichi et al., 2005)
prr79	Long	(Farré et al., 2005; Nakamichi et al., 2005)
PRR7-ox	Long	(Matsushika et al., 2007b)
PRR5-ox	Long	(Matsushika et al., 2007a)
ELF3-ox	Long	(Covington et al., 2001)
CCA1-ox	Arrhythmic	(Matsushika et al., 2007b; Wang and Tobin, 1998)
elf3	Arrhythmic	(Hicks et al., 1996)
prr579	Arrhythmic	(Nakamichi et al., 2005)
lux	Arrhythmic	(Hazen et al., 2005)
TOC1-ox	Arrhythmic	(Más et al., 2003; Matsushika et al., 2007b)
cca1lhy	Arrhythmic	(Alabadí et al., 2002)

2.2. Circadian input pathways

As mentioned above, the plant circadian clock is highly sensitive to environmental stimuli and can be entrained or modulated by light, temperature, and changes in humidity (Gutiérrez et al., 2008; McClung, 2006; Mwimba et al., 2018b). Among those, light and temperature are the main central environmental stimuli entraining the circadian rhythms. Under simulated natural conditions, humidity oscillation also increases the amplitude of the circadian clock and improves plant fitness-related traits (Mwimba et al., 2018b).

2.2.1. Light sensing

As one of the central environmental stimuli entraining circadian rhythms, light has a profound impact on the circadian clock function. Light quality and light quantity change at varying rates along the day and night cycle, and these changes affect the phase and period of the clock. As mentioned above, the period of the clock decreases as the intensity of light to the oscillator increases (Aschoff, 1979). In plants growing under DD conditions, a light pulse changes the phase of the rhythms by varying degrees depending on the length and intensity of light. The time of day that the light signal is given also determines whether the circadian phase will advance or delay (Millar, 2004).

Plants have evolved a set of photoreceptors that precisely monitor the light conditions (Galvão and Fankhauser, 2015). In *Arabidopsis*, 5 groups of photoreceptors are responsible to the detection of light signals. They are phytochromes, cryptochromes, phototropins, the ZEITLUPE (ZTL) family of proteins, and UVR8 protein. These photoreceptors sense light quality and quantity (Möglich et al., 2010). Overall, phytochromes perceive red (R) and far-red (FR) light (660-730) (Franklin and Quail, 2010), cryptochromes perceive blue light and UV-A radiation (300-500nm) (Christie et al., 2015), phototropins perceive blue light (Briggs and Christie, 2002; Christie et al., 2015), the ZTL family of proteins perceive blue light (Christie et al., 2015), and the UVR8 protein perceives ultraviolet B-radiation (UV-B) (Tilbrook et al., 2013).

Photoreceptors in the phytochrome family (PHYA–E in *Arabidopsis*) detect R/FR light and transmit this information to the central circadian oscillator. A delayed phase and lower

amplitude in the rhythmic expression of *CHLOROPHYLLA/B-BING PROTEIN 2* is observed in *phyB* mutant plants (Salomé et al., 2002), while the circadian rhythms in *phyA* mutant are not reset under low fluence red or blue light (Somers et al., 1998a). PHYA and PHYB act additively in red light input to the clock (Devlin and Kay, 2000). The *OUT OF PHASE 1*(*oop1*) mutation, which is a nonsense mutation of PHYB, shows a strong defect in the perception of red light and exhibits an altered circadian phase that similar to the characteristic of *phyB* mutants (Salomé et al., 2002). A longer period is observed in the *phyCDE* triple mutant compared to wild-type (WT) plants across a range of red light fluences (Devlin, 2002; Jones et al., 2015). *phyABCDE* quintuple mutant is nearly unable to perceive red light. Under red light conditions, *phyABCDE* mutants show a shorter period at low fluence rates but show a longer period at higher red light fluence rates, which indicates that other components might also contribute to sustain circadian period by the clock under red light conditions (Hu et al., 2013). Altogether, the results indicate that phytochrome family act as photoreceptors that perceive and transmit red light to the central oscillator.

Regarding cryptochromes, CRY1 mediates high-intensity blue light input signals for period length control (Somers et al., 1998a) whereas CRY2 transmits the low-intensity blue light cues into the clock (Somers et al., 1998a). The *cry1 cry2* double mutant plants also show longer periods than the WT plants at all fluences of blue light and low fluences of red light (Devlin and Kay, 2000; Somers et al., 1998a). Consistently, different sets of photoreceptors interact to regulate the overall light input to the clock. For example, PHYA and CRY1 act together to perceive and transmit low-fluence blue light to the clock (Devlin and Kay, 2000; Somers et al., 1998a)(Somers et al., 1998; Devlin et al., 2000) while PHYB interacts with CRY2 in nuclear speckles that are important for proper control of circadian period by the clock (Más et al., 2000). Evidence for the interaction between CRYs and PHYs in regulating circadian clock input is provided by the synchronization of their expression pattern (Más et al., 2000; Tóth et al., 2001). For instance, a requirement of CRY1 for PHYA signaling to the clock in both red and blue light is identified (Devlin et al., 2000). Further studies are required to elaborate the complete molecular and functional interconnections between these two families of photoreceptors.

2.2.2. Temperature input to the clock

Temperature is another well-known input signal to the circadian clock, with the warm/cold diurnal cycles serving as environmental cues entraining the clock (Boikoglou et al., 2011; Hazen et al., 2005; McClung and Davis, 2010; McWatters et al., 2000; Michael et al., 2003; Somers et al., 1998b). Interestingly, even 4ºC fluctuations within a day can reset the clock (Somers et al., 1998b). Somehow counterintuitively, the circadian clock also shows the property known as temperature compensation, which allows the clock to sustain a stable circadian period over a broad range of physiological temperatures (McClung and Davis, 2010).

Several studies have shown that temperature signals are integrated into the circadian clock through both transcriptional and post-transcriptional mechanisms. For example, CRY1 and CRY2 differentially control circadian period and sustain rhythmicity across the physiological temperature range (Gould et al., 2013). PHYB is also found to participate in temperature sensing through its temperature-dependent reversion from the Pfr form to the Pr form (Jung et al., 2016; Legris et al., 2016). Thermal reversion provides a unique temperature sensing property of PHYB that is independent of light (Huang et al., 2019; Jung et al., 2016; Legris et al., 2016). The thermomorphogenic response is not completely lost in the *phyb* mutant but it is in the quintuple *phyabcd* mutant, suggesting that other phytochromes are also important for temperature sensing (Jung et al., 2016). Although PHYs are considered important integrators of temperature signals, whether these molecules can integrate temperature signals into circadian clock have not been explored yet. The PHYTOCHROME INTERACTING FACTOR 4 (PIF4) protein, a downstream target of PHYB (Pedmale et al., 2016), can be activated by high temperature (Proveniers and van Zanten, 2013). Meanwhile, the expression of *PIF4* is tightly regulated by the clock via the EC (Nusinow et al., 2011).

It has been proposed that PRR5, PRR7 and PRR9 are also involved temperature input to the clock (Farré *et al.*, 2005; Nakamichi *et al.*, 2005; Salome and McClung, 2005). In addition, temperature cues might intersect with the circadian clock through the EC complex, providing rhythmicity and temperature responsiveness to growth (Box et al., 2015; Mizuno

et al., 2014a; Nusinow et al., 2011). The EC complex regulates expression of *PRR9* and *PRR7* in response to a range of physiologically relevant temperatures (Mizuno et al., 2014b; Salomé et al., 2010b). Further studies show that ELF3 also functions in thermocycle entrainment independently from the other *EC* genes (Nusinow et al., 2011; Zhu et al., 2022). The temperature sensing of ELF3 is dependent on a variable length polyQ repeat region located within a prion-like domain in ELF3 protein. The length of the polyQ repeat correlates with thermal responsiveness, which is responsible for the temperature-dependent liquid-liquid phase separation of ELF3. The results show that temperature rapidly shifts ELF3 between active and inactive states (Jung et al., 2020).

2.3. Circadian output pathways

The circadian clock pervasively regulates essential biological processes in plants (Gehan et al., 2015). As mentioned above, about 30% of the *Arabidopsis* transcriptome (Covington et al., 2008; Harmer et al., 2000), 23% of the maize transcriptome (Hayes et al., 2010; Khan et al., 2010), 16% of the poplar transcriptome (Filichkin et al., 2011; Hoffman et al., 2010), 24.6% of the rice transcriptome (Filichkin et al., 2011), and 21% of the soybean transcriptome (Locke et al., 2018) are regulated by the circadian clock.

The circadian-controlling transcriptome participates in the regulation of multiple biological processes (e.g. leaf movement, hypocotyl elongation, photosynthesis, flowering time as well as petal opening) (Singh and Mas, 2018; Xu et al., 2022), through modulating cell proliferation, phytohormone signaling, cell cycle progression, reactive oxygen species homeostasis (Fung-Uceda et al., 2018; Jiménez et al., 2021; Li et al., 2019a; Yang et al., 2021).

Studies in crops have also shown the importance of the circadian clock controlling flowering time, tiller growth and panicle development in rice (Liang et al., 2021; Wang et al., 2020), as well as plant height, internode length, flowering time, seed weight, yield and stress tolerance in soybean (Cheng et al., 2019; Qin et al., 2023; Sun et al., 2023). Other examples include the regulation of anthocyanin content of tuber skin and tuberization time in potato (*Solanum tuberosum*) (Niu et al., 2022; Odgerel et al., 2022), and the heading date, plant height and grain weight in wheat (*Triticum aestivum*) (Sun et al., 2020). Recent

studies using natural accessions of tomato (*Solanum lycopersicum L.*), soybean, oilseed rape (*Brassica napus L.*) have also revealed that altering circadian timing due to natural variation in circadian clock genes could contribute to their adaptation to local environments and maximize yield (Greenham et al., 2017; Lu et al., 2017; Müller et al., 2018; Yarkhunova et al., 2016), which further emphasizes the importance of the circadian clock for its potential application to improve agricultural production.

2.3.1. Photoperiodic regulation of flowering time by the circadian clock

Flowering time, a transition from vegetative to reproductive development, is a key factor determining the yield and quality for agricultural production (Reganold and Wachter, 2016). Several regulatory pathways such as photoperiod, age, gibberellins and vernalization are involved in controlling flowering time (Peer et al., 2021). The photoperiodic regulation of flowering time is regulated by the circadian clock (Gendron and Staiger, 2023). In many plant species, flowering time largely depends on the seasonal changes in the expression of the *FLOWERING LOCUS T* (*FT*) gene, which encodes a mobile florigen that is synthesized in leaves and moves to the shoot apex to induce flowering. The mechanism of photoperiodic flowering induced by FT is well studied in *Arabidopsis thaliana*, which is a facultative long-day plant that flowers earlier under long-day than under short-day conditions. In *Arabidopsis*, high expression of *FT* induced under long-days accelerates flowering, whereas short-day conditions lead to low expression of *FT* (Kobayashi et al., 1999). The day-length-dependent induction of *FT* is governed by activator CONSTANS (CO) (Samach et al., 2000; Song et al., 2012; Valverde et al., 2004), whose expression is also controlled by the circadian clock. In addition, TEMPRANILLO (TEM) acts as a repressor that directly repress the expression of *FT*(Castillejo and Pelaz, 2008). Therefore, the expression level of *FT* is determined by the quantitative balance between TEM and CO proteins (Castillejo and Pelaz, 2008).

The length of the darkness period is a crucial factor determining the formation of plant flowers. Far-red and blue light stabilize CO protein so CO protein accumulates in the nucleus in daytime but it is degraded in the dark (Valverde et al., 2004). Under long-day conditions, the accumulation of CO under light outweigh its degradation in dark and the

gradually accumulated CO protein activates the transcription *FT* (Samach et al., 2000). In contrast, under short day conditions, the accumulation of CO protein during daytime is almost completely degraded during the night in long-day plants (Suárez-López et al., 2001). Precise clock control of the timing of *CO* expression leads to high expression of *CO* during daytime only in long-day conditions, which is critical for daylength discrimination (Yanovsky and Kay, 2002). Interestingly, the components involved in the photoperiodic regulation of flowering pathway are conserved, even in plants having different developmental responses to day-length. For instance, the CO homolog (Hd1) in rice, a typical short-day plant, also functions through the integration between the circadian clock and day-length signals. However, under long-day conditions, accumulation of Hd1 protein represses *FT* homolog (*Hd3a*) instead of activating it (Hayama et al., 2003; Kojima et al., 2002), thus delays the heading date of rice.

Some core clock components regulate the timing of flowering by modulating the accumulation of the CYCLING DOF FACTOR (CDF) family members (CDF1–CDF5) (Gendron et al., 2012; Ito et al., 2008; Nakamichi et al., 2010a; Nakamichi et al., 2007), which function as repressors of flowering time through direct repression of *CO* transcription in the morning (Imaizumi et al., 2005; Rosas et al., 2014; Sawa et al., 2007). Highly elevated *CO* expression in the morning in *cdf1cdf2cdf3cdf5* quadruple mutant cannot distinguish changes in day-length (Fornara et al., 2009). The core clock components CCA1 and LHY induce *CDF1* expression in the morning (Nakamichi et al., 2007; Schaffer et al., 1998; Wang and Tobin, 1998) while PRRs repress *CDF* transcription through the direct binding of PRR5, PRR7, and PRR9 proteins to the promoters of the *CDFs* (Gendron et al., 2012; Nakamichi et al., 2010a; Nakamichi et al., 2012).

3. Arabidopsis floral organs

The successful establishment of angiosperms on land is in part determined by their floral design. Because plants cannot move to find the ideal mate, they have developed a high variety of flowers to provide different mechanisms of pollen release, pollen transfer, and deposition of the pollen from the male (anther) to the female (pistil) sexual organs. Pollination ensures the maintenance of the species, but it is also a means to increase genetic

diversity and, with it, the potential to adapt to new environments (Sanchez et al., 2004). The position and morphology of the anthers and the pistil have often coevolved with the mode of pollen dispersal and pollen receipt, aided either by wind or by animals. Nevertheless, pollination can fail at various points during these processes, causing the extinction of rare plants and lower crop yields (Wilcock and Neiland, 2002).

When *Arabidopsis* grows to the flowering stage, the shoot apical meristem (SAM) converts into an inflorescence meristem, which produces floral meristems on its flanks in an organized pattern (Alvarez-Buylla et al., 2010). Each floral meristem produces one flower. *Arabidopsis* flowers show the typical structure of the *Brassicaceae*, which consist of four sepals, four petals, six stamens, and one gynoecium (Figure 3). The four sepals arise in the outermost whorl and these leaflike organs enclose the flower bud during its development. Four white petals arise in the second whorl, in positions that alternate with the sepals. Six stamens, which consist of a filament and an anther at the tip that produces the pollen, arise in the third whorl. The gynoecium, the female reproductive structure, contains two fused carpels, and generates from the central fourth whorl (Drews et al., 1991; Sablowski, 2015; Weigel, 1995; Wellmer et al., 2014). The two fused carpels are separated by a false septum that divide the ovary into two compartments (Herrera-Ubaldo and de Folter, 2022; Zúñiga-Mayo et al., 2019). The ovules develop into seeds after pollination.

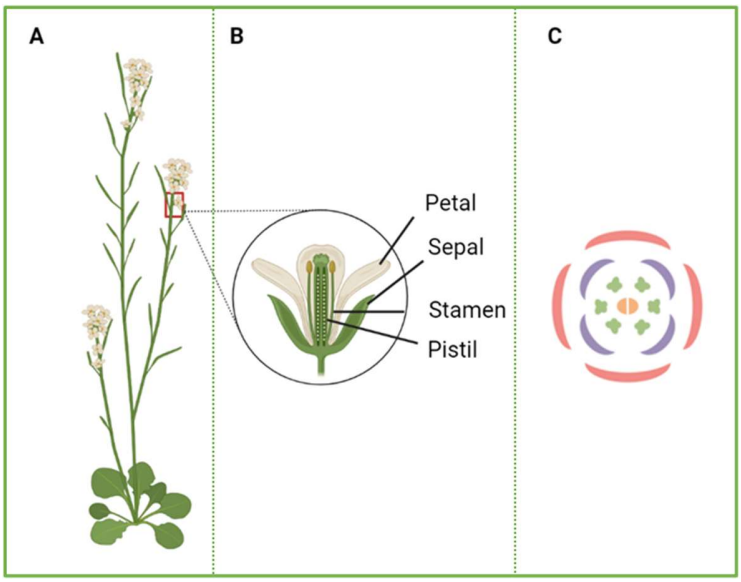


Figure 3. The *Arabidopsis* Flower. (A) Cartoon depicting an *Arabidopsis* plant at the flowering stage. (B) Mature flower at anthesis with organ types are indicated. (C) Cartoon diagram showing the relative placement of floral organs. Modified from (Irish, 2010) using BioRender..

3.1. Circadian gating of flower activities

Many plant species exhibit diurnal flower opening and closing, which is an adaptation influenced by the lifestyle of pollinators and herbivores (Kessler et al., 2010). The activity of pollinators on floral organs enable to transfer pollen to stigmas, thereby inducing plant fertilization. Since the rhythmically active properties of pollinators, movement of floral organs in many plants is gated by the circadian clock (Bai and Kawabata, 2015; Kaihara and Takimoto, 1979; Kessler et al., 2010; Muroya et al., 2021). The regulation by the circadian clock allows the synchronization of flower opening and closing with the diurnal activity of pollinators (Hasegawa et al., 2006; Kessler and Chautá, 2020; Kessler et al., 2010; Kessler et al., 2008). In *Arabidopsis*, the time duration of flower opening or closing also appears to be controlled by the circadian clock (Muroya et al., 2021). Indeed, the opening duration of WT flowers sustains around 0.7-1.4h under long-day conditions. However, the *prr975* mutant flowers remain open during the whole cycle (Muroya et al., 2021).

The coordination of light-signaling pathways and the circadian clock generates heliotropism in the common sunflower (*Helianthus annuus*) (Atamian et al., 2016). Differential elongation on opposite sides of stems confers the solar tracking in young sunflower, showing an *INDOLE-3-ACETICACID19–like* (*IAA19-like*) gene more highly expressed on the west side at night and a *SMALLAUXIN-UPREGULATED 50)–like* (*SAUR50–like*) gene, whose proteins promote cell elongation (Farquharson, 2014), more highly expressed on the east side during the day (Atamian et al., 2016). Furthermore, mature sunflowers exhibit eastward orientation to warm up the floral organs through increased morning interception of solar radiation that enhances the attractiveness to pollinators (Atamian et al., 2016).

The circadian clock may also function in the development of floral organs. The rhythmic expression of *LONG NON-CODING RNAs* (*lncRNAs*) is involved in floral development (Yadav et al., 2022). The circadian clock controls the temporal and spatial patterns of floral development in sunflower (Marshall et al., 2023). Senescence is a tightly controlled process during which nutrients in plants are remobilized from senescing organs to developing organs (Rogers and Munné-Bosch, 2016; Rogers, 2006; Schippers et al., 2015; van Doorn and Woltering, 2004). Petal senescence in rose flowers is regulated by the circadian-controlled PIF8-BBX28 module that governs mitochondrial ROS homeostasis at night (Zhang et al., 2021). These findings open the way for further exploration of the molecular mechanisms controlling the timing in flower opening and closing, floral development, and senescence of floral organs.

3.2. The *Arabidopsis* female reproductive organ

3.2.1. Pistil in Arabidopsis

Pistil is the floral female reproductive organ, which is also termed as gynoecium. At stage 6 of flower development in *Arabidopsis*, a rim in the central dome of the flower primordium grows upward to produce an oval and hollow tube, which is the pistil (Smyth et al., 1990). *Arabidopsis* pistils are composed of stigma, style, and ovary (Figure 4). The ovary of pistils is divided longitudinally into two chambers by a septum (Ferrándiz et al., 1999). The two

ovary wall regions of the ovary are valves, and the external part of the septum is the replum. At the apical end of the ovary are the style and the stigma (Balanzà et al., 2014). When the style is elongating, transmitting tissue within the hollow cylinder of the style is formed (Balanzà et al., 2014). The style is then crowned by stigmatic papillae, which functions in receiving and inducing germination of pollen grains. The pollen tubes grow through the transmitting tract and reach the ovules in the mature gynoecium (Alvarez and Smyth, 2002; Bowman et al., 1999; Roeder and Yanofsky, 2006; Sundberg and Ferrándiz, 2009).

The pollen tube is responsible for delivering the two sperm cells to the ovule, where double fertilization takes place (Johnson et al., 2019). This process requires a very complex and coordinated communication between pollen tubes and the different tissues of the pistil. After fertilization, the fruit elongates synchronically as the seed develops. Stigmatic papillae degenerate, and the valve margin are matured (Ferrándiz, 2002; Marsch-Martínez et al., 2012). Arabidopsis gynoecium is transformed into an elongated bilocular fruit called silique. Siliques open at maturity to release the seeds along four dehiscence zones defined by longitudinal furrows of smaller cells on either side of the replum. The lignification of specific cells in these zones contributes to the dehiscence process (Ferrándiz, 2002).

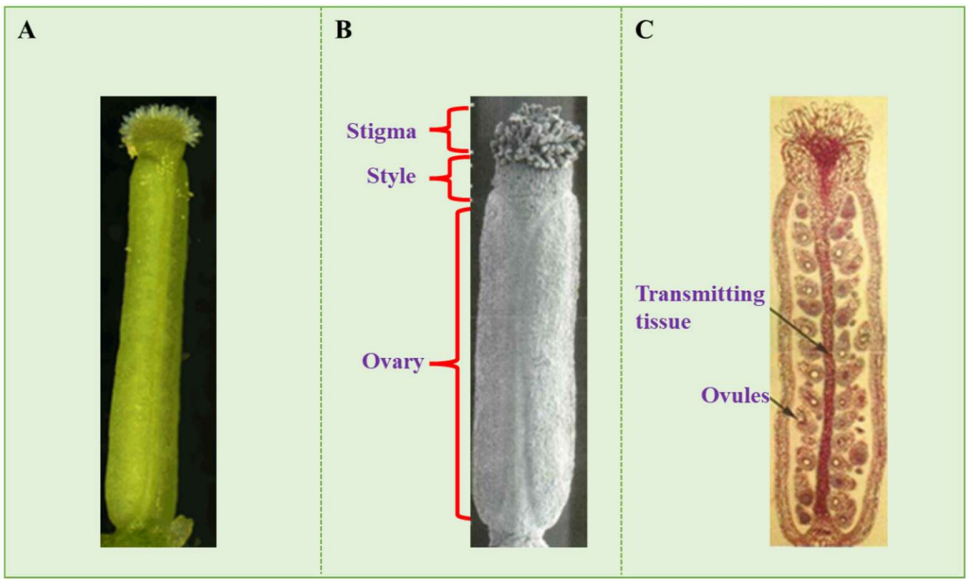


Figure 4. Pistils of *Arabidopsis*. (A) Representative photograph of a pistil. (B) Scanning electron micrographs of the *Arabidopsis* pistil. (C) Bright-field photomicrographs of longitudinal sections of the *Arabidopsis* pistil. Modified from (Gasser and Robinson-Beers,

1993).

Phytohormones play important role in the development of the carpel marginal tissues. Mutations in *YUCCA* (*YCA*) and *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS* (*TAA*) genes, involved in the auxin biosynthesis, alter gynoecium development and patterning, showing obvious defects in marginal tissues (Cheng et al., 2006; Stepanova et al., 2008). Ovules produce low IAA concentrations, and free auxin will boost in the developing embryos after fertilization (Aloni et al., 2006). Clear defects in marginal tissues are also found in plants lacking the auxin efflux transporter *PINFORMED* (*PIN*) genes (Benková et al., 2003; Okada et al., 1991), or the AUXIN RESPONSE FACTOR (ARF) transcription factors (Crawford and Yanofsky, 2011; Sessions and Zambryski, 1995).

In addition, mutations in components involved in Brassinosteroids (BRs) and Cytokinins (CKs) pathways indicate their participation in marginal tissue development. The gynoecium in plants lacking CYP85A2, a cytochrome P450 required for the BRs biosynthesis, shows split apex and horn-like protuberances (Nole-Wilson et al., 2010). Conversely, altering cytokinin catabolism of *Arabidopsis* by knock outing *CYTOKININ OXIDASE/DEHYDROGENASES* (*CKXs*) produce more seeds owing to the increased activity of the placenta that leads to more ovules (Ashikari et al., 2005; Bartrina et al., 2011). CKs affect the differentiation of the reproductive meristems cells and regulate the activity of the placenta (Bartrina et al., 2011). CKs also play important roles in fruit patterning and morphogenesis (Marsch-Martínez et al., 2012). A moderate increase in style length and stigma width is observed in the quintuple mutant of the Arabidopsis DELLA transcriptional repressors of gibberellin (GA) signaling, which may be caused by the alternation in cell elongation instead of carpel marginal tissue development (Fuentes et al., 2012). In some cases, combined mutations of phytohormones pathways are required to produce altered phenotypes, which suggests the redundant functions of genes involved in these pathways in controlling pistil development.

3.2.2 Silique and seed in *Arabidopsis*

After fertilization, pistils develop into siliques and ovules in pistils form seeds (Vivian-Smith and Koltunow, 1999). The expansion, division, and differentiation of cells form the exocarp, mesocarp, structural sclerenchyma and endocarp of the silique (Vivian-Smith and Koltunow, 1999). Silique dehisces along the replum carpel-valve boundary to release mature seeds (Meinke and Sussex, 1979). Mature seeds consist of the embryo, endosperm, and the seed coat (Demonsais et al., 2020). After double fertilization, the fertilized egg cell (zygote) divides and differentiates progressively through several stages including four cell stage, globular stage, heart stage, torpedo stage, walking-stick stage, curled cotyledon stage, and green cotyledon stage to form embryo (Becerra et al., 2006), in the process known as embryogenesis. The endosperm and seed coat arises from the differentiation of ovular integuments (Demonsais et al., 2020).

The development of siliques and seeds are highly coordinated processes that involve development of embryogenesis and endosperm, as well as the maternal of the seed coat and siliques hulls (de Folter et al., 2004; Hennig et al., 2004). Silique development in *Arabidopsis* depends on fertilization (Chaudhury et al., 1997), as the carpel of the unfertilized pistil expands slightly in length following a terminal senescence phase instead of tissue differentiation (Vivian-Smith et al., 2001). Even though the development of seed is initiated in the absence of fertilization, the seeds are abortive or inactive because of the lack of a functional embryo (Grossniklaus et al., 1998). However, developmental processes that have been previously described in developing *Arabidopsis* fruits also occur in the unfertilized pistils (Carbonell-Bejerano et al., 2010). Analyses of the *FERTILISATION INDEPENDENT SEED* (*FIS*) *Arabidopsis* mutants show that seed and fruit development are uncoupled from fertilization (Spillane et al., 2000).

3.3. The *Arabidopsis* male reproductive organ

Stamens, which are composed of a filament and an anther (Figure 5A), are subject to a specific developmental process (Ma, 2005; Regan and Moffatt, 1990; Sanders et al., 1999; Smyth et al., 1990). Pollen grains develop in the stamen and, once they mature, they are

released from the anthers and interact with the stigma. Filament connects the anther to the rest of the flower, providing physical support, water, nutrients, and signals. Before the flower opens, there is a fast filament elongation to support the anther releasing viable pollens onto the stigma where they hydrate and eventually germinate, generating the pollen tube (Edlund et al., 2004). Disruptions of stamen development will lead to male sterility (Ge et al., 2010; Sanders et al., 1999).

3.3.1. *Arabidopsis* anthers and pollen

The *Arabidopsis* anther is a bilaterally symmetrical four-lobed structure (Figure 5B) for pollen production. Each lobe develops from successive divisions of sub-epidermal archesporial cells formed in the anther primordium that give rise to three morphologically distinct cell layers, including endothecium, middle layer, and tapetum. The pollen mother cells in anther lobe undergo meiosis and thereby form the haploid microspores (Sanders et al., 1999). Mature pollens are released along with the process of anther dehiscence, which is essential to ensure the proper fertilization. Anther dehiscence involves a switch of anther cells from cell differentiation to cell degeneration program. The process of anther dehiscence has three steps (Figure 5C): (i) lignification of endothecium cells; (ii) degradation of septum cells leading to a bi-locular anther; and (iii) rupture of the stomium, which is made from modified epidermal cells (Goldberg et al., 1993). Endothecium lignification is necessary for anther breakage because lignification of endothecium generates the tension which is responsible for stomium breakage (Bonner and Dickinson, 1989; Keijzer, 1987; Mitsuda et al., 2005; Yang et al., 2007).

Inside the anthers, sporogenous, the reproductive cell that is also named as pollen mother cell, finally develops into pollen. The development of pollen grain includes microsporogenesis and microgametogenesis stages. During microsporogenesis stage, the pollen mother cells firstly generate tetrads of haploid microspores by meiosis (Borg et al., 2009; Sanders et al., 1999). Microsporogenesis stage is completed when individual microspores are released after the degeneration of the callose wall surrounding tetrads (Borg et al., 2009; Sanders et al., 1999). Microspores further go through two rounds of mitotic divisions. Firstly, the microspore divides asymmetrically to produce a large

vegetative and a small generative cell. Then, a second mitosis produces two twin sperm cells enabling double fertilization to produce the embryo and endosperm. The non-reproductive cells in anther differentiate into tapetum, which is one of the specialized tissue layers that surround the pollen mother cell (Sanders et al., 1999; Scott et al., 2004). Tapetum provides essential nutrients and structural components for microspore expansion. The programmed cell death (PCD) of tapetum as the pollen matures emits tapetal contents to help the formation of a pollen coat (Sanders et al., 1999). Defects in tapetum result in abnormal pollen formation that causes male sterility (Ye et al., 2010).

3.3.2. *Arabidopsis* filaments

The filament has a simple radicalized structure with a single vascular strand, through which conduct water and nutrients to the anther (Cardarelli and Cecchetti, 2014). Filaments also provide structural support to the anther and anchors the stamen to the receptacle that the floral organs are attached to (Cardarelli and Cecchetti, 2014). The differentiation of filaments and anthers emerge around at stage 7 of flower development (Smyth et al., 1990). The filaments exhibit fast elongation during stages 10 to stage13 of flower development, which is coordinated with anther dehiscence (Cardarelli and Cecchetti, 2014; Smyth et al., 1990). Under continue light conditions, the growth of filament between stage 10 to stage 13 consists of three exponential phases (Tashiro et al., 2009). During the second exponential phase, the growth rate of stamen filaments is about 10 times greater than the growth rates in the other two phases (Tashiro et al., 2009).

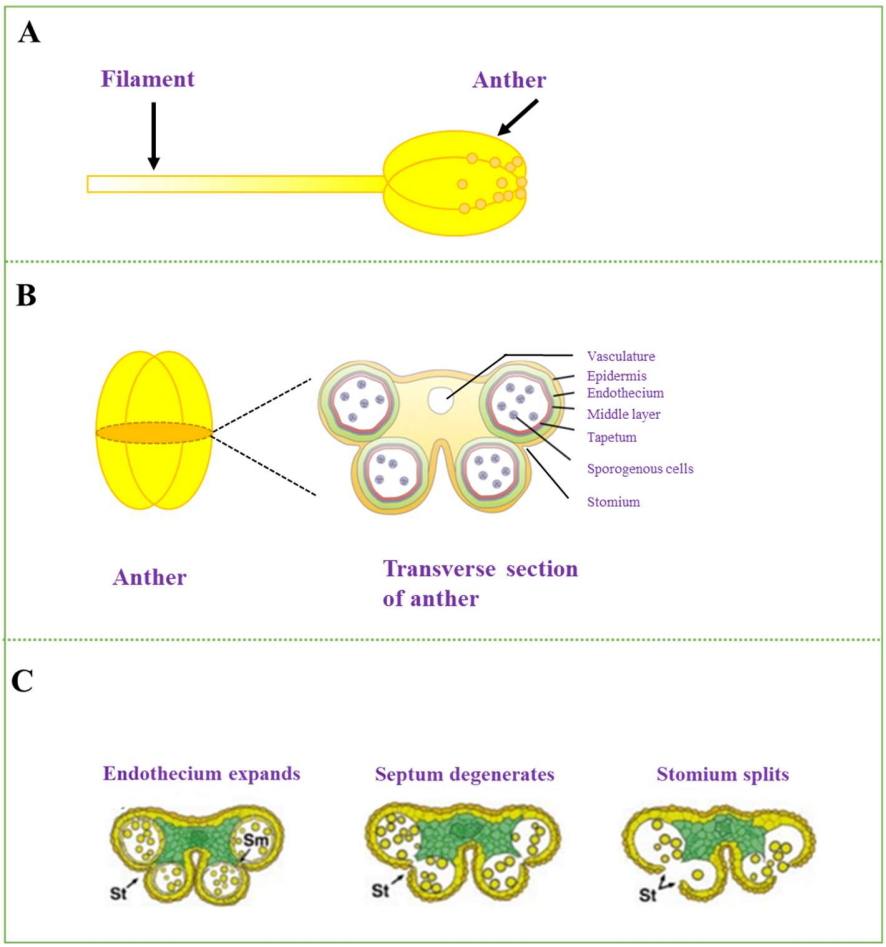


Figure 5. The *Arabidopsis* male reproductive organ. (A) A mature stamen including one filament and one anther. (B) Cartoon of a stamen at a stage after meiosis (left) and of the stamen transverse section, at the level of the anther, with differentiated tissues indicated in different colors (right). (C) Transverse sections of late anther development stages that represent key steps in the anther dehiscence program. St, Stomium. Modified from (Dinneny et al., 2004; Wilson et al., 2011; Sanders et al., 2000).

3.4. Coordination between pistil and stamen for fertilization

Precisely coordinated growth of stamens and pistils determines the fertility of *Arabidopsis* (Song et al., 2014; Tashiro et al., 2009). Fertilization involves a continuous and active communication between female and male tissues over several steps: pollen hydration, pollen germination, pollen tube growth, pollen tube attraction to the ovule, pollen tube

reception, sperm cell delivery and gamete activation (Cascallares et al., 2020). Appropriate stage of stigma development is crucial for receptivity. For example, on immature stigmas of pear flowers, mature pollen can adhere but do not hydrate and germinate. On a degenerating stigma, pollen can adhere, hydrate, and germinate, but pollen tube growth arrests abruptly (Sanzol et al., 2003).

The coordinated elongation between the stamen filament and pistil is also required for self-pollination (Cecchetti et al., 2008; Nagpal et al., 2005). Pistil growth consists of a single exponential growth, while stamen growth consists of three exponential phases. During the second exponential phase, the growth rate of stamen filaments is much higher than the growth rates in the other two phases. Consequently, stamens finally draw level with pistils or exceed pistils in length, thus supporting the release of pollen grains on the stigma (Tashiro et al., 2009).

The dialogue between pollen and pistil is critical in the earlier stages because the desiccated pollen grain on the stigma needs water and other components required for hydration, germination, and pollen tube entry into the stigma barrier (Doucet et al., 2016; Johnson et al., 2019; Rozier et al., 2020). Subsequently, the pollen tube elongates through the densely packed tissue of the female reproductive to the adjacent of an unfertilized ovule (Elleman et al., 1992; Johnson et al., 2019). These processes require the tight communication between the pollen grain/tube and the surrounding pistil tissues to confer compatible pollen grains germination and the penetration in pistil barriers, as well as precise pollen tube navigation that would eventually lead to the release of the sperm cells in the ovule.

OBJECTIVES

Objectives

The general aim of this Doctoral Thesis is to **elucidate the circadian function** and the **transcriptional network** at the core of the **pistil clock**. We also aim to identify the role of the circadian **clock** controlling **pistil and stamen growth and seed production**. This general objective will be accomplished through different specific aims:

1. **To understand the circadian function in buds and flowers.** We will perform bioluminescence analyses using core clock reporter lines to analyze the phase, period, and amplitude of circadian rhythms in buds and flowers excised from plants at different developmental stages.

2. **To examine circadian rhythms in different floral organs.** We will perform bioluminescence analyses using core clock reporter lines to analyze the phase, period, and amplitude of circadian rhythms in sepals, petals, stamens, and pistils excised from the flower.

3. **To elucidate the role of the circadian clock in the control of pistil growth and seed production.** We will use a battery of clock mutants and over-expressing lines to examine if proper circadian function is important in the control of pistil elongation and plant productivity.

4. **To uncover the transcriptional regulatory network at the core of the pistil clock.** We will use a battery of clock mutants and over-expressing lines to examine clock gene expression by RT-QPCR to identify organ-specific transcriptional regulatory functions of the pistil clock.

5. **To identify functional hierarchies within the repressive modules at the core of the pistil clock.** We will perform genetic interaction studies with plants over-expressing a clock component in a mutant background of another clock gene expressed at a similar phase. We will examine their repressive regulatory function on target clock genes and identify the dominance of their repressive function.

6. **To examine the developmental and circadian coordination of pistil and stamen growth.** We will use clock mutants and over-expressing lines to examine the effect of light: dark cycles and the circadian clock on the coordination of pistil and stamen growth. We will also examine the developmental changes of pistil and stamen growth.

RESULTS

Results

1. Self-sustained circadian rhythms in detached buds and flowers

To investigate the circadian function in floral organs, we first performed bioluminescence analyses of plants expressing the morning-phased (*CCA1*) and the evening-phased *TOC1* and *GIGANTEA* (*GI*) gene promoters fused to the LUCIFERASE (*LUC*). Samples from plants synchronized under light: dark (16h: 8h) cycles at 22 °C were transferred to 96-well plates and released into continuous light (LL) at 22 °C. Bioluminescent rhythms of promoter activities in buds and flowers at different developmental stages (Figures 5A-D) were examined in a luminometer. The waveforms of *CCA1* promoter activity displayed robust circadian rhythms with high amplitude in young and mature buds (Figure 6A, C). Similarly, young buds also sustained high-amplitude and robust circadian rhythms of *TOC1::LUC* and *GI::LUC* with circadian periods close to 24-hour (Figure 6B, C).

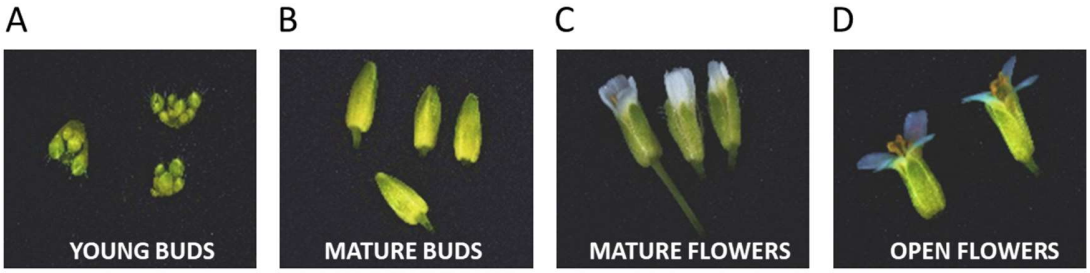


Figure 5. Representative photographs of buds and flowers at different developmental stages. (A) Young buds, (B) Mature buds, (C) Mature flowers, (D) Open flowers.

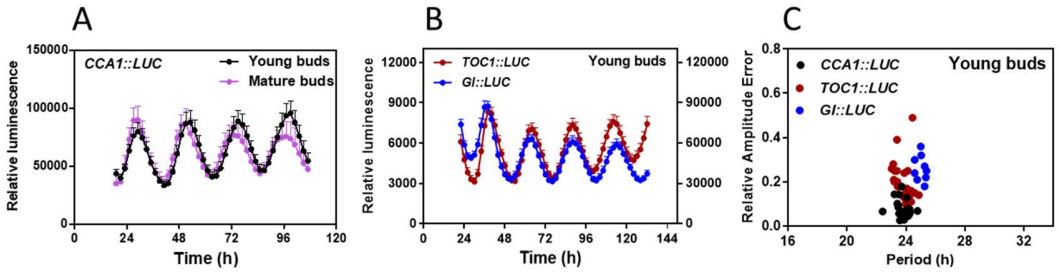


Figure 6. Self-sustained circadian rhythms in buds. (A) Luminescence of *CCA1*::luciferase (*LUC*) rhythms simultaneously measured in young buds (*n=16*) and mature buds (*n=4*). (B) Luminescence of *TOC1::LUC* (left axis) (*n=30*) and *GI::LUC* (right axis) (*n=14*) rhythms measured in young buds. (C) Period, and relative amplitude error estimates of *CCA1::LUC* (*n=16*), *TOC::LUC* (*n=24*) and *GI::LUC* (*n=9*) in young buds. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

Circadian rhythms of *CCA1* promoter activity were also analyzed in mature and open flowers under both free-running (LL) and entraining (light: dark, LD) conditions. The analyses showed that *CCA1:LUC* in mature and open flowers sustained circadian rhythms under both conditions (Figure 7A, B). Consistent with the bioluminescence results, the rhythmic messenger RNA (mRNA) accumulation assayed by RT-qPCR (reverse transcription-quantitative polymerase chain reaction) confirmed the circadian expression of *CCA1* in open flowers (Figure 7C). Analyses of the evening-phased reporter *GI::LUC* in mature and open flowers also showed high-amplitude rhythms under LL (Figures 8A-B). Altogether, our results showed that buds and flowers detached from the plant are able to autonomously sustain circadian rhythms.

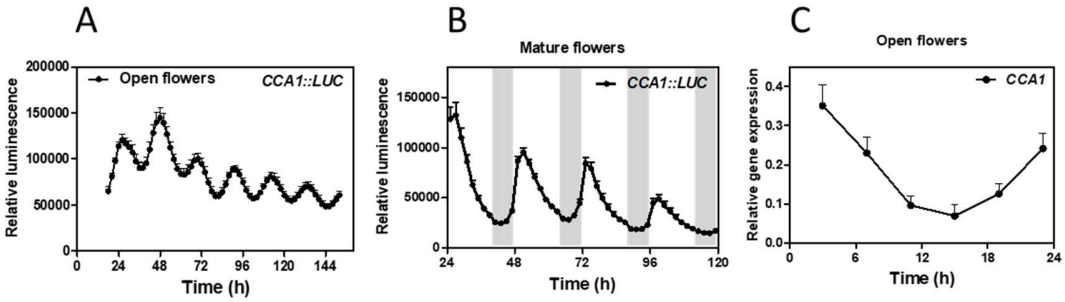


Figure 7. Self-sustained circadian rhythms in flowers. (A) Luminescence of *CCA1::LUC* oscillation measured in open flowers *(n=15).* (B) *CCA1:LUC* rhythms measured in mature flowers *(n=16)* under entraining light: dark cycles (16h light: 8h dark). (C) Circadian time-course analyses of *CCA1* mRNA expression in wild-type (WT) open flowers. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

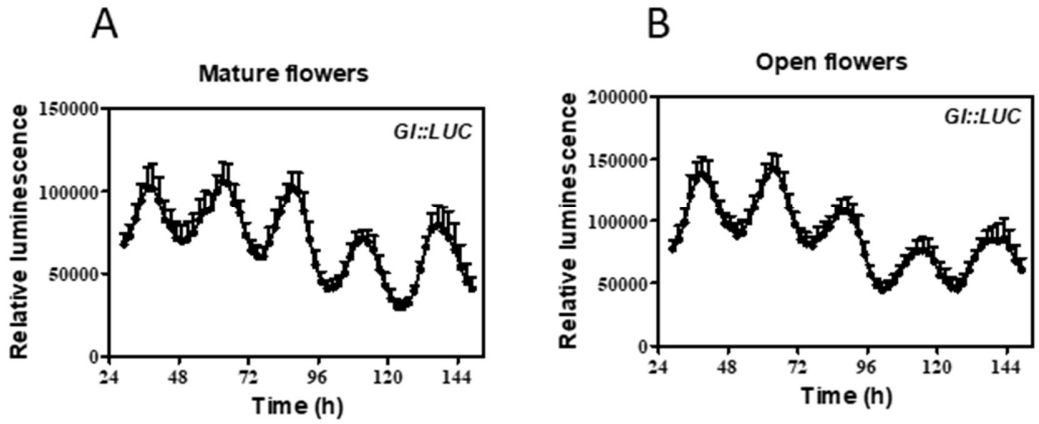


Figure 8. Circadian rhythms in WT flowers. (A) Luminescence of *GI::LUC* oscillation measured in mature flowers (n=6). (B) Luminescence of *GI::LUC* oscillation measured in open flowers (n=6). Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

2. Detached pistils show precise and robust rhythms

We next examined the circadian oscillation of *CCA1::LUC* in sepals, petals, stamens, and pistils excised from the flower (Figure 9A-D). In WT sepals, bioluminescence rhythms were robustly sustained, albeit with a shorter period than 24 h (Figure 10A, D) (Okada et al., 2022). Rhythms in WT petals and stamens also showed short circadian periods for 3 or 4 days, dampening low afterward (Figure 10B, C, E, F) (Okada et al., 2022). In contrast, the circadian waveforms in pistils robustly oscillated for more than 5 days, with a circadian period close to 24 h (Figure 10A-F) (Okada et al., 2022). Similarly, *GI::LUC* in pistils also sustained high-amplitude robust circadian rhythms (Figure 11A) whereas rhythms of *GI::LUC* in stamens were sustained for few days, dampening low afterwards (Figure 11B). *PRR9::LUC* also exhibited robust circadian rhythms in pistils and dampening rhythms in stamens (Figure 11C).

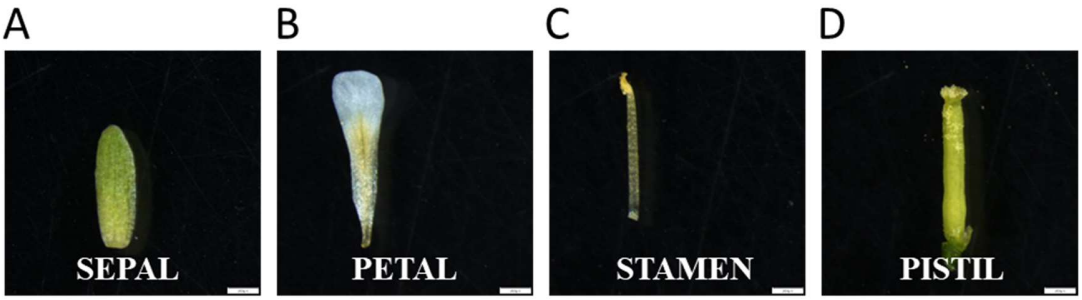


Figure 9. Representative photographs of floral organs from open flowers. (A) sepal, (B) petal, (C) stamen, and (D) pistil.

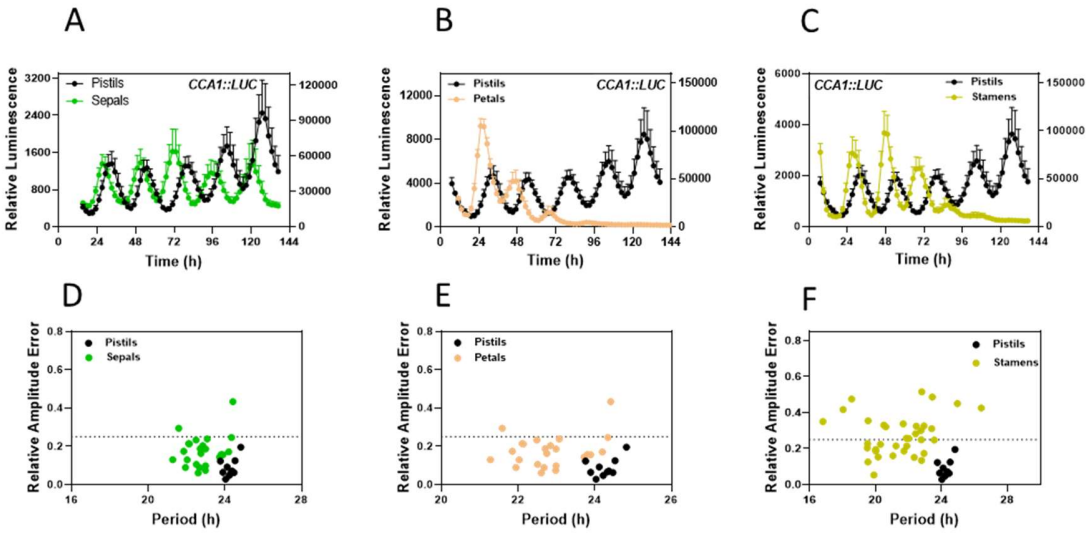


Figure 10. Robust circadian oscillations in pistils. Luminescence of *CCA1*::*LUC* rhythms simultaneously measured in pistils (right axis) and sepals (left axis) (A), petals (left axis) (B), stamen (left axis) (C). Period, and relative amplitude error estimates of *CCA1::LUC* in pistils and sepals (D), petals (E), stamen (F). Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment (This work was done by Dr. Okada).

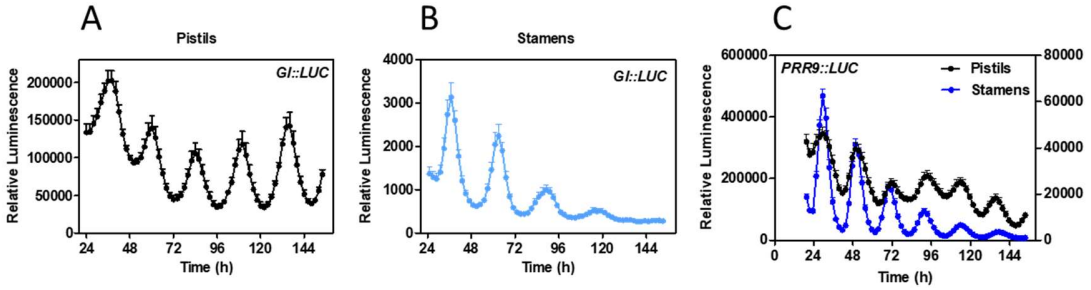


Figure 11. Robust circadian oscillations in pistils. (A) Luminescence of *GI*::*LUC* rhythms measured in pistils (*n=7*). (B) Luminescence of *GI*::*LUC* rhythms measured in stamens (*n=10*). (C) Comparative waveform analyses of *PRR9::LUC* rhythms in WT pistils (left axis) (*n=8*) and stamens (right axis) (*n=16*). Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

CCA1::LUC rhythms in *toc1-2* sepals were similar to those observed in WT sepals for the first 3 days, albeit with slightly reduced amplitude in *toc1-2* (Figure 12A, B). Thus, the characteristic short-period phenotype of *toc1-2* observed in seedlings (Más et al., 2003) and buds was only evident in sepals after several days under LL (Figure 12A, B). We observed a similar trend in *toc1-2* petals and stamens, although the dampened rhythms precluded a clear view of the possible period shortening over time (Figure 12C-F). Analyses of rhythms at early time points before dampening showed that the circadian period of *toc1-2* sepals and petals was not significantly different from WT, whereas the circadian period of *toc1-2* stamens was significantly longer than WT. In pistils, the short-period oscillation observed during the first day rapidly transitioned to very-low-amplitude rhythms (Figure 12G, H), following a similar trend to that observed in whole flowers (Okada et al., 2022). The comparative analyses of rhythms in the different floral organs revealed the organ-specific behavior of *toc1-2* mutant.

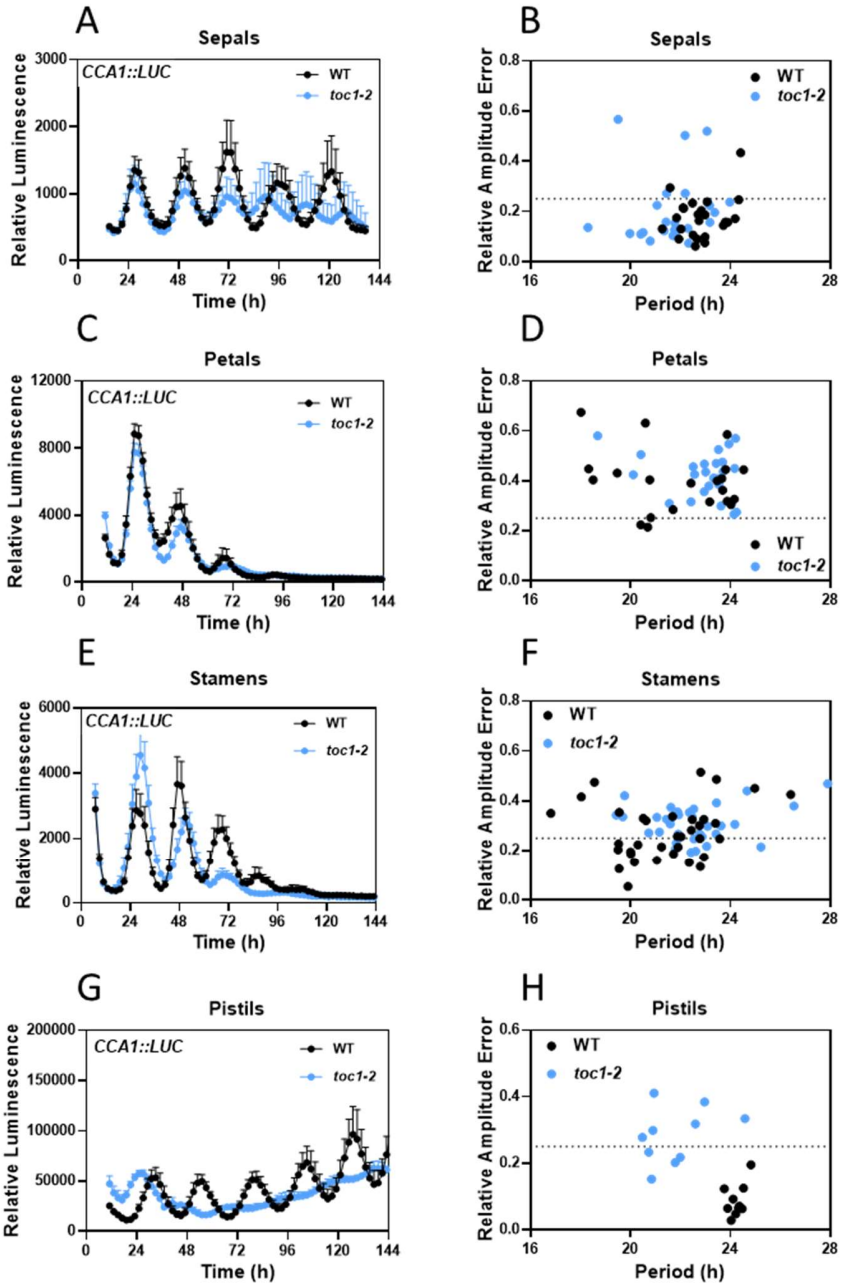


Figure 12. Distinct phenotypes of *toc1-2* mutant in sepals, petals, stamens, and pistils. In vivo luminescence assays of *CCA1::LUC* rhythms in WT and *toc1-2* (A) sepals, (C) petals, (E) stamens, and (G) pistils. Period and relative amplitude error estimates of *CCA1::LUC* rhythms in WT and *toc1-2* (B) sepals, (D) petals, (F) stamens, and (H) pistils. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment (This work was done by Dr. Okada).

3. The circadian clock controls pistil growth and seed production

To explore the physiological role of the circadian clock in the regulation of pistil function, we used plants over-expressing CCA1 (CCA1-ox) in which the clock does not run properly. Our analyses showed that pistil length of CCA1-ox plants examined at different stages of development were significantly shorter than those of WT (Figure 13A). The number of siliques per plant was also significantly reduced in CCA1-ox plants compared to WT (Figure 13B). The results indicate that the circadian clock affects pistil growth and development. CCA1-ox plants also showed shorter siliques (Figure 14A), less seeds per silique (Figure 14B), and smaller seed size, including smaller sectional area and smaller seed weight than WT plants (Figure 15A-C). The inspection of seeds in siliques also suggested developmental defects, with an increased number of abortive ovules in CCA1-ox (Figure 14C).

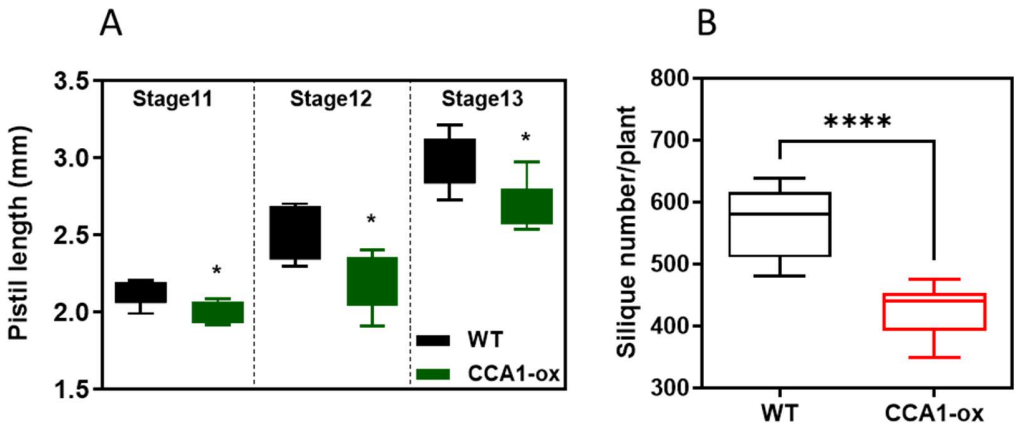


Figure 13. Over-expression of *CCA1* affects pistil elongation and silique productions. (A) analysis of pistil lengths in WT and CCA1-ox (n=6). (B) Analysis of silique number per plant of WT and CCA1-ox (n=10). Two-tailed Student's t-test analyses were performed using the GraphPad Prism software. Data is presented as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

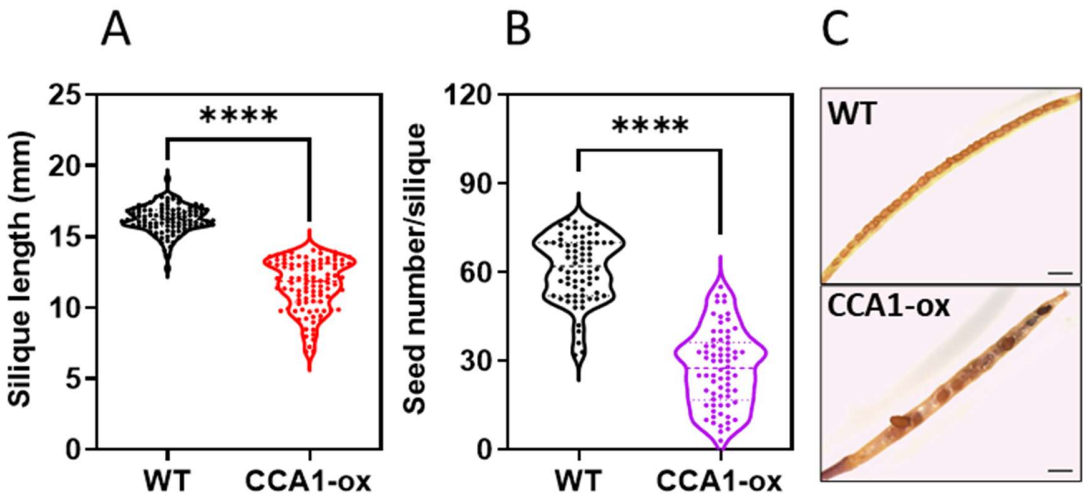


Figure 14. Over-expression of circadian *CCA1* affected seeds production. (A) Analysis of silique lengths in WT and CCA1-ox (n=100). (B) Analysis of seed number per silique of WT and CCA1-ox (n=100). (C) Representative photographs of mature silique from WT and CCA1-ox. Two-tailed Student's t-test analyses were performed using the GraphPad Prism software. Data is presented as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

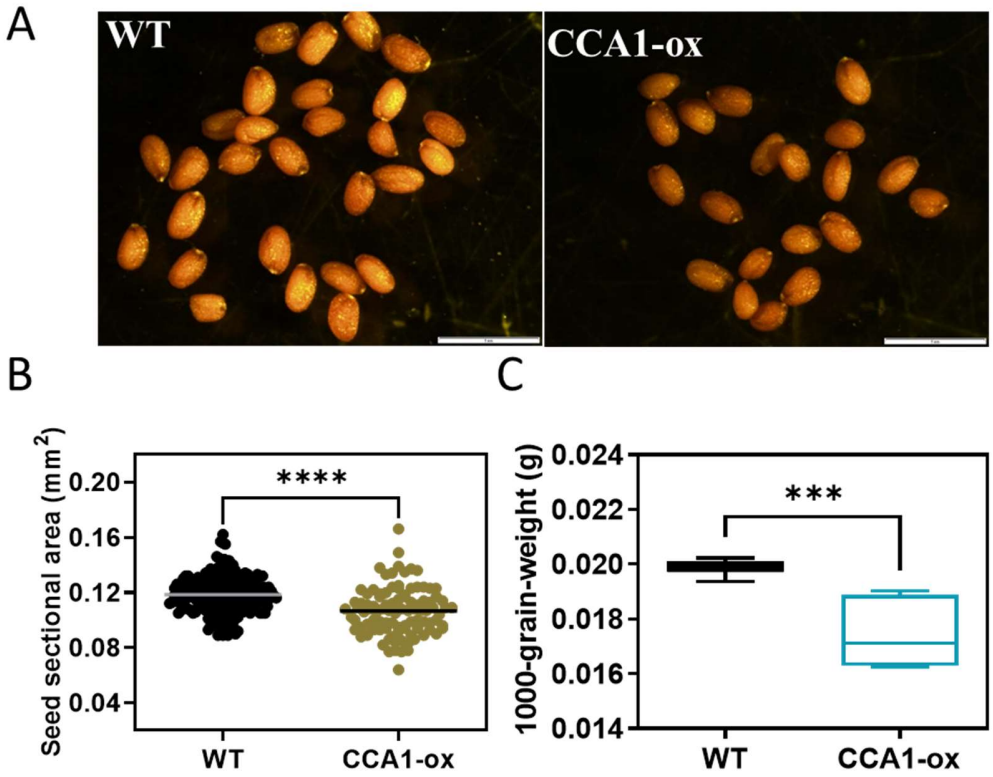


Figure 15. Altered expression of circadian genes affects seed size. (A) Representative photographs of full developed seeds from WT and CCA1-ox. (B) Analysis of seed sectional area in WT and CCA1-ox (n=100). (C) Analysis of seed weight of WT and CCA1-ox (n=8). Two-tailed Student's t-test analyses were performed using the GraphPad Prism software. Data is presented as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

To examine whether the effects are restricted to CCA-ox, or the circadian function is overall important for pistil growth and seed production, we examined mutant plants of different clock components in which the clock is still running, although at a faster or slower pace than in WT. Our analyses showed that pistil length of the *prr* mutant plants was also shortened, particularly in double mutants, displaying significantly reduced pistil length compared with that observed in WT (Figure 16A). Mutation and over-expression of *TOC1* led to reduced and increased pistil length, respectively, and the phenotypes were sustained at different stages of pistil development (Figure 16B). The gradual increase in pistil length

observed in the *prr* mutant plants correlated well with a gradual increment in silique length and seed weight (Figure 17A, B). Altogether, the results indicate that proper function of the circadian clock is important for pistil and silique growth as well as for seed weight and production.

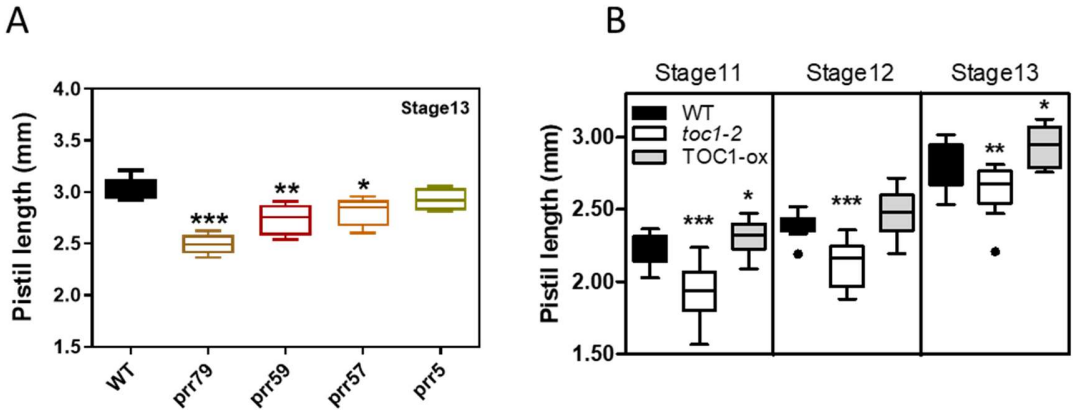


Figure 16. Altered expression of circadian genes affected pistil elongation and seeds production. (A) Analysis of pistil lengths in WT, *toc1-2* and TOC1-ox (n=6). (B) Analysis of pistil lengths in different *Arabidopsis* lines (n=6). Two-tailed Student's t-test analyses were performed using the GraphPad Prism software. Data is presented as mean ±SEM. (*** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

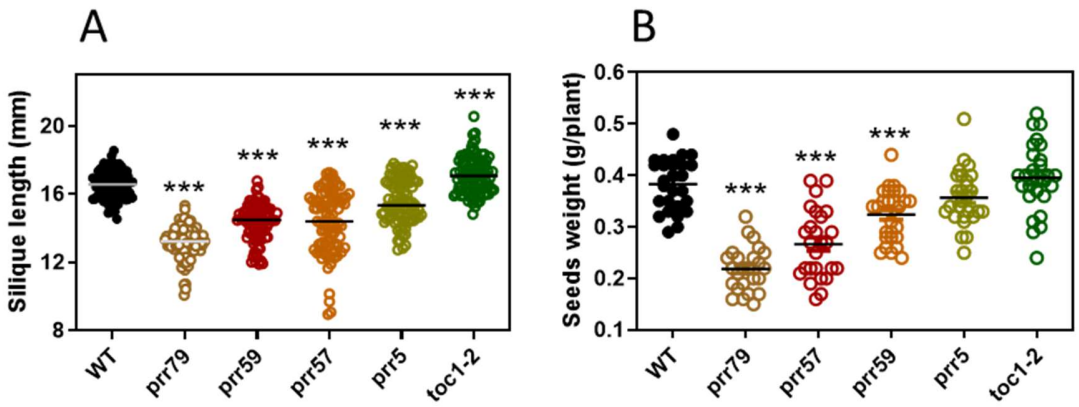


Figure 17. Altered expression of circadian genes affected silique length and seeds production. (A) Analysis of silique lengths in different *Arabidopsis* lines (n=100). (B) Analysis of seed weight per plant in different *Arabidopsis* lines (n=8). Two-tailed Student's t-test analyses were performed using the GraphPad Prism software. Data is presented as mean ±SEM. (*** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

4. Transcriptional regulatory network at the core of the oscillator in pistils

To understand the circadian regulatory network in pistils, we analyzed clock gene expression of clock mutants and over-expressing lines and performed chromatin immunoprecipitation (ChIP) assays of key clock components. Similar to seedlings, the over-expression of *CCA1* down-regulated the expression of *TOC1* and the *EC* genes (Figure 18A-C), suggesting that CCA1 acts as repressor of evening-expressed clock genes in pistils. Repression might occur through the direct binding of CCA1 to the gene promoters as showed by ChIP assays in pistils (Figure 18D). Moreover, *cca1*/*lhy* double mutants led to a marked down-regulation of *PRR7* and *PRR9* expression in pistils (Okada et al., 2022). In turn, an up-regulation of *CCA1* expression was observed in the *prr79* mutant (Figure 19A), suggesting a direct repression of *CCA1* by PRR9 and PRR7, similar to previous results in seedlings (Nakamichi, 2020). The evening-expressed genes were up-regulated during the subjective day but down-regulated during the subjective night in the

prr79 mutant (Figure 19B-E) suggesting a complex direct and indirect regulation during the subjective day and subjective night.

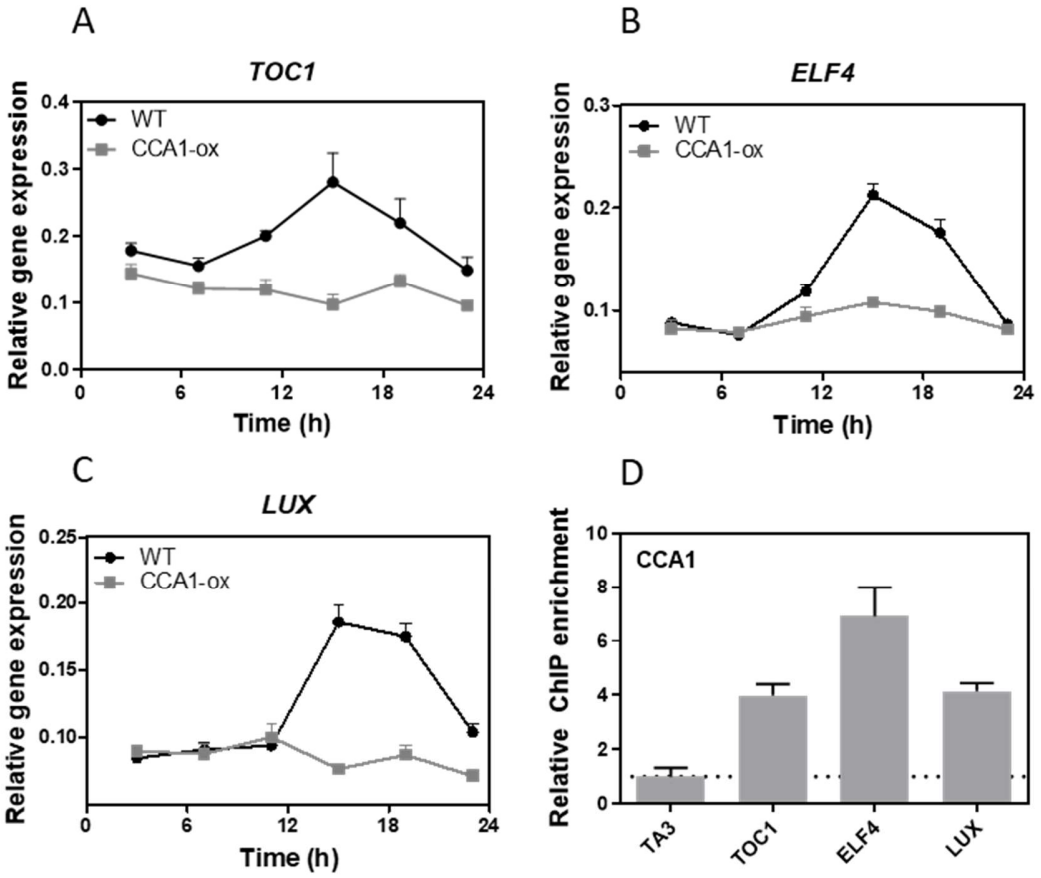


Figure 18. Regulatory network at the core of the pistil oscillator in clock mutants. Time-course analyses of *TOC1* mRNA expression (A), *ELF4* mRNA expression (B), and *LUX* mRNA expression (C) in WT and CCA1-ox pistils. (D) ChIP assays were performed with CCA1::CCA1-YFP pistils collected at ZT3. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

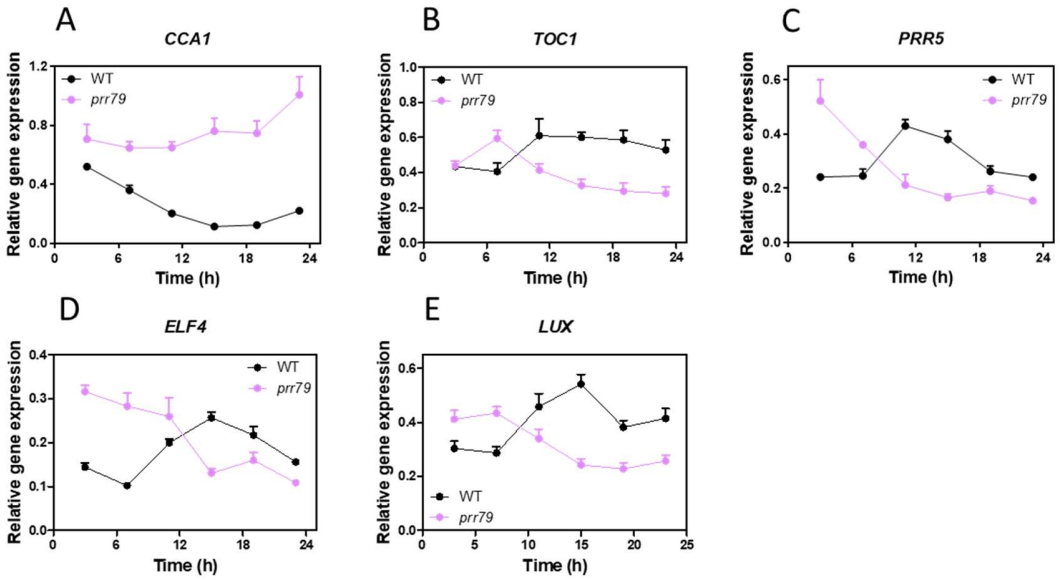


Figure 19. Regulatory network at the core of the pistil oscillator in clock mutants. Time-course analyses of *CCA1* mRNA expression (A), *TOC1* mRNA expression (B), *PRR5* mRNA expression (C), *ELF4* mRNA expression (D), and *LUX* mRNA expression (E) in WT and *prr79* pistils. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

Over-expression of the EC component *ELF3* led to down-regulation of the morning genes *PRR9* and *PRR7* (Figure 20A, B) but up-regulation of *CCA1* (Figure 20C) suggesting that in pistils ELF3 represses the expression of *PRR9* and *PRR7* and, directly or indirectly, activates the expression of *CCA1*, as in seedlings (Chow et al., 2012). However, and contrarily to seedlings and roots, the mutation of the EC component *ELF4* did not lead to a relevant activation of *PRR9* expression (Figure 20D), which suggests that ELF4 might not be part of the EC in the repression of *PRR9* or that an additional function of ELF4 overcomes its EC-dependent regulation of *PRR9*. Consistent with the gene expression results, the ChIP assays showed a significant binding of ELF3 and LUX to the promoters of the *PRR9* and *PRR7* genes in pistils, whereas ELF4 was not significantly enriched on these promoter regions of the *PRR9* and *PRR7* genes (Figure 21A, B). Analyses of the *toc1-2* mutation led to a reduced accumulation of *CCA1* (Figure 22A) but an increased expression of *PRR7* (Figure 22B). The evening-expressed genes were up-regulated in

pistils of *toc1-2* mutant (Figure 22C, D), similarly to the expression patterns observed in the *ec* mutants (Figure 20D).

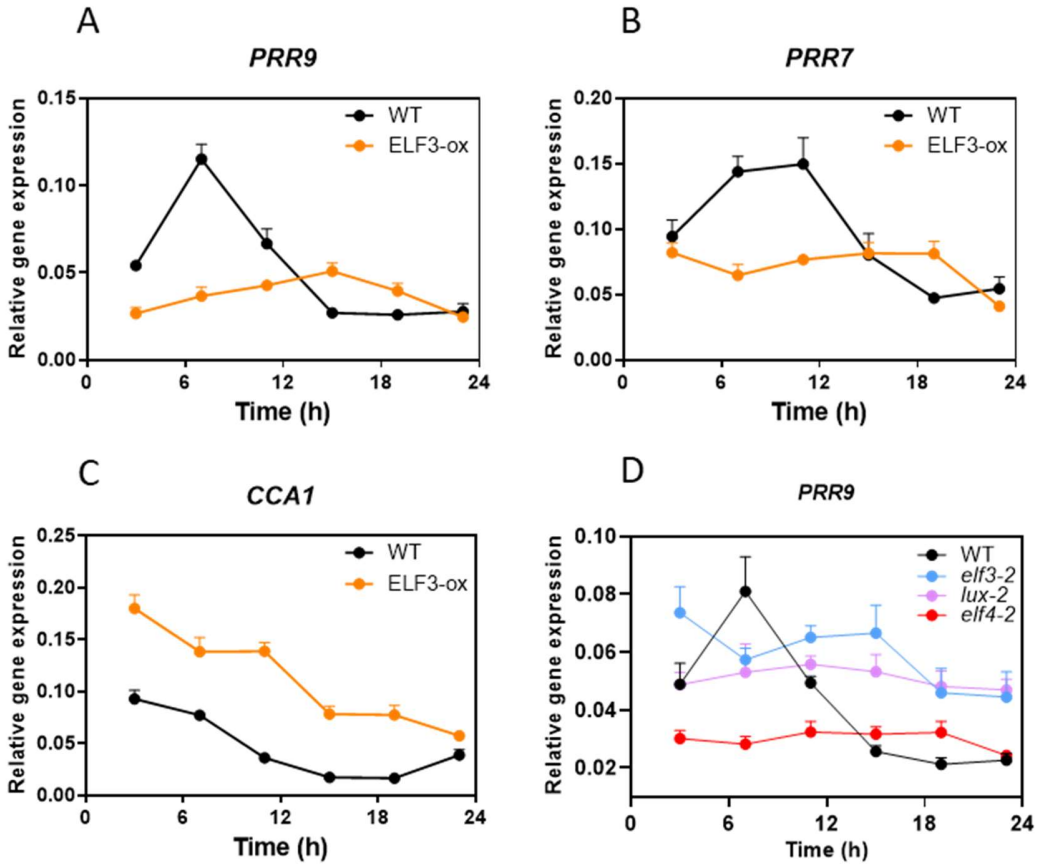


Figure 20. Regulatory network at the core of the pistil oscillator in ELF3 overexpressing line. Time-course analyses of *PRR9* mRNA expression (A), *PRR7* mRNA expression (B), and *CCA1* mRNA expression (C) in WT and ELF3-ox pistils. (D) Time-course analyses of *PRR9* mRNA expression in WT, *elf3-2*, *lux-2*, and *elf4-2* pistils (Okada et al., 2022). Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

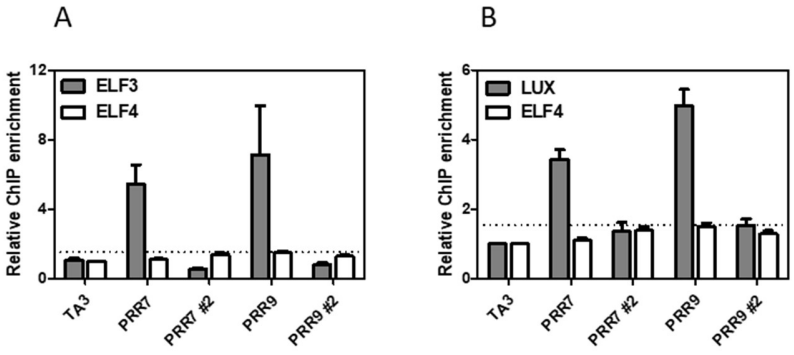


Figure 21. Different enrichment of EC complex components in the promoters of PRRs. (A) Comparative analyses ChIP assays that performed with ELF3-ox-YFP and ELF4-ox-YFP pistils collected at ZT15. (B) Comparative analyses ChIP assays that performed with LUX::LUX-YFP and ELF4-ox-YFP pistils collected at ZT15. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

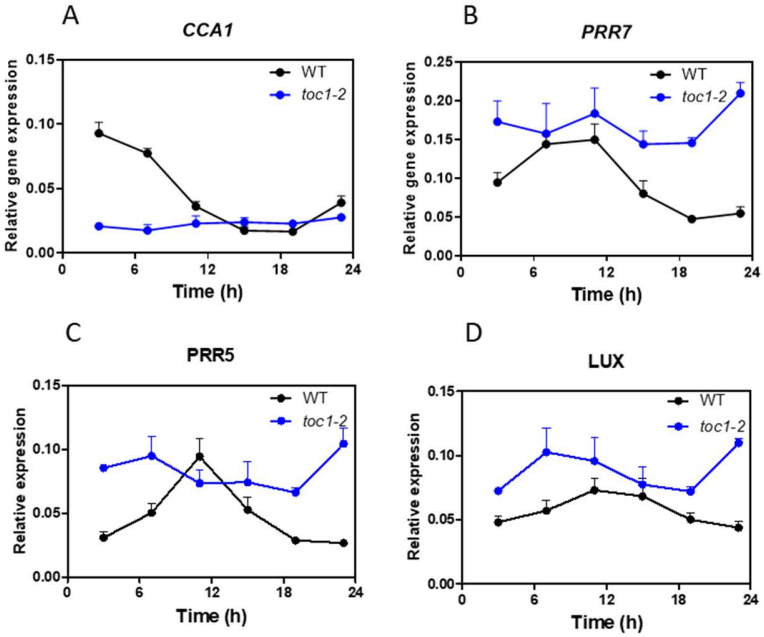


Figure 22. Regulatory network at the core of the pistil oscillator in clock mutants. Time-course analyses of *CCA1* mRNA expression(A), *PRR7* mRNA expression (B), *PRR5* mRNA expression (C), *LUX* mRNA expression (D in WT and *toc1-2* pistils. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

5. Genetic interaction studies on the repressive function of oscillator components

5.1. Genetic interaction studies of evening-phased clock components in pistils

To further understand the circadian regulatory network at the core of the circadian clock in pistils, we generated plants over-expressing a clock component in a mutant background of a clock gene expressed at a similar phase. We first analyzed clock gene expression in pistils of TOC1-ox/*elf3-2* plants. The studies using TOC1-ox pistils showed a highly repressing function of the *PRR* genes (Figures 23A-C and 25A, B). Comparative analyses using TOC1-ox/*elf3-2* pistils showed that over-expression of TOC1 was still able to down-regulate the *PRR* genes even in the absence of ELF3 (Figure 23A-C). Thus, TOC1 repression of the *PRR* genes does not require a functional ELF3. A dominance of TOC1-ox repressive function on *PRRs* over the EC might explain these results. Comparative analyses using whole seedlings showed that repression of the *PRR* genes by TOC1 was alleviated by the *elf3* mutation (Figure 24A-C). Furthermore, *CCA1* gene expression in pistils was fully repressed in TOC1-ox/*elf3-2* resembling the phenotype observed in *elf3-2* mutant, although CCA1 was still repressed in TOC1-ox (Figure 25A). Similarly, *ELF4* expression more closely resembled that observed in *elf3-2* mutant (Figure 20D) than that in TOC1-ox (Figure 25B), which suggests an *elf3-2* dominant phenotype and a possible hierarchy of the EC auto-repression over the repressing function of TOC1-ox. It is also possible that TOC1 requires ELF3 for full repression of the *EC*. The expression pattern of *ELF4* and *CCA1* in TOC1-ox/*elf3-2* seedlings (Figure 26A, B) were similar to those observed in TOC1-ox/*elf3-2* pistils (Figure 25A, B).

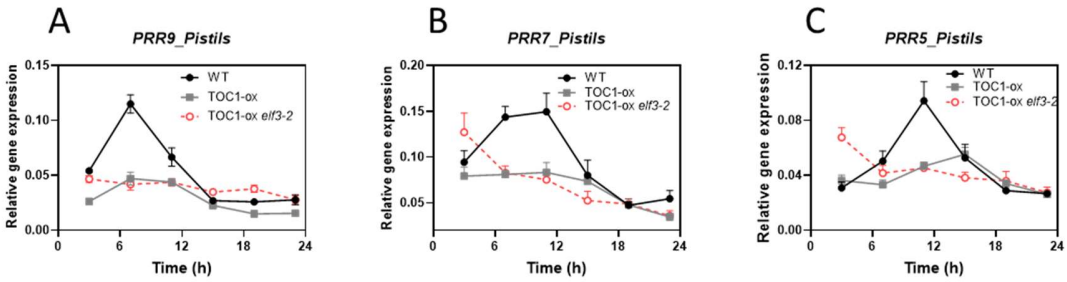


Figure 23. Genetic interaction analyses of the transcriptional repressive networks at the core of the pistil oscillator. Comparative analyses of *PRR9* mRNA expression (A), *PRR7* mRNA expression (B), and *PRR5* mRNA expression(C), in WT, TOC1-ox, and TOC1-ox/*elf3-2* pistils. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

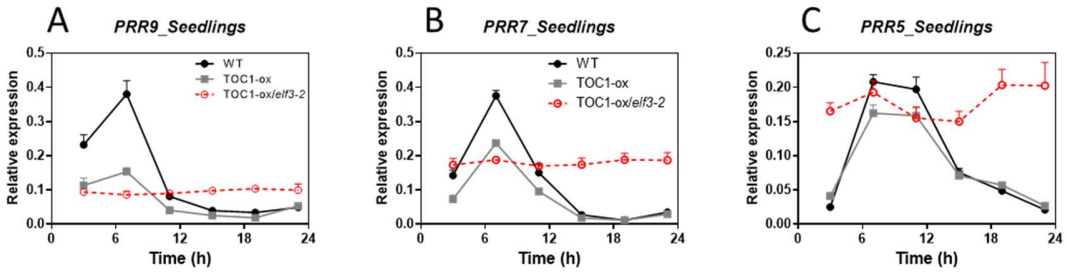


Figure 24. Genetic interaction analyses of the transcriptional repressive networks at the core of the seedling oscillator. Comparative analyses of *PRR9* mRNA expression (A), *PRR7* mRNA expression (B), and *PRR5* mRNA expression (C), in WT, TOC1-ox, and TOC1-ox/*elf3-2* seedlings. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

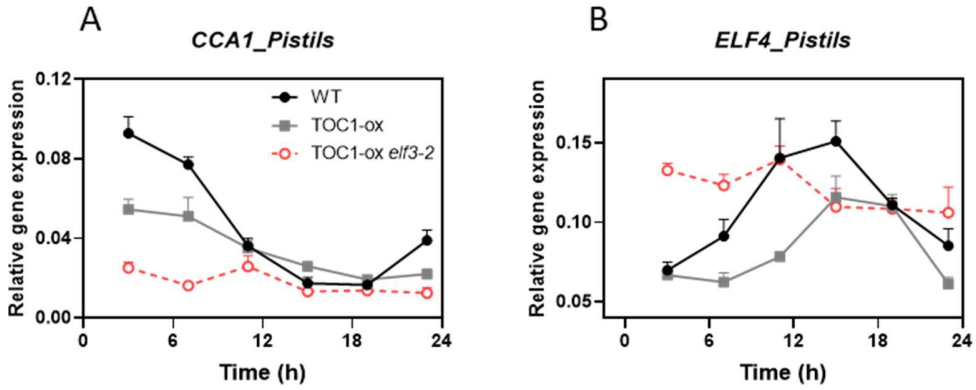


Figure 25. Genetic interaction analyses of the transcriptional repressive networks at the core of the pistil oscillator. Comparative analyses of *CCA1* mRNA expression (A) and *ELF4* mRNA expression (B) in WT, TOC1-ox, and TOC1-ox/*elf3-2* pistils. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

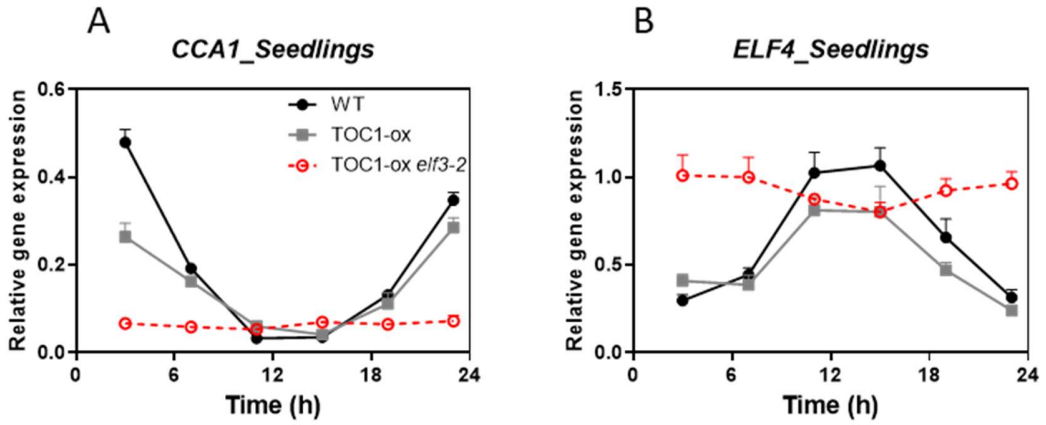


Figure 26. Genetic interaction analyses of the transcriptional repressive networks at the core of the seedling oscillator. Comparative analyses of *CCA1* mRNA expression (A) and *ELF4* mRNA expression (B) in WT, TOC1-ox, and TOC1-ox/*elf3-2* seedlings. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

5.2. Genetic interaction studies of morning-phased clock components in pistils

We also generated CCA1-ox/*prr97* plants to examine the circadian regulatory network in pistils and seedlings. Analyses of CCA1-ox in pistils also showed a repressing function of CCA1 that was not effectively overcome by the *prr79* mutation in the regulation of *TOC1* expression (Figure 27A). However, the repression of *TOC1* expression by CCA1 was effectively overcome by the *prr79* mutation in seedlings (Figure 27B), showing a similar expression pattern as in *prr79* seedlings (Farré et al., 2005). The results suggest that CCA1 might require functional PRR9 and PRR7 for repression of *TOC1* in seedlings but not in pistils. However, in both pistils and seedlings, *ELF4* in CCA1-ox/*prr79* showed an up-regulation during the subjective day like the one observed in *prr79* mutant (Figure 19D) but not in CCA1-ox (Figure 27C, D). The results suggest that CCA1 might require functional PRR9 and PRR7 for repression of *ELF4* in both seedlings and pistils or that the lack of (direct or indirect) repressing function of the PRRs can overcome the repression by CCA1-ox.

Notably, an evident accumulation of *PRR9* expression was observed in CCA1-ox/*prr7* (Figure 28A), suggesting that PRR7 might function as a repressor of *PRR9* expression. However, there was no up-regulation of *PRR5* expression in CCA1-ox/*prr7* (Figure 28B), which indicates that the repression by PRR7 is specific for *PRR9* and not for other closely related *PRR* genes.

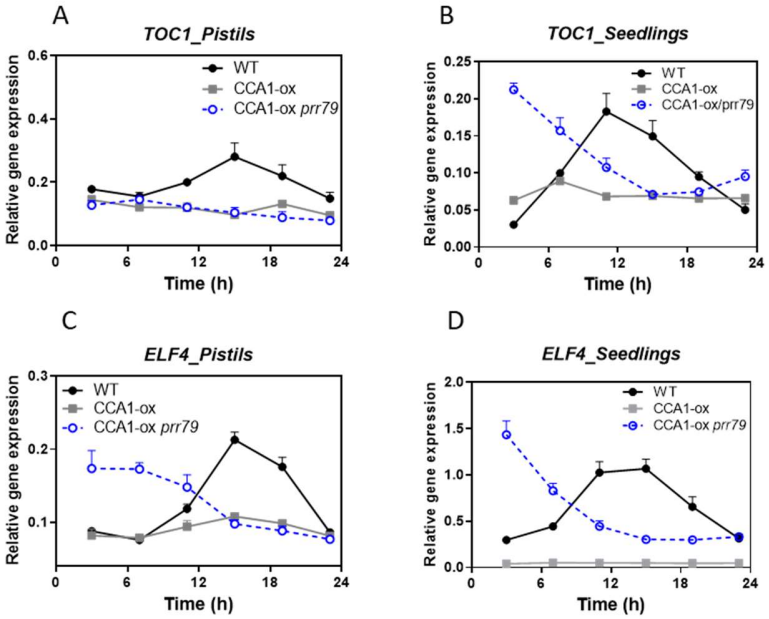


Figure 27. Genetic interaction analyses of the transcriptional repressive networks at the core of the pistil oscillator. Comparative analyses of *TOC1* mRNA expression in WT, CCA1-ox, and CCA1-ox/*prr79* pistils (A) and seedlings (B). Comparative analyses of *ELF4* mRNA expression in WT, CCA1-ox, and CCA1-ox/*prr79* pistils (C) and seedlings (D). Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

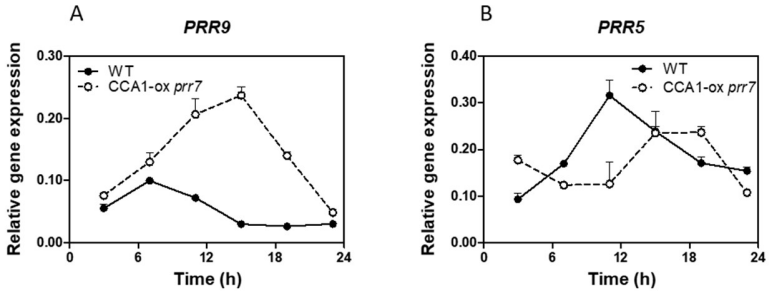


Figure 28. Genetic interaction analyses of the transcriptional repressive networks at the core of the pistil oscillator. Comparative analyses of *PRR9* mRNA expression (A) and *PRR5* mRNA expression (B) in WT and CCA1-ox/*prr7* pistils. Data are presented as the mean ± SEM. At least two biological replicates were performed per experiment.

6. The circadian clock affects the distribution pattern of pistil length

We next examined in further detail the diurnal regulation of pistil length and its circadian control. To that end, we analyzed pistil length of buds and flowers from primary inflorescences during the day (ZT3) and at night (ZT19) from plants growing under long day (LgD) (16h light: 8h dark) conditions. Overall, around five hundred buds and flowers from primary inflorescences of 6-7-weeks-old plants were sampled and used to measure the pistil length. Experiments were performed following the scheme shown in Figure 29. The analyses showed that the WT pistil population at ZT3 displayed a peak around 1.8±0.2 mm length that progressively decreased to reach a minimum at 2.5±0.2 mm length (Figure 30A), which is consistent with previous studies (Tashiro et al., 2009). The distribution of pistil population at ZT19 was similar to that observed at ZT3 (Figure 30A). Analyses of the CCA1-ox pistil population also showed a peak around 1.8±0.2 mm length, but more rapidly decreased to a minimum of 2.5±0.2 mm length (Figure 30B, C). A more rapid decrease from peak to afterward in CCA1-ox pistil population was observed at ZT19 compared that at ZT3 (Figure 30B, C). However, a considerable proportion of pistils in TOC1-ox at ZT3 clustered in the group of 1.3 to 2.3mm, after which decreased slowly to the minimum of 2.5±0.2 mm length (Figure 30D). In contrast, a peak enrichment of pistils round 1.6±0.1mm, following a progressive decrease, was observed at ZT19 in TOC1-ox plants (Figure 30D, E). The results suggest that proper circadian function might be important in the control of pistil length during the day and night.

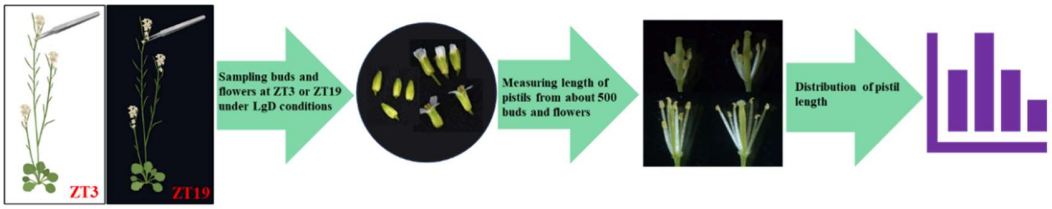


Figure 29. Schematic diagram depicting the population pattern of pistils assays in *Arabidopsis thaliana*. About 100 primary inflorescences were selected at ZT3 or at ZT19 (1) and were separated into about 500 buds and flowers at different developmental stages (2), then lengths of stamens or pistils were measured using Image J software (3). Analyses of distribution pattern of stamen population or pistil population by statistic category of stamens or pistils lengths within 1mm intervals (4).

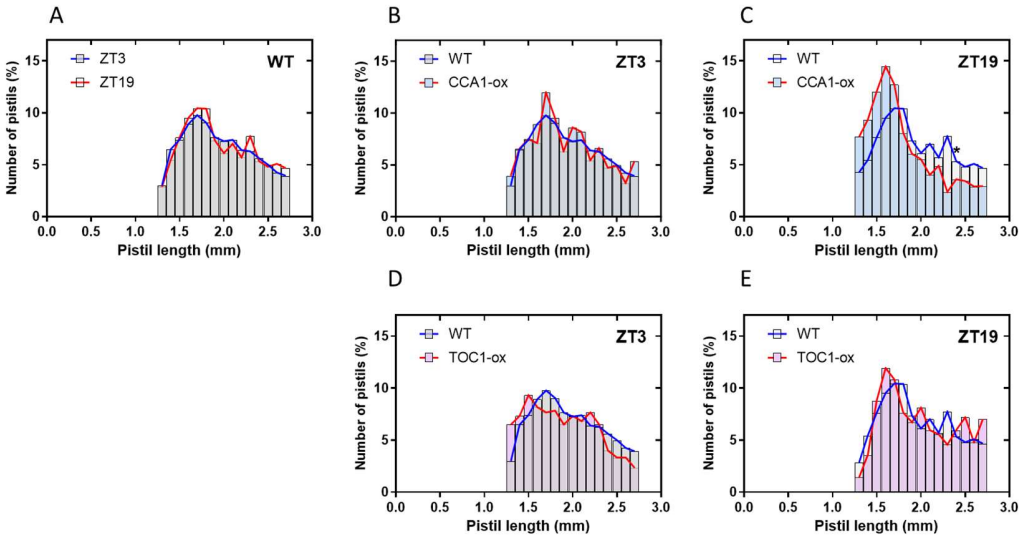


Figure 30. Distribution of pistil lengths in different circadian lines. (A) The distribution of pistil lengths in WT at ZT3 and at ZT19. (B) The distribution of pistil length in CCA1-ox at ZT3. (C) The distribution of pistil length in CCA1-ox at ZT19. (D) The distribution of pistil length in TOC1-ox at ZT3. (E) The distribution of pistil length in TOC1-ox at ZT19. About 100 primary inflorescences including around 500 buds and flowers were obtained from 6-7week-old plants. Pistils from buds and flowers were measured and those flowers with pistils longer than 3 mm were excluded. Pistil length was determined as the distance from the base of the pistil to the tip of the stigma papillae. Data are presented as the mean. At least two biological replicates were performed per experiment.

7. Kinetics of stamen and pistil growth

Based on the observed length distribution, we next analyzed the kinetics of both stamen and pistil growth in buds and flowers at different developmental stages (from stage11 to stage14 according to Smyth et al., 1990) of plants growing under LgD (16h light: 8h dark) cycles. Our studies showed that stamens were shorter than pistils at stages 11 and 12, while stamens rapidly grew afterwards (stage13) reaching a similar length than pistils (stages 13-14) (Figure 31A, B). The results suggest that stamens have different growth rates at different developmental stages, as previously described (Chae et al., 2012). Pistil growth from stages 11 to 14 followed a constant growth rate (Figure 31A, C), which is also consistent with previous results (Tashiro et al., 2009).

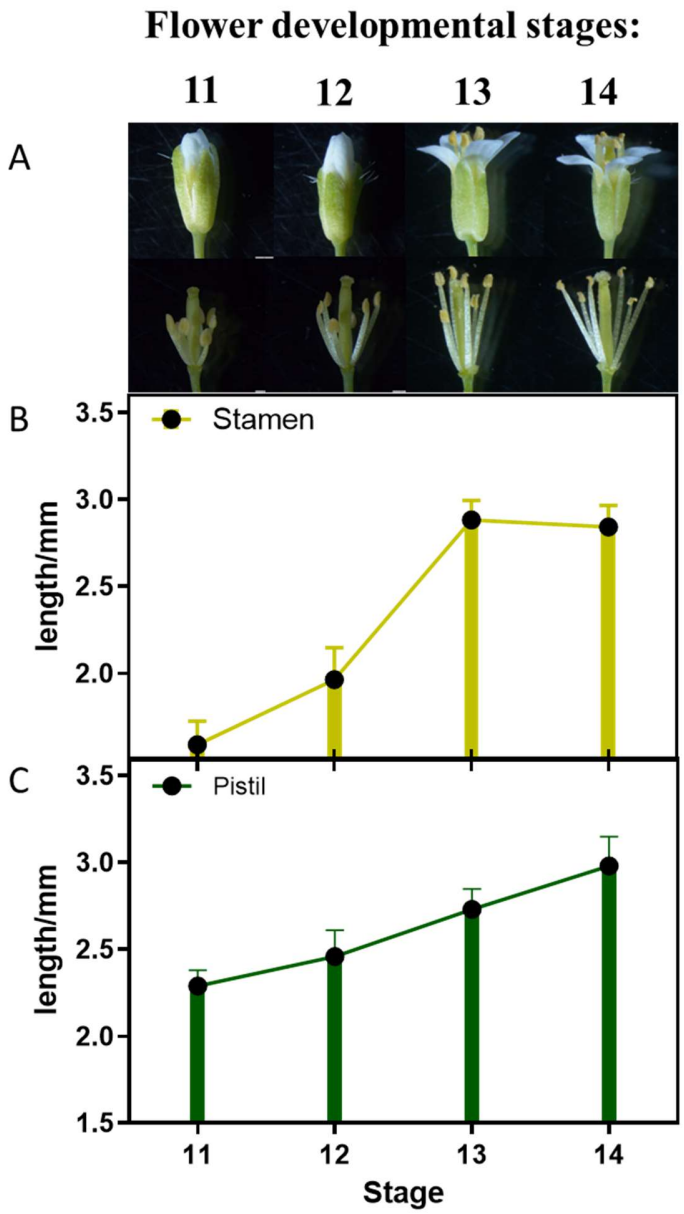


Figure 31. The elongation of stamen and pistil in WT flowers. (A) Wild-type flowers at stages 11–14. Top, whole flower; bottom, sepals and petals removed to show stamen and pistil length. Scale bar = 0.5 mm. (B) Stamen length in wild-type flowers at stages 11–14. (C) Pistil length in wild-type flowers at stages 11–14. Stamen length was determined as the distance from the base of the stamen filament to the tip of the anther. Pistil length was determined as the distance from the base of the pistil to the tip of the stigma papillae.

8. Circadian coordination of stamen and pistil elongation

Based on the different growth rates during development and to examine the possible role of the circadian clock in the coordination of stamen and pistil elongation, we analyzed the coordinated growth of stamens and pistils at ZT3 and ZT19. Experiments were conducted following the experimental design schematically depicted in Figure 32. About five hundred buds and flowers from one hundred primary inflorescences were selected and lengths of the stamens and pistils were measured. The coordination of stamen and pistil length was analyzed by comparing the frequency of stamens longer than pistils. Our results showed that in WT plants, more than 33% stamens around 2.4 ±0.2 mm long, exceeded pistil length in samples analyzed at ZT3 (Figure 33A, C). In contrast, only 13% of stamens were longer than pistils at ZT19 (Figure 33B, C). The results suggest that the elongation of stamens and pistils relative to each other might vary between the day and night.

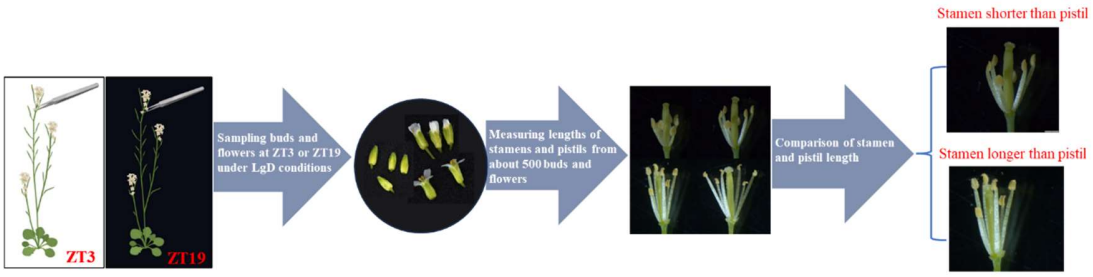


Figure 32. Schematic diagram depicting the coordination of stamen and pistil length in *Arabidopsis thaliana*. About 100 primary inflorescences were selected at ZT3 or at ZT19 (1) and were separated into about 500 buds and flowers at different developmental stages (2), then lengths of stamens and pistils were measured using Image J software (3). Comparing the lengths between stamens and pistil (4).

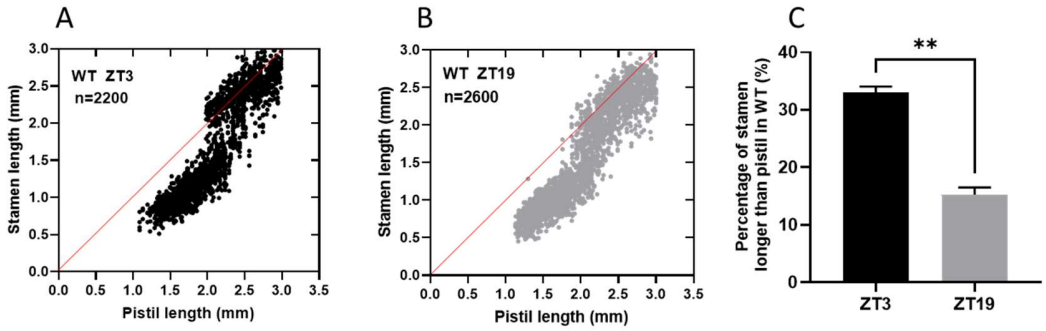


Figure 33. The coordination of stamen and pistil length in WT buds and flowers changed among day and night. Correlation between stamen length and pistil length in WT at ZT3 (A), and at ZT19 (B). (C) The percentage of stamens longer than pistils in WT at different time points. Note: a thin line is drawn in (A-B) panels, having a slope of 45^{o} and passing through the origin. Stamen and pistil lengths were measured in each flower whose pistil was longer than 1.0mm. About 500 buds and flowers were obtained from 100 primary inflorescences of 6-7week-old plants. All the obtained flowers were measured and those flowers with pistils longer than 3 mm or shorter than 1.0 mm were excluded. Data is presented in (C) as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

We also used CCA1-ox and *cca1-1lhy-20* mutant plants to compare stamen and pistil length at ZT3 and ZT19. CCA1-ox plants showed a significant higher percentage of stamens longer than pistils at ZT3 compared to that at ZT19 (Figures 34A-C), similarly to WT. Meanwhile, *cca1-1lhy-20* plants also showed more stamens exceeding pistils in length at ZT3 than at ZT19 (35A-C). The comparative analyses of stamen and pistil length in WT and CCA1-ox plants at ZT3 showed that the CCA1-ox plants had a significant lower percentage of stamens longer than pistils compared to WT (Figure 36A) while no significant difference was observed in *cca1-1lhy-20* mutant plants (although the high variability precluded reaching a clear conclusion) (Figure 36A). At ZT19, CCA1-ox plants also showed a reduced number of stamens longer than pistils compared to WT (Figure 36B), while a similar percentage in WT and *cca1-1lhy-20* mutant plants was observed at ZT19 (also showing high variability in the *cca1-1lhy-20* samples) (Figure 36B).

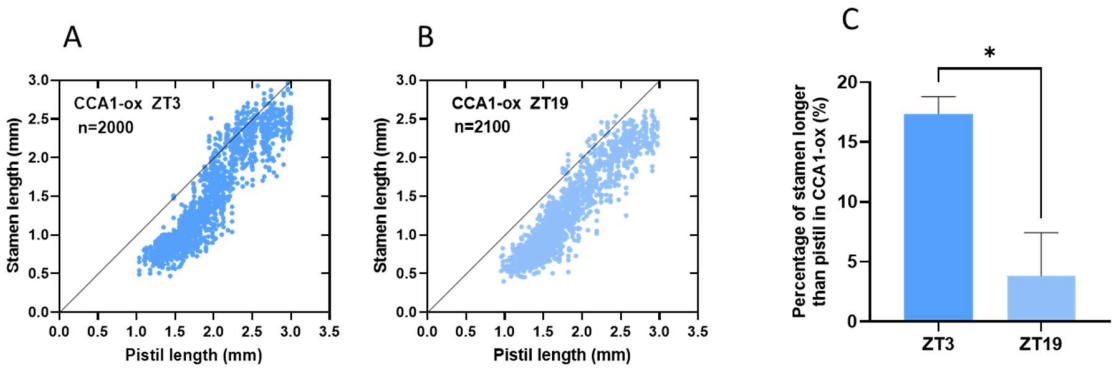


Figure 34. The coordination of stamen and pistil length in CCA1-ox buds and flowers changed among day and night. Correlation between stamen length and pistil length in CCA1-ox at ZT3 (A), and at ZT19 (B). (C) The percentage of stamens longer than pistils in CCA1-ox at different time points. Note: a thin line is drawn in (A-B) panels, having a slope of 45o and passing through the origin. Stamen and pistil lengths were measured in each flower whose pistil was longer than 1.0mm. About 500 buds and flowers were obtained from 100 primary inflorescences of 6-7week-old plants. All the obtained flowers were measured and those flowers with pistils longer than 3 mm or shorter than 1.0 mm were excluded. Data is presented in (C) as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

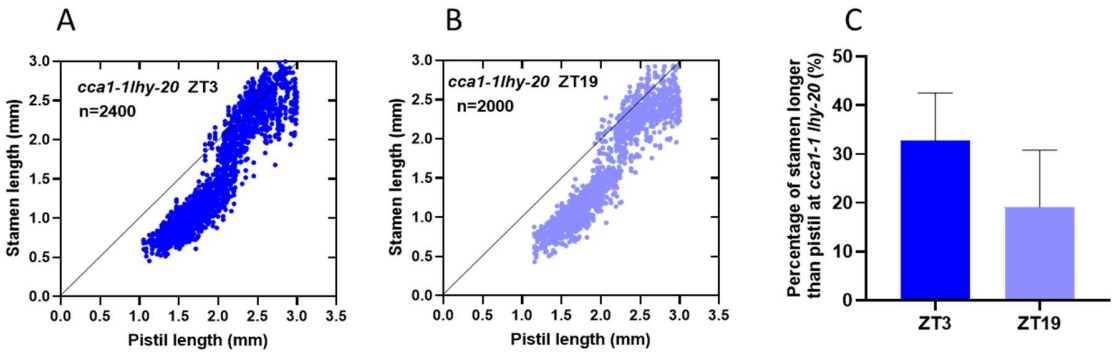


Figure 35. The coordination of stamen and pistil length in *cca1-1lhy-20* buds and flowers changed among day and night. Correlation between stamen length and pistil length in *cca1-1lhy-20* at ZT3 (A), and at ZT19 (B). (C) The percentage of stamens longer than pistils in *cca1-1lhy-20* at different time points. Note: a thin line is drawn in (A-B) panels, having a slope of 45^{o} and passing through the origin. Stamen and pistil lengths were measured in each flower whose pistil was longer than 1.0mm. About 500 buds and flowers were obtained from 100 primary inflorescences of 6-7week-old plants. All the obtained flowers were measured and those flowers with pistils longer than 3 mm or shorter than 1.0 mm were excluded. Data is presented in (C) as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

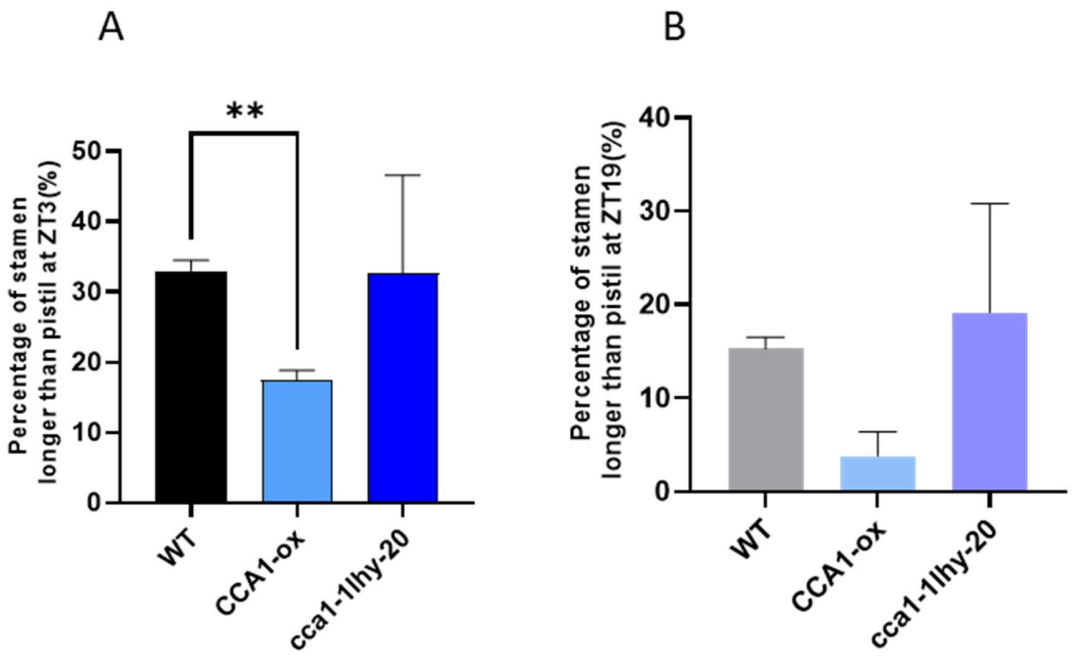


Figure 36. CCA1 affects the coordination of stamen and pistil length. (A) Comparative analyses of the percentage of stamens longer than pistils in WT, CCA1, and *cca1-1lhy-20* flowers at ZT3. (B) Comparative analyses of the percentage of stamens longer than pistils in WT, CCA1, and *cca1-1lhy-20* flowers at ZT19. Data is presented as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

Similar analyses in TOC1-ox and *toc1-2* flowers showed more stamens exceeding pistil length at ZT3 than at ZT19 for both TOC1-ox and *toc1-2* flowers (Figures 37A-C, 38A-C). Comparative analyses of stamen and pistil length between WT and TOC1-ox showed a significant higher percentage of stamens exceeding pistil length at ZT3 in TOC1-ox compared WT (Figure 39A), but a significantly smaller number of stamens exceeding pistil length in TOC1-ox than in WT at ZT19 (Figure 39B). The reverse phenotypes were observed for *toc1-2*, particularly at ZT3 (Figure 39A, B). Altogether, our results suggest that the circadian clock components CCA1 and TOC1 are important for the diurnal coordination of stamen and pistil growth.

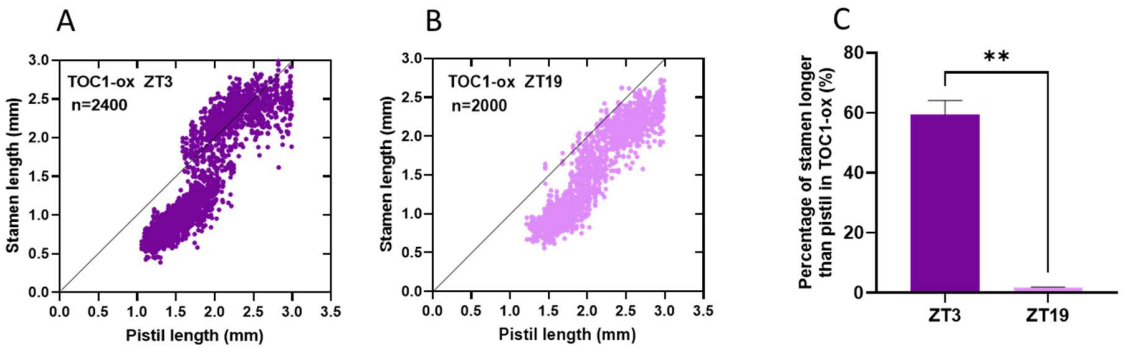


Figure 37. The coordination of stamen and pistil length in TOC1-ox buds and flowers changed among day and night. Correlation between stamen length and pistil length in TOC1-ox at ZT3 (A), and at ZT19 (B). (C) The percentage of stamens longer than pistils in TOC1-ox at different time points. Note: a thin line is drawn in (A-B) panels, having a slope of 45^{o} and passing through the origin. Stamen and pistil lengths were measured in each flower whose pistil was longer than 1.0mm. About 500 buds and flowers were obtained from 100 primary inflorescences of 6-7week-old plants. All the obtained flowers were measured and those flowers with pistils longer than 3 mm or shorter than 1.0 mm were excluded. Data is presented in (C) as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

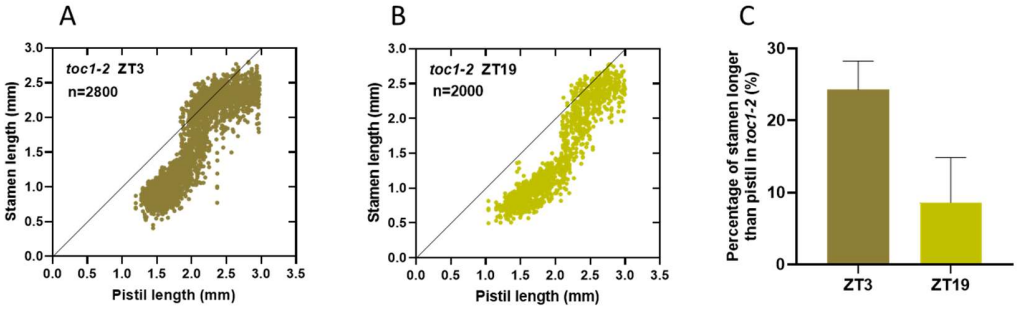


Figure 38. The coordination of stamen and pistil length in *toc1-2* buds and flowers changed among day and night. Correlation between stamen length and pistil length in *toc1-2* at ZT3 (A), and at ZT19 (B). (C) The percentage of stamens longer than pistils in *toc1-2* at different time points. Note: a thin line is drawn in (A-B) panels, having a slope of 45^{o} and passing through the origin. Stamen and pistil lengths were measured in each flower whose pistil was longer than 1.0mm. About 500 buds and flowers were obtained from 100 primary inflorescences of 6-7week-old plants. All the obtained flowers were measured and those flowers with pistils longer than 3 mm or shorter than 1.0 mm were excluded. Data is presented in (C) as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

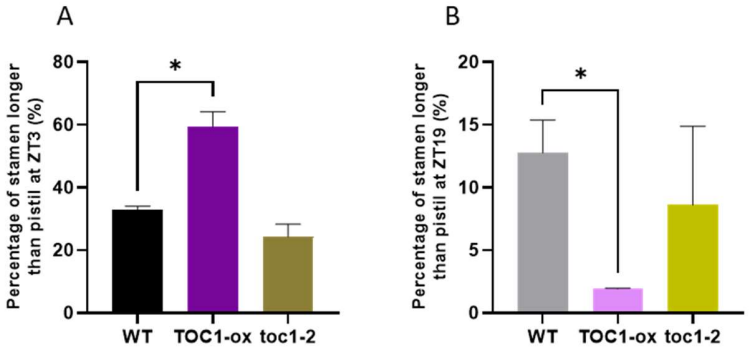


Figure 39. TOC1 affects the coordination of stamen and pistil length. (A) Comparative analyses of the percentage of stamens longer than pistils in WT, TOC1, and *toc1-2* flowers at ZT3. (B) Comparative analyses of the percentage of stamens longer than pistils in WT, TOC1, and *toc1-2* flowers at ZT19. Data is presented as mean ±SEM. (**** p-value<0.0001; ** p-value < 0.001; * p-value<0.05). At least two biological replicates were performed per experiment.

DISCUSSION

Discussion

Knowledge about environmental adaptations and biological rhythms in plants is helpful for ensuring a more sustainable agriculture within climate change and resource scarcity scenarios. When the rhythmic physiological processes in crops are taken into account in agriculture, more efficient and cost-effective management practices can be designed for food production. In medicine, chronotherapy is used to increase drug efficacy, reduce toxicity, and understand the health effects of circadian clock disruption. Meanwhile, circadian agriculture, also known as agro-chronobiology, agricultural chronotherapy, or chronoculture (Belbin et al., 2019; Gottlieb, 2019; Steed et al., 2021), can be used for food production through coordinating the advantages of circadian biology with agricultural practices. For instance, recent studies have focused on the manipulation of the circadian system to improve plant productivity (reviewed in Steed et al., 2021). Flowers, produced from the reproductive shoot apical meristem (SAM), known as the inflorescence meristem, form seeds after fertilization and are central for crop yield. Understanding the circadian determinants in flowers will provide insightful clues for future applications in chronoculture.

Regarding the circadian system, several studies on specific organs and tissues are uncovering both the circadian autonomy of some organs (Bordage et al., 2016; Endo et al., 2014; Fukuda et al., 2012; James et al., 2008; Thain et al., 2002; Wenden et al., 2012; Yakir et al., 2011b) as well as the coupling and coordination of rhythms within the plant (Chen et al., 2020; Gould et al., 2018; Greenwood et al., 2019; Takahashi et al., 2015). Therefore, the plant circadian system comprises autonomous tissue-specific rhythms complemented with cell-to-cell coupling and long-distance coordination (Nakamichi, 2020; Sorkin and Nusinow, 2021). Despite this knowledge, the components and regulatory mechanisms of clock architecture and function in flowers remain to be elucidated. Similarly, there is little information about the direct participation of the circadian clock in reproductive floral organs, such as pistils and stamens, as well as on seed production. Therefore, understanding the circadian clock function in pistils and stamens, and its role in the regulation of pollination and seed production might be useful for improvement of plant productivity.

Our studies have shown that buds and flowers detached from the rest of the plant display rhythmic oscillations, which indicates the presence of self-sustained functional clocks. The rhythmic differences in buds and open flowers may be due to differences in sensitivities to environmental cues for different organs (Atamian et al., 2016). Under the same conditions, other excised organs, for example roots, also sustain rhythms but with a long period and delayed phase compared with shoots (Chen et al., 2020; Takahashi et al., 2015). Notably, excised pistils showed self-sustained rhythms with precise 24-h oscillations. These results indicate that the circadian clock in pistils is able to precisely run without signals from the rest of the plant. In other floral organs, the dampened rhythms could be due to reduced viability or lack of energy after excision from the flower.

The circadian clock plays an important role controlling hypocotyl length in *Arabidopsis* and other crops (Li et al., 2020; Wang and Tobin, 1998). Our studies showed that proper circadian function is also essential for pistil and silique growth as well as for seed quality and production. The circadian factors and regulatory mechanisms controlling growth appear to be organ-specific, judging by the opposite hypocotyl and pistil length phenotypes observed in plants miss-expressing clock components. For instance, CCA1-ox plants displayed a long hypocotyl (Wang and Tobin, 1998), but produced shorter pistil compared to WT. TOC1 miss-expressing plants also regulate both pistil and hypocotyl lengths but show reverse roles in these two organs. Our study showed longer pistil in TOC1-ox and shorter pistils in *toc1-2* mutant plants compared to WT. In contrast, TOC1-ox showed short hypocotyl, and *toc1-2* displayed longer hypocotyls than the WT (Más et al., 2003). The circadian clock regulates hypocotyl elongation by modulating among others, the expression of *PIF4* and *PIF5* (Niwa et al., 2009). The EC binds to the promoters of *PIF4* and *PIF5* to repress their expression at night (Nusinow et al., 2011). CCA1 represses *ELF3* by binding to its promoter and acts upstream of ELF3, mediating the repression of *PIF4* and *PIF5* in the regulation hypocotyl elongation (Lu et al., 2012). In CCA1-ox plants, the *PIF4* and *PIF5* expression are elevated throughout the night owing to the low expression of *ELF3*, leading to long hypocotyls (Lu et al., 2012; Niwa et al., 2009; Nozue et al., 2007). In addition, TOC1 is also able to interact with PIF4 to repress the hypocotyl elongation (Yamashino et al., 2003; Zhu et al., 2016). CYCLING DOF FACTOR (CDF) transcription factors CDF5 controls hypocotyl elongation by promoting cell elongation through

regulating expression of *IAA19* and cell-wall-related genes (Martín et al., 2018). The CDF5 protein is antagonistically targeted by the interplay between PRRs (including TOC1) and PIFs to restrict growth of hypocotyl (Martín et al., 2018). It would be interesting to determine the molecular mechanisms and downstream signaling pathways by which the circadian clock regulates pistil growth and function. Other circadian mutant lines with altering the expression of circadian genes also showed diverse roles of circadian genes in the regulation of hypocotyl and pistil elongations (Table 2).

Table 2. Summary view of circadian roles of clock genes hypocotyl and pistil lengths

Line	Circadian period (LL)	Hypocotyl length	References	Pistil length	References
WT	24 h	WT	(Takahashi et al., 2015)	WT	This study
CCA1-ox	Arrhythmic	Long	(Wang and Tobin, 1998)	Short	This study
toc1-2	Short	Long	(Mas et al., 2003)	Short	This study
TOC1-ox	Arrhythmic	Short	(Mas et al., 2003)	Long	This study
prr5	Short	Long	((Nakamichi et al., 2007)	No change	This study
prr57	Short	Long	(Nakamichi et al., 2007)	Short	This study
prr59	No change	Long	(Nakamichi et al., 2007)	Short	This study
prr79	Long	Long	(Farre et al., 2005)	Short	This study

The size of seeds, a key factor in grain yield of crops, is determined by the integrated signals of maternal and zygotic tissues, which control the coordinated growth of the embryo, endosperm, and seed coat (Li et al., 2019b). The ubiquitin-proteasome pathway, G-protein signaling, mitogen-activated protein kinase (MAPK) signaling, phytohormone perception and homeostasis, and some additional transcriptional regulators participate in the determination of seed size (Li et al., 2019b). Our results showed that plants miss-expressing clock components displayed altered seed size and seed abortion. The role of the circadian clock in the control of seed production also opens interesting possibilities for biotechnological application by improving seed yield, arguably one of the most important traits for plant breeding.

Our studies have focused on elucidating the transcriptional regulatory network at the core of the pistil clock. The analyses of mutant and over-expressing lines point out to a complex regulatory network. Our data provides a pattern in which CCA1 represses the expression of the evening-phased genes, and in turn, these components repress *PRR9* and *PRR7*. TOC1 also represses the expression of the *EC* genes, whereas PRR9 and PRR7 components repress *CCA1*. The marked up-regulation of *CCA1* in ELF3-ox pistils is likely the consequence of the reduced accumulation of the morning-phased repressor genes *PRR7* and *PRR9*. Similarly, the analyses of the *prr79* mutant suggest that PRR9 and PRR7 might repress (directly or indirectly) the evening-phased gene expression during the subjective day. The down-regulation of evening-phased gene expression during the subjective night might be a consequence of the up-regulation of *CCA1* in the *prr79* mutant. Thus, the analyses of the mutants and over-expressing lines suggest that many of the clock components that function as repressors also shape the oscillations by repressing other repressors.

Altogether, the analyses of mutants, over-expressing lines and the genetic interaction studies show a complex regulatory circuitry in pistils (Figure 40) with CCA1-ox repressing *TOC1* over the morning PRRs (1), PRR7 repressing *PRR9* over CCA1-ox (2), morning PRRs repressing *CCA1* (3), and *EC* (4) over CCA1-ox. Within the evening-expressed components, TOC1-ox represses the *PRRs* over the EC (5), the EC auto-represses itself over TOC1-ox (6), and the EC activates *CCA1* over TOC1-ox (7). Current models of the *Arabidopsis* clock in seedlings include the reciprocal regulation of morning and evening oscillator genes that results in their time-of-day specific peak of expression (Avello et al., 2021). The highly repressive interactions between circadian components, autoregulation patterns, and three-node feedback loops configure a circadian network using data from whole seedlings (Avello et al., 2021) that differs from the one observed in pistils. Other models also group several circadian components together to explore the clock responses to various light cues as well as clock control in hypocotyl growth (Avello et al., 2021; De Caluwé et al., 2016). We proposed that the particular circadian architecture that we found in pistils might provide robustness to the pistil clockwork (Figure 40).

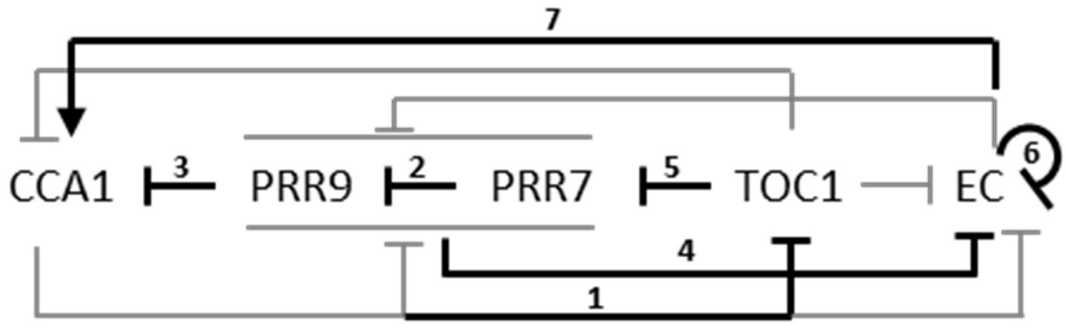


Figure 40. The transcriptional repressive network at the core of the pistil oscillator. Circadian regulatory network comprising dominant regulatory functions (thick black lines) as inferred by the genetic interaction studies. Arrows indicate activation and lines ending in perpendicular dashes indicate repression. See the Results (section 4 and section 5) for a further explanation.

Clock repressors and activators have specific regulatory functions in pistils. In sepals, petals, and stamens, the *toc1-2* mutant showed similar waveforms than WT at least for the first days under LL. These results suggest that the lack of a functional TOC1 can be overcome for few days. In pistils, on the other hand, the short-period phenotype was evident from the initial days, but the rhythms dampened low over time. Thus, TOC1 circadian function is different in the floral organs, with a prevalent role in pistils. We also observed that gene expression of *TOC1* (and also *ELF3*) didn't sustain robust high-amplitude circadian rhythms in pistils (Okada et al., 2022). Translational and/or post-translational mechanisms of regulation might contribute to the rhythmic oscillation of clock protein activity. For example, TOC1 protein is regulated by degradation through the proteasome pathway, thus providing a mechanism for controlling TOC1 protein oscillation and period length by the clock (Más et al., 2003). The degradation of TOC1 protein requires a functional ZTL protein as mutation of ZTL results in constitutive accumulation of TOC1 protein (Más et al., 2003). In addition, GI protein also participates in increasing the amplitude of the daily TOC1 protein oscillation by stabilizing ZTL protein (Kim et al., 2007). The B-BOX family of protein 19 (BBX19), a critical protein fine-tuning circadian rhythms (Yuan et al., 2021), reduces ELF3 protein stability without altering the transcriptional levels of *ELF3* expression (Wang et al., 2015). It would be interesting to

examine if these mechanisms of regulation for TOC1 and ELF3 proteins are also observed in pistils.

Compared with pistils, rhythms in stamens displayed a short-period phenotype, with an advanced phase. It would be interesting to determine the biological relevance of such phase variation between the reproductive organs. Similar differences were reported between hypocotyls and cotyledons, showing earlier rhythmic peaks in hypocotyls compared to cotyledons (Gould et al., 2018). The rhythms of hypocotyls also peaked earlier than the rhythms in upper roots (Gould et al., 2018). These results suggest that the *Arabidopsis* clock may have multiple coordination points (Gould et al., 2018). Circadian differences in reproductive organs are not exclusive of plants. For instance, the expression of core clock genes is also rhythmic in ovarian tissues (Kennaway et al., 2012), and female and male rats show sex differences in daily rhythms and in responses to endogenous and exogenous cues (Bailey and Silver, 2014). The sex-dependent circadian differences are relevant to humans in many areas, most notably those related to reproduction and overall health (Bailey and Silver, 2014). Understanding the circadian differences in reproductive organs in flowers may prove essential for optimizing plant reproduction and productivity.

Several studies performed in *Arabidopsis* hypocotyls and petioles have been informative for understanding the circadian function in the regulation of elongation in plant organs (Favero et al., 2021). Analogously, our work also suggests a role for the circadian clock in the coordinated regulation of stamen and pistil elongation. Light and temperature are two key external signals inducing the elongation of plant organs, but the mechanisms of elongation in various organs are quite different (Ichihashi et al., 2011). For instance, hypocotyl elongation occurs mostly through cell expansion (Gendreau et al., 1997). However, petiole elongation relies on the correct spatial regulation of the proliferative region of leaf primordia (Ichihashi et al., 2011).

Previous studies focusing on stamen and pistil growth revealed three growth phases for stamens while pistils displayed a single exponential growth under LL conditions (Tashiro et al., 2009). The stamens with length around 1.6 to 2.0 mm grew fastest to catch up with pistils or extend above pistils. Our studies of pistil length populations showed that the

frequency of pistil length gradually decreased over development under LgD conditions. However, the relative frequency of pistil length in each group would be inversely proportional to the growth rate as the relative frequency of pistils in each group is proportional to the time during which a pistil grows from the lower end point to the upper end point of the group, if production of flowers is in a steady state (Tashiro et al., 2009). Together, our results indicate that the growth rate of pistil gradually increased with the development of pistil, showing a single exponential growth as previously described (Tashiro et al., 2009). However, this characteristic distribution pattern of WT pistils was slightly altered in both CCA1-ox and TOC1-ox plants, suggesting a role for the circadian clock in the regulation of pistil elongation.

The kinetics of stamen and pistil growth showed that stamens were shorter than pistils at stages 11 and 12, while at a later stage, stamens rapidly grew reaching a similar length than pistils. Previous studies also showed that at floral stages 12-13, filaments elongated rapidly to facilitate pollination and subsequent fertilization (Marzi et al., 2020; Smyth et al., 1990; Tashiro et al., 2009). Coordination of stamen and pistil length is essential for fertilization in *Arabidopsis*. Viable pollens from dehisced anthers cannot be properly released onto the stigma if stamen or pistil growth are not properly coordinated (Sanders et al., 1999). Understanding the molecular mechanism whereby the circadian clock controls stamen and pistil elongation would be important to elucidate how the plant coordinate reproduction in synchronization with the environment.

The involvement of the circadian system in the regulation of flowering time have been extensively reported (Shim et al., 2017). Recent studies also showed that flower opening is redundantly regulated by the circadian clock and an unknown light-sensing pathway, whereas flower closing relies exclusively on circadian control (Muroya et al., 2021). The activities of pollinators on floral organs transfer pollen to stigmas, thereby inducing plant fertilization. In order to adapt the activities of pollinators, the circadian rhythms of movements of floral organs (Bai and Kawabata, 2015; Kaihara and Takimoto, 1979; Muroya et al., 2021) synchronize with the rhythms of activities of pollinators (Hasegawa et al., 2006). For instance, mature sunflowers exhibit eastward orientation to warm up the floral organs through increased morning interception of solar radiation that enhances the

attractiveness to pollinators (Atamian et al., 2016). Flower opening and closure are important for pollen removal to complete pollination process (van Doorn and Van Meeteren, 2003). As a self-pollinating plant, precisely coordinated growth of stamens and pistils determines the fertility in *Arabidopsis* plants (Song et al., 2014).

Under LgD conditions, our studies showed that there are more stamens longer than pistils at ZT3 than at ZT19, suggesting a different coordination between stamen and pistil elongation during the day. Altering circadian components impact the coordination of stamen and pistil length. CCA1-ox showed significant lower percentage of stamens longer than pistils and *cca1-1lhy-20* had more stamens longer than pistil at both day and night compared to WT. However, TOC1-ox showed significant higher percentage of stamens longer than pistils at day and significant lower percentage of stamens longer than pistils at night compared to WT. These results may be explained by the repression by TOC1 of stamen elongation at later developmental stages preferably during the day. The coordination between stamen and pistil length is surely regulated by many internal and external factors (Huang et al., 2014; Marciniak and Przedniczek, 2019). It would be interesting to identify all these factors and their interplay with the circadian clock.

Altogether, our studies have identified the circadian transcriptional network at the core of the pistil clock, showing that pistils can autonomously sustain organ-specific circadian rhythms. The circadian clock controls the coordination of stamen and pistil lengths and consequently, proper circadian function is important for pistil and silique growth as well as seed production.

CONCLUSIONS

Conclusions

In this Doctoral Thesis, we have elucidated the **circadian function** and the **transcriptional network** at the core of the **pistil clock**. We have also identified a role of the **circadian clock** controlling **pistil and stamen growth** and **seed production**. The specific conclusions are summarized in Figure 41 and briefly described below:

1. A **functional circadian clock sustains rhythms in buds and flowers** detached from the rest of the plant, although the rhythmic oscillations appeared more robust in buds than in flowers.

2. Detached **pistils** from the plant show **precise and robust rhythms** and display **organ-specific circadian autonomy**. The robust circadian oscillations in pistils **require a functional TOC1**.

3. Proper **circadian function** is important in the control of **pistil and silique growth** and **seed weight and production**, which altogether opens up new avenues for agro-chronobiology in crops of agronomical relevance.

4. **Clock repressors** show distinctive **organ-specific** regulatory functions in **pistils** and define a **particular regulatory network** at the core of the **pistil clock**.

5. An **organ-specific hierarchy** of **clock repressing activities** might provide **robustness**, and **precision** to the **pistil clock**.

6. **Diurnal** and **developmental regulations** are essential for the **coordinated control** of **pistil and stamen growth**.

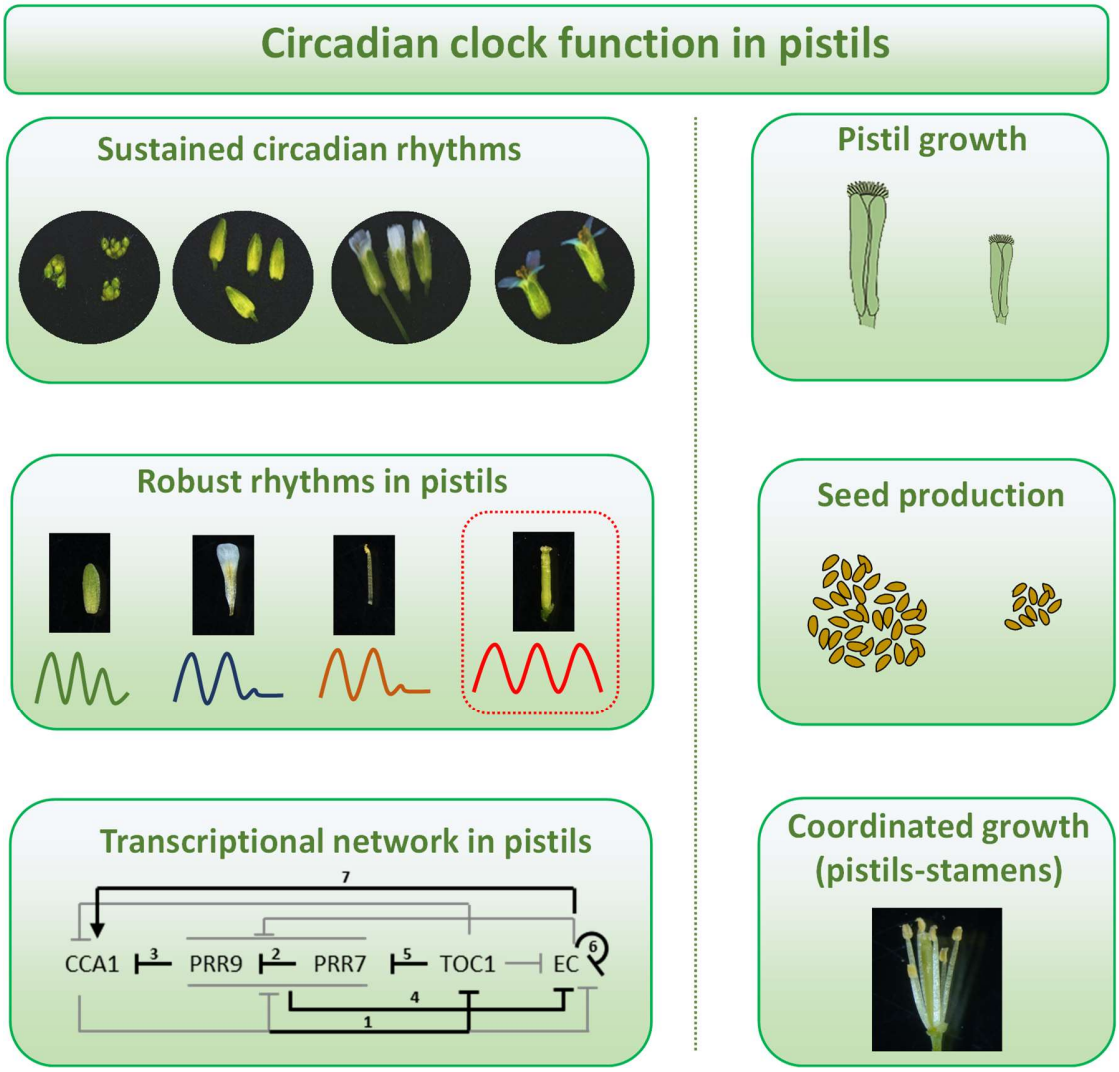


Figure 41. Schematic diagram depicting the main conclusions in this Doctoral Thesis.

SUMMARY

Summary

The circadian clock is an endogenous timekeeping mechanism that generates biological rhythms with a period of 24-hours. In plants, the circadian system regulates many essential processes including among many others, flowering time, and petal movement. In this Doctoral Thesis, we have focused on how the circadian clock functions in *Arabidopsis thaliana* flowers, specifically, in pistils, the female reproductive organ. When detached from the rest of the flower, pistils sustain highly precise rhythms, indicating organ-specific circadian autonomy. In contrast, stamens, and other floral organs, display a short circadian period or dampening rhythms over time. Analyses of clock mutants and chromatin immunoprecipitation assays show distinct expression patterns and specific regulatory functions for clock repressors in pistils. Genetic interaction studies also suggest a hierarchy of the repressing activities that might provide robustness and precision to the pistil clock. Analyses of clock mutant and over-expressing lines indicate that proper circadian function is important for the coordinated regulation of pistil and stamen growth, which is essential for effective pollination. Consequently, clock mutant plants show alterations in seed production. Understanding the circadian intricacies in reproductive organs may prove useful for optimizing plant reproduction and productivity, opening new avenues for manipulation of clock function in crops of agronomic relevance.

RESUMEN

Resumen

El reloj circadiano es un mecanismo celular endógeno que genera ritmos biológicos con un período de 24 horas. En las plantas, el sistema circadiano regula muchos procesos esenciales, incluidos, entre otros, el tiempo de floración y el movimiento de los pétalos. En esta Tesis Doctoral nos hemos centrado en cómo funciona el reloj circadiano en las flores de *Arabidopsis thaliana*, concretamente en los pistilos, el órgano reproductor femenino. Cuando se separan del resto de la flor, los pistilos mantienen ritmos circadianos muy precisos, lo que indica su autonomía circadiana específica. Por el contrario, los estambres y otros órganos florales muestran o bien un período circadiano corto o bien una pérdida de amplitud con el tiempo. Los análisis de mutantes de reloj y ensayos de inmunoprecipitación de cromatina muestran patrones de expresión distintos y funciones reguladoras específicas para los principales represores de reloj en los pistilos. Los estudios de interacción genética también sugieren una jerarquía de las actividades represoras que podrían proporcionar robustez y precisión al reloj en pistilos. Los análisis de líneas mutantes de reloj y de sobre-expresión indican que la función circadiana adecuada es importante para la regulación coordinada del crecimiento del pistilos y el estambres, lo cuál es esencial para una polinización eficaz. En consecuencia, las plantas con mutaciones en componentes importantes del reloj circadiano muestran alteraciones en la producción de semillas. Comprender las complejidades circadianas de los órganos reproductivos puede resultar útil para optimizar la reproducción y la productividad de las plantas, abriendo así nuevas vías para la manipulación de la función del reloj circadiano en cultivos de relevancia agronómica.

MATERIALS AND METHODS

Materials and Methods

1. Plant material, growth conditions and organ dissection

Seedlings were grown on half-strength Murashige and Skoog (MS) agar medium without sucrose, and synchronized under light:dark cycles (LgD, 16h light:8h dark) with 60-100 μmol $m^{-2}s^{-1}$ of cool white fluorescent light at 22°C for about 7-10 days (unless otherwise specified). For experiments using flowering plants, seedlings were transplanted to soil and cultivated throughout the reproductive stage under light:dark cycles (LgD, 16h light:8h dark) with 150-200 μmol $m^{-2}s^{-1}$ of white light emitting diodes (LEDs) at 22°C. The *CCA1::LUC* (Salome and McClung, 2005), *TOC1::LUC* (Perales and Más, 2007) and *GI::LUC* (Wu et al., 2008) reporter lines as well as the CCA1-ox (Wang and Tobin, 1998), TOC1-ox (Huang et al., 2012), and *toc1-2/CCA1::LUC* (NASC, N2107710) (Cervela-Cardona et al., 2021), *cca1-1 lhy-20* (Nitschke et al., 2016), *prr5-11/CCA1::LUC*, *prr57*, *prr59* (Nakamichi *et al.*, 2005), *prr79* (Farré et al., 2005), ELF3-ox-YFP (Herrero et al., 2012a), YFP-ELF4-ox (Herrero et al., 2012a), LUX-GFP (LUXpro::LUX-GFP *lux-4*) (Ezer et al., 2017), CCA1-HA-EYFP/*cca1-1* (Yakir et al., 2009) lines were described elsewhere. All the lines are in Columbia (Col-0) background. Matching WT backgrounds were used for each mutant line. The TOC1-ox/*elf3-2* lines were generated by crossing the TOC1-ox plants (Huang et al., 2012) with the *elf3-2* mutant (Hicks et al., 1996). The CCA1-ox/*prr* lines were generated by transforming the CCA1-ox construct into the prr79 plants and by crossing the CCA1-ox plants (Wang and Tobin, 1998) with the *prr7* mutant plants (Farré et al., 2005). For the luminescence assays (see below) and for the RT-QPCR (Reverse Transcription Quantitative Polymerase Chain Reaction) analysis of floral organs, sterile dissecting forceps were used to carefully excise young buds, mature buds, mature flowers, and open flowers from flowering plants. Similarly, sepals, petals, stamens, and pistils were carefully excised from open flowers.

2. In vivo luminescence assays

Buds, flowers or floral organs from luciferase-expressing plants synchronized under light:dark cycles (LgD, 16h light:8h dark) with 150-200 μmol $m^{-2}s^{-1}$ of white LEDs at 22°C were excised and immediately placed in 96-well microplates with half-strength MS liquid medium with 1% sucrose and 290μM D-luciferin (Biothema). Bioluminescence rhythms, were examined as previously described (Okada and Mas, 2022) under constant light (LL) conditions or entraining Light:Dark cycles (LgD, 16h light:8h dark) as specified for each experiment. A microplate luminometer LB-960 (Berthold Technologies) and the software Microwin, version 4.34 (Mikrotek 2 Laborsysteme) were used for the bioluminescence analyses. Amplitude, period, and relative amplitude error (RAE) were estimated with the fast Fourier transform non-linear least squares (FFT-NLLS) method (Zielinski et al., 2014). The analyses were performed in the statistical environment of R 3.3.2. Data from samples that appeared damaged or that eventually died in the wells were excluded from the analyses. Three biological replicates were performed per experiment.

3. Gene expression analysis by RT-QPCR

About 5-6 seedlings (12-day old) or about 6-8 flowers were collected, snap-frozen and ground using TissueLyser II (QIAGEN). About 6-8 pistils were collected, snap-frozen and ground using plastic grinding pestles. RNA from seedlings, flowers, and pistils was isolated using the Maxwell RSC Plant RNA kit (Promega). Single strand cDNA was synthesized using iScript™ Reverse Transcription Supermix for RT-qPCR (BioRad) following the manufacturer recommendations. For QPCR analysis, cDNAs were diluted 30-50-fold with nuclease-free water and QPCR was performed with Brilliant III ultrafast SYBR qRT-PCR Master Mix (Agilent) in a 96-well CFX96 Touch Real-Time PCR Detection System (BioRad). The *IPP2* gene (Fung-Uceda et al., 2018) was used as control in seedlings and *PP2AA3* (AT1G13320) (Takahashi et al., 2015) was used as control in pistils and flowers. A list of primers used for gene expression analyses is shown in Table 4. Three biological replicates were performed per experiment.

4. Phenotypic analyses of pistils, siliques, and seeds

For pistil analyses, at least 5 pistils for each genotype were selected at stages 11, 12 and 13 (Müller, 1961; Smyth et al., 1990), and pictures were taken with a stereo microscope (SZX16, Olympus) after careful removal of petals and sepals. Measurements of pistil length was performed using the software package *Image J*. For silique and seed analyses, 100 fully developed siliques from the main inflorescences (starting at the fifth silique from the bottom) were collected and photographed using a stereo microscope (SZX16, Olympus). Seeds from each silique were spread on white paper and photographs were taken. Silique length and seed number per silique were quantified using the software package *Image J*. For seed size and weight analyses, seeds were harvested and sieved to remove plant debris. Following incubation at 25°C for 7 days, randomly selected groups of seeds for each line were weighted (W). Seed number (N), sectional area, length and width were quantified using the software package *Image J*. The grain weight was calculated as: *1000-grain weight (g)= W /N *1000*. Two-tailed Student's *t*-test analyses were performed using the GraphPad Prism software.

5. Phenotypic analyses of pistil and stamen length

5.1. Analyses of the distribution of pistil and stamen length

For the circadian coordination between stamen and pistil analyses, we used about 100 primary inflorescencs from 6-7 weeks old plants growing under LgD conditions (16h light:8h dark). Samples were collected at ZT3 and ZT19 and fixed in 70% ethanol for 7-10 days to remove pigments. About 500 buds and flowers at different developmental stages were collected from the fixed inflorescences and stored in a clearing solution (chloral hydrate/glycerol/water=100g/10g/25ml) for 3 days. Pictures of buds and flowers immersed in clearing solution were taken using a stereo microscope (SZX16, Olympus). Measurements of the length of four long stamens and the pistil was performed by software package *Image J*. Stamen length was determined as the distance from the base of the stamen filament to the tip of the anther. Pistil length was determined as the distance from the base of the pistil to the tip of the stigma papillae.

5.2. Kinetics of stamen or pistil growth.

For pistil and stamen growth kinetic analyses, 50 flowers or buds from about 10 plants growing under LgD conditions (16h light:8h dark) were collected at different stages (from stage 11 to stage14) (Smyth et al., 1990). Pictures were taken with a stereo microscope (SZX16, Olympus) after careful removal of petals and sepals. Measurements of pistil and four long stamens length were performed by using the software package *Image J* why sometimes you use italics and sometimes not?.

6. Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Yamaguchi et al., 2014). About 100 mg of pistils from open flowers were sampled, and vacuum infiltrated 3 times for 15 min in 30 ml cross-linking solution (1% formaldehyde in 1×PBS) at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M and vacuum infiltrated for 5 min. Samples were washed three times with cold deionized water, dried with paper towels and snapped-frozen in liquid nitrogen. Samples were ground to fine powder and extracted with 2.5 ml of Nuclei extraction buffer. After filtering the samples through Miracloth (475855, Merck), the chromatin solution was sonicated until obtaining sheared DNA of about 200-600 bp. Soluble chromatin was incubated overnight at 4°C with theAnti-GFP antibody (#A-11122, Thermo Fisher Scientific) for the samples of ELF3-ox-YFP, YFP-ox-ELF4, LUX-GFP and CCA1-HA-EYFP/*cca1-1*. Samples were then incubated with Protein G-Dynabeads beads (10004D, Thermo Fisher Scientific) for 4 hours at 4°C with rotation. The beads were washed thrice with Low salt wash buffer, High salt wash buffer, 250 mM LiCl wash buffer and 0.5×TE, respectively. The samples were eluted from the beads with elution buffer by incubating for 30 min at 65°C. The purified DNA was diluted 10-fold with nuclease-free water and QPCR was performed with Brilliant III ultrafast SYBR qRT-PCR Master Mix (Agilent) in a 96-well CFX96 Touch Real-Time PCR Detection System (BioRad). Primers used for ChIP-QPCR are shown in Table 4.

Table 3. *Arabidopsis thaliana* lines used in this study.

Line	Ecotype	Source
WT	Col-0	This study
CCA1::LUC	Col-0	(Salomé and McClung, 2005)
TOC1::LUC	Col-0	(Perales and Más, 2007)
GI::LUC	Col-0	(Wu et al., 2008)
CCA1-ox	Col-0	(Wang and Tobin, 1998)
toc1-2/CCA1::LUC	Col-0	NASC, (Cervela-Cardona et al., 2021)
cca1-1/lhy-20	Col-0	(Alabadi et al., 2002)
elf3-2	Col-0	(Hicks et al., 1996)
prr5-11/CCA1::LUC	Col-0	(Nakamichi et al. 2005)
prr7-3	Col-0	(Farré et al., 2005)
prr57/CCA1:LUC	Col-0	(Nakamichi et al. 2005)
prr59	Col-0	(Nakamichi et al. 2005)
prr79	Col-0	(Farré et al., 2005)
ELF3-ox-YFP	Col-0	(Herrero et al., 2012)
YFP-ELF4-ox	Col-0	(Herrero et al., 2012)
LUXpro::LUX-GFP/ *lux-4*	Col-0	(Ezer et al., 2017)
CCA1-HA-EYFP/*cca1-1*	Col-0	(Yakir et al., 2009)
TOC1-ox	Col-0	(Huang et al., 2012)
TOC1-ox/*elf3-2*	Col-0	This study
CCA1-ox/*prr7*	Col-0	This study
CCA1-ox/*prr79*	Col-0	This study

Table 4. A list of primers used in this study

Gene		Sequence	Experiment
IPP2	Forward	CCAGGACAATGCACTAGGTGTG	Expression analysis
	Reverse	AGGGAGTGAACTCATCGACTGG	
PP2AA3	Forward	AAGCGGTTGTGGAGAACATGATACG	Expression analysis
	Reverse	TGGAGAGCTTGATTTGCGAAATACCG	
CCA1	Forward	TCGAAAGACGGGAAGTGGAACG	Expression analysis
	Reverse	GTCGATCTTCATTGGCCATCTCAG	
LHY	Forward	AAGTCTCCGAAGAGGGTCGT	Expression analysis
	Reverse	GGCGAAAAGCTTTGAGGCAA	
TOC1	Forward	TCTTCGCAGAATCCCTGTGAT	Expression analysis
	Reverse	GCTGCACCTAGCTTCAAGCA	
ELF3	Forward	ACCGAGATGGTGGCAAAACT	Expression analysis
	Reverse	ACTGCCATGACCCTCTTGTG	
ELF4	Forward	AGTTTCTCGTCGGGCTTTCACG	Expression analysis
	Reverse	TAAGCTCTAGTTCCGGCAGCAC	
LUX	Forward	CGCTACGTGGTGGATCTTCA	Expression analysis
	Reverse	CGAATCCGATCCAGGACTGC	
PRR5	Forward	AATGGTGGTGATGCCCAGAG	Expression analysis
	Reverse	GCACTCCATCTGTACTGCGT	
PRR7	Forward	AAGTAGTGATGGGAGTGGCG	Expression analysis
	Reverse	GAGATACCGCTCGTGGACTG	
PRR9	Forward	ACCAATGAGGGGATTGCTGG	Expression analysis
	Reverse	TGCAGCTTCTCTCTGGCTTC	
TOC1	Forward	ATAAACGAAACGAAGCCGAATC	ChIP analysis
	Reverse	CAAACATATCAAAAGGTCGACAGAA	
ELF3	Forward	GAAGCTTATTGTTGTGAAAGTTGGAG	ChIP analysis
	Reverse	CTTGCAAACTTCTCAAACCCCA	
ELF4	Forward	GACACCGAGGCGAGTAAGTT	ChIP analysis
	Reverse	ACCCAATCACTTCACAGCTTCA	
LUX	Forward	CTCATCACCGAATCTTTCTCCTC	ChIP analysis

	Reverse	CCAAGTCAGCTATTTGTGAGGGT	
PRR7#2	Forward	TTCCTTACCCACCATTACACG	ChIP analysis
	Reverse	CAGAGCGGATATTTCCACATC	
PRR7	Forward	TCAATGGGGCTGGTCTTTAAG	ChIP analysis
	Reverse	GCAAGGACATACACTTTGGCATC	
PRR9	Forward	GTGTTAAGGTGGACCTGCGA	ChIP analysis
	Reverse	CTTCACTGAGCTGACGTGGC	
PRR9#2	Forward	AACGAGCAGCAACCAGGAG	ChIP analysis
	Reverse	GCTTCTGATTCGTTACTGTGGAC	
TA3	Forward	CTGCGTGGAAGTCTGTCAAA	ChIP analysis
	Reverse	CTATGCCACAGGGCAGTTTT	
elf3-2 control	Forward	TGAGTATTTGTTTCTTCTCGAGC	Genotyping PCR
	Reverse	CATATGGAGGGAAGTAGCCATTAC	
elf3-2	Forward	TGGTTATTTATTCTCCGCTCTTTC	
	Reverse	TTGTTCCATTAGCTGTTCAACCTA	
T-DNA	LBb1.3	ATTTTGCCGATTTCGGAAC	Genotyping PCR
prr7-3	LP	AGCAAGGACATACACTTTGGC	Genotyping PCR
	RP	TGAGAATTCGTCGTTCTTCAAC	
prr9-1	LP	TTTCGTGGTTGTGATCGAAAG	Genotyping PCR
	RP	AGGATCATCACGCAACTGATC	

REFERENCES

References

Adams, S., Manfield, I., Stockley, P., and Carre, I.A. (2015). Revised Morning Loops of the Arabidopsis Circadian Clock Based on Analyses of Direct Regulatory Interactions. PLoS One *10*, e0143943. 10.1371/journal.pone.0143943.

Alabadí, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Más, P., and Kay, S.A. (2001). Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science *293*, 880-883.

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.

Aloni, R., Aloni, E., Langhans, M., and Ullrich, C.I. (2006). Role of auxin in regulating Arabidopsis flower development. Planta *223*, 315-328.

Alvarez-Buylla, E.R., Benítez, M., Corvera-Poiré, A., Cador, Á.C., de Folter, S., de Buen, A.G., Garay-Arroyo, A., García-Ponce, B., Jaimes-Miranda, F., and Pérez-Ruiz, R.V. (2010). Flower development. The Arabidopsis Book/American Society of Plant Biologists *8*.

Alvarez, J., and Smyth, D.R. (2002). CRABS CLAW and SPATULA genes regulate growth and pattern formation during gynoecium development in Arabidopsis thaliana. International journal of plant sciences *163*, 17-41.

Aschoff, J. (1979). Circadian rhythms: influences of internal and external factors on the period measured in constant conditions 1. Zeitschrift für Tierpsychologie *49*, 225-249.

Ashikari, M., Sakakibara, H., Lin, S., Yamamoto, T., Takashi, T., Nishimura, A., Angeles, E.R., Qian, Q., Kitano, H., and Matsuoka, M. (2005). Cytokinin oxidase regulates rice grain production. Science *309*, 741-745.

Atamian, H.S., Creux, N.M., Brown, E.A., Garner, A.G., Blackman, B.K., and Harmer, S.L. (2016). Circadian regulation of sunflower heliotropism, floral orientation, and pollinator visits. Science *353*, 587-590.

Avello, P., Davis, S.J., and Pitchford, J.W. (2021). Temperature robustness in Arabidopsis circadian clock models is facilitated by repressive interactions, autoregulation, and three-node feedbacks. Journal of Theoretical Biology *509*, 110495.

Azzi, A., Evans, J.A., Leise, T., Myung, J., Takumi, T., Davidson, A.J., and Brown, S.A. (2017). Network dynamics mediate circadian clock plasticity. Neuron *93*, 441-450.

Bai, J., and Kawabata, S. (2015). Regulation of diurnal rhythms of flower opening and closure by light cycles, wavelength, and intensity in Eustoma grandiflorum. The Horticulture Journal *84*, 148-155.

Bailey, M., and Silver, R. (2014). Sex differences in circadian timing systems: implications for disease. Frontiers in neuroendocrinology *35*, 111-139.

Balanzà, V., Martínez-Fernández, I., and Ferrándiz, C. (2014). Sequential action of FRUITFULL as a modulator of the activity of the floral regulators SVP and SOC1. Journal of experimental botany *65*, 1193-1203.

Bartrina, I., Otto, E., Strnad, M., Werner, T., and Schmülling, T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in Arabidopsis thaliana. The Plant Cell *23*, 69-80.

Becerra, C., Puigdomenech, P., and Vicient, C.M. (2006). Computational and experimental analysis identifies Arabidopsis genes specifically expressed during early seed development. Bmc Genomics *7*, 1-11.

Belbin, F.E., Hall, G.J., Jackson, A.B., Schanschieff, F.E., Archibald, G., Formstone, C., and Dodd, A.N. (2019). Plant circadian rhythms regulate the effectiveness of a glyphosate-based herbicide. Nature Communications *10*, 3704.

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.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell *115*, 591-602.

Boikoglou, E., Ma, Z., von Korff, M., Davis, A.M., Nagy, F., and Davis, S.J. (2011). Environmental memory from a circadian oscillator: the Arabidopsis thaliana clock differentially integrates perception of photic vs. thermal entrainment. Genetics *189*, 655-664.

Bonner, L.J., and Dickinson, H. (1989). Anther dehiscence in Lycopersicon esculentum Mill. I. Structural aspects. New Phytologist *113*, 97-115.

Bordage, S., Sullivan, S., Laird, J., Millar, A.J., and Nimmo, H.G. (2016). Organ specificity in the plant circadian system is explained by different light inputs to the shoot and root clocks. New Phytologist *212*, 136-149.

Borg, M., Brownfield, L., and Twell, D. (2009). Male gametophyte development: a molecular perspective. Journal of experimental botany *60*, 1465-1478.

Bowman, J.L., Baum, S.F., Eshed, Y., Putterill, J., and Alvarez, J. (1999). 4 molecular genetics of gynoecium development in arabidopsis. Current topics in developmental biology *45*, 155-205.

Box, M.S., Huang, B.E., Domijan, M., Jaeger, K.E., Khattak, A.K., Yoo, S.J., Sedivy, E.L., Jones, D.M., Hearn, T.J., and Webb, A.A. (2015). ELF3 controls thermoresponsive growth in Arabidopsis. Current biology *25*, 194-199.

Briggs, W.R., and Christie, J.M. (2002). Phototropins 1 and 2: versatile plant blue-light receptors. Trends in plant science *7*, 204-210.

Caldeira, C.F., Jeanguenin, L., Chaumont, F., and Tardieu, F. (2014). Circadian rhythms of hydraulic conductance and growth are enhanced by drought and improve plant performance. Nat Commun *5*, 5365. 10.1038/ncomms6365.

Campoli, C., Shtaya, M., Davis, S.J., and von Korff, M. (2012). Expression conservation within the circadian clock of a monocot: natural variation at barley Ppd-H1 affects circadian expression of flowering time genes, but not clock orthologs. BMC plant biology *12*, 1-15.

Carbonell-Bejerano, P., Urbez, C., Carbonell, J., Granell, A., and Perez-Amador, M.A. (2010). A fertilization-independent developmental program triggers partial fruit development and senescence processes in pistils of Arabidopsis. Plant Physiology *154*, 163-172.

Cardarelli, M., and Cecchetti, V. (2014). Auxin polar transport in stamen formation and development: how many actors? Frontiers in plant science *5*, 333.

Cascallares, M., Setzes, N., Marchetti, F., López, G.A., Distéfano, A.M., Cainzos, M., Zabaleta, E., and Pagnussat, G.C. (2020). A complex journey: cell wall remodeling, interactions, and integrity during pollen tube growth. Frontiers in Plant Science *11*, 599247.

Castillejo, C., and Pelaz, S. (2008). The balance between CONSTANS and TEMPRANILLO activities determines FT expression to trigger flowering. Current Biology *18*, 1338-1343.

Cecchetti, V., Altamura, M.M., Falasca, G., Costantino, P., and Cardarelli, M. (2008). Auxin regulates Arabidopsis anther dehiscence, pollen maturation, and filament elongation. The Plant Cell *20*, 1760-1774.

Cervela-Cardona, L., Yoshida, T., Zhang, Y., Okada, M., Fernie, A., and Mas, P. (2021). Circadian control of metabolism by the clock component TOC1. Frontiers in Plant Science *12*, 683516.

Chae, K., Isaacs, C.G., Reeves, P.H., Maloney, G.S., Muday, G.K., Nagpal, P., and Reed, J.W. (2012). Arabidopsis SMALL AUXIN UP RNA63 promotes hypocotyl and stamen filament elongation. The Plant Journal *71*, 684-697.

Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in Arabidopsis thaliana. Proceedings of the National Academy of Sciences *94*, 4223-4228.

Chen, W.W., Takahashi, N., Hirata, Y., Ronald, J., Porco, S., Davis, S.J., Nusinow, D.A., Kay, S.A., and Mas, P. (2020). A mobile ELF4 delivers circadian temperature information from shoots to roots. Nature plants *6*, 416-426.

Cheng, Q., Dong, L., Su, T., Li, T., Gan, Z., Nan, H., Lu, S., Fang, C., Kong, L., and Li, H. (2019). CRISPR/Cas9-mediated targeted mutagenesis of GmLHY genes alters plant height and internode length in soybean. BMC plant biology *19*, 1-11.

Cheng, Y., Dai, X., and Zhao, Y. (2006). Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. Genes & development *20*, 1790-1799.

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.

Christie, J.M., Blackwood, L., Petersen, J., and Sullivan, S. (2015). Plant flavoprotein photoreceptors. Plant and Cell Physiology *56*, 401-413.

Covington, M.F., Maloof, J.N., Straume, M., Kay, S.A., and Harmer, S.L. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome biology *9*, 1-18.

Crawford, B.C., and Yanofsky, M.F. (2011). HALF FILLED promotes reproductive tract development and fertilization efficiency in Arabidopsis thaliana. Development *138*, 2999-3009.

De Caluwé, J., Xiao, Q., Hermans, C., Verbruggen, N., Leloup, J.-C., and Gonze, D. (2016). A compact model for the complex plant circadian clock. Frontiers in plant science *7*, 74.

de Folter, S., Busscher, J., Colombo, L., Losa, A., and Angenent, G.C. (2004). Transcript profiling of transcription factor genes during silique development in Arabidopsis. Plant molecular biology *56*, 351-366.

Demonsais, L., Utz-Pugin, A., Loubery, S., and Lopez-Molina, L. (2020). Identification of tannic

cell walls at the outer surface of the endosperm upon Arabidopsis seed coat rupture. The Plant Journal *104*, 567-580.

Devlin, P.F. (2002). Signs of the time: environmental input to the circadian clock. Journal of Experimental Botany *53*, 1535-1550.

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 *12*, 2499-2509.

Dodd, A.N., Salathia, N., Hall, A., Kévei, E., Tóth, R., Nagy, F., ... & Webb, A. A. (2005). Plant Circadian Clocks Increase

Photosynthesis, Growth, Survival,

and Competitive Advantage. science *5734*.

Doherty, C.J., and Kay, S.A. (2010). Circadian control of global gene expression patterns. Annual review of genetics *44*, 419-444.

Doucet, J., Lee, H.K., and Goring, D.R. (2016). Pollen acceptance or rejection: a tale of two pathways. Trends in plant science *21*, 1058-1067.

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.

Drews, G.N., Bowman, J.L., and Meyerowitz, E.M. (1991). Negative regulation of the Arabidopsis homeotic gene AGAMOUS by the APETALA2 product. Cell *65*, 991-1002.

Edlund, A.F., Swanson, R., and Preuss, D. (2004). Pollen and stigma structure and function: the role of diversity in pollination. The plant cell *16*, S84-S97.

Elleman, C., Franklin-Tong, V., and Dickinson, H. (1992). Pollination in species with dry stigmas: the nature of the early stigmatic response and the pathway taken by pollen tubes. New Phytologist *121*, 413-424.

Endo, M., Shimizu, H., Nohales, M.A., Araki, T., and Kay, S.A. (2014). Tissue-specific clocks in Arabidopsis show asymmetric coupling. Nature *515*, 419-422.

Ezer, D., Jung, J.-H., Lan, H., Biswas, S., Gregoire, L., Box, M.S., Charoensawan, V., Cortijo, S., Lai, X., and Stöckle, D. (2017). The evening complex coordinates environmental and endogenous signals in Arabidopsis. Nature plants *3*, 1-12.

Farquharson, K.L. (2014). SAUR19 links auxin and plasma membrane H+-ATPases in cell

expansion. American Society of Plant Biologists.

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.

Favero, D.S., Lambolez, A., and Sugimoto, K. (2021). Molecular pathways regulating elongation of aerial plant organs: a focus on light, the circadian clock, and temperature. The Plant Journal *105*, 392-420.

Ferrándiz, C. (2002). Regulation of fruit dehiscence in Arabidopsis. Journal of Experimental Botany *53*, 2031-2038.

Ferrándiz, C., Pelaz, S., and Yanofsky, M.F. (1999). Control of carpel and fruit development in Arabidopsis. Annual review of biochemistry *68*, 321-354.

Filichkin, S.A., Breton, G., Priest, H.D., Dharmawardhana, P., Jaiswal, P., Fox, S.E., Michael, T.P., Chory, J., Kay, S.A., and Mockler, T.C. (2011). Global profiling of rice and poplar transcriptomes highlights key conserved circadian-controlled pathways and cis-regulatory modules. PloS one *6*, e16907.

Finger, A.M., and Kramer, A. (2021). Mammalian circadian systems: Organization and modern life challenges. Acta Physiologica *231*, e13548.

Footitt, S., Olcer-Footitt, H., Hambidge, A.J., and Finch-Savage, W.E. (2017). A laboratory simulation of Arabidopsis seed dormancy cycling provides new insight into its regulation by clock genes and the dormancy-related genes DOG1, MFT, CIPK23 and PHYA. Plant Cell Environ *40*, 1474-1486. 10.1111/pce.12940.

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.

Frank, A., Matiolli, C.C., Viana, A.J., Hearn, T.J., Kusakina, J., Belbin, F.E., Newman, D.W., Yochikawa, A., Cano-Ramirez, D.L., and Chembath, A. (2018). Circadian entrainment in Arabidopsis by the sugar-responsive transcription factor bZIP63. Current Biology *28*, 2597-2606. e2596.

Franklin, K.A., and Quail, P.H. (2010). Phytochrome functions in Arabidopsis development. Journal of experimental botany *61*, 11-24.

Fuentes, S., Ljung, K., Sorefan, K., Alvey, E., Harberd, N.P., and Østergaard, L. (2012). Fruit growth in Arabidopsis occurs via DELLA-dependent and DELLA-independent gibberellin

responses. The Plant Cell *24*, 3982-3996.

Fukuda, S., Shimada, K., Maeda, K., Kawasaki, T., Kono, Y., Miyahana, R., Jissho, S., Taguchi, H., Yoshiyama, M., and Fujita, M. (2012). Circadian variation in coronary flow velocity reserve and its relation to α1-sympathetic activity in humans. International journal of cardiology *157*, 216-220.

Fung-Uceda, J., Lee, K., Seo, P.J., Polyn, S., De Veylder, L., and Mas, P. (2018). The circadian clock sets the time of DNA replication licensing to regulate growth in Arabidopsis. Developmental Cell *45*, 101-113. e104.

Galvão, V.C., and Fankhauser, C. (2015). Sensing the light environment in plants: photoreceptors and early signaling steps. Current opinion in neurobiology *34*, 46-53.

Ge, X., Chang, F., and Ma, H. (2010). Signaling and transcriptional control of reproductive development in Arabidopsis. Current Biology *20*, R988-R997.

Gehan, M.A., Greenham, K., Mockler, T.C., and McClung, C.R. (2015). Transcriptional networks—crops, clocks, and abiotic stress. Current opinion in plant biology *24*, 39-46.

Gendreau, E., Traas, J., Desnos, T., Grandjean, O., Caboche, M., and Hofte, H. (1997). Cellular basis of hypocotyl growth in Arabidopsis thaliana. Plant physiology *114*, 295-305.

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.

Gendron, J.M., and Staiger, D. (2023). New Horizons in Plant Photoperiodism. Annual Review of Plant Biology *74*, 481-509.

Goldberg, R.B., Beals, T.P., and Sanders, P.M. (1993). Anther development: basic principles and practical applications. The Plant Cell *5*, 1217.

Gottlieb, D. (2019). Agro-chronobiology: Integrating circadian clocks/time biology into storage management. Journal of stored products research *82*, 9-16.

Gould, P.D., Domijan, M., Greenwood, M., Tokuda, I.T., Rees, H., Kozma-Bognar, L., Hall, A.J., and Locke, J.C. (2018). Coordination of robust single cell rhythms in the Arabidopsis circadian clock via spatial waves of gene expression. Elife *7*, e31700.

Gould, P.D., Locke, J.C., Larue, C., Southern, M.M., Davis, S.J., Hanano, S., Moyle, R., Milich, R., Putterill, J., and Millar, A.J. (2006). The molecular basis of temperature compensation in the Arabidopsis circadian clock. The Plant Cell *18*, 1177-1187.

Gould, P.D., Ugarte, N., Domijan, M., Costa, M., Foreman, J., MacGregor, D., Rose, K., Griffiths, J., Millar, A.J., and Finkenstädt, B. (2013). Network balance via CRY signalling controls the Arabidopsis circadian clock over ambient temperatures. Molecular systems biology *9*, 650.

Green, R., and Tobin, E. (1999). Loss of the circadian clock-associated protein 1 in Arabidopsis results in altered clock-regulated gene expression. Proceedings of the National Academy of Sciences *96*, 4176-4179.

Green, R.M., Tingay, S., Wang, Z.Y., and Tobin, E.M. (2002). Circadian rhythms confer a higher level of fitness to Arabidopsis plants. Plant Physiol *129*, 576-584. 10.1104/pp.004374.

Greenham, K., Lou, P., Puzey, J.R., Kumar, G., Arnevik, C., Farid, H., Willis, J.H., and McClung, C.R. (2017). Geographic variation of plant circadian clock function in natural and agricultural settings. Journal of Biological Rhythms *32*, 26-34.

Greenham, K., and McClung, C.R. (2015). Integrating circadian dynamics with physiological processes in plants. Nature Reviews Genetics *16*, 598-610.

Greenwood, M., Domijan, M., Gould, P.D., Hall, A.J., and Locke, J.C. (2019). Coordinated circadian timing through the integration of local inputs in Arabidopsis thaliana. PLoS biology *17*, e3000407.

Greenwood, M., and Locke, J.C. (2020). The circadian clock coordinates plant development through specificity at the tissue and cellular level. Curr Opin Plant Biol *53*, 65-72. 10.1016/j.pbi.2019.09.004.

Grossniklaus, U., Vielle-Calzada, J.-P., Hoeppner, M.A., and Gagliano, W.B. (1998). Maternal control of embryogenesis by MEDEA, a polycomb group gene in Arabidopsis. Science *280*, 446-450.

Gutiérrez, R.A., Stokes, T.L., Thum, K., Xu, X., Obertello, M., Katari, M.S., Tanurdzic, M., Dean, A., Nero, D.C., and McClung, C.R. (2008). Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene CCA1. Proceedings of the National Academy of Sciences *105*, 4939-4944.

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.

Hasegawa, M., Yahara, T., Yasumoto, A., and Hotta, M. (2006). Bimodal distribution of flowering time in a natural hybrid population of daylily (Hemerocallis fulva) and nightlily (Hemerocallis citrina). Journal of Plant Research *119*, 63-68.

Hayama, R., Yokoi, S., Tamaki, S., Yano, M., and Shimamoto, K. (2003). Adaptation of photoperiodic control pathways produces short-day flowering in rice. Nature *422*, 719-722.

Haydon, M.J., Mielczarek, O., Robertson, F.C., Hubbard, K.E., and Webb, A.A. (2013a). Photosynthetic entrainment of the Arabidopsis thaliana circadian clock. Nature *502*, 689-692. 10.1038/nature12603.

Haydon, M.J., Mielczarek, O., Robertson, F.C., Hubbard, K.E., and Webb, A.A. (2013b). Photosynthetic entrainment of the Arabidopsis thaliana circadian clock. Nature *502*, 689-692.

Hayes, K.R., Beatty, M., Meng, X., Simmons, C.R., Habben, J.E., and Danilevskaya, O.N. (2010). Maize global transcriptomics reveals pervasive leaf diurnal rhythms but rhythms in developing ears are largely limited to the core oscillator. PloS one *5*, e12887.

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 *102*, 10387-10392.

Hennig, L., Gruissem, W., Grossniklaus, U., and Köhler, C. (2004). Transcriptional programs of early reproductive stages in Arabidopsis. Plant Physiology *135*, 1765-1775.

Herrera-Ubaldo, H., and de Folter, S. (2022). Gynoecium and fruit development in Arabidopsis. Development *149*, dev200120.

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

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

Hicks, K.A., Millar, A.J., Carre, 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.

Hoffman, D.E., Jonsson, P., Bylesjö, M., Trygg, J., Antti, H., Eriksson, M.E., and Moritz, T. (2010).

Changes in diurnal patterns within the Populus transcriptome and metabolome in response to photoperiod variation. Plant, cell & environment *33*, 1298-1313.

Hotta, C.T., Gardner, M.J., Hubbard, K.E., Baek, S.J., Dalchau, N., Suhita, D., Dodd, A.N., and Webb, A.A. (2007). Modulation of environmental responses of plants by circadian clocks. Plant, cell & environment *30*, 333-349.

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.

Huang, H., McLoughlin, K.E., Sorkin, M.L., Burgie, E.S., Bindbeutel, R.K., Vierstra, R.D., and Nusinow, D.A. (2019). PCH1 regulates light, temperature, and circadian signaling as a structural component of phytochrome B-photobodies in Arabidopsis. Proceedings of the National Academy of Sciences *116*, 8603-8608.

Huang, W., Pérez-García, 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.

Huang, Z., Footitt, S., and Finch-Savage, W.E. (2014). The effect of temperature on reproduction in the summer and winter annual Arabidopsis thaliana ecotypes Bur and Cvi. Annals of botany *113*, 921-929.

Ichihashi, Y., Kawade, K., Usami, T., Horiguchi, G., Takahashi, T., and Tsukaya, H. (2011). Key proliferative activity in the junction between the leaf blade and leaf petiole of Arabidopsis. Plant Physiology *157*, 1151-1162.

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., Niwa, Y., Nakamichi, N., Kawamura, H., Yamashino, T., and Mizuno, T. (2008). Insight into missing genetic links between two evening-expressed pseudo-response regulator genes TOC1 and PRR5 in the circadian clock-controlled circuitry in Arabidopsis thaliana. Plant and cell physiology *49*, 201-213.

Iwasaki, H., and Kondo, T. (2000). The current state and problems of circadian clock studies in cyanobacteria. Plant and cell physiology *41*, 1013-1020.

James, A.B., Monreal, J.A., Nimmo, G.A., Kelly, C.n.L., Herzyk, P., Jenkins, G.I., and Nimmo, H.G. (2008). The circadian clock in Arabidopsis roots is a simplified slave version of the clock in

shoots. Science *322*, 1832-1835.

Jiménez, A., Sevilla, F., and Martí, M.C. (2021). Reactive oxygen species homeostasis and circadian rhythms in plants. Journal of Experimental Botany *72*, 5825-5840.

Johnson, M.A., Harper, J.F., and Palanivelu, R. (2019). A fruitful journey: pollen tube navigation from germination to fertilization. Annual review of plant biology *70*, 809-837.

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., Hu, W., Litthauer, S., Lagarias, J.C., and Harmer, S.L. (2015). A constitutively active allele of phytochrome B maintains circadian robustness in the absence of light. Plant Physiology *169*, 814-825.

Jung, J.-H., Barbosa, A.D., Hutin, S., Kumita, J.R., Gao, M., Derwort, D., Silva, C.S., Lai, X., Pierre, E., and Geng, F. (2020). A prion-like domain in ELF3 functions as a thermosensor in Arabidopsis. Nature *585*, 256-260.

Jung, J.-H., Domijan, M., Klose, C., Biswas, S., Ezer, D., Gao, M., Khattak, A.K., Box, M.S., Charoensawan, V., and Cortijo, S. (2016). Phytochromes function as thermosensors in Arabidopsis. Science *354*, 886-889.

Kaihara, S., and Takimoto, A. (1979). Environmental factors controlling the time of flower-opening in Pharbitis nil. Plant and cell physiology *20*, 1659-1666.

Keijzer, C. (1987). The processes of anther dehiscence and pollen dispersal: ii. the formation and the transfer mechanism of pollenkitt, cell-wall development of the loculus tissues and a function of orbicules in pollen dispersal. New Phytologist *105*, 499-507.

Kennaway, D.J., Boden, M.J., and Varcoe, T.J. (2012). Circadian rhythms and fertility. Molecular and cellular endocrinology *349*, 56-61.

Kessler, A., and Chautá, A. (2020). The ecological consequences of herbivore-induced plant responses on plant–pollinator interactions. Emerging Topics in Life Sciences *4*, 33-43.

Kessler, D., Diezel, C., and Baldwin, I.T. (2010). Changing pollinators as a means of escaping herbivores. Current Biology *20*, 237-242.

Kessler, D., Gase, K., and Baldwin, I.T. (2008). Field experiments with transformed plants reveal the sense of floral scents. Science *321*, 1200-1202.

Khan, S., Rowe, S.C., and Harmon, F.G. (2010). Coordination of the maize transcriptome by a

conserved circadian clock. BMC plant biology *10*, 1-15.

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.

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.

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T., and Yano, M. (2002). Hd3a, a rice ortholog of the Arabidopsis FT gene, promotes transition to flowering downstream of Hd1 under short-day conditions. Plant and cell physiology *43*, 1096-1105.

Lee, S.-J., Kang, K., Lim, J.-H., and Paek, N.-C. (2022). Natural alleles of CIRCADIAN CLOCK ASSOCIATED1 contribute to rice cultivation by fine-tuning flowering time. Plant Physiology *190*, 640-656.

Legris, M., Klose, C., Burgie, E.S., Rojas, C.C.R., Neme, M., Hiltbrunner, A., Wigge, P.A., Schäfer, E., Vierstra, R.D., and Casal, J.J. (2016). Phytochrome B integrates light and temperature signals in Arabidopsis. Science *354*, 897-900.

Li, B., Wang, Y., Zhang, Y., Tian, W., Chong, K., Jang, J.-C., and Wang, L. (2019a). PRR5, 7 and 9 positively modulate TOR signaling-mediated root cell proliferation by repressing TANDEM ZINC FINGER 1 in Arabidopsis. Nucleic Acids Research *47*, 5001-5015.

Li, C., Li, Y.-h., Li, Y., Lu, H., Hong, H., Tian, Y., Li, H., Zhao, T., Zhou, X., and Liu, J. (2020). A domestication-associated gene GmPRR3b regulates the circadian clock and flowering time in soybean. Molecular plant *13*, 745-759.

Li, N., Xu, R., and Li, Y. (2019b). Molecular networks of seed size control in plants. Annual review of plant biology *70*, 435-463.

Liang, L., Zhang, Z., Cheng, N., Liu, H., Song, S., Hu, Y., Zhou, X., Zhang, J., and Xing, Y. (2021). The transcriptional repressor OsPRR73 links circadian clock and photoperiod pathway to control heading date in rice. Plant, Cell & Environment *44*, 842-855.

Locke, A.M., Slattery, R.A., and Ort, D.R. (2018). Field-grown soybean transcriptome shows diurnal patterns in photosynthesis-related processes. Plant Direct *2*, e00099.

Lou, P., Xie, Q., Xu, X., Edwards, C., Brock, M., Weinig, C., and McClung, C. (2011). Genetic architecture of the circadian clock and flowering time in Brassica rapa. Theoretical and applied genetics *123*, 397-409.

Lu, H., McClung, C.R., and Zhang, C. (2017). Tick tock: circadian regulation of plant innate immunity. Annual review of phytopathology *55*, 287-311.

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.

Ma, H. (2005). Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. Annu. Rev. Plant Biol. *56*, 393-434.

Marciniak, K., and Przedniczek, K. (2019). Comprehensive insight into gibberellin-and jasmonate-mediated stamen development. Genes *10*, 811.

Marcolino-Gomes, J., Rodrigues, F.A., Fuganti-Pagliarini, R., Bendix, C., Nakayama, T.J., Celaya, B., Molinari, H.B.C., de Oliveira, M.C.N., Harmon, F.G., and Nepomuceno, A. (2014). Diurnal oscillations of soybean circadian clock and drought responsive genes. PLoS One *9*, e86402.

Marsch-Martínez, N., Ramos-Cruz, D., Irepan Reyes-Olalde, J., Lozano-Sotomayor, P., Zúñiga-Mayo, V.M., and De Folter, S. (2012). The role of cytokinin during Arabidopsis gynoecia and fruit morphogenesis and patterning. The Plant Journal *72*, 222-234.

Marshall, C.M., Thompson, V.L., Creux, N.M., and Harmer, S.L. (2023). The circadian clock controls temporal and spatial patterns of floral development in sunflower. Elife *12*, e80984.

Martín, G., Rovira, A., Veciana, N., Soy, J., Toledo-Ortiz, G., Gommers, C.M., Boix, M., Henriques, R., Minguet, E.G., and Alabadí, D. (2018). Circadian waves of transcriptional repression shape PIF-regulated photoperiod-responsive growth in Arabidopsis. Current Biology *28*, 311-318. e315.

Marzi, D., Brunetti, P., Mele, G., Napoli, N., Calò, L., Spaziani, E., Matsui, M., De Panfilis, S., Costantino, P., and Serino, G. (2020). Light controls stamen elongation via cryptochromes, phytochromes and COP1 through HY5 and HYH. The Plant Journal *103*, 379-394.

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.

Matsushika, A., Murakami, M., Ito, S., Nakamichi, N., Yamashino, T., and Mizuno, T. (2007). Characterization of circadian-associated pseudo-response regulators: I. Comparative studies on a series of transgenic lines misexpressing five distinctive PRR genes in Arabidopsis thaliana. Bioscience, biotechnology, and biochemistry *71*, 527-534.

McClung, C.R. (2006). Plant circadian rhythms. The Plant Cell *18*, 792-803.

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.

Mehra, A., Shi, M., Baker, C.L., Colot, H.V., Loros, J.J., and Dunlap, J.C. (2009). A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. Cell *137*, 749-760.

Meinke, D.W., and Sussex, I.M. (1979). Embryo-lethal mutants of Arabidopsis thaliana: a model system for genetic analysis of plant embryo development. Developmental Biology *72*, 50-61.

Michael, T.P., Salomé, P.A., and McClung, C.R. (2003). Two Arabidopsis circadian oscillators can be distinguished by differential temperature sensitivity. Proceedings of the National Academy of Sciences *100*, 6878-6883.

Millar, A.J. (2004). Input signals to the plant circadian clock. Journal of experimental botany *55*, 277-283.

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.

Mitsuda, N., Seki, M., Shinozaki, K., and Ohme-Takagi, M. (2005). The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. The Plant Cell *17*, 2993-3006.

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

Möglich, A., Yang, X., Ayers, R.A., and Moffat, K. (2010). Structure and function of plant photoreceptors. Annual review of plant biology *61*, 21-47.

Müller, A. (1961). Zur Charakterisierung der Blüten und Infloreszenzen von Arabidopsis thaliana (L.) Heynh. Die Kulturpflanze *9*, 364-393.

Müller, N.A., Zhang, L., Koornneef, M., and Jiménez-Gómez, J.M. (2018). Mutations in EID1 and

LNK2 caused light-conditional clock deceleration during tomato domestication. Proceedings of the National Academy of Sciences *115*, 7135-7140.

Muroya, M., Oshima, H., Kobayashi, S., Miura, A., Miyamura, Y., Shiota, H., Onai, K., Ishiura, M., Manabe, K., and Kutsuna, S. (2021). Circadian clock in Arabidopsis thaliana determines flower opening time early in the morning and dominantly closes early in the afternoon. Plant and Cell Physiology *62*, 883-893.

Mwimba, M., Karapetyan, S., Liu, L., Marques, J., McGinnis, E.M., Buchler, N.E., and Dong, X. (2018a). Daily humidity oscillation regulates the circadian clock to influence plant physiology. Nat Commun *9*, 4290. 10.1038/s41467-018-06692-2.

Mwimba, M., Karapetyan, S., Liu, L., Marqués, J., McGinnis, E.M., Buchler, N.E., and Dong, X. (2018b). Daily humidity oscillation regulates the circadian clock to influence plant physiology. Nature Communications *9*, 4290.

Nagpal, P., Ellis, C.M., Weber, H., Ploense, S.E., Barkawi, L.S., Guilfoyle, T.J., Hagen, G., Alonso, J.M., Cohen, J.D., and Farmer, E.E. (2005). Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation.

Nakamichi, N. (2020). The transcriptional network in the Arabidopsis circadian clock system. Genes *11*, 1284.

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

Nakamichi, N., Kiba, T., Henriques, R., Mizuno, T., Chua, N.H., and Sakakibara, H. (2010b). PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the Arabidopsis circadian clock. Plant Cell *22*, 594-605. 10.1105/tpc.109.072892.

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.

Nakamichi, N., Kita, M., Niinuma, K., Ito, S., Yamashino, T., Mizoguchi, T., and Mizuno, T. (2007). Arabidopsis clock-associated pseudo-response regulators PRR9, PRR7 and PRR5 coordinately and

positively regulate flowering time through the canonical CONSTANS-dependent photoperiodic pathway.

Nitschke, S., Cortleven, A., Iven, T., Feussner, I., Havaux, M., Riefler, M., and Schmülling, T. (2016). Circadian stress regimes affect the circadian clock and cause jasmonic acid-dependent cell death in cytokinin-deficient Arabidopsis plants. The Plant Cell *28*, 1616-1639.

Niu, Y., Li, G., Jian, Y., Duan, S., Liu, J., Xu, J., and Jin, L. (2022). Genes related to circadian rhythm are involved in regulating tuberization time in potato. Horticultural Plant Journal *8*, 369-380.

Niwa, Y., Yamashino, T., and Mizuno, T. (2009). The circadian clock regulates the photoperiodic response of hypocotyl elongation through a coincidence mechanism in Arabidopsis thaliana. Plant and Cell Physiology *50*, 838-854.

Nohales, M.A., and Kay, S.A. (2016). Molecular mechanisms at the core of the plant circadian oscillator. Nature structural & molecular biology *23*, 1061-1069.

Nole-Wilson, S., Rueschhoff, E.E., Bhatti, H., and Franks, R.G. (2010). Synergistic disruptions in seuss cyp85A2 double mutants reveal a role for brassinolide synthesis during gynoecium and ovule development. BMC Plant Biology *10*, 1-7.

Nozue, K., Covington, M.F., Duek, P.D., Lorrain, S., Fankhauser, C., Harmer, S.L., and Maloof, J.N. (2007). Rhythmic growth explained by coincidence between internal and external cues. Nature *448*, 358-361.

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.

Odgerel, K., Jose, J., Karsai-Rektenwald, F., Ficzek, G., Simon, G., Végvári, G., and Bánfalvi, Z. (2022). Effects of the repression of GIGANTEA gene StGI. 04 on the potato leaf transcriptome and the anthocyanin content of tuber skin. BMC Plant Biology *22*, 1-18.

Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y. (1991). Requirement of the auxin polar transport system in early stages of Arabidopsis floral bud formation. The Plant Cell *3*, 677-684.

Okada, M., Yang, Z., and Mas, P. (2022). Circadian autonomy and rhythmic precision of the Arabidopsis female reproductive organ. Developmental Cell *57*, 2168-2180. e2164.

Parsons, R., Parsons, R., Garner, N., Oster, H., and Rawashdeh, O. (2020). CircaCompare: a method

to estimate and statistically support differences in mesor, amplitude and phase, between circadian rhythms. Bioinformatics *36*, 1208-1212.

Pedmale, U.V., Huang, S.-s.C., Zander, M., Cole, B.J., Hetzel, J., Ljung, K., Reis, P.A., Sridevi, P., Nito, K., and Nery, J.R. (2016). Cryptochromes interact directly with PIFs to control plant growth in limiting blue light. Cell *164*, 233-245.

Peer, L.A., Bhat, M.Y., Ahmad, N., and Mir, B.A. (2021). Floral induction pathways: Decision making and determination in plants to flower-A comprehensive review. Journal of Applied Biology and Biotechnology *9*, 7-17.

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

Proveniers, M.C., and van Zanten, M. (2013). High temperature acclimation through PIF4 signaling. Trends in plant science *18*, 59-64.

Qin, C., Li, H., Zhang, S., Lin, X., Jia, Z., Zhao, F., Wei, X., Jiao, Y., Li, Z., and Niu, Z. (2023). GmEID1 modulates light signaling through the Evening Complex to control flowering time and yield in soybean. Proceedings of the National Academy of Sciences *120*, e2212468120.

Regan, S.M., and Moffatt, B.A. (1990). Cytochemical analysis of pollen development in wild-type Arabidopsis and a male-sterile mutant. The Plant Cell *2*, 877-889.

Reganold, J.P., and Wachter, J.M. (2016). Organic agriculture in the twenty-first century. Nature plants *2*, 1-8.

Roeder, A.H., and Yanofsky, M.F. (2006). Fruit development in Arabidopsis. The Arabidopsis Book/American Society of Plant Biologists *4*.

Roenneberg, T., Kumar, C.J., and Merrow, M. (2007). The human circadian clock entrains to sun time. Current Biology *17*, R44-R45.

Rogers, H., and Munné-Bosch, S. (2016). Production and scavenging of reactive oxygen species and redox signaling during leaf and flower senescence: similar but different. Plant Physiology *171*, 1560-1568.

Rogers, H.J. (2006). Programmed cell death in floral organs: how and why do flowers die? Annals of Botany *97*, 309-315.

Rosas, U., Mei, Y., Xie, Q., Banta, J.A., Zhou, R.W., Seufferheld, G., Gerard, S., Chou, L., Bhambhra, N., and Parks, J.D. (2014). Variation in Arabidopsis flowering time associated with cis-

regulatory variation in CONSTANS. Nature communications *5*, 3651.

Rozier, F., Riglet, L., Kodera, C., Bayle, V., Durand, E., Schnabel, J., Gaude, T., and Fobis-Loisy, I. (2020). Live-cell imaging of early events following pollen perception in self-incompatible Arabidopsis thaliana. Journal of Experimental Botany *71*, 2513-2526.

Sablowski, R. (2015). Control of patterning, growth, and differentiation by floral organ identity genes. Journal of Experimental Botany *66*, 1065-1073.

Salome, P.A., and McClung, C.R. (2005). 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., Michael, T.P., Kearns, E.V., Fett-Neto, A.G., Sharrock, R.A., and McClung, C.R. (2002). The out of phase 1 mutant defines a role for PHYB in circadian phase control in Arabidopsis. Plant Physiology *129*, 1674-1685.

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

Salomé, P.A., Weigel, D., and McClung, C.R.J.T.P.C. (2010b). The role of the Arabidopsis morning loop components CCA1, LHY, PRR7, and PRR9 in temperature compensation. *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, A.M., Bosch, M., Bots, M., Nieuwland, J., Feron, R., and Mariani, C. (2004). Pistil factors controlling pollination. The Plant Cell *16*, S98-S106.

Sanders, P.M., Bui, A.Q., Weterings, K., McIntire, K., Hsu, Y.-C., Lee, P.Y., Truong, M.T., Beals, T., and Goldberg, R. (1999). Anther developmental defects in Arabidopsis thaliana male-sterile mutants. Sexual plant reproduction *11*, 297-322.

Sanzol, J., Rallo, P., and Herrero, M. (2003). Asynchronous development of stigmatic receptivity in the pear (Pyrus communis; Rosaceae) flower. American Journal of Botany *90*, 78-84.

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.

Scheer, F.A., Wright Jr, K.P., Kronauer, R.E., and Czeisler, C.A. (2007). Plasticity of the intrinsic period of the human circadian timing system. PloS one *2*, e721.

Schippers, J.H., Schmidt, R., Wagstaff, C., and Jing, H.-C. (2015). Living to die and dying to live: the survival strategy behind leaf senescence. Plant Physiology *169*, 914-930.

Scott, R.J., Spielman, M., and Dickinson, H. (2004). Stamen structure and function. The plant cell *16*, S46-S60.

Sessions, R.A., and Zambryski, P.C. (1995). Arabidopsis gynoecium structure in the wild type and in ettin mutants. Development *121*, 1519-1532.

Shim, J.S., Kubota, A., and Imaizumi, T. (2017). Circadian clock and photoperiodic flowering in Arabidopsis: CONSTANS is a hub for signal integration. Plant physiology *173*, 5-15.

Shin, J., Sánchez-Villarreal, A., Davis, A.M., Du, S.x., Berendzen, K.W., Koncz, C., Ding, Z., Li, C., and Davis, S.J. (2017). The metabolic sensor AKIN10 modulates the Arabidopsis circadian clock in a light-dependent manner. Plant, cell & environment *40*, 997-1008.

Singh, M., and Mas, P. (2018). A functional connection between the circadian clock and hormonal timing in Arabidopsis. Genes *9*, 567.

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in Arabidopsis. The Plant Cell *2*, 755-767.

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., Webb, A.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, S., Qi, T., Wasternack, C., and Xie, D. (2014). Jasmonate signaling and crosstalk with gibberellin and ethylene. Current Opinion in Plant Biology *21*, 112-119.

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.

Sorkin, M.L., and Nusinow, D.A. (2021). Time will tell: intercellular communication in the plant clock. Trends in plant science *26*, 706-719.

Spillane, C., MacDougall, C., Stock, C., Köhler, C., Vielle-Calzada, J., Nunes, S.M., Grossniklaus, U., and Goodrich, J. (2000). Interaction of the Arabidopsis polycomb group proteins FIE and MEA mediates their common phenotypes. Current Biology *10*, 1535-1538.

Steed, G., Ramirez, D.C., Hannah, M.A., and Webb, A.A. (2021). Chronoculture, harnessing the circadian clock to improve crop yield and sustainability. Science *372*, eabc9141.

Stepanova, A.N., Robertson-Hoyt, J., Yun, J., Benavente, L.M., Xie, D.-Y., Doležal, K., Schlereth, A., Jürgens, G., and Alonso, J.M. (2008). TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell *133*, 177-191.

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.

Sun, H., Zhang, W., Wu, Y., Gao, L., Cui, F., Zhao, C., Guo, Z., and Jia, J. (2020). The circadian clock gene, TaPRR1, is associated with yield-related traits in wheat (Triticum aestivum L.). Frontiers in Plant Science *11*, 285.

Sun, J., Zheng, Y., Guo, J., Zhang, Y., Liu, Y., Tao, Y., Wang, M., Liu, T., Liu, Y., and Li, X. (2023). GmGAMYB-BINDING PROTEIN 1 promotes Small Auxin-Up RNA gene transcription to modulate soybean maturity and height. Plant Physiology, kiad293.

Sundberg, E., and Ferrándiz, C. (2009). Gynoecium patterning in Arabidopsis: a basic plan behind a complex structure. Annual Plant Reviews Volume 38: Fruit Development and Seed Dispersal *38*, 35-69.

Takahashi, N., Hirata, Y., Aihara, K., and Mas, P. (2015). A hierarchical multi-oscillator network orchestrates the Arabidopsis circadian system. Cell *163*, 148-159.

Tashiro, S., Tian, C.e., Watahiki, M.K., and Yamamoto, K.T. (2009). Changes in growth kinetics of stamen filaments cause inefficient pollination in massugu2, an auxin insensitive, dominant mutant of Arabidopsis thaliana. Physiologia plantarum *137*, 175-187.

Thain, S.C., Hall, A., and Millar, A.J. (2000). Functional independence of circadian clocks that regulate plant gene expression. Current biology *10*, 951-956.

Thain, S.C., Murtas, G., Lynn, J.R., McGrath, R.B., and Millar, A.J. (2002). The circadian clock that controls gene expression in Arabidopsis is tissue specific. Plant Physiology *130*, 102-110.

Tilbrook, K., Arongaus, A.B., Binkert, M., Heijde, M., Yin, R., and Ulm, R. (2013). The UVR8 UV-B photoreceptor: perception, signaling and response. The Arabidopsis Book/American Society

of Plant Biologists *11*.

Tóth, R., Kevei, E., Hall, A., Millar, A.J., Nagy, F., and Kozma-Bognár, L. (2001). Circadian clock-regulated expression of phytochrome and cryptochrome genes in Arabidopsis. Plant physiology *127*, 1607-1616.

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 Doorn, W.G., and Van Meeteren, U. (2003). Flower opening and closure: a review. Journal of experimental botany *54*, 1801-1812.

van Doorn, W.G., and Woltering, E.J. (2004). Senescence and programmed cell death: substance or semantics? Journal of experimental botany *55*, 2147-2153.

Vitaterna, M.H., Takahashi, J.S., and Turek, F.W. (2001). Overview of circadian rhythms. Alcohol research & health *25*, 85.

Vivian-Smith, A., and Koltunow, A.M. (1999). Genetic analysis of growth-regulator-induced parthenocarpy in Arabidopsis. Plant Physiology *121*, 437-452.

Vivian-Smith, A., Luo, M., Chaudhury, A., and Koltunow, A. (2001). Fruit development is actively restricted in the absence of fertilization in Arabidopsis.

Wang, C.-Q., Sarmast, M.K., Jiang, J., and Dehesh, K. (2015). The transcriptional regulator BBX19 promotes hypocotyl growth by facilitating COP1-mediated EARLY FLOWERING3 degradation in Arabidopsis. The Plant Cell *27*, 1128-1139.

Wang, F., Han, T., Song, Q., Ye, W., Song, X., Chu, J., Li, J., and Chen, Z.J. (2020). The rice circadian clock regulates tiller growth and panicle development through strigolactone signaling and sugar sensing. Plant Cell *32*, 3124-3138.

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.

Weigel, D. (1995). The genetics of flower development: from floral induction to ovule morphogenesis. Annual review of genetics *29*, 19-39.

Wellmer, F., Graciet, E., and Riechmann, J.L. (2014). Specification of floral organs in Arabidopsis. Journal of experimental botany *65*, 1-9.

Wenden, B., Toner, D.L., Hodge, S.K., Grima, R., and Millar, A.J. (2012). Spontaneous spatiotemporal waves of gene expression from biological clocks in the leaf. Proceedings of the National Academy of Sciences *109*, 6757-6762.

Wilcock, C., and Neiland, R. (2002). Pollination failure in plants: why it happens and when it matters. Trends in plant science *7*, 270-277.

Wu, J.-F., Wang, Y., and Wu, S.-H. (2008). Two new clock proteins, LWD1 and LWD2, regulate Arabidopsis photoperiodic flowering. Plant physiology *148*, 948-959.

Xu, X., Yuan, L., Yang, X., Zhang, X., Wang, L., and Xie, Q. (2022). Circadian clock in plants: Linking timing to fitness. Journal of integrative plant biology *64*, 792-811.

Yadav, V.K., Sawant, S.V., Yadav, A., Jalmi, S.K., and Kerkar, S. (2022). Genome-wide analysis of long non-coding RNAs under diel light exhibits role in floral development and the circadian clock in Arabidopsis thaliana. International Journal of Biological Macromolecules *223*, 1693-1704.

Yakir, E., Hassidim, M., Melamed-Book, N., Hilman, D., Kron, I., and Green, R.M. (2011a). Cell autonomous and cell-type specific circadian rhythms in Arabidopsis. Plant J *68*, 520-531. 10.1111/j.1365-313X.2011.04707.x.

Yakir, E., Hassidim, M., Melamed-Book, N., Hilman, D., Kron, I., and Green, R.M. (2011b). Cell autonomous and cell-type specific circadian rhythms in Arabidopsis. The Plant Journal *68*, 520-531.

Yakir, E., Hilman, D., Harir, Y., and Green, R.M. (2007). Regulation of output from the plant circadian clock. The FEBS journal *274*, 335-345.

Yamashino, T., Matsushika, A., Fujimori, T., Sato, S., Kato, T., Tabata, S., and Mizuno, T. (2003). A link between circadian-controlled bHLH factors and the APRR1/TOC1 quintet in Arabidopsis thaliana. Plant and Cell Physiology *44*, 619-629.

Yang, C., Xu, Z., Song, J., Conner, K., Vizcay Barrena, G., and Wilson, Z.A. (2007). Arabidopsis MYB26/MALE STERILE35 regulates secondary thickening in the endothecium and is essential for anther dehiscence. The Plant Cell *19*, 534-548.

Yang, M., Han, X., Yang, J., Jiang, Y., and Hu, Y. (2021). The Arabidopsis circadian clock protein PRR5 interacts with and stimulates ABI5 to modulate abscisic acid signaling during seed germination. The Plant Cell *33*, 3022-3041.

Yang, Y., Peng, Q., Chen, G.-X., Li, X.-H., and Wu, C.-Y. (2013). OsELF3 is involved in circadian clock regulation for promoting flowering under long-day conditions in rice. Molecular Plant *6*, 202-

215.

Yanovsky, M.J., and Kay, S.A. (2002). Molecular basis of seasonal time measurement in Arabidopsis. Nature *419*, 308-312.

Yarkhunova, Y., Edwards, C.E., Ewers, B.E., Baker, R.L., Aston, T.L., McClung, C.R., Lou, P., and Weinig, C. (2016). Selection during crop diversification involves correlated evolution of the circadian clock and ecophysiological traits in Brassica rapa. New Phytologist *210*, 133-144.

Ye, Q., Zhu, W., Li, L., Zhang, S., Yin, Y., Ma, H., and Wang, X. (2010). Brassinosteroids control male fertility by regulating the expression of key genes involved in Arabidopsis anther and pollen development. Proceedings of the National Academy of Sciences *107*, 6100-6105.

Yuan, L., Yu, Y., Liu, M., Song, Y., Li, H., Sun, J., Wang, Q., Xie, Q., Wang, L., and Xu, X. (2021). BBX19 fine-tunes the circadian rhythm by interacting with PSEUDO-RESPONSE REGULATOR proteins to facilitate their repressive effect on morning-phased clock genes. The Plant Cell *33*, 2602-2617.

Zdepski, A., Wang, W., Priest, H.D., Ali, F., Alam, M., Mockler, T.C., and Michael, T.P. (2008). Conserved daily transcriptional programs in Carica papaya. Tropical plant biology *1*, 236-245.

Zhang, Y., Wu, Z., Feng, M., Chen, J., Qin, M., Wang, W., Bao, Y., Xu, Q., Ye, Y., and Ma, C. (2021). The circadian-controlled PIF8–BBX28 module regulates petal senescence in rose flowers by governing mitochondrial ROS homeostasis at night. The Plant Cell *33*, 2716-2735.

Zhu, J.-Y., Oh, E., Wang, T., and Wang, Z.-Y. (2016). TOC1–PIF4 interaction mediates the circadian gating of thermoresponsive growth in Arabidopsis. Nature Communications *7*, 13692.

Zhu, Z., Quint, M., and Anwer, M.U. (2022). Arabidopsis EARLY FLOWERING 3 controls temperature responsiveness of the circadian clock independently of the evening complex. Journal of Experimental Botany *73*, 1049-1061.

Zúñiga-Mayo, V.M., Gómez-Felipe, A., Herrera-Ubaldo, H., and de Folter, S. (2019). Gynoecium development: networks in Arabidopsis and beyond. Journal of experimental botany *70*, 1447-1460.

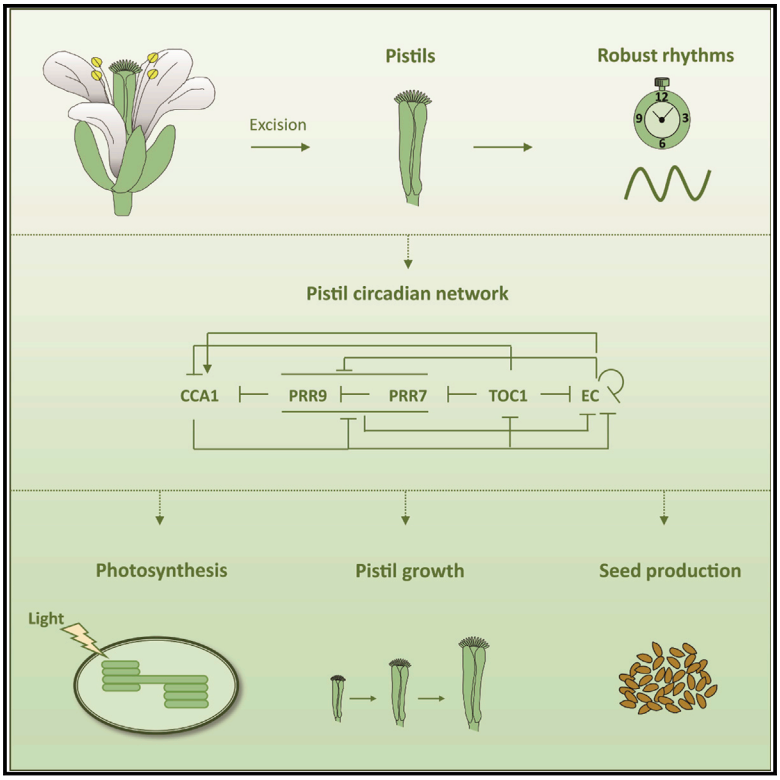
ANNEXES

Article

Developmental Cell

Circadian autonomy and rhythmic precision of the *Arabidopsis* female reproductive organ

Graphical abstract



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In brief

Okada and Yang et al. show that pistils, the plant female reproductive organs, survive for several days after detachment from the flower and generate robust circadian rhythms, which indicate high circadian autonomy and precision. Understanding the circadian clock function in reproductive organs is useful for improving plant reproduction and productivity.

Highlights

- Pistils sustain robust rhythms and display organ-specific circadian autonomy
- The pistil clock controls pistil and silique growth and seed weight and production
- Clock activators and repressors show specific regulatory functions in pistils
- A hierarchy of repressing activities might provide robustness to the pistil clock

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Developmental Cell

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Article

Circadian autonomy and rhythmic precision of the *Arabidopsis* female reproductive organ

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SUMMARY

The plant circadian clock regulates essential biological processes including flowering time or petal movement. However, little is known about how the clock functions in flowers. Here, we identified the circadian components and transcriptional networks contributing to the generation of rhythms in pistils, the female reproductive organ. When detached from the rest of the flower, pistils sustain highly precise rhythms, indicating organ-specific circadian autonomy. Analyses of clock mutants and chromatin immunoprecipitation assays showed distinct expression patterns and specific regulatory functions for clock activators and repressors in pistils. Genetic interaction studies also suggested a hierarchy of the repressing activities that provide robustness and precision to the pistil clock. Globally, the circadian function in pistils primarily governs responses to environmental stimuli and photosynthesis and controls pistil growth and seed weight and production. Understanding the circadian intricacies in reproductive organs may prove useful for optimizing plant reproduction and productivity.

INTRODUCTION

The circadian clock generates 24-h biological rhythms in synchrony with external and internal cues (Young and Kay, 2001). At its basis, generation of the rhythms relies on a precise rhythmic regulation of clock gene expression and protein function (Chen and Mas, 2019; Crosby and Partch, 2020; Seo and Mas, 2014; Takahashi, 2017). The circadian molecular network has been extensively investigated in the model plant *Arabidopsis thaliana*, most notably using whole seedlings (Nakamichi, 2020). Recent studies on specific organs and tissues are uncovering both the circadian autonomy of some organs (e.g., Thain et al., 2000, 2002; James et al., 2008; Yakir et al., 2011; Fukuda et al., 2012; Wenden et al., 2012; Endo et al., 2014; Bordage et al., 2016) as well as the coupling and coordination of rhythms within the plant (Chen et al., 2020; Endo et al., 2014; Fukuda et al., 2007; Gould et al., 2018; Greenwood et al., 2019; Takahashi et al., 2015). Therefore, the plant circadian system comprises autonomous tissue-specific rhythms complemented with cell-to-cell coupling and long-distance coordination (Nakamichi, 2020; Sorkin and Nusinow, 2021).

Transcriptional feedback loops at the core of the *Arabidopsis* oscillator delineate a time-of-day specific expression of the main oscillator genes (McClung, 2019; Nakamichi, 2020; Sanchez and Kay, 2016). The morning-expressed core clock components include the single-MYB transcription factors CIRCADIAN CLOCK ASSOCIATED1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), and the members of the PSEUDO-RESPONSE REGULATOR (PRR) family, PRR9 and PRR7, which act during the day primarily to repress clock gene expression (McClung, 2019; Nakamichi, 2020; Sanchez and Kay, 2016). Core clock components expressed close to dusk or at night include additional members of the PRR family, such as PRR5 and TIMING OF CAB EXPRESSION1/PRR1 (TOC1/PRR1) as well as the components of the evening complex (EC), comprising EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO/PHYTOCLOCK1 (LUX/PCL1) (McClung, 2019; Nagel and Kay, 2012; Nakamichi, 2020). The evening-expressed components function as repressors of morning genes to ensure that they are repressed during the night.

In addition to the clock repressors, several activators shape the rhythmic oscillations. Some of the activators include chromatin marks contributing to an open chromatin conformation (Chen and Mas, 2019) and additional clock components such as LIGHT-REGULATED WD1 and 2 (LWD1 and LWD2) (Wang et al., 2011; Wu et al., 2008, 2016), or members of the REVEILLE (RVE) protein family (Farinas and Mas, 2011; Hsu et al., 2013; Rawat et al., 2011; Shalit-Kaneh et al., 2018). The RVE proteins form a co-activating protein complex with the LNK (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED GENE) proteins (Ma et al., 2018; Rugnone et al., 2013; Xie et al., 2014) and activate clock gene expression by timely recruiting the transcriptional machinery to control the rhythms of nascent RNAs (Ma et al., 2018). Altogether, current models of the *Arabidopsis* oscillator depict the transcriptional regulation

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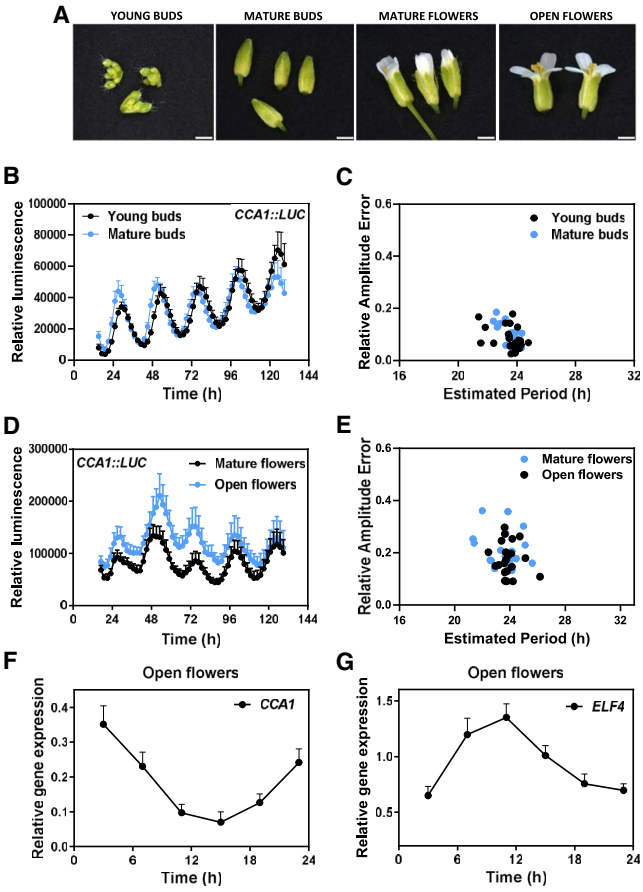

Figure 1. Self-sustained circadian rhythms in buds and flowers

(A) Representative photographs of buds and flowers at different developmental stages.

(B and D) *In vivo* luminescence assays of *CCA1:LUC* rhythms in (B) young and mature buds and (D) young and mature flowers.

(C and E) Period, and relative amplitude error estimates of circadian rhythms of (C) young and mature buds and (E) mature and open flowers. Circadian time course analyses by RT-qPCR of (F) *CCA1* and (G) *ELF4* mRNA expression in open flowers. Samples were examined under constant light (LL) following synchronization under light:dark cycles (16 h light:8 h dark). Data are presented as the mean + SEM. Scale bars, 1 mm. At least two biological replicates were performed per experiment.

See also Figure S1.

of morning-expressed clock components that specifically regulate evening clock genes and vice versa (Avello et al., 2021; Caluwé et al., 2016).

The time-of-day specific expression of oscillator genes and proteins defines the timing of biological processes or outputs controlled by the clock. The circadian clock intersects with the function of major organelles and cellular pathways including among many others, hormonal pathways (Sanchez and Kay, 2016), the cell cycle (Fung-Uceda et al., 2018), chloroplasts (Atkins and Dodd, 2014; Flis et al., 2019; Fukushima et al., 2009), or mitochondria (Cervela-Cardona et al., 2021; Fukushima et al., 2009; Sanchez-Villarreal et al., 2013). Consequently, the circadian system regulates nearly every aspect of development, growth, metabolism, and responses to biotic and abiotic stresses (Adams and Carré, 2011; Kinmonth-Schultz et al., 2013; Sanchez and Kay, 2016). The photoperiodic regulation of flowering time has been firmly established as an important clock output (Shim et al., 2017). Daily rhythms of scent emission, pollinator attraction, flower closing, and orientation have been also documented (Atamian et al., 2016; Creux et al., 2021; Fenske and Imaizumi, 2016; Fenske et al., 2018; Muroya et al., 2021). However, there is limited information about how the clock actually works in flowers and what specific molecular and cellular pathways regulates within the flower.

Arabidopsis flowers show the typical structure of the *Brassicaceae*, which consist of concentric whorls, including four sepals, a corolla of four petals, the androecium with six stamens, and the gynecium at the center (Drews et al., 1991; Sablowski, 2015; Weigel, 1995; Wellmer et al., 2014). The gynecium contains two fused carpels separated by a false septum that divide the ovary into two compartments (Ferrándiz et al., 1999; Zúñiga-Mayo et al., 2019). The gynecium allows pollen fertilization of the ovules, which eventually will develop into seeds. Here, we have studied the circadian function in flowers and reproductive organs and identified the specific regulatory network at the core of the clock in pistils, arguably one of the more complex and evolutionary innovative organs of flowering plants (Simonini and Østergaard, 2019). We found a distinct functional network that confers precision and robustness to the pistil clock.

RESULTS

Self-sustained circadian rhythms in detached buds and flowers

To understand the circadian clock function in floral organs, we examined rhythms in buds and flowers at different developmental stages (Figure 1A). Young and mature buds (stages 6–12) (Müller, 1961; Smyth et al., 1990) sustained high-amplitude and robust circadian rhythms of *CCA1:LUC* activity (*CCA1* promoter fused to the LUCIFERASE) with circadian periods close to 24h under constant light (LL) conditions (Figures 1B and 1C). Mature and fully open flowers (stages 13–15) (Müller, 1961; Smyth et al., 1990) also sustained rhythms under both LL (Figures 1D and 1E) and entraining conditions (Figure S1A). Evening-phased circadian reporters such as *TOC1::LUC* and *GIGANTEA::LUC* (*GI::LUC*) also showed high-amplitude rhythms in flowers (Figures S1B–S1F). Consistent with the bioluminescence results, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses confirmed the rhythmic circadian expression of oscillator genes in flowers (Figures 1F and 1G). Thus, a functional circadian clock sustains rhythms in buds and flowers detached from the rest of the plant, although the rhythmic oscillations appeared more robust in buds than in flowers.

Analyses of circadian rhythms in *toc1-2* mutant showed that *toc1-2* buds sustained the rhythmic oscillations, albeit with a short period (~20 h) (Figures 2A, 2C and 2E), and thus, displaying a phenotype similar to that previously reported in seedlings (Millar et al., 1995). In flowers, *toc1-2* showed short-period oscillations only the first 2 or 3 days, dampening low afterward (Figures 2B, 2D and 2F) (higher relative amplitude error values indicate weaker rhythms). Comparative analyses confirmed the altered *toc1-2* rhythms in flowers compared with buds (Figures 2G and 2H). Altogether, the circadian phenotype of *toc1-2* buds resembles that previously reported in seedlings, but rhythms dampen low after few days in *toc1-2* flowers.

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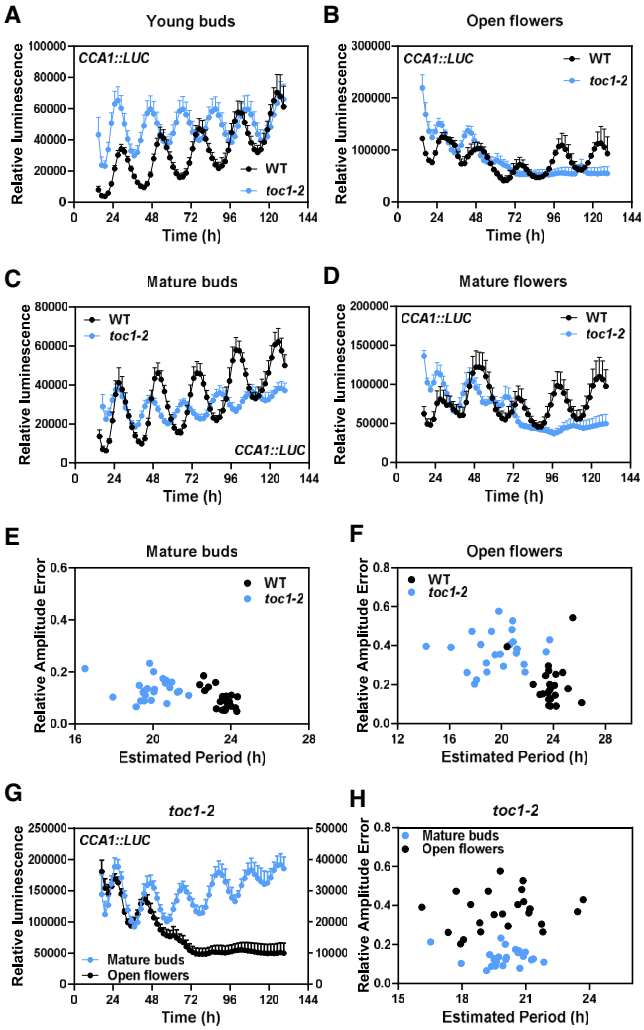

Figure 2. Distinct phenotypes of *toc1-2* mutant in buds and in flowers

(A–D) *In vivo* luminescence assays of *CCA1:LUC* rhythms in WT and *toc1-2* (A) young buds, (B) open flowers, (C) mature buds, and (D) mature flowers.

(E and F) Period and relative amplitude error estimates of *CCA1:LUC* rhythms in WT and *toc1-2* (E) mature buds and (F) open flowers.

(G) Comparative luminescence analyses of *CCA1:LUC* rhythms in *toc1-2* mature buds and open flowers.

(H) Period and relative amplitude error estimates of *CCA1:LUC* rhythms in *toc1-2* mature buds and open flowers. Samples were examined under LL following synchronization under light:dark cycles (16 h light:8 h dark). Data are presented as the mean + SEM. At least two biological replicates were performed per experiment. Data are repeated in different graphs to facilitate comparisons.

Detached female reproductive organs show precise and robust rhythms

Variations of rhythms in the different floral organs can contribute to the rhythmic dampening in *toc1-2* flowers. Thus, we examined the circadian oscillation in sepals, petals, stamens, and pistils (Figure 3A). In WT sepals, bioluminescence rhythms were robustly sustained, albeit with a shorter period than 24 h (Figures 3B and 3H). Rhythms in WT petals and stamens showed short circadian periods for 3 or 4 days, dampening low afterward (Figures 3C, 3D, 3I, S2A, and S2B). In contrast, the circadian waveforms in pistils robustly oscillated for more than 5 days, with a circadian period close to 24 h (Figures 3E–3H and 3J). We observed similar results with different clock reporters (Figures S2C–S2F). Time course analyses by RT-qPCR also confirmed the differences observed between stamens and pistils (Figures S2G and S2H). When we followed individual open flowers in intact plants that were maintained for several days under LL, we found that apart from pistils, the other floral organs disappeared very rapidly, indicating that floral organs other than pistils are short-lived *in planta* (Figure S2I). Therefore, reduced viability and/or the lack of energy after excision might contribute to the dampening of the rhythms that we observed in the bioluminescence assays. In any case, our results showed that pistils can survive for several days after excision from the rest of the flower and that the circadian rhythms in pistils robustly oscillate. Fertilization appears not to be a major factor contributing to the robustness of the pistil clock as similar patterns of gene expression were observed in pistils from flowers before and after fertilization (Figures S2J–S2M).

In *toc1-2* sepals, rhythms were similar to WT for the first 3 days, albeit with slightly reduced amplitude. Thus, the characteristic short-period phenotype of *toc1-2* observed in seedlings and buds was only evident in sepals after several days under LL (Figures 3B and S3A). We observed a similar trend in *toc1-2* petals and stamens, although the dampened rhythms precluded a clear view of the possible period shortening over time (Figures 3C, 3D, S3B, and S3C). As mentioned above, the reduced viability or the lack of energy might contribute to the dampening of the rhythms. In any case, analyses of rhythms at early time points before dampening showed that the circadian period of *toc1-2* sepals and petals was not significantly different from WT, whereas the circadian period of *toc1-2* stamens was significantly longer than WT (p value 0.0003 in samples with relative amplitude error [RAE] < 0.4). In pistils, the short-period oscillation observed during the first day rapidly transitioned to very-low-amplitude rhythms (Figures 3E and S3D), following a similar trend to that observed in whole flowers. Comparison of the different floral organs revealed the organ-specific behavior of *toc1-2* mutant (Figures S3E and S3F).

The pistil clock regulates the circadian expression of genes involved in photosynthesis and responses to stimuli and controls pistil growth and seed production

We next performed time course analyses by RNA sequencing (RNA-seq) to obtain a genome-wide view of the circadian transcriptional landscape in pistils. We first verified the reliability of the RNA-seq data by comparing our dataset with a previously published analysis of pistil-enriched genes (Klepikova et al., 2016; Martínez-Fernández et al., 2014, GEO: GSM1359146, GSM1359147). Initial comparisons revealed that the similarities were high despite the different sampling, growing conditions, and mode of analyses (Figure S4A). For example, the highest and lowest expressed genes were highly conserved in both datasets (examples in Figure S4B). The trends of expression for many of the genes was also quite similar (Figures S4C–S4F). We also found that the expression of genes characteristic of other floral organs was absent or much reduced compared with canonical pistil genes (Figure S4G), suggesting that our dataset was specific and reliably reflected the transcriptional landscape in pistils.

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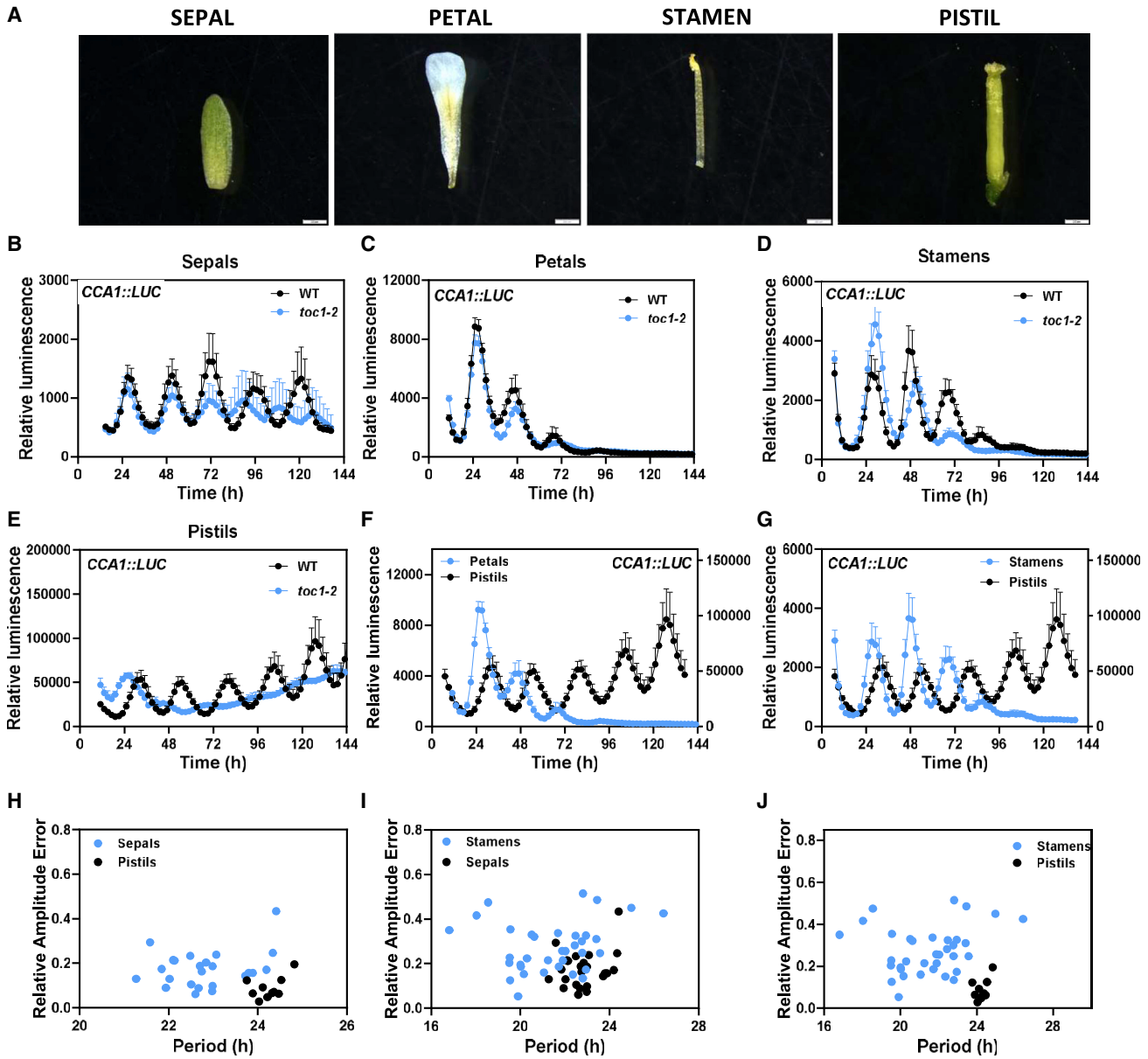

Figure 3. Robust circadian oscillations in pistils require a functional TOC1

(A) Representative photographs of sepals, petals, stamens, and pistils.

(B–E) *In vivo* luminescence assays of *CCA1:LUC* rhythms in WT and *toc1-2* (B) sepals, (C) petals, (D) stamens, and (E) pistils.

(F and G) Comparative waveform analyses of *CCA1:LUC* rhythms in WT (F) petals and pistils and (G) stamens and pistils.

(H–J) Period and relative amplitude error estimates of circadian rhythms in WT (H) sepals and pistils, (I) stamens and sepals, and (J) stamens and pistils. Samples were examined under LL following synchronization under light:dark cycles (16 h light:8 h dark). Data are presented as mean + SEM. At least two biological replicates were performed per experiment. Data are repeated in different graphs to facilitate comparisons among floral organs. Scale bars, 0.5 mm.

See also Figures S2 and S3.

Analyses of the rhythmic genes in pistils using the JTK_CYCLE algorithm (Hughes et al., 2010) (adjusted p value < 0.05) uncovered around 1,000 circadian genes (Table S1) with a range of amplitudes that were similar or slightly lower than the ones previously described at the shoot apex (Takahashi et al., 2015; Figures 4A and 4B). Overall, low-amplitude rhythmic genes showed lower expression than high-amplitude genes (Figure S4H). The circadian phases of rhythmic genes in pistils expanded across the whole circadian cycle but were slightly enriched during the day, particularly at circadian time 4 (CT4) (Figure 4C) as opposed to the enrichment after subjective dusk observed at the shoot apex (Takahashi et al., 2015; Figure S4I). In addition to the organ-specificities, different entrainment regimes (pistils: 16 h light:8 h dark versus shoot apexes: 12 h light:12 h dark) can contribute to peak-phase differences.

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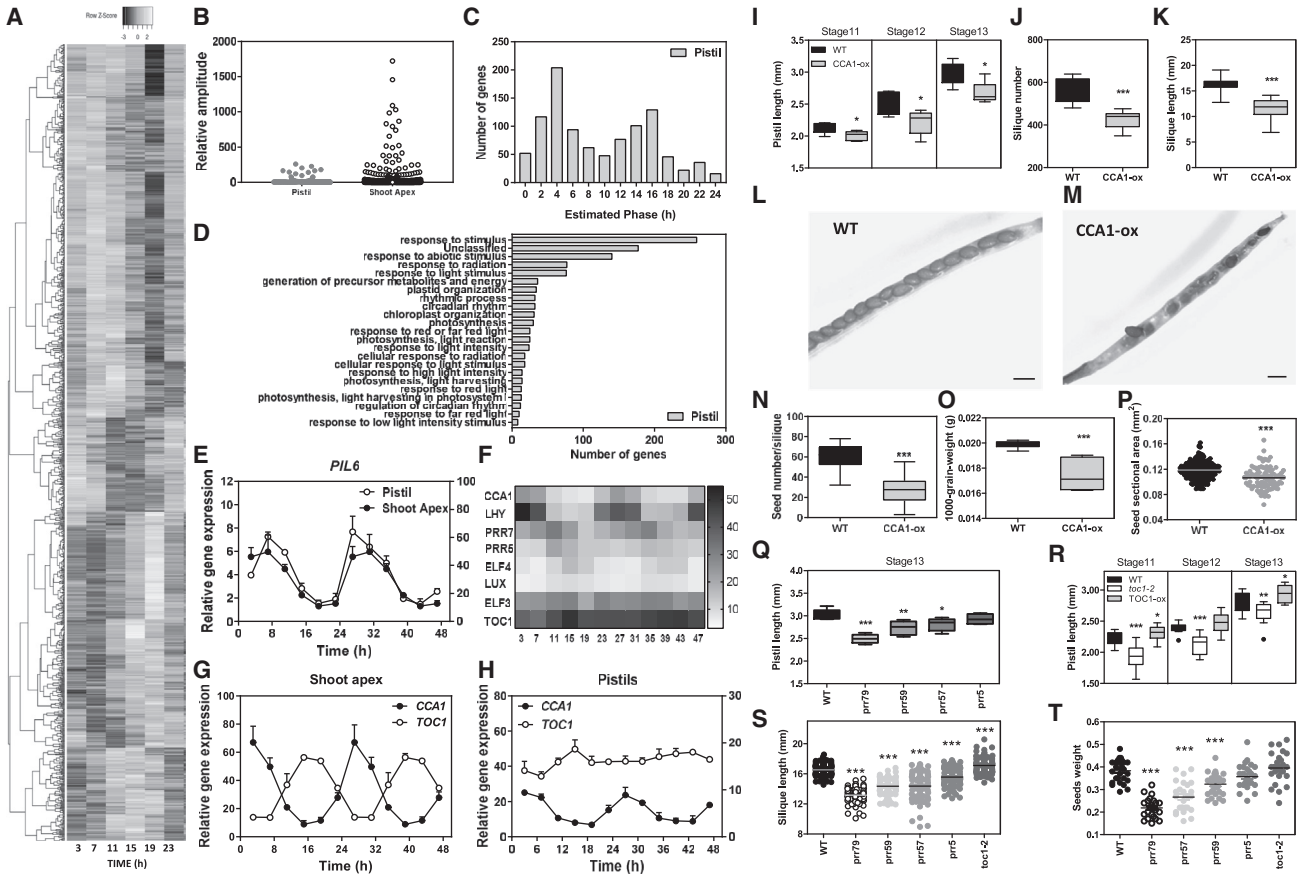

Figure 4. Circadian transcriptional landscape in pistils and regulation of pistil growth and seed production

(A) Expression-based heatmap from transcriptomic RNA-seq data at different circadian times.

(B and C) Analyses of (B) Relative amplitude and (C) phase estimates of oscillating genes in pistils.

(D) Functional categorization of the main circadian genes in pistils.

(E) Comparative time course analysis of *PIL6* gene expression from RNA-seq data in pistils and shoot apex.

(F) Expression-based heatmap from RNA-seq data of the main oscillator genes at different circadian times.

(G and H) Comparative time course analysis of *CCA1* and *TOC1* gene expression from RNA-seq data in (G) shoot apex and (H) pistils.

(I–K) Analyses of (I) pistil length, (J) silique number, and (K) silique length in WT and CCA1-ox plants.

(L and M) Representative images of seeds in siliques of (L) WT and (M) CCA1-ox plants. Siliques are not displayed in full length to facilitate the visualization of the seeds.

(N–P) Analyses of (N) seed number per silique, (O) seed weight, and (P) seed sectional area in WT and CCA1-ox plants.

(Q and R) Analyses of pistil length in (Q) the *prr* mutants and (R) *toc1-2* and TOC1-ox plants.

(S and T) Analyses of (S) silique length and (T) seed weight in the *prr* mutants. Data are presented as mean ± SEM. At least two biological replicates were performed per experiment. (***p value < 0.0001; **p value < 0.001; *p value < 0.05.) Scale bars, 0.5 mm.

See also Figure S4.

Functional categorization of the rhythmic genes showed significant enrichment in photosynthetic processes, circadian rhythms, and responses to stimuli, most prominently light and radiation (Table S1; Figure 4D). Although leaves are the primary organs for photosynthesis, reproductive organs in many plant species are also photosynthetically active (Brazel and Ó'Maoiléidigh, 2019). The circadian control of photosynthesis in pistils might ensure the appropriate timing of carbon sources needed for reproductive success. Analyses of selected genes within these functional categories confirmed robust oscillations that were similar to the ones previously observed at the shoot apex (Figures 4E and S4J–S4L). The rhythmic genes in pistils also included most of the morning- and evening-expressed core clock components (Figures 4F and S4M–S4O). The waveforms oscillated with similar phases and amplitudes to those previously reported in whole seedlings or shoot apexes (Takahashi et al., 2015; Figures 4F and 4G). However, the evening-expressed core clock genes *TOC1* and *ELF3* showed weaker or no oscillation (Figures 4H and S4P). Despite the weak rhythms, TOC1 and ELF3 have a relevant function within the pistil clock (see below), which suggest that translational and post-translational regulation might be important mechanisms for the circadian activity of the proteins.

To determine whether the circadian clock is indeed important for pistil function, we first used arrhythmic plants in which the clock is not able to properly run due to over-expression of CCA1 (CCA1-ox). Our results showed that pistil length was significantly shorter in CCA1-ox compared with WT. The shorter

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pistil length was sustained at different pistil developmental stages (11, 12, and 13) (Müller, 1961; Smyth et al., 1990; Figure 4I). The results suggest that proper circadian function is important for pistil growth and that CCA1 over-expression reduces the slope of pistil growth. To check whether changes in clock function also affect silique and seed production, we analyzed silique and seed number, length, weight, and area. Our results showed that CCA1-ox produced less siliques that were significantly shorter than in WT (Figures 4J and 4K). The inspection of seeds in siliques also suggested developmental defects, with an increased number of abortive ovules in CCA1-ox (Figures 4L and 4M). Consistently, the number of seeds per silique, the seed mass and area were significantly reduced in CCA1-ox (Figures 4N–4P).

To examine whether the effects were restricted to CCA1-ox or the circadian function is overall important for pistil growth and seed production, we examined mutant plants of different clock components in which the clock is still running, although at a faster or slower pace than in WT. Our analyses showed that pistil length was also affected, particularly in double mutants, displaying reduced pistil size compared with that observed in WT (Figure 4Q). Mutation and over-expression of TOC1 led to reduced and increased pistil length, respectively, and the phenotypes were sustained at different stages of pistil development (Figure 4R). TOC1 miss-expressing plants also regulate hypocotyl length but show the reverse phenotypes, with *toc1-2* displaying long hypocotyls, and TOC1-ox showing longer than WT hypocotyl length (Mas et al., 2003a), which is in clear contrast with the pistil length phenotypes of TOC1 miss-expressing plants. It is worth noting that the gradual increase in pistil length observed in the *prr* mutant plants correlated with a gradual increment in silique length and seed weight (Figures 4S and 4T). Altogether, the results indicate that proper function of the circadian clock is important for pistil and silique growth as well as for seed weight and production.

Transcriptional regulatory network at the core of the oscillator in pistils

To understand the circadian regulatory network in pistils, we examined gene expression in clock mutants and performed chromatin immunoprecipitation (ChIP) assays of key clock components. As in seedlings, the expression of *TOC1* and the *EC* genes was upregulated in *cca1/lhy* double mutant (Figures 5A–5C), suggesting that CCA1 and LHY act as repressors of evening-expressed clock genes in pistils. The repression likely occurs through direct binding to the gene promoters as suggested by ChIP assays in pistils (Figure 5J). *cca1* and *lhy* double mutation also led to a marked downregulation of *PRR7* and *PRR9* expression in pistils (Figures S5A and S5B). In turn, analyses of the *prr79* mutant showed an upregulation of *CCA1* expression (Figure 5D), suggesting a direct repression of *CCA1* by PRR9 and PRR7, as previously described in seedlings (Nakamichi, 2020). The evening-expressed genes were upregulated during the subjective day but downregulated during the subjective night in the *prr79* mutant (Figures S5C–S5F).

Mutation of the EC components ELF3 and LUX resulted in increased expression of *PRR7* and *PRR9* (Figures 5E and 5F) and downregulation of *CCA1* (Figure 5G). However, and contrarily to seedlings and roots, the mutation of *ELF4* did not lead to a relevant activation of *PRR9* expression (Figure 5E), which suggests that ELF4 might not be part of the EC in the repression of *PRR9* or that an additional function of ELF4 overcomes its EC-dependent regulation of *PRR9*. Consistent with the gene expression data, ChIP assays in pistils confirmed a significant binding of ELF3 and LUX to the promoters of the *PRR9* and *PRR7* genes, whereas ELF4 was not significantly enriched on these promoter regions (Figure 5K). Evening-expressed genes were upregulated in the *ec* mutants (Figures S5G–S5J) as well as in the *toc1-2* mutant (Figure S5K). The *toc1-2* mutation also led to a reduced expression of *CCA1* and *LHY* (Figures 5H and S5L) but an increased accumulation of *PRR7* (Figure 5I). Overall, we found that in the absence of functional CCA1 and LHY, the morning-expressed *PRR* genes are repressed, whereas the evening-expressed genes are activated. On the other hand, mutation of evening-expressed genes results in downregulation of *CCA1* and *LHY* and upregulation of *PRR7*, *PRR9*, and evening-expressed genes.

The data fit a model in which CCA1 represses the expression of the evening-phased genes, and in turn, these components repress *PRR9* and *PRR7*. TOC1 also represses the expression of the *EC* genes, whereas PRR9 and PRR7 components repress *CCA1*. Analyses of mutants also provided interesting clues about the repressing functions. For instance, mutation of *CCA1* results in the marked downregulation of *PRR9* and *PRR7* expression, which is likely the consequence of the upregulation of the evening-phased repressor genes in the mutant. Similarly, the analyses of the *prr79* mutant suggest that PRR9 and PRR7 might repress (directly or indirectly) the evening-phased gene expression during the subjective day. The downregulation of evening-phased gene expression during the subjective night might be a consequence of the upregulation of *CCA1* in the *prr79* mutant. Similarly, by repressing *PRR9* and *PRR7* expression, the EC components upregulate *CCA1* and *LHY* expression. Thus, the analyses of the mutants suggest that many of the clock components that function as repressors also shape the oscillations by repressing other repressors.

To identify canonical activators of clock gene expression in pistils, we examined the function of members of the RVE protein family, previously documented to be activators in whole seedlings (Ma et al., 2018; Rugnone et al., 2013; Xie et al., 2014). As previously described (Hsu et al., 2013), we found that in seedlings, the *rve4,6,8* triple mutant showed a clear phase-shift in the expression of oscillator genes (Figures 6A–6C and S6A–S6C). However, in pistils, the *rve4,6,8* triple mutant showed very weak amplitude or arrhythmia and resulted in a predominant downregulation of *PRR5* and *TOC1* expression at nearly all time points (Figures 6D and 6E). Notably, the expression of morning genes was also clearly affected with downregulation during the subjective day and slight upregulation during the subjective night (Figure 6F). Comparative analyses revealed the different effects of *rve4,6,8* triple mutant in seedlings versus pistils (Figures 6G, 6H, and S6D–S6F). The different phenotypes were not due to the different nature of the two samples as mainly changes in amplitude were observed in comparisons of WT seedlings versus WT pistils (Figures S6G–S6I). The expression of *RVEs* was also reasonably similar in seedlings and pistils (Figure 6I), with *RVE6* showing low amplitude and *RVE8* displaying a phase-shift in pistils (Figures S6J–S6L). Together, the results

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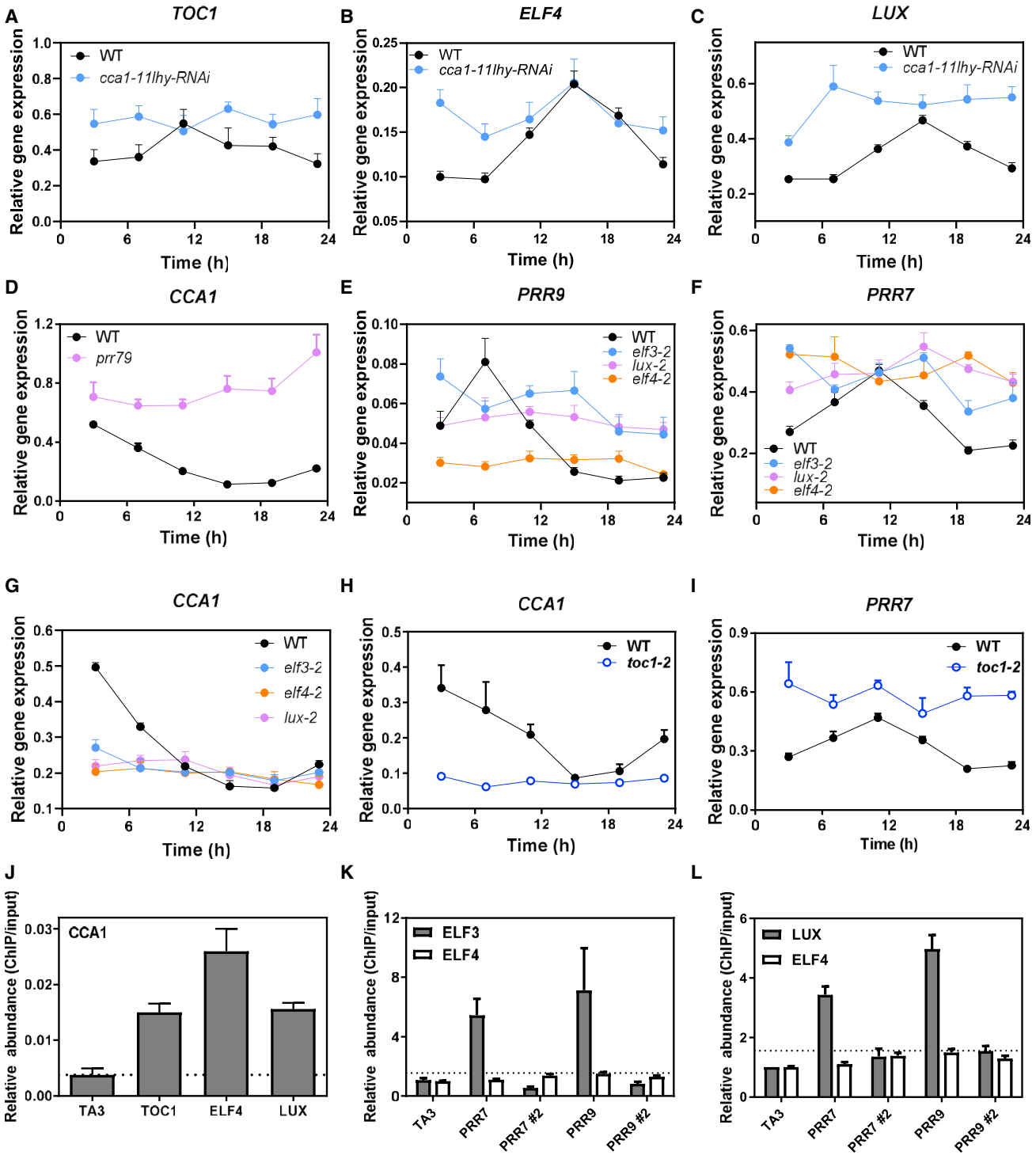

Figure 5. Regulatory network at the core of the pistil oscillator in clock mutants

(A–C) Time course analysis by RT-qPCR of (A) *TOC1*, (B) *ELF4*, and (C) *LUX* gene expression in WT and *cca1-1lhy-RNAi* pistils.

(D) *CCA1* gene expression in WT and *prr79* mutant pistils.

(E–G) Time course analysis by RT-qPCR of (E) *PRR9*, (F) *PRR7*, and (G) *CCA1* gene expression in WT and *elf3-2*, *lux-2*, and *elf4-2* pistils.

(H and I) Time course analysis by RT-qPCR of (H) *CCA1* and (I) *PRR7* in WT and *toc1-2* pistils.

(J–L) ChIP analyses of (J) CCA1 at ZT3, (K) ELF3, and ELF4 at ZT15, and (L) LUX and ELF4 at ZT15 showing the enrichment (relative to the input) to the target promoters. For comparative analyses ChIP assays of ELF4 are shown in (K) and (L).

Data are presented as the mean + SEM. At least two biological replicates were performed per experiment.

See also Figure S5.

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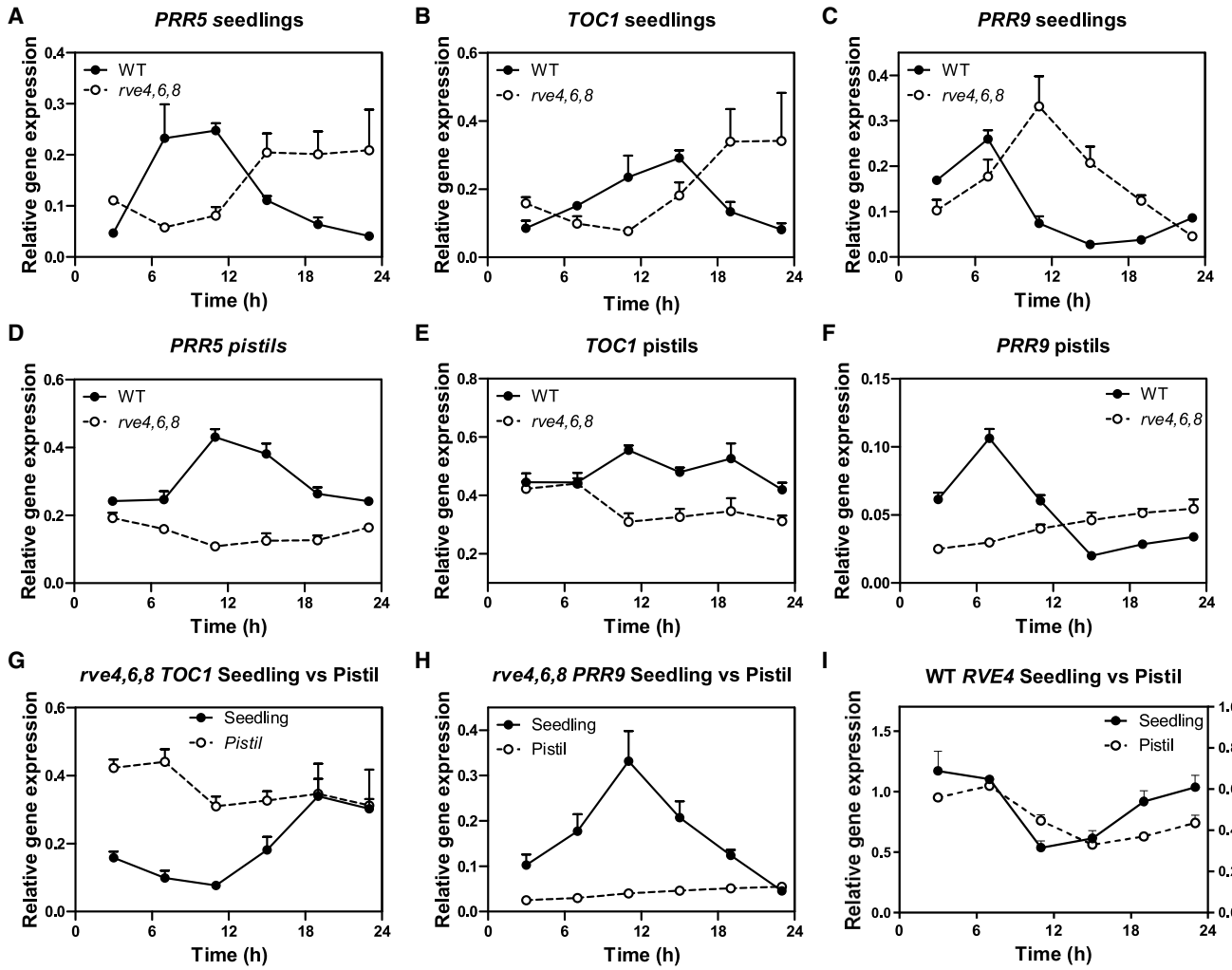

Figure 6. Distinct regulatory role of RVEs at the core of the pistil oscillator

Comparative time course analysis by RT-qPCR in WT and *rve4,6,8* seedlings (A–C) and WT and *rve4,6,8* pistils (D–F).

(A and D) *PRR5* gene expression.

(B, E, and G) *TOC1* gene expression.

(C, F, and H) *PRR9* gene expression.

(G and H) Comparison of (G) *TOC1* and (H) *PRR9* gene expression in *rve4,6,8* seedlings and *rve4,6,8* pistils.

(I) Comparison of *RVE4* expression in seedlings and pistils. Data are repeated in different graphs to facilitate comparisons. Data are presented as the mean + SEM. Two biological replicates were performed for the analyses in seedlings, whereas three biological replicates were performed for the analyses in pistils. See also Figure S6.

suggest that in pistils, RVEs display a prevalent function controlling the expression of oscillator genes.

Genetic interaction studies on the repressive function of oscillator components

As the oscillator components regulate each other and share common targets, it is rather difficult to discern their specific function. To get insights into the morning and evening regulatory network, we performed genetic interaction studies and analyzed clock gene expression in pistils of TOC1-ox/*elf3-2* and in CCA1-ox/*prr79* plants. Studies with TOC1-ox pistils showed a highly repressing function of TOC1-ox (Figures 7A–7D, S7A, and S7B). Comparative analyses using TOC1-ox/*elf3-2* pistils showed that over-expression of TOC1 was able to overcome the upregulation of the *PRR* genes in *elf3-2* mutants (Figures 7A, 7B, and S7B). Thus, TOC1 repression of the *PRR* genes does not require a functional ELF3. A dominance of TOC1-ox repressive function on *PRRs* over the EC might explain these results. On the other hand, *ELF4* expression more closely resembled that observed in *elf3-2* mutant (see Figure S5H) than that in TOC1-ox (Figure 7C), which suggests an *elf3-2* dominant phenotype and a possible hierarchy of the EC auto-repression over the repressing function of TOC1-ox. It is also possible that TOC1 requires ELF3 for full repression of the EC. Similarly, *CCA1* gene expression was fully repressed in TOC1-ox/*elf3-2* resembling the phenotype observed in *elf3-2* mutant, although *CCA1* was still repressed in TOC1-ox (Figure 7D).

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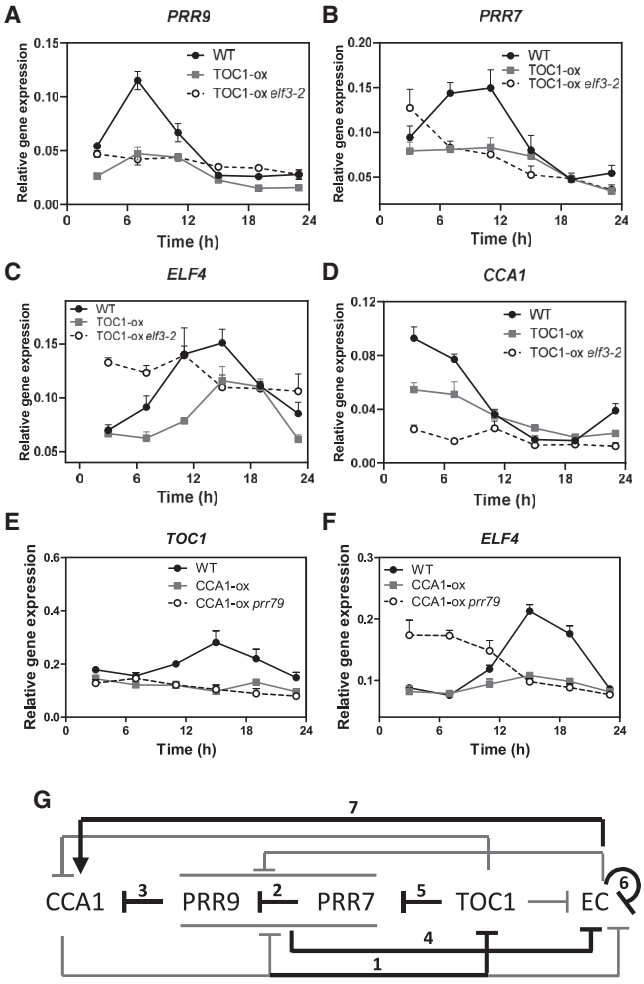

Figure 7. Genetic interaction analyses of the transcriptional repressive networks at the core of the pistil oscillator

(A–D) Time course analysis by RT-qPCR of (A) *PRR9*, (B) *PRR7*, (C) *ELF4*, and (D) *CCA1* gene expression in WT, TOC1-ox, and TOC1-ox/*elf3-2* pistils.

(E and F) Time course analysis by RT-qPCR of (E) *TOC1* and (F) *ELF4* gene expression in WT and CCA1-ox/*prr79* pistils. Data are presented as the mean + SEM. Three biological replicates were performed per experiment.

(G) Circadian regulatory network comprising dominant regulatory functions (thick black lines) as inferred by the genetic interaction studies. See the main text for a further explanation.

See also Figure S7.

Analyses of CCA1-ox (Figure S7C) also showed a repressing function of CCA1 that was not effectively overcome by the *prr79* mutation in the regulation of *TOC1* expression (Figure 7E). However, *ELF4* in CCA1-ox/*prr79* showed an upregulation during the subjective day like the one observed in *prr79* mutant (Figure S5E) but not in CCA1-ox (Figure 7F and S7D). The results suggest that CCA1 might require functional PRR9 and PRR7 for repression of *ELF4* or that the lack of the direct or indirect repressing function of PRRs can overcome the repression by CCA1-ox. Notably, analyses of CCA1-ox/*prr7* showed an evident upregulation of *PRR9* expression (Figure S7E), suggesting that PRR7 acts as a repressor of *PRR9* expression. Repression by PRR7 is specific for *PRR9*, as *PRR5* expression was not upregulated in CCA1-ox/*prr7* (Figure S7F). Analyses of clock gene expression in seedlings reinforced the differences on the regulation of the morning *PRRs* by the EC, particularly *PRR7* during the subjective night and of *TOC1* and *ELF3* by the morning-expressed components during the subjective day (compare Figures S7G–S7L with Figures 7B and 7E). Altogether, the analyses of mutants, over-expressing lines and the genetic interaction studies show a complex regulatory circuitry in pistils (Figure 7G) with CCA1-ox repressing *TOC1* over the morning PRRs (1), PRR7 repressing *PRR9* over CCA1-ox (2), morning PRRs repressing *CCA1* (3), and *EC* (4) over CCA1-ox. Within the evening-expressed components, TOC1-ox represses the *PRRs* over the EC (5), the EC auto-represses itself over TOC1-ox (6), and the EC activates *CCA1* over TOC1-ox (7).

DISCUSSION

Studies of the circadian regulatory networks in plants are increasingly shifting from whole seedlings to specific organs and tissues (Nakamichi, 2020; Sorkin and Nusinow, 2021). Key questions arise about the degree of circadian autonomy of tissues and organs, and the relevance of cell-to-cell coupling and long-distance circadian communication (Sorkin and Nusinow, 2021). *Arabidopsis* tissues with high cell density such as those at the shoot and root meristems favor circadian coupling (Sorkin and Nusinow, 2021). Consistently, the shoot apex and the tip of the root clocks have been proposed as coordinating signaling nodes influencing rhythms in other parts of the plant (Gould et al., 2018; Takahashi et al., 2015). Overall, the results thus far fit the notion of tissue-specific clocks that also require cell-to-cell and long-distance coordination for circadian precision and responses to environmental cues (Nakamichi, 2020; Sorkin and Nusinow, 2021). To fully understand the circadian function and communication, it is important to elucidate the similarities and differences of the circadian regulatory network in cells, tissues, and organs.

Our studies have shown that buds and flowers detached from the rest of the plant display rhythmic oscillations, which indicate the presence of self-sustained functional clocks. The developmental differences (e.g., in buds and open flowers) may be due to different sensitivities to environmental cues (Atamian et al., 2016). Under our conditions, other excised organs, for example roots, also sustain rhythms but with a long period and delayed phase compared with shoots (Chen et al., 2020; Takahashi et al., 2015). Notably, excised pistils showed self-sustained rhythms with precise 24-h oscillations. These results indicate the presence of a clock that is able to precisely run even in the absence of signals from the rest of the plant. In other floral organs, the dampened rhythms could be due to reduced viability or lack of energy after excision from the flower. Future studies should focus on understanding the circadian regulatory network in sepals, petals, and stamens as well as on their circadian robustness after several days under LL. Compared with pistils, rhythms in stamens displayed a short-period phenotype. It would be interesting to determine the biological relevance of such variation between the reproductive organs. Circadian differences in reproductive organs are not exclusive of plants. For instance, the expression of core clock genes is also rhythmic in ovarian tissues (Kennaway et al., 2012), and female and

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male rats show sex differences in daily rhythms and in responses to endogenous and exogenous cues (Bailey and Silver, 2014). The sex-dependent circadian differences are relevant to humans in many areas, most notably those related to reproduction and overall health (Bailey and Silver, 2014). Understanding the circadian clockwork in flowers may prove essential for optimizing plant reproduction and productivity.

Clock repressors and activators have specific regulatory functions in pistils. For instance, RVE proteins appear to have a prevalent activating function in pistils. The low amplitude or arrhythmic phenotypes of the *rve4,6,8* triple mutant in pistils are in sharp contrast with the clear oscillations observed in seedlings. In sepals, petals, and stamens, the *toc1-2* mutant showed similar waveforms than WT at least for the first days under LL. These results suggest that the lack of a functional TOC1 can be overcome for few days. In pistils, on the other hand, the short-period phenotype is evident from the initial days, but the rhythms dampened low over time. Thus, TOC1 circadian function is different in the floral organs, with a prevalent role in pistils. The expression of oscillator genes in pistils is similar to the one previously described with some exceptions like *ELF3*. Translational and/or post-translational mechanisms of regulation might contribute to the rhythmic oscillation of clock protein activity. For example, TOC1 protein is regulated by degradation through the proteasome pathway, thus providing a mechanism for controlling TOC1 protein oscillation and period length by the clock (Mas et al., 2003b).

Genome-wide analyses of the circadian transcriptional landscape in pistils showed the importance of the circadian clock regulating photosynthesis and responses to environmental signals. Photosynthesis is not exclusive of leaves, as it is also present in reproductive organs (Brazel and Ó'Maoiléidigh, 2019). It has been suggested that photosynthesis in reproductive organs may represent and adaptive trait, not only by balancing the carbon cost of reproduction but also by conferring resistance to abiotic stresses (Raven and Griffiths, 2015). Some possible disadvantages of the photosynthetic activity include the increased DNA damage and the production of reactive oxygen species. Thus, proper timing of photosynthesis in pistils by the circadian clock might be beneficial for ensuring enough energy resources when needed but may also activate responses to cope with DNA damage. Consistently, the circadian clock in pistils coordinates responses to radiation, light, and abiotic stimulus. In humans and animal models, increasing evidence is pointing out relevant processes controlled by the clock that show sex differences in daily rhythms including, among others, the sleep-wake cycle and hormonal and metabolic oscillations (Mong et al., 2011). Proper circadian function is also important for pistil and silique growth as well as for seed quality and production. It would be interesting to determine the molecular mechanisms and downstream signaling pathways by which the circadian clock regulates pistil growth and function. The circadian factors and regulatory mechanisms controlling growth appear to be organ-specific, judging by the opposite hypocotyl and pistil length phenotypes observed in plants miss-expressing clock components. The circadian clock implication in the control of seed production opens interesting possibilities for biotechnological application of improving seed yield, arguably one of the most important traits for plant breeding.

Analyses of mutant and over-expressing lines point out to a complex regulatory network for the pistil clock. The transcriptional regulatory activity does not sustain robust amplitude of *TOC1* and *ELF3* mRNA expression in pistils, as opposed to their rhythms observed in seedlings. Furthermore, the specific phenotypes of *toc1-2*, the different behavior of the *ec* mutants regulating *PRR9* and *PRR7* circadian expression, and the clock gene expression patterns in *rve* mutant, all indicate some specificities of the pistil clock. The changes were also confirmed in the seedling analyses of the clock over-expressing/mutant lines. The genetic interaction studies also suggest dominant repressive phenotypes, able to overcome either the over-expression or the mutation of other oscillator components. Current models of the *Arabidopsis* clock in whole seedlings include the reciprocal regulation of morning and evening oscillator genes that results in their time-of-day specific peak of expression (Avello et al., 2021). Other models also group several circadian components together (Avello et al., 2021; Caluwé et al., 2016). The particular circadian architecture that we found in pistils might provide robustness to the pistil clockwork. Our results pave the way for a better understanding of the circadian system in the reproductive organs, which might likely provide biotechnological tools to manipulate plant reproduction and hence productivity.

Limitations of the study

In this study, we have focused on the transcriptional regulatory network at the core of the pistil clock. However, we have not elucidated the network in other floral organs including sepals, petals, and stamens. Floral organs other than pistils are short-lived *in planta* so that reduced viability and/or the lack of energy after excision might contribute to the dampening of the rhythms that we observed in the bioluminescence assays. We propose that detailed time course analyses by RT-qPCR will provide useful information about the circadian networks in other floral organs. We have also not provided evidence of the biological relevance of the different circadian periods observed in pistils and stamens. Likewise, we have not identified the molecular mechanisms responsible for the circadian control of pistil growth. These interesting aspects are important to fully understand the flower clock in *Arabidopsis*.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.devcel.2022.08.013.

AUTHOR CONTRIBUTIONS

M.O. and Z.Y. performed the experiments. P.M. conceived the project, designed the experiments, and wrote the manuscript. All authors read, revised, and approved the manuscript.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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REFERENCES

Adams, S., and Carré, I.A. (2011). Downstream of the plant circadian clock: output pathways for the control of physiology and development. Essays Biochem *49*, 53–69. https://doi.org/10.1042/bse0490053.

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. Curr. Biol. *12*, 757–761. https://doi.org/10.1016/S0960-9822(02)00815-1.

Atamian, H.S., Creux, N.M., Brown, E.A., Garner, A.G., Blackman, B.K., and Harmer, S.L. (2016). Circadian regulation of sunflower heliotropism, floral orientation, and pollinator visits. Science *353*, 587–590. https://doi.org/10.1126/science.aaf9793.

Atkins, K.A., and Dodd, A.N. (2014). Circadian regulation of chloroplasts. Curr. Opin. Plant Biol. *21*, 43–50. https://doi.org/10.1016/j.pbi.2014.06.008.

Avello, P.A., Davis, S.J., and Pitchford, J.W. (2021). Temperature robustness in Arabidopsis circadian clock models is facilitated by repressive interactions, autoregulation, and three-node feedbacks. J. Theor. Biol. *509*, 110495. https://doi.org/10.1016/J.JTBI.2020.110495.

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J.R., Maciejewski, A., and Wishart, D.S. (2016). Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res *44*, W147–W153. https://doi.org/10.1093/nar/gkw419.

Bailey, M., and Silver, R. (2014). Sex differences in circadian timing systems: implications for disease. Front. Neuroendocrinol. *35*, 111–139. https://doi.org/10.1016/J.YFRNE.2013.11.003.

Bordage, S., Sullivan, S., Laird, J., Millar, A.J., and Nimmo, H.G. (2016). Organ specificity in the plant circadian system is explained by different light inputs to the shoot and root clocks. New Phytol *212*, 136–149. https://doi.org/10.1111/nph.14024.

Brazel, A.J., and Ó'Maoiléidigh, D.S. (2019). Photosynthetic activity of reproductive organs. J. Exp. Bot. *70*, 1737–1754. https://doi.org/10.1093/JXB/ERZ033.

De Caluwé, J., Xiao, Q., Hermans, C., Verbruggen, N., Leloup, J.C., and Gonze, D. (2016). A compact model for the complex plant circadian clock. Front. Plant Sci. *7*, 74. https://doi.org/10.3389/FPLS.2016.00074.

Cervela-Cardona, L., Yoshida, T., Zhang, Y., Okada, M., Fernie, A., and Mas, P. (2021). Circadian control of metabolism by the clock component TOC1. Front. Plant Sci. *12*, 683516. https://doi.org/10.3389/fpls.2021.683516.

Chen, W.W., Takahashi, N., Hirata, Y., Ronald, J., Porco, S., Davis, S.J., Nusinow, D.A., Kay, S.A., and Mas, P. (2020). A mobile ELF4 delivers circadian temperature information from shoots to roots. Nat. Plants *6*, 416–426. https://doi.org/10.1038/s41477-020-0634-2.

Chen, Z.J., and Mas, P. (2019). Interactive roles of chromatin regulation and circadian clock function in plants. Genome Biol *20*, 62. https://doi.org/10.1186/s13059-019-1672-9.

Creux, N.M., Brown, E.A., Garner, A.G., Saeed, S., Scher, C.L., Holalu, S.V., Yang, D., Maloof, J.N., Blackman, B.K., and Harmer, S.L. (2021). Flower orientation influences floral temperature, pollinator visits, and plant fitness. New Phytol *232*, 868–879. https://doi.org/10.1111/nph.17627.

Crosby, P., and Partch, C.L. (2020). New insights into non-transcriptional regulation of mammalian core clock proteins. J. Cell Sci. *133*, jcs241174. https://doi.org/10.1242/jcs.241174.

Drews, G.N., Weigel, D., and Meyerowitz, E.M. (1991). Floral patterning. Curr. Opin. Genet. Dev. *1*, 174–178. https://doi.org/10.1016/S0959-437X(05)80066-8.

Endo, M., Shimizu, H., Nohales, M.A., Araki, T., and Kay, S.A. (2014). Tissue-specific clocks in Arabidopsis show asymmetric coupling. Nature *515*, 419–422. https://doi.org/10.1038/nature13919.

Ezer, D., Jung, J.H., Lan, H., Biswas, S., Gregoire, L., Box, M.S., Charoensawan, V., Cortijo, S., Lai, X., Stöckle, D., et al. (2017). The evening complex coordinates environmental and endogenous signals in Arabidopsis. Nat. Plants *3*, 17087. https://doi.org/10.1038/nplants.2017.87.

Farinas, B., and Mas, P. (2011). Functional implication of the MYB transcription factor RVE8/LCL5 in the circadian control of histone acetylation. Plant J *66*, 318–329. https://doi.org/10.1111/j.1365-313X.2011.04484.x.

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. Curr. Biol. *15*, 47–54. https://doi.org/10.1016/j.cub.2004.12.067.

Fenske, M.P., and Imaizumi, T. (2016). Circadian rhythms in floral scent emission. Front. Plant Sci. *7*, 462. https://doi.org/10.3389/fpls.2016.00462.

Fenske, M.P., Nguyen, L.P., Horn, E.K., Riffell, J.A., and Imaizumi, T. (2018). Circadian clocks of both plants and pollinators influence flower seeking behavior of the pollinator hawkmoth Manduca sexta. Sci. Rep. *8*, 2842. https://doi.org/10.1038/s41598-018-21251-x.

Ferrándiz, C., Pelaz, S., and Yanofsky, M.F. (1999). Control of carpel and fruit development in Arabidopsis. Annu. Rev. Biochem. *68*, 321–354. https://doi.org/10.1146/annurev.biochem.68.1.321.

Flis, A., Mengin, V., Ivakov, A.A., Mugford, S.T., Hubberten, H.M., Encke, B., Krohn, N., Höhne, M., Feil, R., Hoefgen, R., et al. (2019). Multiple circadian clock outputs regulate diel turnover of carbon and nitrogen reserves. Plant Cell Environ *42*, 549–573. https://doi.org/10.1111/pce.13440.

Fukuda, H., Nakamichi, N., Hisatsune, M., Murase, H., and Mizuno, T. (2007). Synchronization of plant circadian oscillators with a phase delay effect of the vein network. Phys. Rev. Lett. *99*, 098102. https://doi.org/10.1103/PhysRevLett.99.098102.

Fukuda, H., Ukai, K., and Oyama, T. (2012). Self-arrangement of cellular circadian rhythms through phase-resetting in plant roots. Phys. Rev. E *86*, 041917. https://doi.org/10.1103/PhysRevE.86.041917.

Fukushima, A., Kusano, M., Nakamichi, N., Kobayashi, M., Hayashi, N., Sakakibara, H., Mizuno, T., and Saito, K. (2009). Impact of clock-associated Arabidopsis pseudoresponse regulators in metabolic coordination. Proc. Natl. Acad. Sci. USA *106*, 7251–7256. https://doi.org/10.1073/pnas.0900952106.

Fung-Uceda, J., Lee, K., Seo, P.J.P.J., Polyn, S., De Veylder, L., and Mas, P. (2018). The circadian clock sets the time of DNA replication licensing to regulate growth in Arabidopsis. Dev. Cell *45*, 101–113.e4. https://doi.org/10.1016/j.devcel.2018.02.022.

Gould, P.D., Domijan, M., Greenwood, M., Tokuda, I.T., Rees, H., Kozma-Bognar, L., Hall, A.J., and Locke, J.C. (2018). Coordination of robust single cell rhythms in the Arabidopsis circadian clock via spatial waves of gene expression. eLife *7*, e31700. https://doi.org/10.7554/eLife.31700.

Greenwood, M., Domijan, M., Gould, P.D., Hall, A.J.W., and Locke, J.C.W. (2019). Coordinated circadian timing through the integration of local inputs in Arabidopsis thaliana. PLoS Biol *17*, e3000407. https://doi.org/10.1371/journal.pbio.3000407.

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. Proc. Natl. Acad. Sci. USA *102*, 10387–10392. https://doi.org/10.1073/pnas.0503029102.

Herrero, E., Kolmos, E., Bujdoso, N., Yuan, Y., Wang, M., Berns, M.C., Uhlworm, H., Coupland, G., Saini, R., Jaskolski, M., et al. (2012). EARLY FLOWERING4 recruitment of EARLY FLOWERING3 in the nucleus sustains the Arabidopsis circadian clock. Plant Cell *24*, 428–443. https://doi.org/10.1105/tpc.111.093807.

Hicks, K.A., Millar, A.J., Carré, I.A., Somers, D.E., Straume, M.D., Meeks-Wagner, D.R., and Kay, S.A. (1996). Conditional circadian dysfunction of the Arabidopsis early-flowering 3 mutant. Science *274*, 790–792. https://doi.org/10.1126/science.274.5288.790.

Hsu, P.Y., Devisetty, U.K., and Harmer, S.L. (2013). Accurate timekeeping is controlled by a cycling activator in Arabidopsis. eLife *2*, e00473. https://doi.org/10.7554/eLife.00473.

Huang, H., Alvarez, S., Bindbeutel, R., Shen, Z., Naldrett, M.J., Evans, B.S., Briggs, S.P., Hicks, L.M., Kay, S.A., and Nusinow, D.A. (2016). Identification of Evening Complex associated proteins in *Arabidopsis* by affinity purification and mass spectrometry. Mol. Cell. Proteomics *15*, 201–217. https://doi.org/10.1074/mcp.M115.054064.

Huang, W., Pérez-García, P., Pokhilko, A., Millar, A.J., 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. https://doi.org/10.1126/science.1219075.

Hughes, M.E., Hogenesch, J.B., and Kornacker, K. (2010). JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. J. Biol. Rhythms *25*, 372–380. https://doi.org/10.1177/0748730410379711.

James, A.B., Monreal, J.A., Nimmo, G.A., Kelly, C.L., Herzyk, P., Jenkins, G.I., and Nimmo, H.G. (2008). The circadian clock in Arabidopsis roots is a simplified slave version of the clock in shoots. Science *322*, 1832–1835. https://doi.org/10.1126/science.1161403.

Katari, M.S., Nowicki, S.D., Aceituno, F.F., Nero, D., Kelfer, J., Thompson, L.P., Cabello, J.M., Davidson, R.S., Goldberg, A.P., Shasha, D.E., et al. (2010). VirtualPlant: A software platform to support systems biology research. Plant Physiol *152*, 500–515. https://doi.org/10.1104/pp.109.147025.

Kennaway, D.J., Boden, M.J., and Varcoe, T.J. (2012). Circadian rhythms and fertility. Mol. Cell. Endocrinol. *349*, 56–61. https://doi.org/10.1016/J.MCE.2011.08.013.

Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat. Biotechnol. *37*, 907–915. https://doi.org/10.1038/s41587-019-0201-4.

Kinmonth-Schultz, H.A., Golembeski, G.S., and Imaizumi, T. (2013). Circadian clock-regulated physiological outputs: dynamic responses in nature. Semin. Cell Dev. Biol. *24*, 407–413. https://doi.org/10.1016/j.semcdb.2013.02.006.

Klepikova, A.V., Kasianov, A.S., Gerasimov, E.S., Logacheva, M.D., and Penin, A.A. (2016). A high resolution map of the Arabidopsis thaliana developmental transcriptome based on RNA-seq profiling. Plant J *88*, 1058–1070. https://doi.org/10.1111/TPJ.13312.

Ma, Y., Gil, S., Grasser, K.D., and Mas, P. (2018). Targeted recruitment of the basal transcriptional machinery by LNK clock components controls the circadian rhythms of nascent RNAs in Arabidopsis. Plant Cell *30*, 907–924. https://doi.org/10.1105/tpc.18.00052.

Martínez-Fernández, I., Sanchís, S., Marini, N., Balanzá, V., Ballester, P., Navarrete-Gómez, M., Oliveira, A.C., Colombo, L., and Ferrándiz, C. (2014). The effect of NGATHA altered activity on auxin signaling pathways within the Arabidopsis gynoecium. Front. Plant Sci. *5*, 210. https://doi.org/10.3389/FPLS.2014.00210.

Más, P., Alabadí, D., Yanovsky, M.J., Oyama, T., and Kay, S.A. (2003a). Dual role of TOC1 in the control of circadian and photomorphogenic responses in Arabidopsis. Plant Cell *15*, 223–236. https://doi.org/10.1105/tpc.006734.

Más, P., Kim, W.Y., Somers, D.E., and Kay, S.A. (2003b). Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. Nature *426*, 567–570. https://doi.org/10.1038/nature02163.

McClung, C.R. (2019). The plant circadian oscillator. Biology *8*, 14. https://doi.org/10.3390/biology8010014.

Michael, T.P., Mockler, T.C., Breton, G., McEntee, C., Byer, A., Trout, J.D., Hazen, S.P., Shen, R., Priest, H.D., Sullivan, C.M., et al. (2008). Network discovery pipeline elucidates conserved time-of-day-specific *cis*-regulatory modules. PLoS Genet *4*, e14. https://doi.org/10.1371/journal.pgen.0040014.

Millar, A.J., Carré, 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. https://doi.org/10.1126/science.7855595.

Mockler, T.C., Michael, T.P., Priest, H.D., Shen, R., Sullivan, C.M., Givan, S.A., McEntee, C., Kay, S.A., and Chory, J. (2007). The DIURNAL project: DIURNAL and circadian expression profiling, model-based pattern matching, and promoter analysis. Cold Spring Harb. Symp. Quant. Biol. *72*, 353–363. https://doi.org/10.1101/sqb.2007.72.006.

Mong, J.A., Baker, F.C., Mahoney, M.M., Paul, K.N., Schwartz, M.D., Semba, K., and Silver, R. (2011). Sleep, rhythms, and the endocrine brain: influence of sex and gonadal hormones. J. Neurosci. *31*, 16107–16116. https://doi.org/10.1523/JNEUROSCI.4175-11.2011.

Müller, A. (1961). Zur Charakterisierung der Blüten und Infloreszenzen von Arabidopsis fhaliana (L.) Heynh. Kulturpflanze *9*, 364–393. https://doi.org/10.1007/BF02095757.

Muroya, M., Oshima, H., Kobayashi, S., Miura, A., Miyamura, Y., Shiota, H., Onai, K., Ishiura, M., Manabe, K., and Kutsuna, S. (2021). Circadian clock in Arabidopsis thaliana determines flower opening time early in the morning and dominantly closes early in the afternoon. Plant Cell Physiol *62*, 883–893. https://doi.org/10.1093/pcp/pcab048.

Nagel, D.H., and Kay, S.A. (2012). Complexity in the wiring and regulation of plant circadian networks. Curr. Biol. *22*, R648–R657. https://doi.org/10.1016/j.cub.2012.07.025.

Nakamichi, N. (2020). The transcriptional network in the Arabidopsis circadian clock system. Genes *11*, 1284. https://doi.org/10.3390/genes11111284.

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 Cell Physiol *46*, 686–698. https://doi.org/10.1093/pcp/pci086.

Okada, M., and Mas, P. (2022). In vivo bioluminescence analyses of circadian rhythms in *Arabidopsis thaliana* using a microplate luminometer. Methods Mol. Biol. *2482*, 395–406. https://doi.org/10.1007/978-1-0716-2249-0_27.

Perales, M., and Más, P. (2007). A functional link between rhythmic changes in chromatin structure and the *Arabidopsis* biological clock. Plant Cell *19*, 2111–2123. https://doi.org/10.1105/tpc.107.050807.

Raven, J.A., and Griffiths, H. (2015). Photosynthesis in reproductive structures: costs and benefits. J. Exp. Bot. *66*, 1699–1705. https://doi.org/10.1093/JXB/ERV009.

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 Genet *7*, e1001350. https://doi.org/10.1371/journal.pgen.1001350.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. *29*, 24–26. https://doi.org/10.1038/nbt.1754.

Rugnone, M.L., Faigón Soverna, A.F., Sanchez, S.E., Schlaen, R.G., Hernando, C.E., Seymour, D.K., Mancini, E., Chernomoretz, A., Weigel, D., Más, P., et al. (2013). LNK genes integrate light and clock signaling networks at the core of the Arabidopsis oscillator. Proc. Natl. Acad. Sci. USA *110*, 12120–12125. https://doi.org/10.1073/pnas.1302170110.

Sablowski, R. (2015). Control of patterning, growth, and differentiation by floral organ identity genes. J. Exp. Bot. *66*, 1065–1073. https://doi.org/10.1093/jxb/eru514.

Salomé, P.A., and McClung, C.R. (2005). PSEUDO-RESPONSE REGULATOR 7 and 9 are partially redundant genes essential for the temperature responsiveness of the Arabidopsis circadian clock. Plant Cell *17*, 791–803. https://doi.org/10.1105/tpc.104.029504.

Sanchez, S.E., and Kay, S.A. (2016). The plant circadian clock: From a simple timekeeper to a complex developmental manager. Cold Spring Harb. Perspect. Biol. *8*, a027728. https://doi.org/10.1101/cshperspect.a027748.

Sanchez-Villarreal, A., Shin, J., Bujdoso, N., Obata, T., Neumann, U., Du, S.-X., Ding, Z., Davis, A.M., Shindo, T., Schmelzer, E., et al. (2013). TIME FOR COFFEE is an essential component in the maintenance of metabolic homeostasis in *Arabidopsis thaliana*. Plant J *76*, 188–200. https://doi.org/10.1111/tpj.12292.

Seo, P.J., and Mas, P. (2014). Multiple layers of posttranslational regulation refine circadian clock activity in Arabidopsis. Plant Cell *26*, 79–87. https://doi.org/10.1105/tpc.113.119842.

Shalit-Kaneh, A., Kumimoto, R.W., Filkov, V., and Harmer, S.L. (2018). Multiple feedback loops of the Arabidopsis circadian clock provide rhythmic robustness across environmental conditions. Proc. Natl. Acad. Sci. USA *115*, 7147–7152. https://doi.org/10.1073/pnas.1805524115.

Shim, J.S., Kubota, A., and Imaizumi, T. (2017). Circadian clock and photoperiodic flowering in Arabidopsis: CONSTANS is a Hub for Signal integration. Plant Physiol *173*, 5–15. https://doi.org/10.1104/pp.16.01327.

Simonini, S., and Østergaard, L. (2019). Female reproductive organ formation: a multitasking endeavor. Curr. Top. Dev. Biol. *131*, 337–371. https://doi.org/10.1016/bs.ctdb.2018.10.004.

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in *Arabidopsis*. Plant Cell *2*, 755–767. https://doi.org/10.1105/tpc.2.8.755.

Sorkin, M.L., and Nusinow, D.A. (2021). Time will tell: intercellular communication in the plant clock. Trends Plant Sci *26*, 706–719. https://doi.org/10.1016/j.tplants.2020.12.009.

Takahashi, J.S. (2017). Transcriptional architecture of the mammalian circadian clock. Nat. Rev. Genet. *18*, 164–179. https://doi.org/10.1038/nrg.2016.150.

Takahashi, N., Hirata, Y., Aihara, K., and Mas, P. (2015). A hierarchical multi-oscillator network orchestrates the *Arabidopsis* circadian system. Cell *163*, 148–159. https://doi.org/10.1016/j.cell.2015.08.062.

Thain, S.C., Hall, A., and Millar, A.J. (2000). Functional independence of circadian clocks that regulate plant gene expression. Curr. Biol. *10*, 951–956. https://doi.org/10.1016/S0960-9822(00)00630-8.

Thain, S.C., Murtas, G., Lynn, J.R., McGrath, R.B., and Millar, A.J. (2002). The circadian clock that controls gene expression in *Arabidopsis* is tissue specific. Plant Physiol *130*, 102–110. https://doi.org/10.1104/pp.005405.

Thorvaldsdóttir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. *14*, 178–192. https://doi.org/10.1093/bib/bbs017.

Wang, Y., Wu, J.-F.F., Nakamichi, N., Sakakibara, H., Nam, H.-G.G., and Wu, S.-H.H. (2011). LIGHT-REGULATED WD1 and PSEUDO-RESPONSE REGULATOR9 form a positive feedback regulatory loop in the Arabidopsis circadian clock. Plant Cell *23*, 486–498. https://doi.org/10.1105/tpc.110.081661.

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. https://doi.org/10.1016/S0092-8674(00)81464-6.

Weigel, D. (1995). The genetics of flower development: From floral induction to ovule morphogenesis. Annu. Rev. Genet. *29*, 19–39. https://doi.org/10.1146/annurev.ge.29.120195.000315.

Wellmer, F., Bowman, J.L., Davies, B., Ferrándiz, C., Fletcher, J.C., Franks, R.G., Graciet, E., Gregis, V., Ito, T., Jack, T.P., et al. (2014). Flower development: open questions and future directions. Methods Mol. Biol. *1110*, 103–124. https://doi.org/10.1007/978-1-4614-9408-9_5.

Wenden, B., Toner, D.L.K., Hodge, S.K., Grima, R., and Millar, A.J. (2012). Spontaneous spatiotemporal waves of gene expression from biological clocks in the leaf. Proc. Natl. Acad. Sci. USA *109*, 6757–6762. https://doi.org/10.1073/pnas.1118814109.

Wu, J.F., Tsai, H.L., Joanito, I., Wu, Y.C., Chang, C.W., Li, Y.H., Wang, Y., Hong, J.C., Chu, J.W., Hsu, C.P., et al. (2016). LWD-TCP complex activates the morning gene CCA1 in Arabidopsis. Nat. Commun. *7*, 13181. https://doi.org/10.1038/ncomms13181.

Wu, J.F., Wang, Y., and Wu, S.H. (2008). Two new clock proteins, LWD1 and LWD2, regulate Arabidopsis photoperiodic flowering. Plant Physiol *148*, 948–959. https://doi.org/10.1104/pp.108.124917.

Xie, Q., Wang, P., Liu, X., Yuan, L., Wang, L., Zhang, C., Li, Y., Xing, H., Zhi, L., Yue, Z., et al. (2014). LNK1 and LNK2 are transcriptional Coactivators in the Arabidopsis circadian oscillator. Plant Cell *26*, 2843–2857. https://doi.org/10.1105/tpc.114.126573.

Yakir, E., Hassidim, M., Melamed-Book, N., Hilman, D., Kron, I., and Green, R.M. (2011). Cell autonomous and cell-type specific circadian rhythms in Arabidopsis. Plant J *68*, 520–531. https://doi.org/10.1111/j.1365-313X.2011.04707.x.

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 Physiol *150*, 844–857. https://doi.org/10.1104/pp.109.137414.

Yamaguchi, N., Winter, C.M., Wu, M.-F., Kwon, C.S., William, D.A., and Wagner, D. (2014). PROTOCOLS: chromatin immunoprecipitation from *Arabidopsis* tissues. Arabidopsis Book *12*, e0170. https://doi.org/10.1199/tab.0170.

Young, M.W., and Kay, S.A. (2001). Time zones: a comparative genetics of circadian clocks. Nat. Rev. Genet. *2*, 702–715. https://doi.org/10.1038/35088576.

Zielinski, T., Moore, A.M., Troup, E., Halliday, K.J., and Millar, A.J. (2014). Strengths and limitations of period estimation methods for circadian data. PLoS One *9*, e96462. https://doi.org/10.1371/journal.pone.0096462.

Zúñiga-Mayo, V.M., Gómez-Felipe, A., Herrera-Ubaldo, H., and De Folter, S. (2019). Gynoecium development: networks in *Arabidopsis* and beyond. J. Exp. Bot. *70*, 1447–1460. https://doi.org/10.1093/jxb/erz026.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-GFP antibody (Anti-GFP, IgG)	Invitrogen by Thermo Fisher Scientific	Cat#A-11122-100uL; RRID: AB_221569
Bacterial and virus strains		
Agrobacterium tumefaciens (strain GV2260)	N/A	N/A
Chemicals, peptides, and recombinant proteins		
D-Luciferin Free Acid 100 mg	Bio Thema	Cat# BT11-100
Pierce 16% Formaldehyde (w/v), Methanol-free	Thermo Scientific	Cat#28908
Protein G Dynabeads for Immunoprecipitation	Life Technologies	Cat#10004D
Lithium chloride	Merck	Cat#1.05679
Glycine	Sigma-Aldrich	Cat#200-272-2
Critical commercial assays		
Maxwell RSC Plant RNA kit	Promega	Cat#AS1500
iScript™ Reverse Transcription Supermix for RT-qPCR	BioRad	Cat#1708841
Brilliant III ultrafast SYBR qRT-PCR Master Mix	Agilent Technologies	Cat#600883
RNA Nano 6000 Assay Kit	Agilent Technologies	Cat#5067-1511
NEBNext® UltraTM RNA Library Prep Kit for Illumina®	NEB	Cat#E7530L
USER Enzyme	NEB	Cat#M5505L
PE Cluster Kit cBot-HS	Illumina	Cat#PE-401-3001
Deposited data		
RNA-seq	This study	SRA: PRJNA858223
Arabidopsis thaliana: WT Col-0	N/A	N/A
Arabidopsis thaliana: WT WS	N/A	N/A
Arabidopsis thaliana: *CCA1:LUC*	Salomé and McClung, 2005	N/A
Arabidopsis thaliana: *TOC1:LUC*	Perales and Más, 2007	N/A
Arabidopsis thaliana: *GI:LUC*	Wu et al., 2008	N/A
Arabidopsis thaliana: CCA1-ox	Wang and Tobin, 1998	N/A
Arabidopsis thaliana: *toc1-2*;CCA1:LUC	NASC; Cervela-Cardona et al., 2021	N2107710
Arabidopsis thaliana: *cca1-1/lhy-RNAi*	Alabadi et al., 2002	N/A
Arabidopsis thaliana: *lux-2*	Hazen et al., 2005	N/A
Arabidopsis thaliana: *elf3-2*	Hicks et al., 1996	N/A
Arabidopsis thaliana: *elf4-2*	Huang et al., 2016	N/A
Arabidopsis thaliana: *prr5-11*;CCA1:LUC	Nakamichi et al., 2005	N/A
Arabidopsis thaliana: *prr57*;CCA1:LUC	Nakamichi et al., 2005	N/A
Arabidopsis thaliana: *prr59*	Nakamichi et al., 2005	N/A
Arabidopsis thaliana: *prr79*	Farré et al., 2005	N/A
Arabidopsis thaliana: *rve4,6,8*	Hsu et al., 2013	N/A
Arabidopsis thaliana: ELF3-ox-YFP	Herrero et al., 2012	N/A
Arabidopsis thaliana: YFP-ELF4-ox	Herrero et al., 2012	N/A
Arabidopsis thaliana: LUXpro::LUX-GFP *lux-4*	Ezer et al., 2017	N/A
Arabidopsis thaliana: CCA1-HA-EYFP/*cca1-1*	Yakir et al., 2009	N/A
Arabidopsis thaliana: TOC1-ox/*elf3-2*	This study	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Arabidopsis thaliana: TOC1-ox	Huang et al., 2012	N/A
Arabidopsis thaliana: CCA1-ox/*prr7*	This study	N/A
Arabidopsis thaliana: CCA1-ox/*prr79*	This study	N/A
Oligonucleotides		
Primers for RT-qPCR	This study Table S2	N/A
Primers for ChIP	This study Table S2	N/A
Software and algorithms		
MikroWin 2010, version 5.15	Mikrotek Laborsysteme GmbH	https://labsis.de
R, version 3.3.2	The R Project for Statistical Computing	https://www.r-project.org
HISAT2	Kim et al., 2019	http://daehwankimlab.github.io/hisat2/
Heatmapper	Babicki et al., 2016	http://www.heatmapper.ca
JTK_Cycle	Hughes et al., 2010	https://openwetware.org/wiki/HughesLab:JTK_Cycle
Integrative Genomics Viewer	Robinson et al., 2011; Thorvaldsdóttir et al., 2013	https://software.broadinstitute.org/software/igv/
DIURNAL database	Michael et al., 2008; Mockler et al., 2007	http://diurnal.mocklerlab.org
PANTHER	GENEONTOLOGY	http://www.pantherdb.org
BIOMAPS	Katari et al., 2010	http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/
Image J	Image J	https://imagej.nih.gov/ij/
GraphPad Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Paloma Mas (paloma.mas@cragenomica.es).

Materials availability

All materials generated in this study will be available upon request from Paloma Mas (paloma.mas@cragenomica.es).

Data and code availability

- The RNA-sequencing data have been deposited at the Sequence Read Archive and are publicly available as of the date of publication. Accession number is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant material, growing conditions and organ dissection

Seedlings were grown on half-strength Murashige and Skoog (MS) agar medium without sucrose, and synchronized under light:dark cycles (LgD, 16h light:8h dark) with 60-100 μmol $m^{-2}s^{-1}$ of cool white fluorescent light at 22°C for about 7-10 days (unless otherwise specified). For experiments using flowering plants, seedlings were then transplanted to soil and cultivated throughout the reproductive stage under light:dark cycles (LgD, 16h light:8h dark) with 150-200 μmol $m^{-2}s^{-1}$ of white light emitting diodes (LEDs) at 22°C. The *CCA1:LUC* (Salomé and McClung, 2005), *TOC1:LUC* (Perales and Más, 2007) and *GI:LUC* (Wu et al., 2008) reporter lines as well as the CCA1-ox (Wang and Tobin, 1998), TOC1-ox (Huang et al., 2012), and *toc1-2;CCA1:LUC* (NASC, N2107710) (Cervela-Cardona et al., 2021), *cca1-1/lhy-RNAi* (Alabadí et al., 2002), *lux-2* (Hazen et al., 2005), *elf3-2* (Hicks et al., 1996), *elf4-2* (Huang et al., 2016), *prr5-11;CCA1:LUC*, *prr57*, *prr59* (Nakamichi et al., 2005), *prr79* (Farré et al., 2005), *rve4,6,8* (Hsu et al., 2013), ELF3-ox-YFP (Herrero et al., 2012), YFP-ELF4-ox (Herrero et al., 2012), LUX-GFP (LUXpro::LUX-GFP *lux-4*) (Ezer et al., 2017), CCA1-HA-EYFP/*cca1-1* (Yakir et al., 2009) lines were described elsewhere. All the lines are in Columbia (Col-0) background except the *cca1-1/lhy-RNAi* plants, which are in Wassilewskija (WS) background. Matching WT backgrounds were used for each mutant line.

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The TOC1-ox/*elf3-2* lines were generated by crossing the TOC1-ox plants (Huang et al., 2012) with the *elf3-2* mutant (Hicks et al., 1996). The CCA1-ox/*prr* lines were generated by transforming the CCA1-ox construct into the *prr79* plants and by crossing the CCA1-ox plants (Wang and Tobin, 1998) with the *prr7* mutant plants (Farré et al., 2005). To follow individual open flowers in intact plants, inflorescences of open flowers were marked by a black marker at day 0 under LL. Pictures of the selected flowers were taken with a stereo microscope (SZX16, Olympus) the following days. For the luminescence assays (see below) and for the RT-QPCR (Reverse Transcription Quantitative Polymerase Chain Reaction) analysis of floral organs, sterile dissecting forceps were used to carefully excise young buds, mature buds, mature flowers and open flowers from flowering plants. Similarly, sepals, petals, stamens, and pistils were carefully excised from open flowers.

METHOD DETAILS

In vivo luminescence assays

Buds, flowers or floral organs from luciferase-expressing plants synchronized under light:dark cycles (LgD, 16h light:8h dark) with 150-200 μmol $m^{-2}s^{-1}$ of white LEDs at 22°C were excised and immediately placed in 96-well microplates with half-strength MS liquid medium with 1% sucrose and 290μM D-luciferin (Biothema). Bioluminescence rhythms, were examined as previously described (Okada and Mas, 2022) under constant light (LL) conditions or entraining Light:Dark cycles (LgD, 16h light:8h dark) as specified for each experiment. A microplate luminometer LB-960 (Berthold Technologies) and the software Microwin, version 4.34 (Mikrotek 2 Laborsysteme) were used for the bioluminescence analyses. Amplitude, period, and relative amplitude error (RAE) were estimated with the fast Fourier transform non-linear least squares (FFT-NLLS) method (Zielinski et al., 2014). The analyses were performed in the statistical environment of R 3.3.2. Data from samples that appeared damaged or that eventually died in the wells were excluded from the analyses. Three biological replicates were performed per experiment.

Gene expression analysis by RT-QPCR

About 5-6 12-day old seedlings or about 6-8 flowers were collected, snap-frozen and ground using TissueLyser II (QIAGEN). About 6-8 pistils were collected, snap-frozen and ground using plastic grinding pestles. About 70 stamens were collected in homogenization solution (Promega, Z305H), snap-frozen, and ground using plastic grinding pestles. RNA from flowers, stamens and pistils was isolated using the Maxwell RSC Plant RNA kit (Promega). Single strand cDNA was synthesized using iScript™ Reverse Transcription Supermix for RT-qPCR (BioRad) following the manufacturer recommendations. For QPCR analysis, cDNAs were diluted 30-50-fold with nuclease-free water and QPCR was performed with Brilliant III ultrafast SYBR qRT-PCR Master Mix (Agilent) in a 96-well CFX96 Touch Real-Time PCR Detection System (BioRad). The *IPP2* gene (Fung-Uceda et al., 2018) was used as control in seedlings and *PP2AA3* (AT1G13320) (Takahashi et al., 2015) was used as control in pistil and flowers. A list of primers used for gene expression analyses is shown in Table S2.

RNA-Seq analysis

Plants were synchronized under LgD conditions (16h light:8h dark) with 150-200 μmol $m^{-2}s^{-1}$ of white LEDs at 22°C and subsequently transferred to LL for two days. About 6-8 pistils from open flowers were collected the third day under LL, every four hours over two circadian cycles. Total RNA was isolated using the Maxwell RSC Plant RNA kit (Promega) following the manufacturer's recommendations. Sequencing was performed by Novogene Co., Ltd. RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and RNA integrity and quantitation were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies). A total of 1 μg RNA per sample was used for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB) following manufacturer's recommendations. Index codes were added to attribute sequences to each sample. mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150∼200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter). Subsequently, 3 μl of USER Enzyme (NEB) were used with size-selected, adaptor ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. The PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After clustering, the library preparations were sequenced on an Illumina platform and paired-end reads were generated. Raw data (raw reads) of FASTQ format were firstly processed through fastp. In this step, clean data (clean reads) were obtained by removing reads containing adapter and poly-N sequences and reads with low quality from raw data. At the same time, Q20, Q30 and GC content of the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Paired-end clean reads were mapped to the reference genome using HISAT2 software (Kim et al., 2019). HISAT2 uses a large set of small GFM indexes that collectively cover the whole genome. These small indexes (called local indexes), combined with several alignment strategies, enable rapid and accurate alignment of sequencing reads. Featurecounts was used to count the read numbers mapped of each gene. RPKM (Reads Per Kilobase of exon model per Million mapped reads) of each gene

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was calculated based on the length of the gene and reads count mapped to this gene. RPKM considers the effect of sequencing depth and gene length for the reads count at the same time and is currently the most commonly used method for estimating gene expression levels. The web-based tool ''Heatmapper'' was used to visualize data as heatmaps (Babicki et al., 2016).

The JTK_Cycle algorithm (Hughes et al., 2010) was used to identify oscillating genes (adjusted p-value < 0.05) with a period ranging from 20 to 28. The Integrative Genomics Viewer (IGV) was used to visualize the data (Robinson et al., 2011; Thorvaldsdóttir et al., 2013). The phases of circadian expression were analyzed using the publicly available Gene Phase Analysis Tool ''PHASER'' of the DIURNAL database (http://diurnal.mocklerlab.org/) (Michael et al., 2008; Mockler et al., 2007). Phase over-representation is calculated as the number of genes with a given phase divided by the total number of genes over the number of genes called rhythmic and divided by the total number of genes in the dataset. Circadian genes were classified into broad functional categories using the PANTHER Over-representation Test (Fisher's Exact Test, Bonferroni correction) and the web tool ''BIOMAPS'' (Katari et al., 2010) (Fisher's Exact Test, cut-off 0.01), which renders over-represented and significant functional terms (Gene Ontology or MIPS) as compared to the frequency of the term in the whole genome.

Phenotypic analyses of pistils, siliques and seeds

For pistil analyses, at least 5 pistils for each genotype were selected at stages 11, 12 and 13 (Müller, 1961; Smyth et al., 1990), and pictures were taken with a stereo microscope (SZX16, Olympus) after careful removal of petals and sepals. Measurements of pistil length was performed by using the software package *Image J*. For silique and seed analyses, 100 fully developed siliques from the main inflorescences (starting at the fifth silique from the bottom) were collected and photographed using a stereo microscope (SZX16, Olympus). Seeds from each silique were spread on white paper and photographs were taken. Silique length and seed number per silique were quantified using the software package *Image J*. For seed size and weight analyses, seeds were harvested and sieved to remove plant debris. Following incubation at 25°C for 7 days, randomly selected groups of seeds for each line were weighted (W). Seed number (N), sectional area, length and width were quantified using the software package *Image J*. The grain weight was calculated as: *1000-grain weight (g)= W /N *1000*. Two-tailed Student's t-test analyses were performed using the GraphPad Prism software.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Yamaguchi et al., 2014). About 100 mg of pistils from open flowers were sampled, and vacuum infiltrated 3 times for 15 min in 30 ml cross-linking solution (1% formaldehyde in 1×PBS) at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M and vacuum infiltrated for 5 min. Samples were washed three times with cold deionized water, dried with paper towels and snapped-frozen in liquid nitrogen. Samples were ground to fine powder and extracted with 2.5 ml of Nuclei extraction buffer. After filtering the samples through Miracloth (475855, Merck), the chromatin solution was sonicated until obtaining sheared DNA of about 200-600 bp. Soluble chromatin was incubated overnight at 4°C with theAnti-GFP antibody (#A-11122, Thermo Fisher Scientific) for the samples of ELF3-ox-YFP, YFP-ox-ELF4, LUX-GFP and CCA1-HA-EYFP/*cca1-1*. Samples were then incubated with Protein G-Dynabeads beads (10004D, Thermo Fisher Scientific) for 4 hours at 4°C with rotation. The beads were washed thrice with Low salt wash buffer, High salt wash buffer, 250 mM LiCl wash buffer and 0.5×TE, respectively. The samples were eluted from the beads with elution buffer by incubating for 30 min at 65°C. The purified DNA was diluted 10-fold with nuclease-free water and QPCR was performed with Brilliant III ultrafast SYBR qRT-PCR Master Mix (Agilent) in a 96-well CFX96 Touch Real-Time PCR Detection System (BioRad). A list of primers used for ChIP analyses is shown in Table S2.

QUANTIFICATION AND STATISTICAL ANALYSIS

For luminescence assays, data represent means + SEM of $n \geq 3$ (Figures 1B, 1D, 2A–2D, 2G, 3B–3G, S1, S2A–S2F, S3E, and S3F). Periods and relative amplitude errors of bioluminescence rhythms were estimated with the fast Fourier transform nonlinear least squares (FFT-NLLS) and plotted with three biological replicates (Figures 1C, 1E, 2E, 2F, 2H, 3H–3J, and S3A–S3D). Statistical analyses were performed by two-tailed Student's t-test to compare period lengths. Quantification of pistil length (Figures 4I, 4Q, and 4R), silique length (Figures 4K and 4S), seed number (Figure 4N), and seed sectional area (Figure 4P) was performed using ImageJ software. For pistil length, data represent median ± SEM of $n \approx 15$ pistils (Figures 4I, 4Q, and 4R). For silique number, data represent median ± SEM of $n \approx 15$ plants (Figure 4J). For silique length, $n \approx 100$ siliques were measured. Data represent median ± SEM (Figure 4K), lengths are plotted with the means (Figure 4S). For seed number per silique, data represent median ± SEM of $n \approx 70$ siliques (Figure 4N). For 1000-grain-weight, more than 500 seeds were weighed, and data represent median ± SEM of $n \approx 12$ (Figure 4O). For seed sectional area, areas of $n \approx 200$ seeds are plotted with those means (Figure 4P). For seed weights per plants, $n \approx 30$ plants were used and weights of seeds from each plant are plotted with those means (Figure 4T). Statistical analyses were performed by two-tailed Student's t-test (*** p-value<0.0001; ** p-value<0.001; * p-value<0.05). For gene expression analysis using qPCR (Figures 1F, 1G, 5, 6, 7A–7F, S2G, S2H, S2J–S2M, and S5–S7), data represent means + SEM of technical duplicates using three biological replicates. Crossing point (Cp) calculation was used for quantification using the Absolute Quantification analysis by the 2nd Derivative Maximum method.