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Molecular characterization of the circadian clock in Solanum lycopersicum

Benjamin Alary

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Molecular characterization of the circadian clock in *Solanum lycopersicum*

Benjamin Alary

Barcelona 2023

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Molecular characterization of the circadian clock in *Solanum lycopersicum*

PhD Thesis

Benjamin Alary

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Universidad Autónoma de Barcelona

Facultad de Biociencias

Departamento de Biología Animal, Biología Vegetal y Ecología

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Molecular characterization of the circadian clock in *Solanum lycopersicum*

Memoria presentada por Benjamin Alary para optar al título de doctor
por la Universidad Autónoma de Barcelona

Directora	Doctorando
Dra. Paloma Más Martínez	Benjamin Alary

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INTRODUCTION

1. Circadian clocks, an adaptive response to daily environmental rhythms

The earth rotates around its axis with a period of 24h leading to the day and night cycles due to the alternation of the sun exposition. This natural rhythm results in a daily temperature oscillation and a cyclic variation of light conditions. The succession of days and night influences the metabolism, physiology and behaviour of most organisms, including prokaryotes. These processes are coordinated by a cellular biochemical oscillator called circadian clock [derived from the Latin *circa* (about) *dies* (a day)]. The circadian clock is synchronized with this environmental changes to generate internal rhythms of 24h (Golden 2003; King and Takahashi 2000; Más and Yanovsky 2009). The proper timing by the circadian clock provides an adaptive advantage and increases the fitness of the organisms (Green *et al.* 2002; Wu *et al.* 2011).

The first record about endogenous clock is credited to Theophrastus (400BC), when he described the diurnal leaf movement of *Tamarindus indicus* (Hort 1916). During the age of enlightenment, de Mairan (1729) conducted the first experiment to demonstrate that leaf movement persisted under constant darkness. Konopka and Benzer (1971) later identified the first mutants affecting the clock in *Drosophila*. In 2017, Hall, Rosbash and Young received the Nobel Prize in Physiology or Medicine for the discovery of the first key genes of the *Drosophila* circadian system.

A classical view of the circadian clock function consists of three main components: (1) input pathways that perceive and transmit the environmental cues to the (2) central oscillator that is responsible for the generation of rhythms of the (3) output pathways or biological processes controlled by the clock. Light and temperature are the principal inputs sensed by the organisms to keep their internal circadian clock synchronised to the day and night cycle (Millar 2004; Simoni *et al.* 2014). Other cues can synchronize the clock including for example feeding in animals (Mistlberger and Antle 2011). In most organisms, the central oscillator is composed of several clock components regulating each other through multiple transcriptional and translational feedback loops (Bell-Pedersen *et al.* 2005). The central oscillator is connected to output processes from individual cells to the whole organism. The importance of the circadian function is exemplified by several facts, including the various severe pathologies that emerge following deregulation of the circadian system (Reinke and Asher 2019).

2. Properties of circadian rhythms

Circadian rhythms exhibit sinusoidal waveforms when plotted over time. Different mathematical terms describe these rhythms (Más 2008) (Figure 1). For instance, the period refers to the duration of one full cycle, whereas the amplitude indicates the difference between the mean and the minimum (trough) or maximum value (peak) of the oscillation. The phase is the state of a rhythm oscillation relative to another rhythm, normally the environmental rhythm (relative to dawn or dusk, for example). Under light-dark cycles, the input cues reset every day the circadian clock so that the period of the clock is exactly 24 hours. The entraining cues under light-dark cycles are considered as Zeitgeber Time (ZT) (or "time giver" from the German), with ZT0 normally defined in diurnal organisms as dawn (Golombek and Rosenstein 2010). In circadian biology, and following synchronization under light:dark (LD) cycles, the experiments are often performed under constant, free running conditions, for example constant light (LL) or constant darkness (DD). With this experimental setup, the truly circadian rhythms are distinguished from the rhythms exclusively regulated by the light-dark cycles.

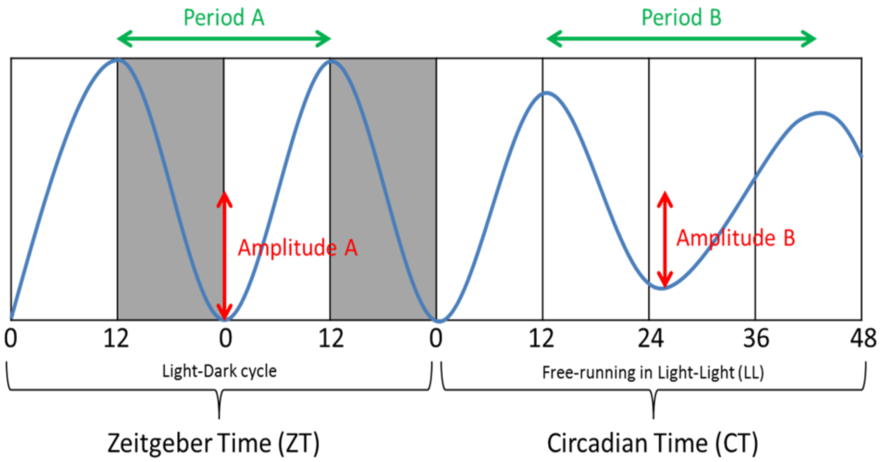


Figure 1: Main properties of the circadian rhythms. The period represents the total time required to complete one cycle. In the Figure, period A is about 24h whereas period B is about 30h. The amplitude is calculated from the difference between the peak and the average of the oscillation; here the amplitude A is higher than the B. White boxes: light phase; Shaded boxes: dark phases. Modified from Harmer (2009).

3. The circadian clock in *Arabidopsis thaliana*

Plants as sessile organisms cannot avoid unfavourable environmental conditions, so they have developed strategies to growth and adapt to their surround environment. The circadian clock plays a major role in the adaptability to the environment and the impact on the plant fitness (Sanchez and Kay 2016). *Arabidopsis thaliana* has been historically used as a plant model to study the circadian clock in plants. However, over the past recent years, many studies have been done in other species, including crops of agronomical interest (Bendix *et al.* 2015; Calixto *et al.* 2015). The following section describes the main tree components of the plant circadian system in *Arabidopsis thaliana*: (3.1) central oscillator, (3.2) inputs and (3.3) output pathways (Figure 2).

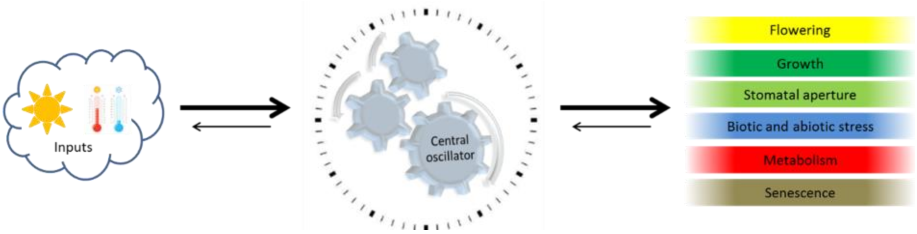


Figure 2: Schematic representation of the circadian clock network. Daily environmental changes in light and temperature are perceived and incorporated as input to the central oscillator. Then the central oscillator generates rhythms in many different output pathways to synchronize the organism with its environment. Modified from Nohales and Kay (2016).

3.1 Central oscillator

Many studies have focused on the *Arabidopsis* circadian components at the core of the clock and the regulatory mechanisms among these clock components (reviewed in Nakamachi 2020). Essentially, the components of the *Arabidopsis* central oscillator regulate each other by transcriptional, post-transcriptional and post-translational regulation as well as by chromatin modifications (Seo and Más 2014; Yang *et al.* 2018; Maric and Más 2020; Más 2008; McClung 2019) (Figures 3 and 4). The particular regulation of each component leads to their sequential gene expression and protein function at specific times during the day and night.

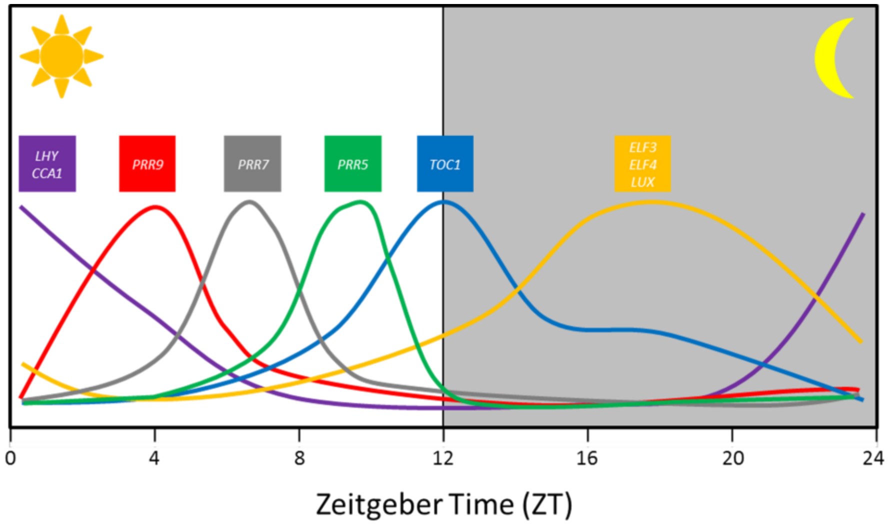


Figure 3: Schematic representation of the expression of oscillator genes showing their sequential expression along the day and night. The expression of the morning genes *CCA1* and *LHY* peaks close to dawn. *PRR9*, *PRR7*, *PRR5* and *PRR3* are sequentially expressed afterwards. *TOC1* has a peak of expression at dusk, whereas *ELF3*, *ELF4* and *LUX* from the EC are expressed just after dusk. Modified from Huang and Nusinow (2016).

3.1.1 Morning-expressed genes

The morning-phased single-MYB transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) have a partially redundant function and heterodimerize (Lu *et al.* 2009) to repress the afternoon- and evening-phased genes like *PSEUDO-RESPONSE REGULATOR 9 (PRR9), PSEUDO-RESPONSE REGULATOR 7 (PRR7), PSEUDO-RESPONSE REGULATOR 5 (PRR5)*, and the evening-phased genes such as *EARLY FLOWERING 4 (ELF4), LUX ARRHYTHMO (LUX)* and *TIME OF CAB1 (TOC1)* [also called *PSEUDO-RESPONSE REGULATOR 1* (*PRR1*)] (Alabadí *et al.* 2001; Li *et al.* 2011; Adams *et al.* 2015; Kamioka *et al.* 2016). Loss of function of *CCA1* or *LHY* leads to shortened period and advanced phase whereas the double mutant *cca1/lhy* shows an advance circadian phase of clock genes (Mizoguchi *et al.* 2002; Alabadí *et al.* 2002). Under LL or DD conditions, the double mutant is arrhythmic (Mizoguchi *et al.* 2002; Alabadí *et al.* 2002). The *cca1/lhy* double mutant plants are also early flowering under Short Days compared to WT but not under Long Days. The *cca1/lhy* plants are smaller than WT, and the leaves are also smaller and paler than WT (Mizoguchi *et al* 2002). Over-expression of *LHY* or *CCA1* leads to arrhythmia (Wang and Tobin 1998).

3.1.2 The Pseudo-Response Regulator family

The PRR clock-related family is composed by five proteins. The genes are sequentially expressed: *PRR9* peaks close to dawn, then *PRR7*, *PRR5*, *PRR3* and finally *PRR1/TOC1* at dusk (Mizuno and Nakamachi 2005; Nakamachi *et al.* 2005) (Figure 3). PRR9, PRR7, PRR5 and TOC1 act as repressors of *CCA1* and *LHY*, thus confining their expression close to dawn (Nakamachi *et al.* 2010; Gendron *et al.* 2012; Huang *et al.* 2012). TOC1 can directly bind to the promoter of *CCA1* and *LHY* by its CONSTANS, Co-LIKE, TOC1 (CCT) domain. Mutation or deletion in the CCT domain prevents this repression showing that DNA-binding is necessary for TOC1 action (Gendron *et al.* 2012). Mutations of *prr9* or *prr7* produce plants with slightly longer circadian periods (Farré *et al.* 2005), while *prr5* mutant plants displayed a shorter circadian period than WT (Fujiwara *et al.* 2008). *prr9/prr5* double mutant plants are slightly late flowering displaying longer petioles and hypocotyls (Nakamachi *et al.* 2005). Triple mutant plants *prr9/prr7/prr5* are arrhythmic under free-running conditions (Nakamachi *et al.* 2005). In turn, *toc1* mutant plants have shortened circadian rhythms (Strayer *et al.* 2000) whereas TOC1 over-expressing

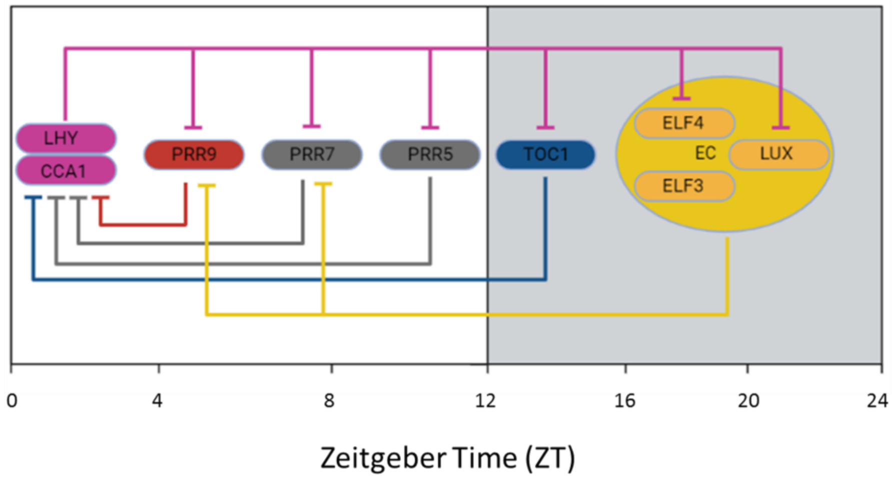


Figure 4: Scheme depicting a simplified view of the main clock components and their regulatory network in *Arabidopsis thaliana*. Perpendicular dashes denote gene repression. EC: Evening Complex. Please see the main text for details. Created with BioRender.

plants lead to arrhythmic phenotypes under free-running conditions (Más *et al.* 2003). *toc1* and *prr5* mutant plants have longer hypocotyls under red light conditions during early photomorphogenesis, whereas the double mutant *toc1/prr5* shows additive effect. These results suggest that TOC1 and PRR5 affect the red light signal transduction pathway (Ito *et al.* 2008). *toc1* plants have an early flowering phenotype under short day conditions, and *prr5* plants have a slightly late flowering phenotype under long day condition. Interestingly, the double mutant *toc1/prr5* plants have a late flowering phenotype under long day (Ito *et al.* 2008). The ZTL protein family are responsible for the degradation of the PRR proteins (Más *et al.* 2003; Kiba *et al.* 2007), with PRR5 regulating the phosphorylation, nuclear import and subnuclear localization of TOC1 (Wang *et al.* 2010). PRR3 seems to interact with TOC1 to increase its stability by hindering the ZTL-dependent degradation of TOC1 (Para *et al.* 2007; Fujiwara *et al.* 2008).

3.1.3 The Evening Complex

The components of the Evening Complex (EC), including the MYB-like transcription factor LUX, ELF3 and ELF4 (Huang and Nusinow 2016) are expressed after dusk. The EC acts as a transcriptional negative regulator directly binding to the promoter of *PRR9*, *PRR7* and *LUX* itself to repress their expression (Dixon *et al.* 2011; Chow *et al.* 2012; Mizuno *et al.* 2014). ELF3 protein contains disordered domain known as Prion domain (PrD) and can form Liquid-Liquid Phase Separation (LLPS) at hight temperature (Jung *et al.* 2020). ELF3 forms insoluble condensates within minutes of exposure to high temperature, turning into inactivate state. These results are consistent with previous study showing that the EC complex is dissociated at high temperature (Ezer *et al.* 2017; Silvia *et al.* 2019). Loss of function of any of the EC components (*elf3*, *elf4* or *lux*) causes arrhythmic phenotype (Hicks *et al.* 1996; Doyle *et al.* 2002, Hazen *et al.* 2005; Onai and Ishiura 2005).The arrhytmia is also accompanied by other phenotypes such as early flowering and long hypocotyl elongation.

3.2 Light input to the clock

The circadian clock allows plants to synchronize essential biological processes with the diurnal variations in the environmental conditions (Dodd *et al.* 2005). Light is the principal input resetting cue of the *Arabidopsis thaliana* circadian clock (Devlin 2002; Harmer 2009). Light intensity affects the central oscillator (Nohales and Kay 2016) as increasing light intensity accelerates the circadian oscillator leading to shortening of periodicity in diurnal organisms (Aschoff 1979). Light quality also regulates the circadian clock. Light quality sensing relies on a set of photoreceptors (Fankhauser and Staiger 2002) that differentially entrain the plant clock (Somers *et al.* 1998). There are four families of circadian-associated photoreceptors in *Arabidopsis thaliana*: PHYTOCHROMES, CRYPTOCHROMES, the ZEITLUPE family and Ultra Violet Resistance Locus 8 (UVR8) (Sanchez *et al.* 2020). Their role in circadian signalling is briefly described below.

3.2.1 Phytochromes

Red (R: 600-700nm) and far-red (FR: 700-750nm) light are sensed in *Arabidopsis* by the PHYTOCHROME family of photoreceptors (Franklin and Quail 2009). There are five PHYTOCHROMES: from PHYA to PHYE, with PHYA and PHYB playing a dominant role in light perception (Whitelam and Devlin 1997). PHYs are involved in different developmental processes, such as elongation of hypocotyls, shoot branching, flowering time and regulation of the circadian clock (Wang and Wang 2015). PHYs exist in two interconvertible isoforms: a R-absorbing biologically inactive Pr form, and a FR-absorbing active Pfr form (Rockwell *et al.* 2006; Pfeiffer *et al.* 2012). PHYB acts not only as a photoreceptor but also as a temperature sensor (Jung *et al.* 2016). The active Pfr form is sensitive to the temperature and can be reconverted to the Pr inactive form by thermal reversion (Legris *et al.* 2019).

The phytochrome signal transducers FAR-RED ELONGATED HYPOCOTYLS 3 (FHY3) and FAR-RED IMPARED RESPONSE 1 (FAR1) promote the expression of *CCA1* after light exposure of only one minute (Liu *et al.* 2020). PHYs also interact with the family PHYTOCHROME INTERACTING FACTORs (PIFs), which are basic helix-look-helix motif (bHLH) containing transcription factors (Shin *et al.* 2013). PIFs can bind the G-box motif of the promoter region of some clock genes like *LHY* and *CCA1* to repress them (Martínez-García *et al.* 2000; Kim *et al.* 2003; Shor *et al.* 2017). Under diurnal cycles, the high levels of FAR1 and FHY3 and low level of PIF5 and TOC1 at the end of the evening promote the peak expression of the morning gene *CCA1* before the dawn (Liu *et al.* 2020). On the other hand, PHYB directly interacts *in planta* with six clock components including CCA1, LHY, GIGANTEA (GI), TOC1, LUX and ELF3 (Liu *et al.* 2001; Yeom *et al.* 2014). There is also a feed-back loop from the evening clock components ELF3, ELF4 and LUX that can directly repress *PIF4* and *PIF5* (Nusinow *et al.* 2011).

3.2.2 Cryptochromes

CRYPTOCHROMES (CRY) are the blue/UVA-light (320-500nm) photoreceptors, first described in *Arabidopsis thaliana* (Ahmad and Cashmore 1993). The CRYs photoreceptor family is present in all evolutionary lineages from bacteria to mammals (Lin and Todo 2005). In *Arabidopsis,* there are three CRYs named as CRY1, CRY2 and CRY3 (also called CRY-DASH because it was found in *Drosophila*, *Arabidopsis*, *Synechocystis* and *Human*) (Lin *et al.* 1996; Brudler *et al.* 2003). CRYs play an important role in the circadian clock (Somers *et al.* 1998; Devlin and Kay 2000). For example, *cry1* mutant plants show a lengthened period compared to Wild Type (WT) plants under constant blue light condition (Somers *et al.* 1998). The double *cry1*/*cry2* mutant seedlings exhibit a longer period compared to WT under continuous blue light (Devlin and Kay 2000). CRYs are also involved in the regulation of other processes such as the inhibition of germination of the dormant seeds, chloroplast development, and gravitropism (Lopez *et al.* 2021). CRYs, like PHYs interact with the PIFs family known to be involved in the regulation of circadian rhythms (Ma *et al.* 2016; Paik *et al.* 2017). CRY2 and PHYB also interact together to control the flowering time, hypocotyl elongation and the circadian period by the clock (Más *et al.* 2000).

3.2.3 Zeitlupe protein family

ZEITLUPE are also blue-light photoreceptors composed of three members: ZEITLUPE (ZTL), FLAVIN-BINDING KELCH REPEAT F-BOX1 (FKF1) and LOVE KELCH PROTEIN 2 (LKP2) (Kami *et al.* 2010). As mentioned above, ZTL can degrade TOC1 and PRR5 (Mas *et al.* 2003; Kiba *et al.* 2007). The clock can directly affect stability of ZTL, in fact the clock protein GIGANTEA (GI) interacts with ZTL to post-translationally stabilize it in blue-light (Kim *et al.* 2007). FKF1 and LKP2 seem to play redundant role with ZTL (Baudry *et al.* 2010).

3.2.4 Ultra Violet Resistance locus 8 (UVR8)

UVR8 is a photoreceptor of the Ultraviolet-B (UV-B) light (280-315nm) (Rizzini *et al.* 2011). Low intensity of UV-B radiation acts as entraining signal for the clock (Fehér *et al.* 2011). UVR8 interacts with the E3 ubiquitin ligase: CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) in a UV-B dependent manner (Favory *et al.* 2009). The co-action of UVR8 and COP1 suppresses transcript abundance of *PIF4*. UV-B also stabilizes the bHLH factor LONG HYPOCOTYL IN FAR-RED 1 (HFR1), which can bind and inhibit PIF4 function (Hayes *et al.* 2017).

3.3 Other inputs to the clock

3.3.1 Temperature

The plant circadian clock can be also entrained by cold/warm temperature cycles associated with the day and night. Differences as small as 4ºC under constant light conditions can synchronize the circadian clock (Thines and Harmon 2010). High temperatures increase the amplitude and peak levels of *TOC1* and *GI*, whereas they decrease the peak expression of *LHY*. In turn, lower temperatures increase the amplitude and peak expression of *CCA1* and *LHY* and decrease the peak expression of *GI* but not for interestingly *TOC1* (Gould *et al.* 2006). The morning-expressed clock components PRR7 and PRR9 have a role in the regulation of CCA1 and LHY activities in response to ambient temperature (Salomé *et al.* 2010). Temperature also affects the activity of the Evening Complex (EC): warm ambient temperatures inhibit the EC function, whereas cooler temperatures stimulate it, specifically at night (Mizuno *et al.* 2014; Ezer *et al.* 2017). Additionally, the ELF4 protein can move from shoots to roots in temperature-dependent manner, affecting the pace of the circadian clock in roots (Chen *et al.* 2020).

3.3.2 Nutrient and humidity

Nutrient assimilation can also affect the plant circadian clock. Lack of iron results in a lengthened circadian period (Hong *et al.* 2012; Chen *et al.* 2013) whereas nitrate assimilation can advance or delay the *CCA1* phase, depending on the nitrate assimilated (Gutiérrez *et al.* 2008). Daily humidity oscillation can also regulate the circadian clock (Mwimba *et al.* 2018). Although the specific signalling pathway has not been established yet, it seems to be different as the ones for light and temperature.

3.4 Output pathways

The circadian clock regulates the transcription of multiple genes. For example, around one-third of the *Arabidopsis* transcriptome is regulated by the circadian clock (Covington *et al.* 2008). The circadian clock also controls multiple developmental and physiological processes, such as seed dormancy (Penfield and Hall 2009), hypocotyl and stem elongation (Dowson-Day and Millar 1999; Ruts *et al.* 2012), rhythmic movement of leaves (Edward and Millar 2007), stomatal aperture (Hassidim *et al.* 2017), flowering (Suárez-López *et al.* 2001), and senescence (Majeed *et al.* 2020). The circadian clock also interacts with hormonal signalling pathways (Robertson *et al.* 2008), immune defence against pathogens (de Leone *et al.* 2020) and is involved is abiotic stress response such as cold, drought and salinity (Nakamachi *et al.* 2016). The circadian system also controls metabolic processes (Farré and Weise 2012) and the timing of mitochondrial activity and cellular energy (Cervela-Cardona *et al.* 2021).

3.4.1 Plant growth

Plant growth is controlled by environmental signals such light, temperature and water availability (Burko *et al.* 2022), but also by endogenous phytohormonal pathways (Gray 2004; Depuydt and Hardtke 2011). The circadian clock regulates the hypocotyl elongation of *Arabidopsis thaliana* under light:dark cycles (Niwa *et al.* 2009). Under constant light conditions, hypocotyl growth shows rhythmic pauses near to the subjective dawn with a rapid elongation at subjective dusk suggesting that this process is also controlled by the circadian clock; this hypothesis was confirmed using clock mutants, such as *toc1* plants, which exhibit a shortened period and a long hypocotyl (Dowson-Day and Millar 1999). Root growth also display circadian rhythms, which continue under both constant light and constant dark conditions. Root growth starts increasing during the night with a peak few hours after dawn (Maizel *et al.* 2011; Yazdanbakhsh *et al.* 2011). The circadian clock also regulates phase-specific growth and cell division, for instance, TOC1 controls the timing of the endocycle and the mitotic cycle at early stages of leaf development, by binding to the promoter of the cell cycle gene *CELL DIVISION CONTROL 6* (*CDC6*) (Fung *et al.* 2018). PIFs are key hubs in the control of plant growth (Nozue *et al.* 2007; Favero 2020). The PRR family represses PIF activity, to regulate plant growth (Martin *et al.* 2018) whereas the EC can directly bind to the promoter of *PIF4* and *PIF5* to repress their expression (Huang *et al.* 2016).

3.4.2 Flowering

The transition from vegetative state to the reproductive state is controlled by the clock (Más 2008). The initiation of flowering is regulated by several pathways (Amasino and Michaels 2010). One of these pathways is known as the photoperiodic pathway, which is based on the fact that plants can measure the duration of the day to determine whether to flower or not. The mechanism responsible for this measurement is the circadian clock (Kobayashi and Weigel 2007). In *A. thaliana*, flowering is controlled by the regulation of the florigen gene, *FLOWERING LOCUS T* (*FT*), which is activated by CONSTANS (CO) (Kobayashi *et al.* 1999; Suárez-López *et al.* 2001). CO is a clock-regulated zinc-finger protein present in phloem companion cells in leaves (Putterill *et al.* 1995; Shim *et al.* 2017). Light stabilizes CO in the evening, whereas in the morning or darkness, the protein is degraded (Valverde *et al.* 2004). Under short days, CO does not accumulate enough protein to start the flowering signal, but under long days, CO protein accumulation increases the amount of *FT* transcripts and FT protein can move from companion cells of leaf phloem to the shoot apical meristem (Shim *et al.* 2017). Once in the shoot apex, FT interacts with the bZIP transcription factor FLOWERING LOCUS D (FD) to induce flowering (Mathieu *et al.* 2007). Interestingly, the homologs of

CO in rice and sorghum (*HEADING DATE 1*) regulate not only flowering time but also grain yield (Liu *et al.* 2015; Li and Xu 2017; Zhang *et al.* 2017).

4. Chronoculture

As mentioned above, the circadian clock controls and modulates many aspects of the plant biology, which pave the way to understand better the circadian clock with the goal to improve important agronomical traits. The use of the circadian clock in agriculture was coined as chronoculture (Steed *et al.* 2021).

Several studies have already shown the relevance of the chronoculture. *Arabidopsis thaliana* shows natural variation in its circadian clock depending to the accessions. In fact, there is a correlation between the period length and the day length at latitude of origin. These natural variations are proposed to enhance the fitness of the plants (Michael *et al.* 2003). In another hand, in *Brassica rapa*, circadian traits and exchange and biomass accumulation seem to be correlated: a shift to shorter circadian period is associated with higher stomatal conductance, lower photosynthetic rate and lower biomass accumulation (Yarkhunova *et al.* 2016). There is also correlation between longer circadian period and the cultivation of elite soybean cultivars in higher latitudes (Greenham *et al.* 2017). In wheat (*Triticum aestivum*), *TaLHY* seems to be involved in the heading of the wheat and in the defence responses against pathogen infection (Zhang *et al.* 2015). Also in wheat, several haplotype and polymorphism of *TaPRR1* were identified and associated to significant differences in yield-related traits (Sun *et al.* 2020). The decrease in transcript levels for the ortholog *PRR3/7* in short day crops such as sorghum *(SbPRR37*), advance flowering under long days (Murphy *et al.*

2011). All these examples show the relevance of studying the circadian clock, to increase the fitness, agronomical trait of our cultivated species.

5. *Solanum lycopersicum*

5.1 Tomato, species of interest

Solanum lycopersicum (tomato) is the second most important vegetable crop, after potato (*Solanum tuberosum*), with approximately 187 million tons of tomato fruits produced on 5 million ha in 2020 (FAOSTAT 2020). Unlike *Arabidopsis*, which have a long-day requirement for flowering, tomato is a day-neutral plant, although some of its wild relatives are short-day plants. Tomato is a climacteric fruit containing several compounds with nutritional traits that are easily integrated to a balanced diet (Marti *et al.* 2016). The nutritional benefits of tomato fruit are explained by their health-promoting compounds, including carotenoids, phenolic compounds and vitamins (Raiola *et al.* 2014). In fact, these molecules work as antioxidant metabolites, providing a protection by neutralizing free radicals, which are unstable molecules linked to the development of a number of diseases (Lobo *et al.* 2010; Sinbad *et al.* 2019). In addition to its economic and nutritional importance, tomatoes are used as model to study fleshy fruit development and plant-pathogen interactions, among many others (Kimura and Sinha 2008; Karlova *et al.* 2014; Arie *et al.* 2007).

5.2 Tomato domestication

The process of tomato domestication started thousands of years ago from Central and South America. Genomic analyses showed that the domestication and improvement focused on two independent sets of quantitative trait loci (QTLs), giving the modern tomato fruit around 100 times bigger than its ancestors: *S. pimpinellifolium* and *S. lycopersicum* cv. Cerasifrome (Bai and Pim 2007; Lin *et al.* 2014). *S. pimpinellifolium* is a small, yellow-fruited wild tomato and *S. lycopersicum* cv. Cerasiforme is a red-fruited wild tomato also known as the wild cherry tomato. Different genes have been identified to be involved in yield traits, such as *SELF PRUNING* (*SP*), *LOCULE NUMBER* (*LC*), *FASCIATED* (*FAS*), or *TERMINATEING FLOWER* (*TMF*) (Mata-Nicolás *et al.* 2020; Ye *et al.* 2021). Interestingly, the circadian clock of the cultivated tomato has slowed down during the domestication process (Kay and Remigereau 2016; Müller *et al.* 2016). Mutations in the clock genes *EMPFINDLICHER IM DUNKELROTEN LICHT 1 (EID1)* and *LNK2* are responsible for the deceleration of the circadian rhythms. In light:dark cycle, only the day fraction is longer because the circadian clock is reset at each dawn and dusk to fit with the 24h period (Müller *et al.* 2018). Xiang *et al.* (2022) created tomato isogenic lines with the wild alleles of *EID1* and *LNK2*, recreating the change observed during the domestication process, this study confirms the hypothesis gave by Müller *et al.* (2016) that *EID6 and LNK2* are responsible for the evolution of the tomato traits during the domestication process.

5.3 Tomato resources

Mueller *et al.* (2005) built a powerful web-tool for the *Solanaceae* family, including tomato, called the SOL Genomics Network (SGN, https://solgenomics.net/). The SGN platform have several objectives: (1) cataloguing genetic maps and markers of the *Solanaceae* family; (2) spreading sequence information, mostly in the form of expressed sequence tags (ESTs); (3) reporting and publishing phenotypic information; and (4) assembling data from the sequencing of the tomato genome. Another web-tool including the expression profiles of the genes have been developed (https://diurnal.sbs.ntu.edu.sg/, Mutwil *et al.* 2011; Rhee and Mutwil 2014; Hansen *et al.* 2014; Ruprecht *et al.* 2016; Ruprecht *et al.* 2017; Proost and Mutwil 2016; Proost *et al.* 2017). Additionally, the genome of the inbred tomato cultivar *Solanum lycopersicum* "Heinz 1706" is sequenced and assembled (Sato *et al.* 2012). Scott and Harbaugh (1989) created the tomato cultivar Micro-Tom for ornamental purposes by crossing Florida Basket and Ohio 4013-3 cultivars. This new cultivar displays a very dwarf phenotype with small and red ripened fruits. The Micro-Tom cultivar has also a rapid growth and easy transformation, making it a convenient model system for research on the regulation of berry fruit development (Meissner *et al.* 1997). The phenotype of the cultivar Micro-Tom has been attributed to the mutations in the *SELF-PRUNING (SP)* and *DWARF (D)* genes (Marti *et al.* 2006). The polymorphism between the cultivars Heinz 1706 and Micro-Tom has been published (Asamizu *et al.* 2012; Kobayashi *et al.* 2014). In addition, Saito *et al.* (2011) have produced a mutant database distributing Micro-Tom mutant collections (https://tomatoma.nbrp.jp/).

5.4 Genome editing and its use to improve tomato trait

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 technology is providing precise genome editing tools with minimal off-target mutations, with high-efficiency and cost-effectiveness (Westermann *et al.* 2021). CRISPR-Cas9 system consists of two main components: the Cas9 protein and one or several guide RNA (gRNA). gRNA is designed to recognize and bind to a specific sequence of the DNA. Then the Cas9 protein cuts the DNA at the targeted site after the

Protospacer Adjacent Motif (PAM), in the case of Cas9, the PAM sequence is NGG (Mojica *et al.* 2009). After the digestion of the DNA, the cell naturally repairs the DNA, during this event it can be introduce changes in the DNA sequence, like gene insertions deletions or replacements (Nidhi *et al.* 2021). In plants, several studies have already shown the potential of this technology to enhance growth based on optimal gene discovery, *de novo* modification, trait improvement and biotic or abiotic stress responses (Chandrasekaran *et al.* 2021). Specifically in tomato, the CRISPR technology is already used to improve several traits, such as fruit ripening (Wang *et al.* 2019), fruit size (Rodríguez-Leal *et al.* 2017), fruit colour in relation with anthocyanin content (Zhi *et al.* 2020) and shelf-life (Casals *et al.* 2012) (see Chandrasekaran *et al.* 2021, for full review). CRISPR/Cas9, and its derivative technologies (Nidhi *et al.* 2021), are efficient and transgene-free gene editing tools used in tomato (Danilo *et al.* 2019).

5.5 Circadian clock components and function in tomato

Several studies have focused on the circadian network in tomato plants. For instance, five phytochrome genes were identified in tomato: *SlPHYA, SlPHYB1, SlPHYB2, SlPHYE and SlPHYF* (Alba *et al.* 2000). Tomato plants with mutations in *SlPHYA* and *SlPHYB* (*B1* and *B2*) are drought tolerant (Abdellatif *et al.* 2023) while SlPHYB1 and SlPHYB2 have been shown to contribute also to blue light sensing (Waller *et al.* 2001).

Two SlCRY1 (SlCRY1a and SlCRY1b), one SlCRY2 and one SlCRY-DASH were reported in tomato (Perrota *et al.* 2001; Facella *et al.* 2006). SlCRY1a is a blue light photoreceptor involved in photomorphogenic phenotypes such as hypocotyl and stem elongation, anthocyanin and carotenoid biosynthesis (Weller *et al.* 2001; Liu *et al.* 2018). *SlCRY2b* encodes a truncated form of *SlCRY1a*, lacking the C-terminal part (Sato *et al.* 2012). Over-expression of SlCRY2 (CRY2ox) in tomato give plants with hypocotyl and internode shortening under low- and high-fluence blue light, showing high-pigment phenotypes, and an over-production of anthocyanin and chlorophyll in leaves, and of flavonoids and lycopene in fruits (Giliberto *et al.* 2005; Lopez *et al.* 2012). CRY2ox plants are late flowering under short- and long-day conditions (Giliberto *et al.* 2005). SlCRY2 is also considered a master gene of transcriptional regulation in the tomato chloroplasts (Facella *et al.* 2017). Interestingly, *cry1a*/cry2 mutant plants show an accumulation of several metabolites in leaves and fruits, such as sterol, tocopherol, quinone and sugar classes (Fantini *et al.* 2019). Tomato plants with an alteration of the cryptochrome expression affect the rhythmic oscillations of several other genes (Facella *et al.* 2008). SlCRY1 and SlPHYA are proposed to be the major photoreceptors for blue light-induced de-etiolation in tomato (under low and high irradiance, respectively) (Weller *et al.* 2001). Under UV-B, SlUVR8 regulates hypocotyl elongation and anthocyanin accumulation (Liu *et al.* 2020).

Other components have been also described. For example, Ewas *et al.* (2017) reported the gene *TOMATO DOF DAILY FLUCTUATIONS 1* (*SlTDDF1*) to be involved in circadian regulation and stress resistance. *SlTDDF1* expression seems to improve growth, salt and various hormones related to stress tolerance. The tomato circadian clock also controls the expression of some salinity-stress related genes. After salinity induction, the expression of *SALT OVERLY SENSISTIVE 2* (*SlSOS2*) and *DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1* (*SlP5CS*) were increased only during the morning, whereas *DEHYDRATATION-RESPONSIVE ELEMENT BINDING PROTEIN 2* (*SlDREB2*) expression increased during the evening

(Coyne *et al.* 2019).Transcriptomic studies from the group of Fukuda (Tanigaki *et al.* 2015; Higashi *et al.* 2016) showed gene expression oscillations of tomato plants growing in a sunlight-type plant factory. They found a weak circadian period and checked if it can be possible to improve crop resistance by modulating the input to the clock.

Tomato plants (*Solanum lycopersicum*) have been used as a model plant system in many studies. However, comprehensive analyses of the components and mechanisms of the circadian clock in tomato are still lacking. Understanding the circadian function in tomato is quite relevant due to its possible application to improve tomato growth and productivity.

OBJECTIVES

OBJECTIVES

The general aim of our research project is to understand the circadian function in tomato and its role controlling important traits in tomato. The specific objectives include:

1. ***In silico* identification of putative clock genes in *Solanum lycopersicum***. The aim is to perform bioinformatics analysis using the *Arabidopsis thaliana* clock genes to search for homologs in *Solanum lycopersicum* (tomato).
2. **Expression analysis of clock genes in *Solanum lycopersicum*.** We aim to decipher the diurnal and circadian expression pattern of selected clock homologs in tomato leaves and fruits.
3. **Utilization of gene editing strategies by CRISPR-Cas9 to knock-down clock genes in *Solanum lycopersicum*.** We aim to knock-down (or knock-out) selected clock candidates in *Solanum lycopersicum* using CRISPR-Cas9 strategies.
4. **Analyses of clock gene expression in the generated clock CRISPR-Cas9 lines.** We aim to decipher the effect of knocking-down specific clock genes on the rhythmic expression of other oscillator genes in tomato leaves and fruits.
5. **Developmental and phenotypic characterization of the tomato CRISPR-Cas9 lines.** We aim to examine circadian clock outputs such as growth and development and investigate the effect of the different tomato CRISPR-Cas9 lines in plant productivity.

RESULTS

1. *In silico* analysis of putative clock genes in tomato

Protein sequences of well-characterized clock components from *Arabidopsis thaliana* were used as reference to find putative clock homolog proteins in *Solanum lycopersicum* (Table 1). Basic Local Alignment Search Tool (BLAST) analysis using the protein sequences of the two morning-expressed components AtCCA1 (AT2G46830) and AtLHY (AT1G01060) both rendered the same candidate in tomato: a protein annotated as SlLHY (Solyc10g005080) with a percentage of identity of 37.23% (for AtCCA1) and 42.23% (for AtLHY). The best candidates of AtPRR9 (AT2G46790) and AtPRR5 (AT5G24470) were the proteins SlPRR9 (Solyc10g005080) and SlPRR5 (Solyc03g081240) with homologies of 74.42% and 67.70%, respectively. AtPRR7 (AT5G02810) and AtPRR3 (AT5G60100) were both very similar to SlPRR37 (Solyc04g049670) with homologies of 44.26% and 42.91%, but also to SlPRR73 (Solyc10g081240) with a homology of 42.65% and 50.37%, respectively. The evening-expressed component AtTOC1 (AT5G61380) showed two homologs in tomato: SlTOC1 (Solyc03g115770) with a homology of 45.73% and SlTOC1-like with a homology of 48.58%. The homology between SlTOC1 and SlTOC1-like was about 65.52% between each other. The BLAST analysis of AtLUX (AT3G46640), from the evening complex, rendered two candidates, SlLUX (Solyc06g076350) and SlLUX-like (Solyc06g005680) (identity of 48.66% and 61.16%, respectively). Lastly, the protein BLAST of AtELF3 (AT2G25930) and AtELF4 (AT2G40080), also from the evening complex, rendered the candidates SlELF3 (Solyc08g065870) and SlELF4 (Solyc06g051680) with homologies of 36.75% and 64.04%, respectively.

Table 1: Clock gene candidates in tomato and their homologs in *A. thaliana*.

S. lycopersicum gene	Gene symbol	*A. thaliana gene*	Gene symbol	Identity (%)	*E*-value
Solyc10g005080	*SlLHY*	AT2G46830	*AtCCA1*	37.23	7e-86
Solyc10g005080	*SlLHY*	AT1G01060	*AtLHY*	42.23	1e-65
Solyc04g049670	*SlPRR37*	AT5G60100	*AtPRR3*	42.91	7e-86
Solyc04g049670	*SlPRR37*	AT5G02810	*AtPRR7*	44.26	2e-153
Solyc10g086000	*SlPRR73*	AT5G60100	*AtPRR3*	50.37	5e-66
Solyc10g086000	*SlPRR73*	AT5G02810	*AtPRR7*	42.65	4e-118
Solyc10g005030	*SlPRR9*	AT5G24470	*AtPRR5*	37.82	3e-102
Solyc10g005030	*SlPRR9*	AT2G46790	*AtPRR9*	74.42	1e-64
Solyc03g081240	*SlPRR5*	AT5G24470	*AtPRR5*	67.70	1e-87
Solyc03g081240	*SlPRR5*	AT2G46790	*AtPRR9*	73.88	2e-70
Solyc03g115770	*SlTOC1*	AT5G61380	*AtTOC1*	45.73	1e-170
Solyc06g069690	*SlTOC1-like*	AT5G61380	*AtTOC1*	48.58	5e-168
Solyc08g065870	*SlELF3*	AT2G25930	*AtELF3*	36.75	7e-107
Solyc06g051680	*SlELF4*	AT2G40080	*AtELF4*	64.04	3e-33
Solyc06g005680	*SlLUX-like*	AT3G46640	*AtLUX*	61.16	2e-86
Solyc06g076350	*SlLUX*	AT3G46640	*AtLUX*	48.66	5e-76

2. Rhythmic expression of morning-expressed clock genes in tomato leaves and fruits

The expression of the candidate genes was examined in leaves, green and red fruits from plants grown under Long Day conditions (LD). To check whether the oscillations were truly controlled by the circadian clock, the expression was also examined under constant light conditions (LL) following the synchronization under LD.

In leaves from plants grown under LD, *LHY* showed a clear peak of expression close to dawn (at Zeitgeber Time 3, ZT3) that declined during the rest of the day to increase again at the end of the night (ZT19-23)

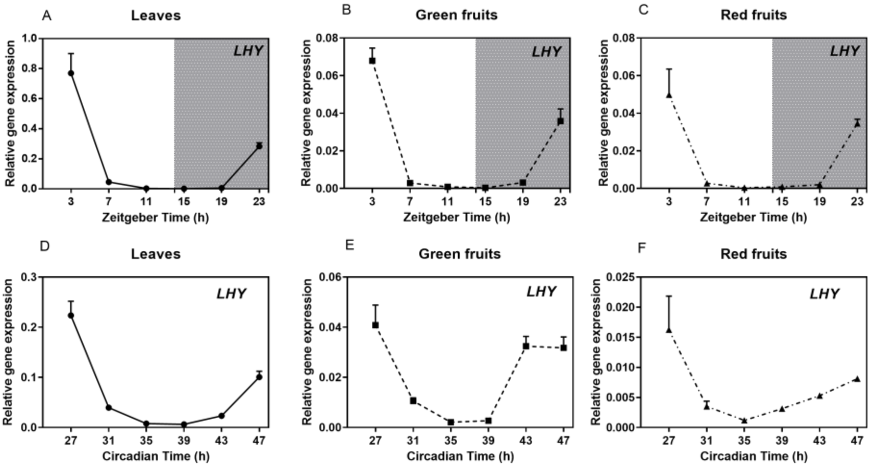


Figure 5: *LHY* mRNA expression in WT tomato plants. Circadian time course analysis of *LHY* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *LHY* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

(Figure 5A). A similar pattern of expression was observed in leaves, green and red fruits (Figure 5A-C). Comparative analyses showed that *LHY* was expressed around 10 times more in leaves than in fruits while the level of expression was quite similar in green and red fruits. Under LL, *LHY* showed a similar pattern of expression than under LD with a peak of expression close to subjective dawn (Circadian Time 27, CT27) that declined during the rest of the subjective day to increase again at the end of the subjective night (CT43-CT47) (Figure 5D). *LHY* expression in leaves was around 3-fold times lower under LL than under LD. In green fruits, *LHY* also peaked at the beginning of the subjective day (CT27) but showed an approximately 4-hour advanced raising phase (CT39) (Figure 5E). In red fruits, the peak of expression was also at subjective dawn but showed a reduced raising phase from CT39 and on (Figure 5F).

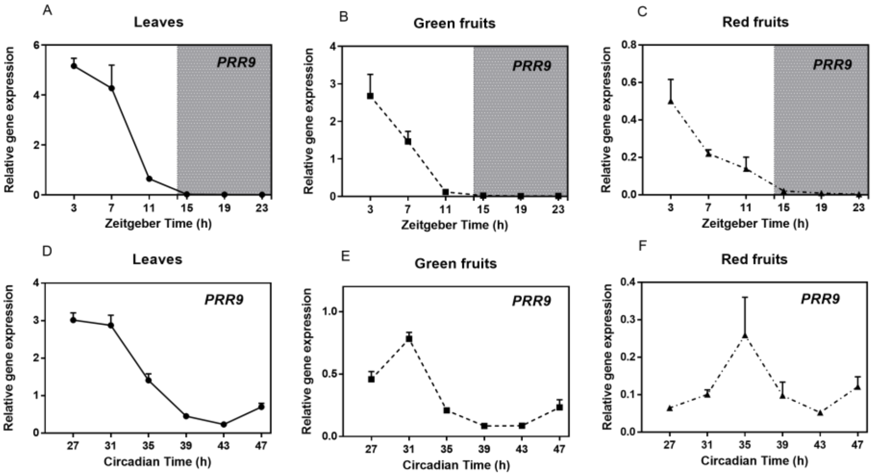


Figure 6: *PRR9* mRNA expression in WT tomato plants. Circadian time course analysis of *PRR9* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *PRR9* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

The analyses of other morning-expressed genes showed that in leaves from plants grown under LD, *PRR9* was highly expressed at the beginning of the day with a peak at ZT3. The expression decreased from ZT7 to ZT11 to reach a level close to 0 at the beginning of the night (ZT15) and remaining low during the night (Figure 6A). Fruits under LD had similar pattern than in leaves (Figure 6B-C). Comparative analyses showed that in leaves and green fruits, *PRR9* expression was higher than in red fruits. In leaves, *PRR9* has a similar pattern of expression under LL than under LD (Figure 6A and D), but with the difference that under LL, *PRR9* showed a lower expression at the beginning of the subjective day and the raising phase started earlier, at the end of the subjective night (Figure 6D). In fruits under LL, green fruits, *PRR9* had similar expression pattern than the one observed in leaves, but with a more pronounced peak at CT31 (Figure 6E). In red fruits, *PRR9* peak expression was delayed compared to leaves and green fruits (Figure 6F). In green fruits under LL, the phase was delayed compared to LD (Figure 6B and E) with the expression being reduced by more than 2-fold. In red fruits under LL, *PRR9* expression was much more delayed compared to LD, showing a peak at the end of the subjective day at CT35 (Figure 6F).

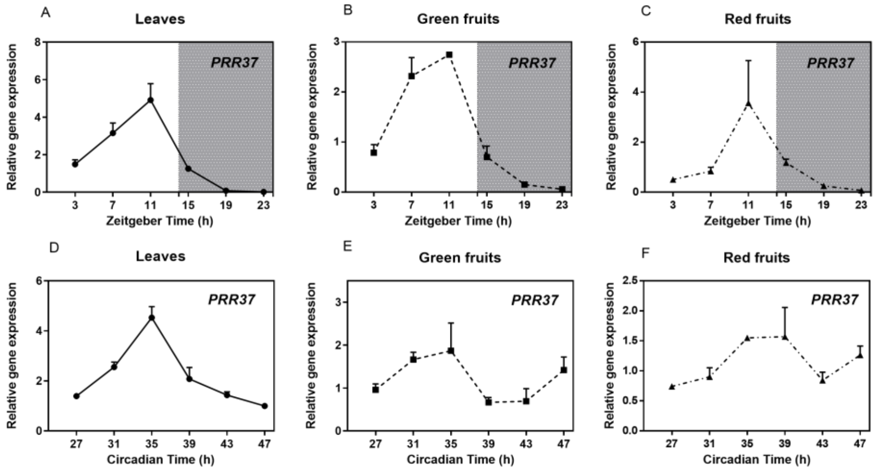


Figure 7: *PRR37* mRNA expression in WT tomato plants. Circadian time course analysis of *PRR37* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *PRR37* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

PRR37 expression followed in time the one observed for *LHY* and *PRR9* (Figure 7). Thus, the peak phase of *PRR37* expression was observed at ZT11, just before dusk in leaves from plants analyzed under LD (Figure 7A) and LL (Figure 7D) conditions. The peak-phase of expression in green

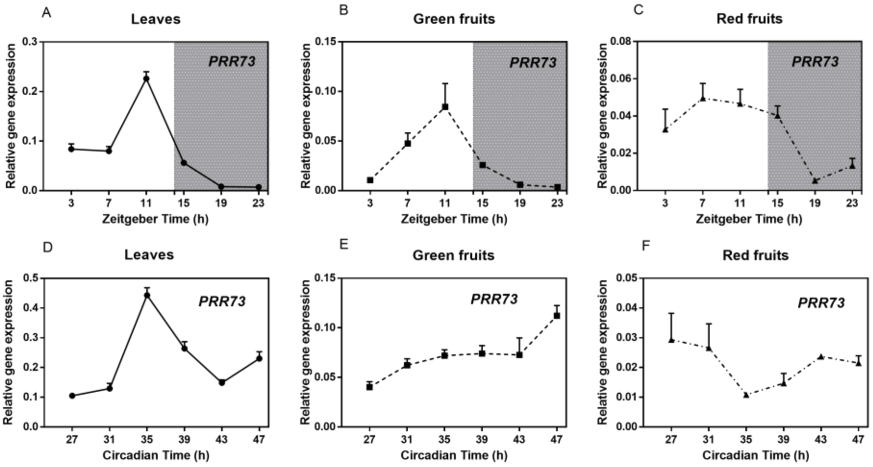


Figure 8: *PRR73* mRNA expression in WT tomato plants. Circadian time course analysis of *PRR73* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *PRR73* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

fruits (Figure 7B and 7E) followed a similar trend to that observed in leaves although the amplitude was reduced under LL and showed an advanced raising phase at the end of the subjective night (Figure 7E). Similar *PRR37* mRNA abundance was observed under LD and LL in the different organs. On the other hand, *PRR73* gene expression showed a low expression but with a similar peak-phase than *PRR37* under LD conditions for leaves and fruits (Figure 8A-C). Under LL, *PRR73* also peaked at ZT11 but showed and advanced raising phase at the end of the subjective night (Figure 8D). No robust oscillation with high amplitude or clear peak of expression was observed in green or red fruits (Figure 8E-F). Notably, the pattern of *PRR5* (Figure 9) expression was quite similar to that observed for *PRR73* and to some extend to *PRR37*. The peak of expression was also at ZT7-ZT11 (Figure 9A-D) while the oscillations were not so robust in fruits, particularly in red fruits under LL conditions (Figure 9B-C and 9E-F).

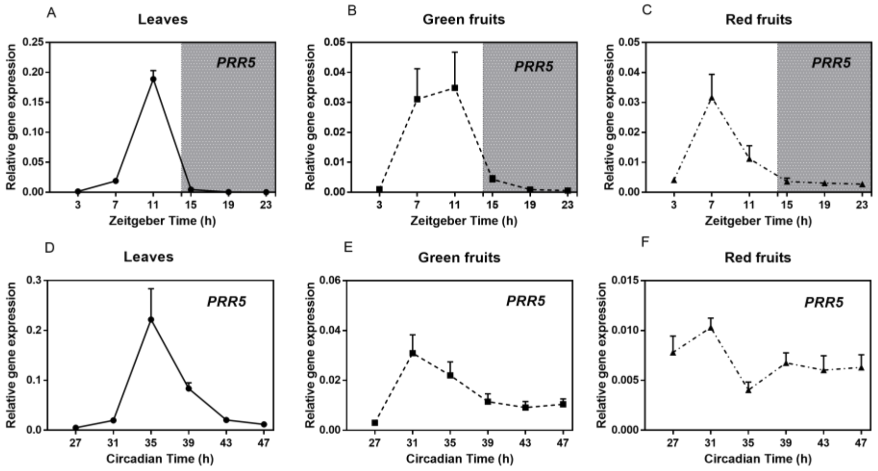


Figure 9: *PRR5* mRNA expression in WT tomato plants. Circadian time course analysis of *PRR5* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *PRR5* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

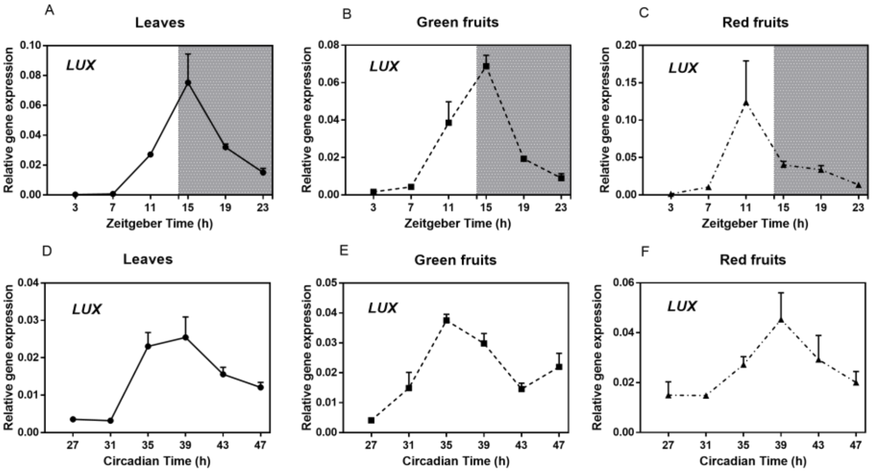


Figure 10: ***LUX*** **mRNA expression in WT tomato plants.** Circadian time course analysis of *LUX* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *LUX* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

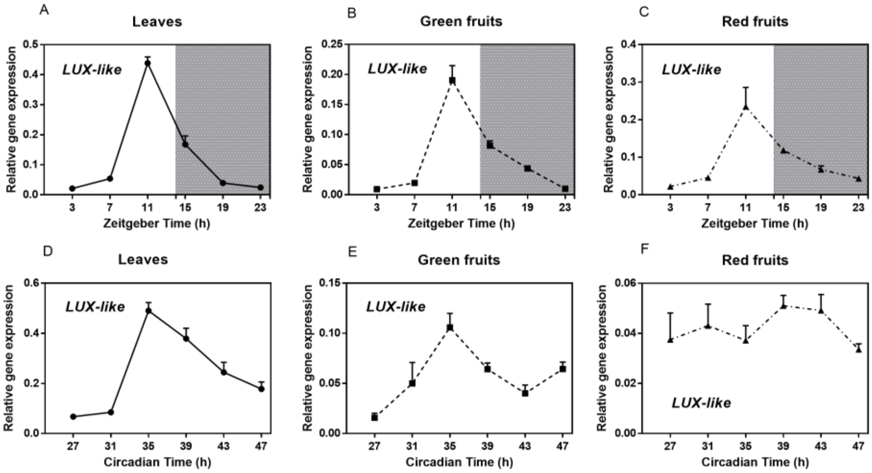


Figure 11: *LUX-like* mRNA expression in WT tomato plants. Circadian time course analysis of *LUX-like* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *LUX-like* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

3. Rhythmic expression of evening-expressed clock genes in tomato

We next examined the pattern of expression of the tomato clock genes whose homologs in *Arabidopsis* have a peak of expression at dusk or during the night. Like in *Arabidopsis*, *LUX* expression in tomato showed a peak-phase just after dusk in leaves and green fruits (Figure 10A-B) and slightly earlier in red fruits (Figure 10C). Contrarily, the peak-phase of *LUX* expression was advanced in leaves and green fruits under LL (Figure 10D-E) and delayed in red fruits (Figure 10F) compared to LD. *LUX* expression was also approximately 2-fold reduced under LL compared to LD. The analyses of *LUX-like* peak of expression showed a peak just slightly earlier than *LUX* under LD conditions in the three organs examined (Figure 11A-C) while the phase was slightly advanced under LL except in red fruits, which showed low amplitude and no clear peak of expression (Figure 11F). Notably, the abundance of *LUX-like* mRNA was about 2-5 fold higher than the one observed for its homolog *LUX*.

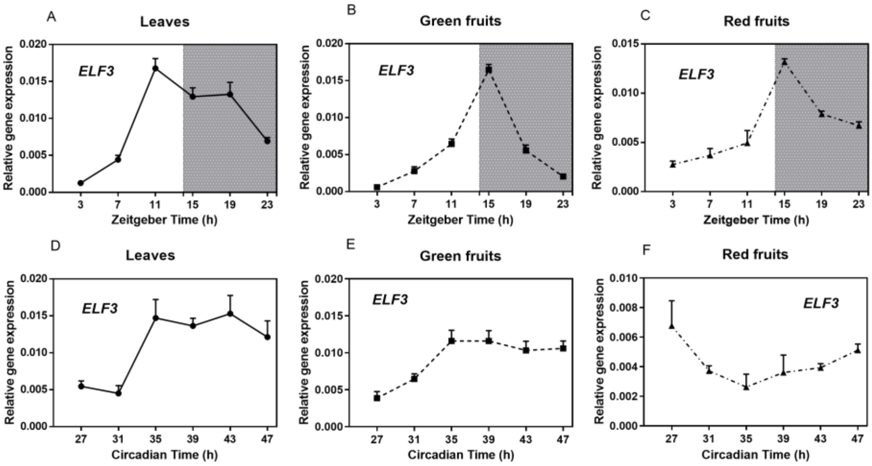


Figure 12: *ELF3* mRNA expression in WT tomato plants. Circadian time course analysis of *ELF3* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long days condition. Circadian time course analysis of *ELF3* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

LUX forms a protein complex, known as Evening Complex (EC), together with ELF3 and ELF4 in *Arabidopsis* (Nusinow *et al.* 2011). The analyses of *ELF3* expression in tomato showed that the overall expression was low and showed a peak close to dusk under LD conditions (Figure 12A-C), and with a small “shoulder” in the mid of the night particularly evident in leaves (Figure 12A). This small peak has been described for

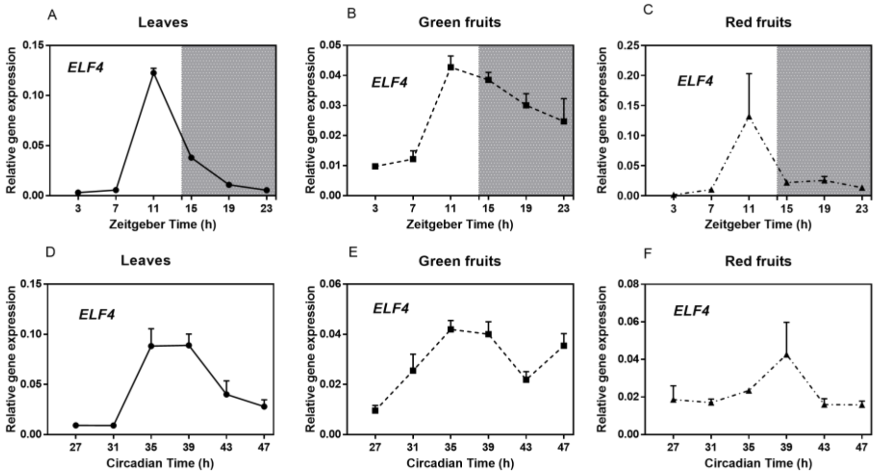


Figure 13: *ELF4* mRNA expression in WT tomato plants. Circadian time course analysis of *ELF4* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *ELF4* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

other evening-expressed genes in *Arabidopsis* (Hicks *et al.* 2001). The "shoulder" peak was high in leaves under LL conditions (Figure 12D) whereas the amplitude was considerably reduced in green fruits under LL (Figure 12E) and arrhythmic in red fruits (Figure 12F). *ELF4* expression analysis in leaves under LD was very low at the beginning of the day (ZT3-7), but then increased drastically to peak at ZT11, and declined during the night, reaching very low level at ZT19-23 (Figure 13A). In green fruits under LD, *ELF4* expression also peaked at ZT11 but by 2-3-fold less than in leaves or red fruits, and then decreased slowly during the night (Figure 13B). In red fruits, under LD, the expression seems similar to the leaves under LD, but the differences between the two biological replicates made difficult to reach a clear conclusion (Figure 13C). In leaves under LL, *ELF4* expression was similar than under LD, with a short delay of about 2h in its phase (Figure 13A-D). In red fruits, it also seems to have a delay in the phase (Figure 13C-F). In green fruits under LL, the expression was quite similar than under LD, but with an increased expression at ZT23 (Figure 13B-E).

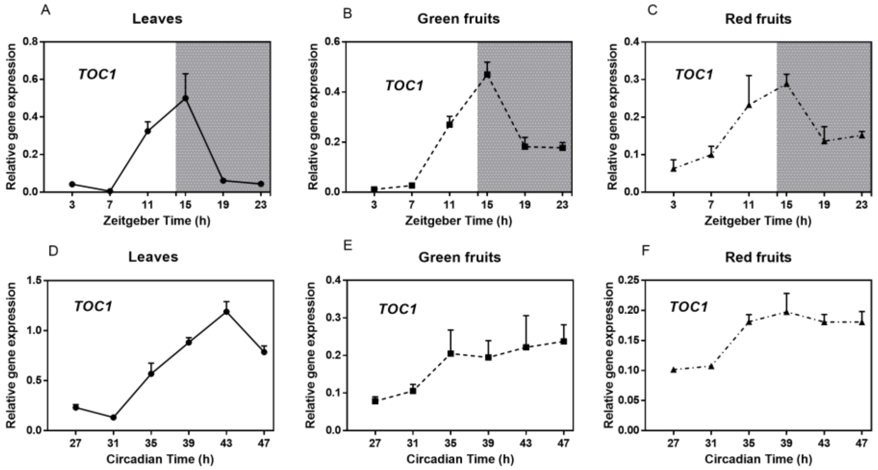


Figure 14: *TOC1* mRNA expression in WT tomato plants. Circadian time course analysis of *TOC1* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *TOC1* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

The analyses of *TOC1* expression under LD showed a peak at dusk in leaves, green and red fruits with trough values at night (Figure 14A-C). The abundance of *TOC1* expression was somewhat similar in leaves and green fruits but slightly lower in red fruits. Under LL, the peak phase of *TOC1* expression was about 4-hour delayed in leaves compared to LD whereas the amplitude was highly reduced in fruits (Figure 14D-F). Analysis of *TOC1-like* expression in leaves under LD showed a peak before dusk in leaves and red fruits but after dusk in green fruits (Figure 15A-C). Under LL, the amplitude of *TOC1-like* expression was clearly reduced compared to LD showing reduced amplitude in leaves and no evident rhythmic oscillation in green and red fruits (Figure 15D-F).

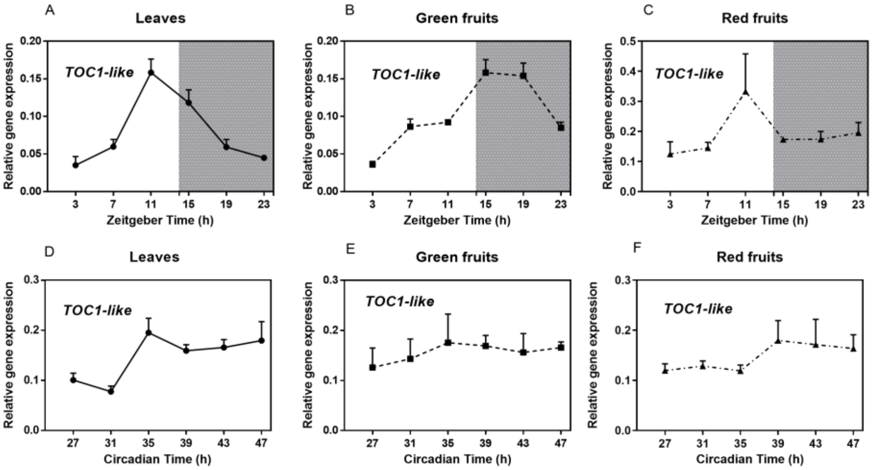


Figure 15: *TOC1-like* mRNA expression in WT tomato plants. Circadian time course analysis of *TOC1-like* in leaves (A), green fruits (B) and red fruits (C) of plants grown under long day condition. Circadian time course analysis of *TOC1-like* in leaves (D), green fruits (E) and red fruits (F) of plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

Overall, we found that the expression of the candidate clock genes in tomato sustained a robust oscillation in leaves from plants grown under LD conditions. The oscillation and expression pattern of these genes in leaves are similar in fruits, with small differences of amplitude and phase for some of them. Under LL conditions, all the genes analyzed sustained rhythmic oscillations in leaves, except *TOC1-Like*. In green fruits, there is an overall loss of oscillation of several genes, such as *PRR73*, *TOC1*, *ELF3* and *TOC1-like*. In red fruits, this phenomenon is more evident with a loss of oscillation for *PRR5*, *PRR73*, *TOC1*, *ELF3*, *LUX-like* and *TOC1-like*.

Together, these results suggest that in leaves, most of the clock genes sustain their rhythmicity under LL, whereas in fruits, the circadian oscillations are not as robust as the diurnal oscillations.

4. Generation and characterization of the CRISPR-Cas9 tomato clock lines

In order to understand the biological function of the identified candidates, we performed genome editing of the main genes using CRISPR-Cas9 strategies. We then characterized how circadian gene expression was affected in these CRISPR lines. The CRISPR lines targeted the clock genes *SlLHY*, *SlPRR9*, *SlPRR37* and *SlTOC1*. The generation and phenotypic characterization of these lines are described in the following sections.

4.1 Generation and molecular characterization of the *lhy*^{crispr} line

For *LHY*, and as specified in Material and Methods (section 2), two guide RNAs (gRNAs) were designed: $^{5'}$AGCAGATAAATGGGGTCCCG$^{3'}$ (cloned in backbone U3) and $^{5'}$GCTTCAGAGCATTCTGGGAC$^{3'}$ (cloned in backbone U6) targeting the 5th exon of *LHY* (Figure 16A). Both gRNAs present 3 off-targets each, with 4 mismatches, but no one presents off-targets following by a Protospacer Adjacent Motif (PAM) sequence (nGG). After selection of homozygous lines, we obtained three *lhy*^{crispr} lines: #1.16, #7.3 and #12.1, each with a deletion of 81, 905 and 303 nucleotides, respectively. Gene expression analyses by RT-QPCR confirmed the lack of

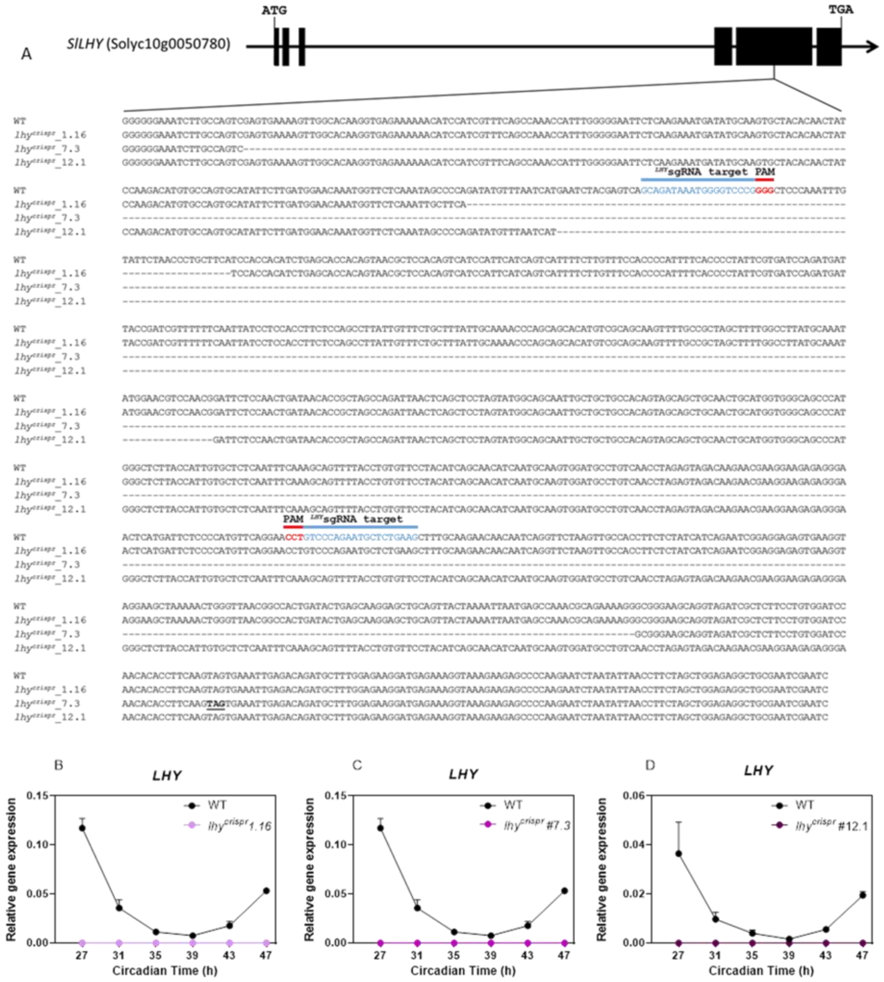


Figure 16: *lhy*^{crispr} lines generated. *LHY* sequences (A) of the new alleles generated. Circadian time course analysis of *LHY* in leaves of WT and *lhy*^{crispr} plants transferred to continuous light conditions after synchronization: (B) WT vs *lhy*^{crispr} #1.16; (C) WT vs *lhy*^{crispr} #7.3; (D) WT vs *lhy*^{crispr} #12.1. Data are represented as the mean ± SEM of two biological *replicates.lhy*^{crispr} #7.3 has change in its frame, giving a truncated form, of the protein with the stop codon: **TAG**

LHY mRNA accumulation in leaves from the three *lhy*^{crispr} #1.16, #7.3 and #12.1 lines using primers amplifying the deleted regions (Figure 16B-D). For subsequent studies, we used the #7.3 line, which in addition to the deletion, the new allele encoded a truncated form of the protein due to an early stop codon (Figure 16A). The sequences of the new alleles are presented in Figure 16A.

We examined circadian gene expression by RT-qPCR in WT and *lhy*^{crispr} in leaves, green and red fruits from plants synchronized under LD cycles followed by LL conditions. Samples were harvested during the second day under LL. We first analyzed the oscillating genes with a peak of expression during the day. Our analyses showed that in leaves, the phase of *PRR9* expression was advanced in *lhy*^{crispr} compared to WT (Figure 17A). Interestingly, in green fruits, the expression of *PRR9* was nearly abolished in *lhy*^{crispr} (Figure 17B) whereas in red fruits, the weak oscillation and the variability among replicates precluded a clear conclusion (Figure 17C). The expression of *PRR37* in leaves was also nearly abolished in the *lhy*^{crispr} line (Figure 17D) while the oscillatory pattern in fruits was too erratic to reach clear conclusions (Figure 17E-F). The phase of *PRR5* expression in *lhy*^{crispr} leaves was clearly advanced, with slight up-regulation of peak expression compared to WT (Figure 17G). In green fruits, the expression of *PRR5* was nearly abolished (Figure 17H) whereas no clear circadian pattern was observed in red fruits (Figure 17I). The results suggest that LHY is required for setting the proper phase and amplitude of morning-gene expression in leaves and green fruits.

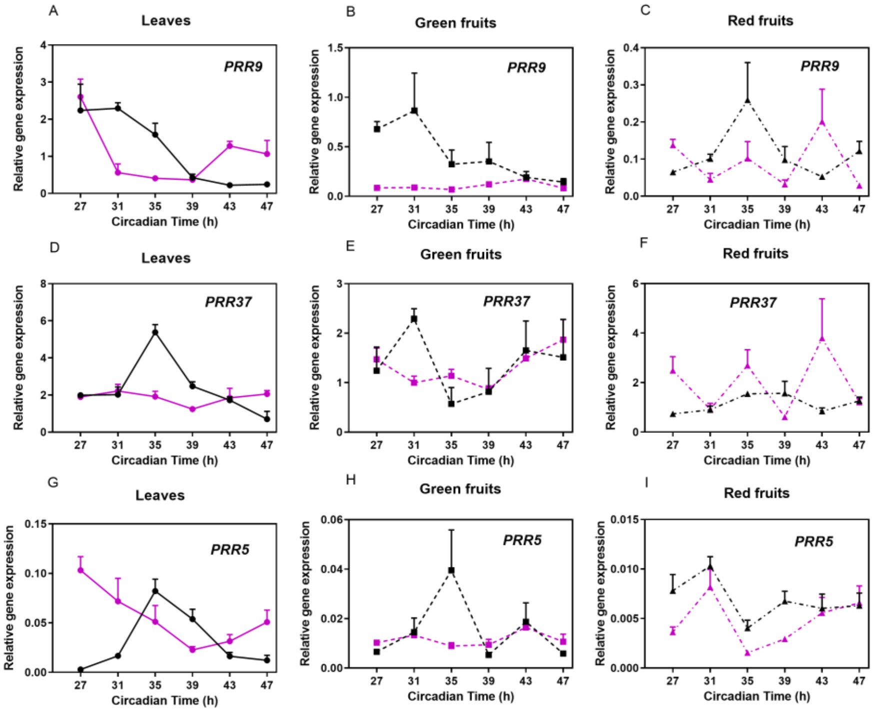


Figure 17: Morning gene mRNA expression in WT and lhy^{crispr} tomato plants. Circadian time course analysis of: *PRR9* in leaves (A), green fruits (B) and red fruits (C); *PRR37* in leaves (D), green fruits (E) and red fruits (F); and *PRR5* in leaves (G), green fruits (H) and red fruits (I); of plants transferred to continuous light conditions after synchronization. WT (-) and lhy^{crispr} (-) plants. Data are represented as the mean ± SEM of two biological replicates.

We next examined the oscillating genes with a peak of expression at dusk or close to dusk. Our analyses showed that in leaves, the phase of *LUX* expression was advanced during the subjective day but nearly arrhythmic in green fruits (Figure 18A and B). In red fruits, we observed a weak oscillation and high variability among replicates (Figure 18C). The expression of *LUX-like* was also nearly arrhythmic in leaves and green fruits whereas no clear oscillation was observed in WT or in *lhy*^{crispr} red fruits (Figure 18D-F). *ELF3* expression declined earlier in *lhy*^{crispr} leaves compared to WT (Figure 18G) whereas in green fruits, the phase of *ELF3* was slightly advanced (Figure 18H) whereas no clear circadian pattern was observed in red fruits (Figure 18I). The phase of *ELF4* and *TOC1* was also advanced in *lhy*^{crispr} leaves (Figure 18J and M) while he oscillatory pattern of *ELF4* in fruits was erratic (Figure 18K-L). In green fruits, the phase of *TOC1* expression was slightly advanced compared to WT (Figure 18N), whereas in red fruits, the weak oscillation and the variability among replicates precluded a clear conclusion (Figure 18O).

Overall, LHY plays an important role regulating the amplitude and phase of most of the examined rhythmic genes in leaves. In green fruits, LHY seems to be required for controlling the circadian phase, particularly for the evening-expressed genes, whereas in red fruits, the oscillations are weak in both WT and *lhy*^{crispr}.

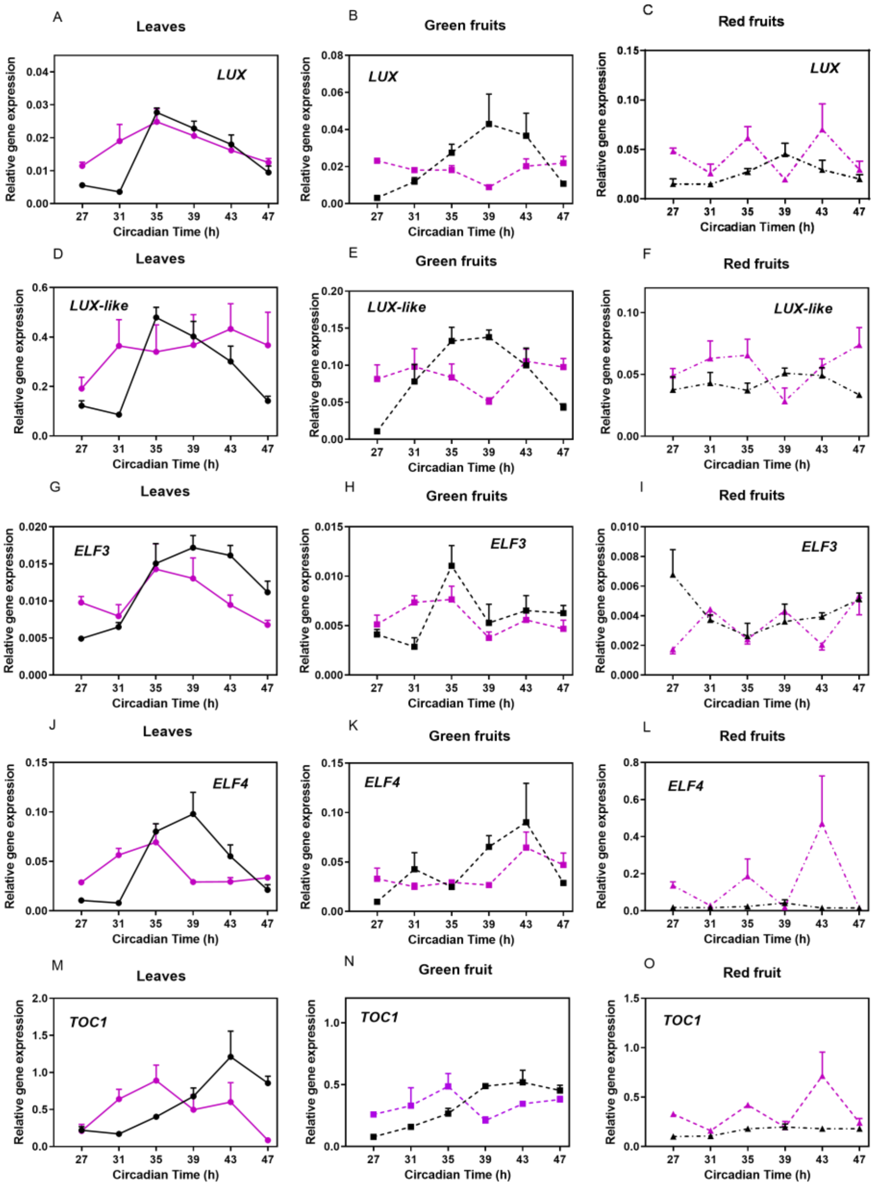


Figure 18: Evening gene mRNA expression in WT and *lhy*^{crispr} tomato plants. Circadian time course analysis of: *LUX* in leaves (A), green fruits (B) and red fruits (C); *LUX-like* in leaves (D), green fruits (E) and red fruits (F); *ELF3* in leaves (G), green fruits (H) and red fruits (I); *ELF4* in leaves (J), green fruits (K) and red fruits (L); and *TOC1* in leaves (M), green fruits (N) and red fruits (O); of plants transferred to continuous light conditions after synchronization. WT (-) and *lhy*^{crispr} (-) plants. Data are represented as the mean ± SEM of two biological replicates.

4.2 Generation and molecular characterization of the *prr9^{crispr}* line

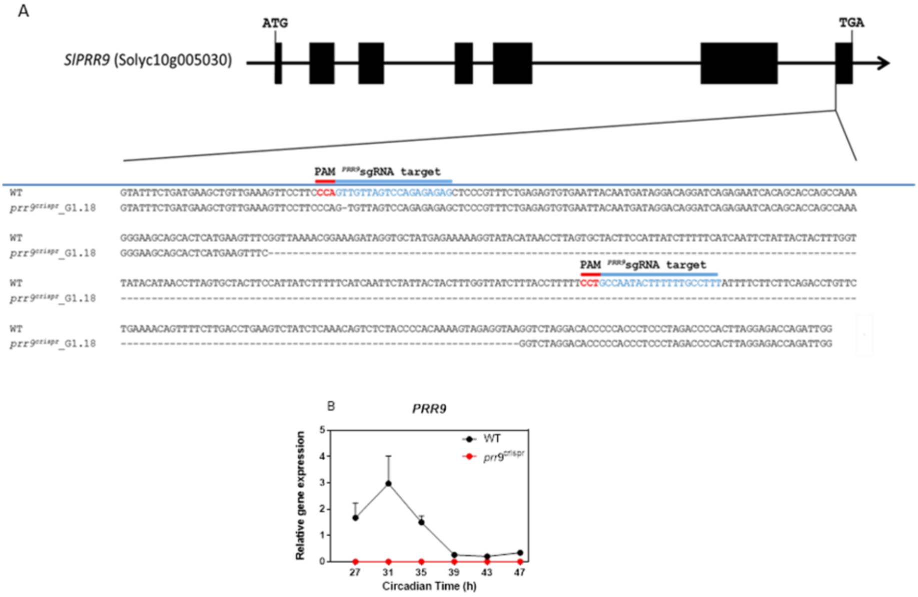


Figure 19: *PRR9* sequence of the new allele generated. *PRR9* sequence (A) of the new allele generated. Circadian time course analysis of *PRR9* (B) in leaves of WT and *prr9^{crispr}* plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

For *PRR9*, and as specified in Material and Methods (section 2), two gRNAs were designed: $^{5'}$AAGGCAAAAAGTATTGGC$^{3'}$ (cloned in backbone U3) and $^{5'}$CTCTCTCTGGACTAACAAC$^{3'}$ (cloned in backbone U6) targeting the CCT domain (Putterill *et al.* 1997) of the 7th exon of *PRR9* (Figure 19A). First gRNA presents 2 off target with 2 mismatches, 15 with 3 and 81 with 4 mismatches. Second gRNA presents 2 off targets with 3 mismatches and 9 with 4. But no one presents off targets following by a PAM sequence. After selection of homozygous lines, we obtained one *prr9*^{crispr} line: #G1.16 with a deletion of 281 nucleotides. Gene expression analyses by RT-QPCR confirmed the lack of *PRR9* mRNA accumulation in leaves using primers amplifying the deleted regions (Figure 19B). The sequences of the new alleles are presented in (Figure 19A).

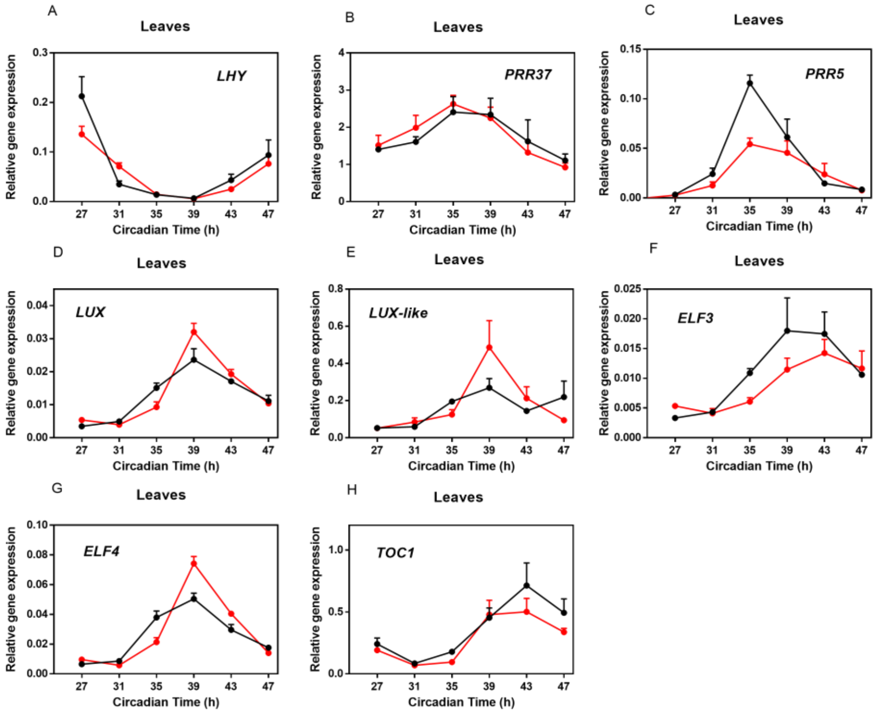


Figure 20: Clock gene mRNA expression in WT and *prr9*^{crispr} tomato plants. Circadian time course analysis in leaves of: *LHY* (A), *PRR37* (B), PRR5 (C), *LUX* (D), *LUX-like* (E), *ELF3* (F), *ELF4* (G) and *TOC1* (H), of plants transferred to continuous light conditions after synchronization. WT (-) and *prr9*^{crispr} (-) plants. Data are represented as the mean ± SEM of two biological replicates.

We examined circadian gene expression by RT-qPCR in WT and *prr9*^{crispr} in leaves. Plants were synchronized under LD cycles followed by two day under LL. Samples were harvested during the second day in LL.

We first analyzed the oscillating genes with a peak of expression during the day. Our analyses showed not very clear changes in the pattern of expression in *prr9*^{crispr} compared to WT (Figure 20A and B) except for a reduction of amplitude for *PRR5* (Figure 20C). Analyses of the evening-expressed genes showed a similar expression to WT but with slight increased amplitude (Figure 20D, E, G). Contrarily, the expression of *ELF3* showed lower amplitude in *prr9*^{crispr} compared to WT, and slight delayed phase (Figure 20F). *TOC1* expression also showed lower amplitude (Figure 20H).

Overall, the lack of *PRR9* or the new allele generated, results in minor alterations of rhythmic gene expression and only some changes in amplitude for a fraction of evening-expressed genes.

4.3 Generation and molecular characterization of the *prr37*^{crispr} line

As specified in Material and Methods (section 2), two gRNAs were designed: $^{5'}$ACAAATTGCCCTCGCATGCG$^{3'}$ (cloned in backbone U3) and $^{5'}$GTACCGGACCTAAAATACAT$^{3'}$ (cloned in backbone U6) targeting the CCT domain of the 7th exon. The first one does not present any off target, the second 58 with 4 mismatches, but only one following by a PAM sequence. After selection of homozygous lines, we obtained two *prr37*^{crispr} lines: #210.6.1-3 and #M18 with a deletion of 82 and 67 nucleotides, respectively. Gene expression analyses by RT-QPCR confirmed the lack of *PRR37* mRNA accumulation in leaves from the *prr37*^{crisprr} #210.6.1-3 and *prr37*^{crisprr} #M18 lines using primers amplifying the deleted regions (Figure 21B-C). For subsequent studies, we used the #210.6.1-3 line. The sequences of the new alleles are presented in Figure 21A.

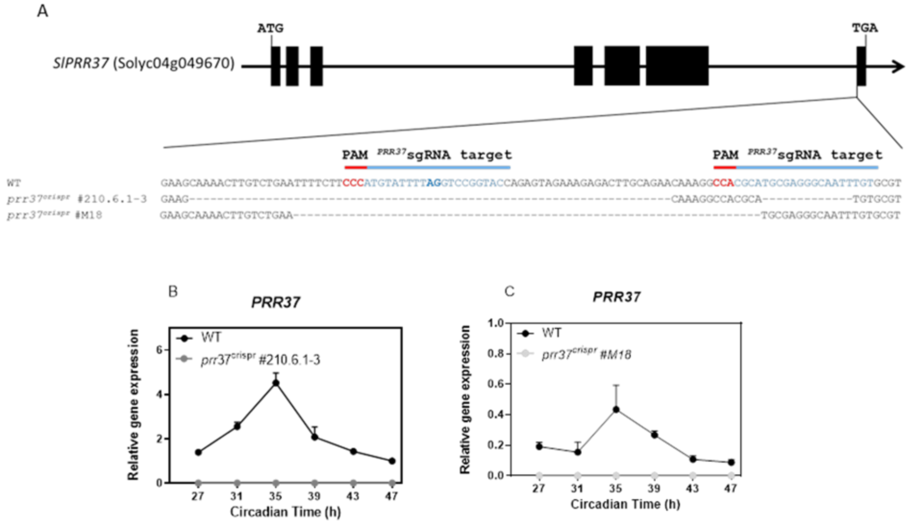


Figure 21: *prr37^{crispr}* lines generated. (A) *PRR37* sequences of the new alleles generated. Circadian time course analysis of *PRR37* in leaves of WT and *prr37^{crispr}* plants transferred to continuous light conditions after synchronization: (B) WT vs *prr37^{crispr}* #210.6.1-3; (C) WT vs *prr37^{crispr}* #M18. Data are represented as the mean ± SEM of two biological replicates.

We examined circadian gene expression by RT-qPCR in WT and *prr37^{crispr}* in leaves, green and red fruits from plants synchronized under LD cycles followed by LL conditions. Samples were harvested during the second day in LL. Our analyses showed that the phase of *LHY* expression was delayed in *prr37^{crispr}* compared to WT in leaves and green fruits (Figure 22A-B). However, in red fruits *LHY* is overexpress along the days compare

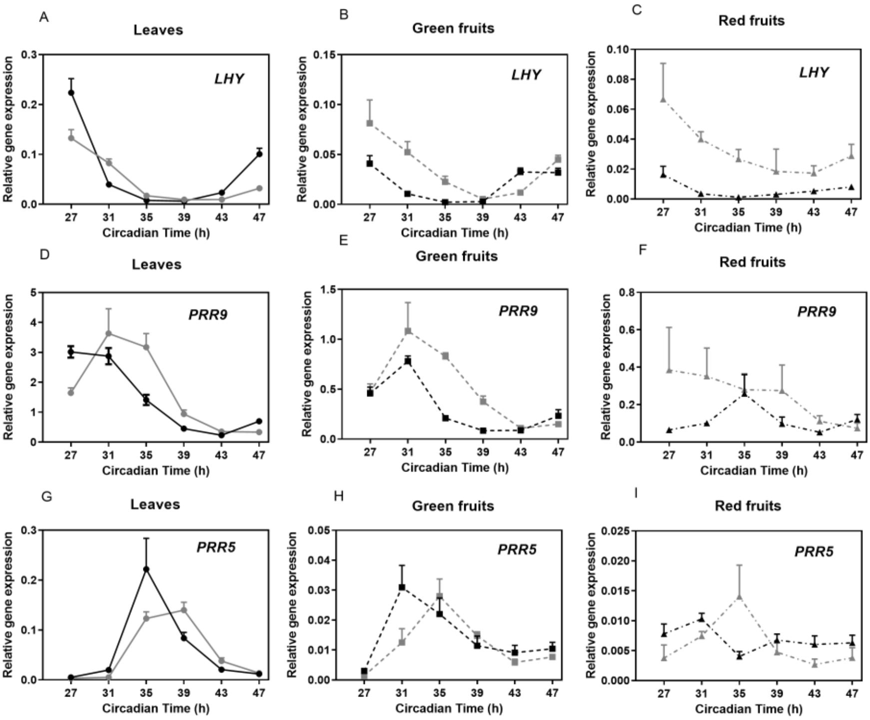


Figure 22: Morning gene mRNA expression in WT and *prr37*^{crispr} tomato plants. Circadian time course analysis of: *LHY* in leaves (A), green fruits (B) and red fruits (C); *PRR9* in leaves (D), green fruits (E) and red fruits (F); and *PRR5* in leaves (G), green fruits (H) and red fruits (I); of plants transferred to continuous light conditions after synchronization. WT (-) and *prr37*^{crispr} (-) plants. Data are represented as the mean ± SEM of two biological replicates.

to WT (Figure 22C). The expression of *PRR9* was also clearly delayed in the *prr37*^{crispr} line (Figure 22D) in leaves and in green fruits (Figure 22E) while the oscillatory pattern in fruits were erratic (Figure 22F). The phase of *PRR5* expression in *prr37*^{crispr} leaves was clearly delayed, with slight down-regulation of peak expression compared to WT (Figure 22G). In green fruits, the expression of *PRR5* was also delayed about 4h compared to WT (Figure 22H-I). The results suggest that PRR37 is required for setting the proper phase and amplitude of morning-gene expression in leaves and fruits.

We next examined the oscillating genes with a peak of expression at dusk or close to dusk. Our analyses showed that in leaves and green fruits, the phase of *LUX* and *LUX-like* expression was similarly affected in *prr37*^{crispr} with a delayed phase and higher amplitude than in WT (Figure 23A-B, D-E). In red fruits, *LUX* and *LUX-like* expression appeared a bit up-regulated compared to WT (Figure 23C). The phase of *ELF3* expression in *prr37*^{crispr} leaves was delayed, with a slightly up-regulation compared to WT (Figure 23G). In green fruits, *ELF3* was slightly down-regulated (Figure 23H) as opposed to its up-regulation in red fruits (Figure 23I). The phase delay in *prr37*^{crisp} leaves and green fruits was also evident for *ELF4* expression (Figure 23J-K) whereas in red fruits *ELF4* appeared up-regulated (Figure 23L). The phase delay of *TOC1* was not so apparent but a decreased rate during the raising phase was evident (Figure 23M). This phenotype was more subtle in green (Figure 23N) whereas in red fruits, *TOC1* was also up-regulated in *prr37*^{crisp} compared to WT (Figure 23O).

prr37^{crispr} shows in almost all genes a delay in the phase in leaves and green fruits, suggesting a role for PRR37 to keep the phase.

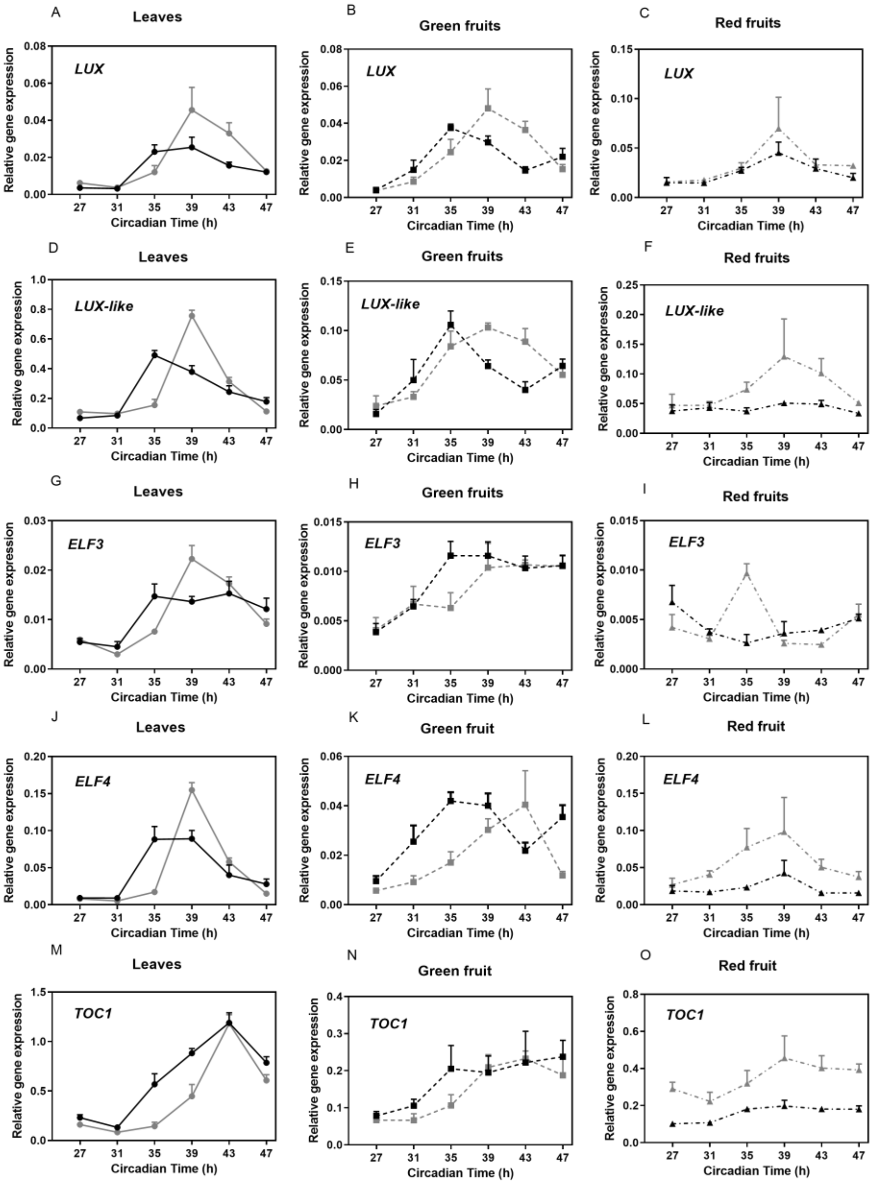


Figure 23: Evening gene mRNA expression in WT and *prr37*^{crispr} tomato plants. Circadian time course analysis of: *LUX* in leaves (A), green fruits (B) and red fruits (C); *LUX-like* in leaves (D), green fruits (E) and red fruits (F); *ELF3* in leaves (G), green fruits (H) and red fruits (I); *ELF4* in leaves (J), green fruits (K) and red fruits (L); and *TOC1* in leaves (M), green fruits (N) and red fruits (O); of plants transferred to continuous light conditions after synchronization. WT (-) and *prr37*^{crispr} (-) plants. Data are represented as the mean ± SEM of two biological replicates.

4.4 Generation and molecular characterization of the *toc1*^{crispr} line

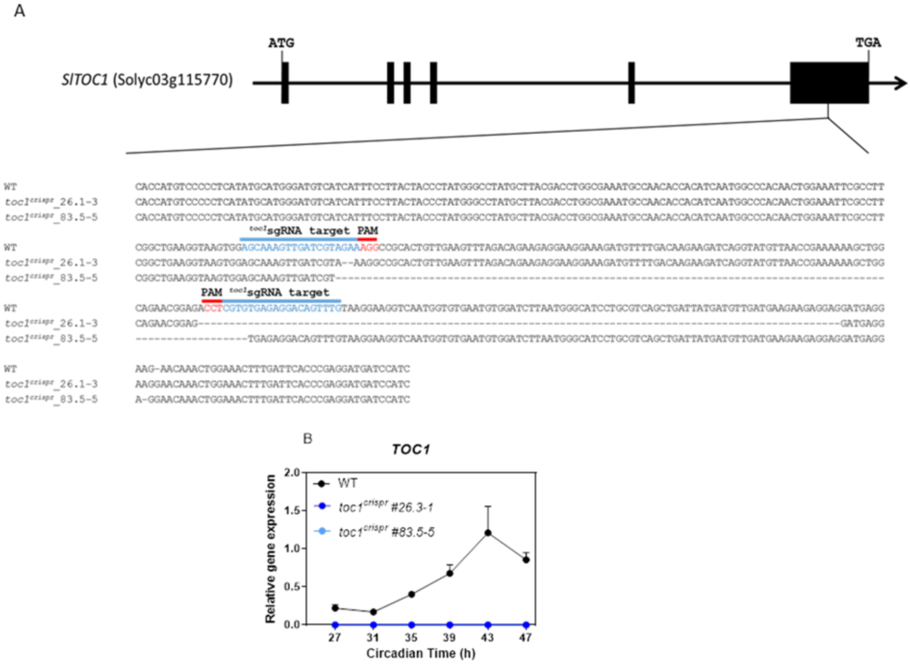


Figure 24: *toc1*^{crispr} lines generated. *TOC1* sequences (A) of the new alleles generated. (B) Circadian time course analysis of *TOC1* (B) in leaves of WT and *toc1*^{crispr} plants transferred to continuous light conditions after synchronization. Data are represented as the mean ± SEM of two biological replicates.

As specified in Material and Methods (section 2), two RNA guides (gRNA) were designed: $^{5'}$CAAACTGTCCTCTCACACG$^{3'}$ (cloned in backbone U3) and $^{5'}$AGCAAAGTTGATCGTAGAA$^{3'}$ (cloned in backbone U6) targeting the CCT domain of the 6th exon (Figure 24A). First gRNA presents 2 off targets with 2 mismatches, 6 with 3 and 9 with 4, respectively. Second gRNA presents off target of 3 mismatches and 39 with 4. But no one presents off targets following by a PAM sequence. After selection of homozygous lines, we obtained two *toc1*^{crispr} lines: #26.1-3 and #83.5-5 with a deletion of 105 and 106 nucleotides, respectively. Gene expression analyses by RT-QPCR confirmed the lack of *TOC1* mRNA accumulation in leaves from the *toc1*^{crisprr} #26.1-3 and *toc1*^{crispr} #83.5-5 lines using primers amplifying the deleted regions (Figure 24B). For subsequent studies, we used the #26.1-3 line. The sequences of the new alleles are presented in Figure 24A.

As for the other *clock*^{crispr} lines, we examined circadian gene expression by RT-qPCR in WT and *toc1*^{crispr} in leaves, green and red fruits from plants synchronized under LD cycles followed by LL conditions. Samples were harvested during the second day in LL. Our analyses showed that in leaves, the phase of *LHY* expression was advanced in *toc1*^{crispr} compared to WT (Figure 25A). Interestingly, in green and red fruits, the expression of *LHY* was nearly abolished in *toc1*^{crispr} (Figure 25B-C). In leaves and green fruits, the phase of *PRR9* expression was advanced in *toc1*^{crispr} compared to WT (Figure 25D-E). The oscillation of *PRR37* in leaves and green fruits was also nearly abolished in the *toc1*^{crispr} line compared to WT (Figure 25G). The phase of *PRR5* expression in *toc1*^{crispr} leaves was clearly advanced compared to WT (Figure 25J). In green fruits, the phase of *PRR5* expression seems advanced with a down regulation (Figure 25K). Overall, not a clear phenotypic pattern was observed in *toc1*^{crispr} red fruits compared to WT (Figures 25F, I, L). The results suggest that TOC1 is required for setting the proper phase and amplitude of morning-gene expression in leaves and green fruits.

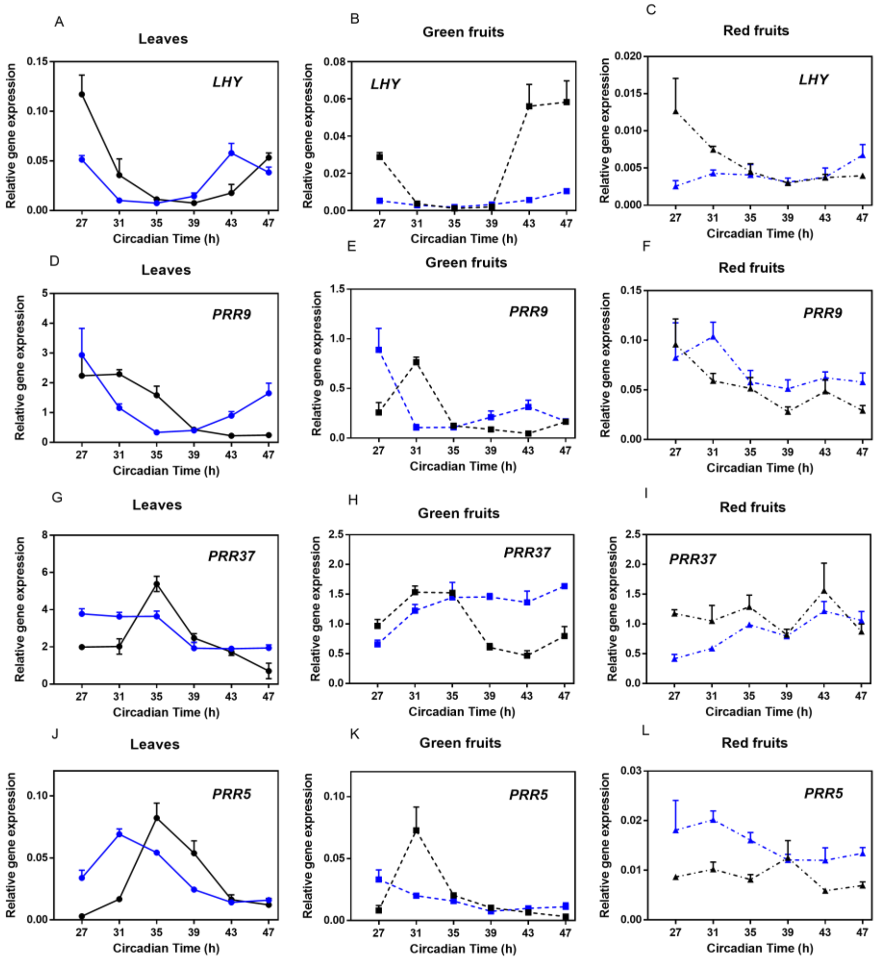


Figure 25: Morning gene mRNA expression in WT and *toc1*^{crispr} tomato plants. Circadian time course analysis of: *LHY* in leaves (A), green fruits (B) and red fruits (C); *PRR9* in leaves (D), green fruits (E) and red fruits (F); *PRR37* in leaves (G), green fruits (H) and red fruits (I); and *PRR5* in leaves (J), green fruits (K) and red fruits (L); of plants transferred to continuous light conditions after synchronization. WT (-) and *toc1*^{crispr} (-) plants. Data are represented as the mean ± SEM of two biological replicates.

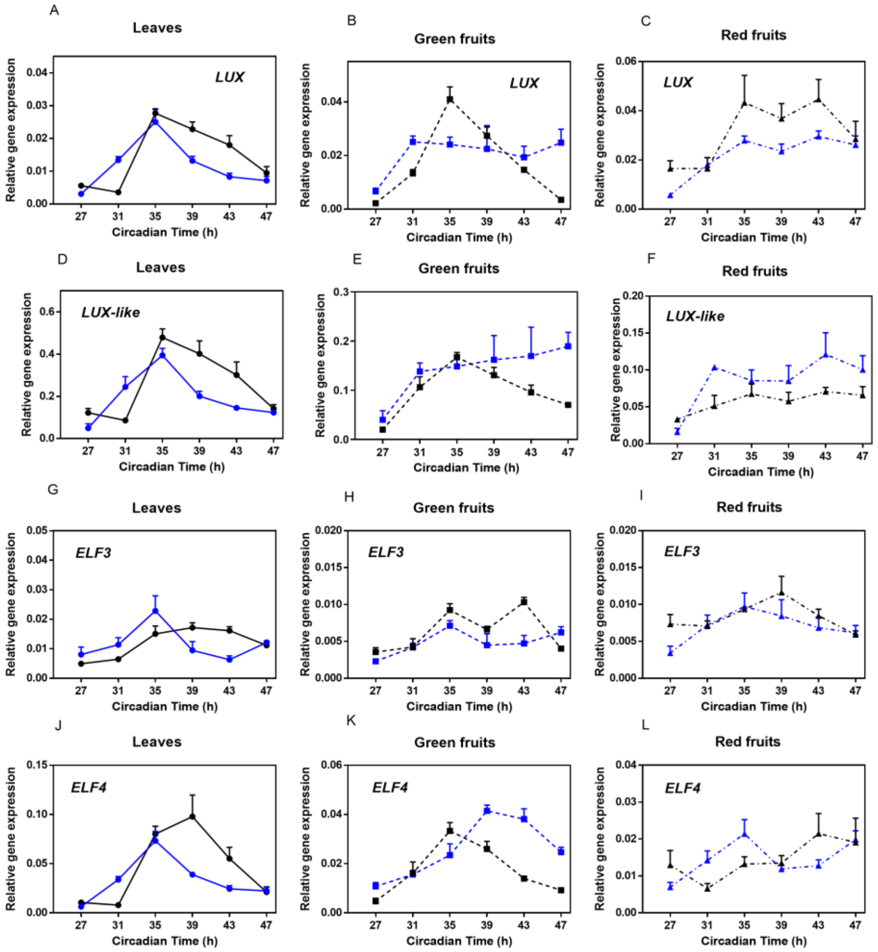


Figure 26: Evening gene mRNA expression in WT and *toc1*^{crispr} tomato plants. Circadian time course analysis of: *LUX* in leaves (A), green fruits (B) and red fruits (C); *LUX-like* in leaves (D), green fruits (E) and red fruits (F); *ELF3* in leaves (G), green fruits (H) and red fruits (I); and *ELF4* in leaves (J), green fruits (K) and red fruits (L); of plants transferred to continuous light conditions after synchronization. WT (-) and *toc1*^{crispr} (-) plants. Data are represented as the mean ± SEM of two biological replicates.

In the case of genes with a peak of expression at dusk or close to dusk, our analyses showed an increased declining phase of *LUX* and *LUX-like* expression in *toc1^{crispr}* leaves (Figure 26A and D) while in green fruits, *LUX* and *LUX-like* expression sustained a steady-state throughout the most part of the cycle (Figure 26B, D). The phase of *ELF3* expression in *toc1^{crispr}* leaves was advanced (Figure 26G) whereas no clear phenotypes were observed in green and red fruits (Figure 26H and I). In leaves, the phase of *ELF4* expression was advanced in *toc1^{crispr}* compared to WT (Figure 26J) as opposed to the delayed phase in green fruits (Figure 26K).

Overall, the results suggest that TOC1 plays an important role regulating the amplitude and phase of most of the clock genes in leaves. In green fruits, TOC1 seems to be required for controlling the oscillation of *LUX* and *LUX-like* expression, whereas in red fruits, the oscillations are weak in both WT and *toc1^{crispr}*.

5. Growth and developmental phenotypes

5.1 Growth phenotypes

5.1.1 Analyses of plant height

To determine the possible implication of the different clock genes in the regulation of plant growth, we measured the overall height of the different lines. Our results showed that the *lhy^{crispr}* plants displayed a significant increase in plant height that was evident at different stages of development, including 20 days after transplanting (20 DAT) (Figure 27C), 30 DAT (Figure 27A and C) and 50 DAT (Figure 27B and C). The *toc1^{crispr}* plants also showed significantly increased height that was more evident at earlier stages (20 and 30 DAT) (Figure 27A and C). The *prr9*^{crispr} and *prr37*^{crispr} plants did not show difference in height compared to WT plants under our growing conditions. Together, our results indicate that proper expression of *LHY* and *TOC1* is important for the regulation of plant growth.

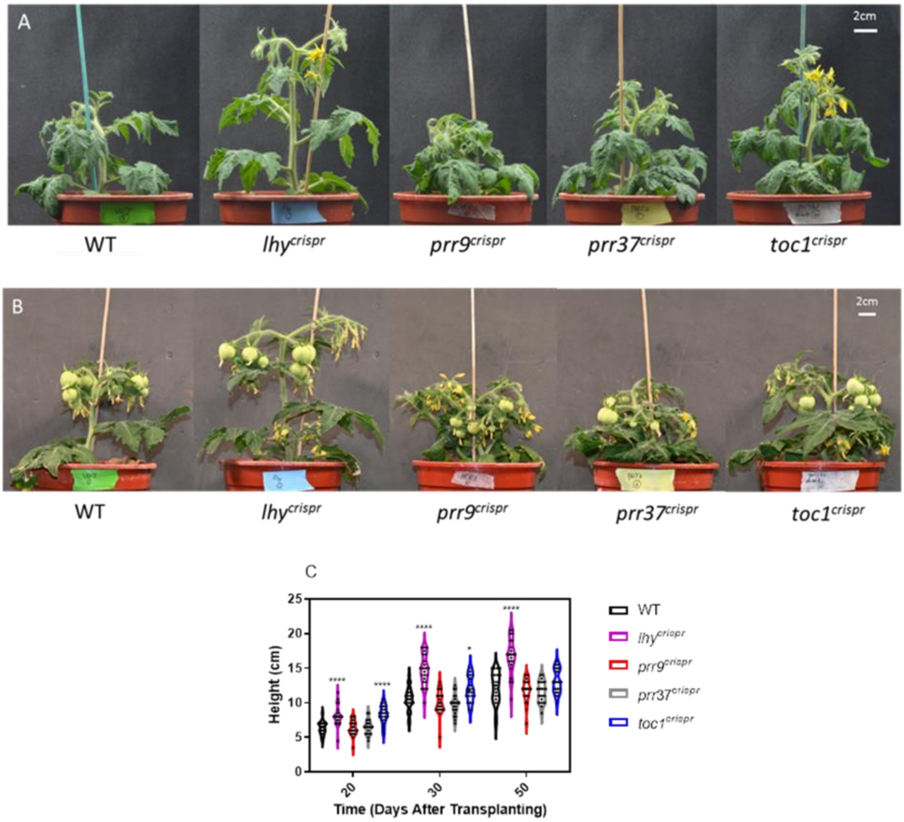


Figure 27: Tomato plant height at different time. Representative picture of the plants at DAT30 (A) and DAT50 (B); Height of the plants (C). Data from >22 plants by lines. Statistical analysis done by ANOVA 1 with Dunnett's multiple comparison test. Signif. Codes: <0.0001 '****' ; <0.001 '***' ; <0.01 '**' ; <0.05 '*' .

5.1.2 Characterization of leaflet size and weight

To determine if the clock genes contributed to leaf growth, we measured fresh weight (FW), dry weight (DW) and area of the highest leaflet. Our analyses showed that the area of *lhy*^{crispr} leaves was higher than WT at both developmental stages examined (21 and 42 DAT) (Figure 28A and F-G). The FW and DW were also significantly higher in *lhy*^{crispr} plants compared to WT (Figure 28B-C). The other *clock*^{crispr} lines did not show significant differences compared to WT plants for these parameters. The Foliar Mass Area (FMA) was similar for all the lines at 21 DAT (including *lhy*^{crispr}) but *prr9*^{crispr} FMA was significantly lower than WT at 42 DAT (Figure 28D). The *prr9*^{crispr} line also showed a higher hydration (H) than WT at 21 and 42 DAT (Figure 28E). The higher H of *prr9*^{crispr} plants might correlate with the reduced FMA observed in these plants.

5.2 Developmental phenotypes

5.2.1 Germination rate

The germination rate of the different CRISPR lines was examined by germinating the seeds of the different CRISPR-Cas9 lines in a growth chamber under LD conditions. The *lhy*^{crispr} and *prr9*^{crispr} did not show any significant difference with WT (Figure 29A). The *prr37*^{crispr} seeds clearly showed an increased germination rate from the third day on (Figure 29B) while *toc1*^{crispr} seeds also showed a significant increase from the fifth day on. Approximately 80% of the *prr37*^{crispr} and *toc1*^{crispr} seeds germinated at day 5 compared to the 7-8 days required for the 80% germination of WT seeds. At day 7, the two lines *prr37*^{crispr} and *toc1*^{crispr} showed a similar cumulative frequency of germination (80% ±10%). The results suggest a

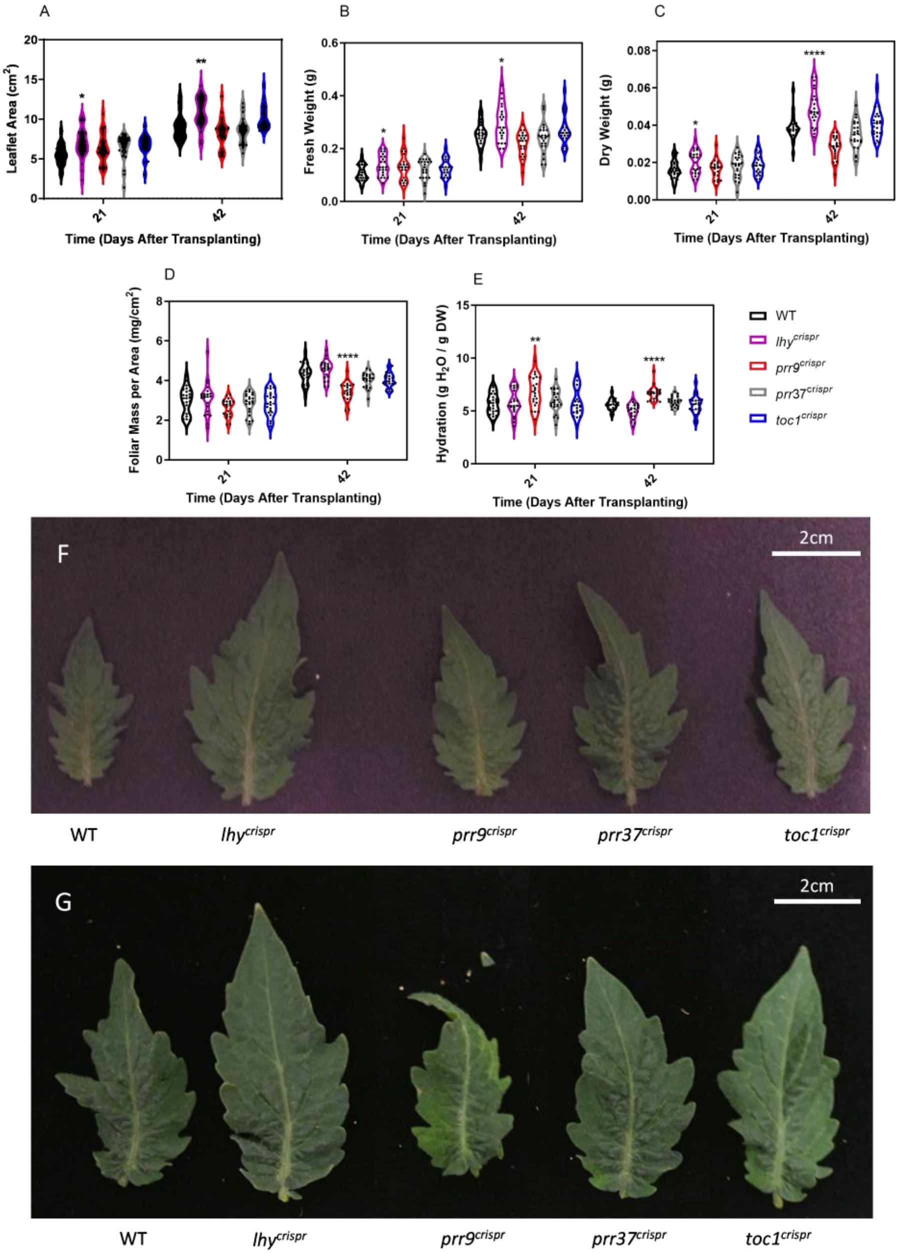


Figure 28: Leaflet phenotypes of the *clock*^{crispr} plants. Area (A), fresh weight (B), dry weight (C), Foliar Mass Area (D) and Hydration (E) of the last leaflet at DAT21 (F) and DAT42 (G). Data from >22 plants by lines. Statistical analysis done by ANOVA 1 with Dunnett's multiple comparison test. Signif. Codes: <0.0001 '****' ; <0.001 '***' ; <0.01 '**' ; <0.05 '*' .

possible role of PRR37 and TOC1 in the regulation of seed germination and/or seed dormancy.

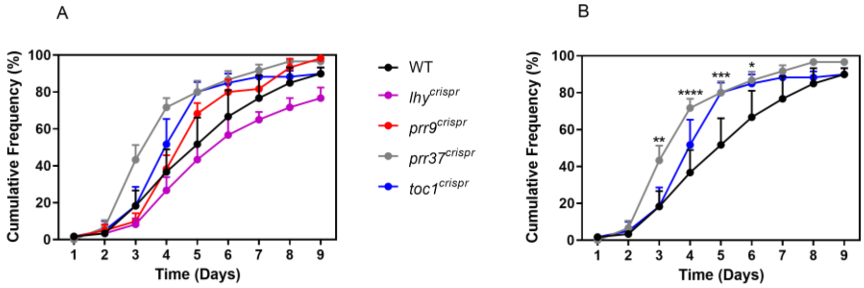


Figure 29: Cumulative germination rate of the *clock*^{crispr} lines. All the *clock*^{crispr} lines are represented in (A), only *prr37*^{crispr} and *toc1*^{crispr} lines compared to WT in (B). Data from >60seeds by lines. Statistical analysis done by ANOVA 2 with Dunnett's multiple comparison test. Signif. Codes: <0.0001 '****' ; <0.001 '***' ; <0.01 '**' ; <0.05 '*' . Data are represented as the mean ± SEM.

5.3 Flowering and fructification

5.3.1 Flowering

To examine whether the clock components influence the developmental transition from vegetative to reproductive stage, we measured the timing of the first flower appearance for each CRISPR-Cas9 line. Our results showed that *toc1*^{crispr} plants displayed a significantly early flowering phenotype, with the first flower appearing around 23 ±3 DAT compared to WT at 29 ±3 DAT (Figure 30A). The other lines did not show significant differences compared to WT. We also measured the number of flowers per plant and found that at 50 DAT, the *prr9*^{crispr} line showed a significantly increased number of flowers (28 ±10) compared to WT plants (13 ±6) (Figure 30B). The other lines did not show significant changes in the number of flowers per plant compared to WT. Together, our results indicate that TOC1 is involved in the regulation of the timing of flowering while PRR9 might have a role controlling reproductive meristem activity.

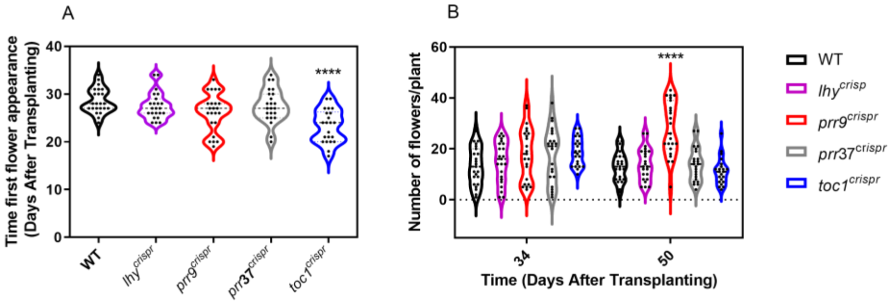


Figure 30: Flower characterization of *clock*^{crispr} plants. First flower appearance (A) and number of flower (B) at DAT34 and DAT50. Data from >22 plants by lines. Statistical analysis done by ANOVA 1 with Dunnett's multiple comparison test. Signif. Codes: <0.0001 '****' ; <0.001 '***' ; <0.01 '**' ; <0.05 '*' . Data are represented as the mean ± SEM.

5.3.2 Fruit development and ripening

We next focused on fruits and examined several parameters including the timing of first fruit appearance (with a diameter of 3mm). Analyses of *toc1*^{crispr} plants showed a significant difference with WT, with a first fruit appearance at 35 DAT ±3 compared to WT (38 DAT ±3) (Figure 31A). We also checked the time when the first fruit started to break (with a 10% yellow). Our results showed a significant early breaking of *lhy*^{crispr} compared to WT (Figure 31B). We found that *lhy*^{crispr} fruits breaked about 64 DAT ±3 compared to 68 DAT±4 for WT. The other lines did not showed differences in breaking time compared to WT.

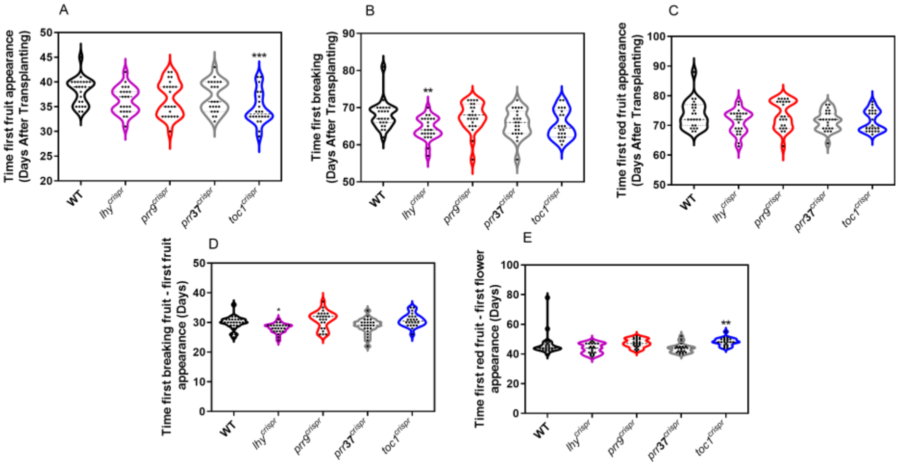


Figure 31: Fruit characterization of *clock*^{crispr} plants. First fruit appearance (A), first fruit breaking and first fruit red (C). Delta between the first green fruit and the first breaking fruit observed (D), and between the first flower opened to get the first red fruit (E). Data from >22 plants by lines. Statistical analysis done by ANOVA 1 with Dunnett's multiple comparison test. Signif. Codes: <0.0001 '****' ; <0.001 '***' ; <0.01 '**' ; <0.05 '*' .

We also examined the time of the first full red fruit appearance but found no significant differences compared to WT (Figure 31C). When we calculated the time that each fruit takes to break (delta between first green fruit and first breaking fruit observed), we found that the *lhy*^{crispr} fruit breaking was significantly earlier than in WT (about 28 days ±2 for *lhy*^{crispr} and 30 days ±2 for WT) (Figure 31D). Similarly, *toc1*^{crispr} showed a significant difference on the time between the first flower appearance and the first red fruit observed (delta between first opened and the first red fruit) (Figure 31E). Overall, our results showed that TOC1 is important in the regulation of flowering time and fruit set but the process of ripening is uncoupled from these regulations. On the other hand, LHY might have a role controlling the timing of ripening, particularly between the formations of the green fruit until the breaking stage.

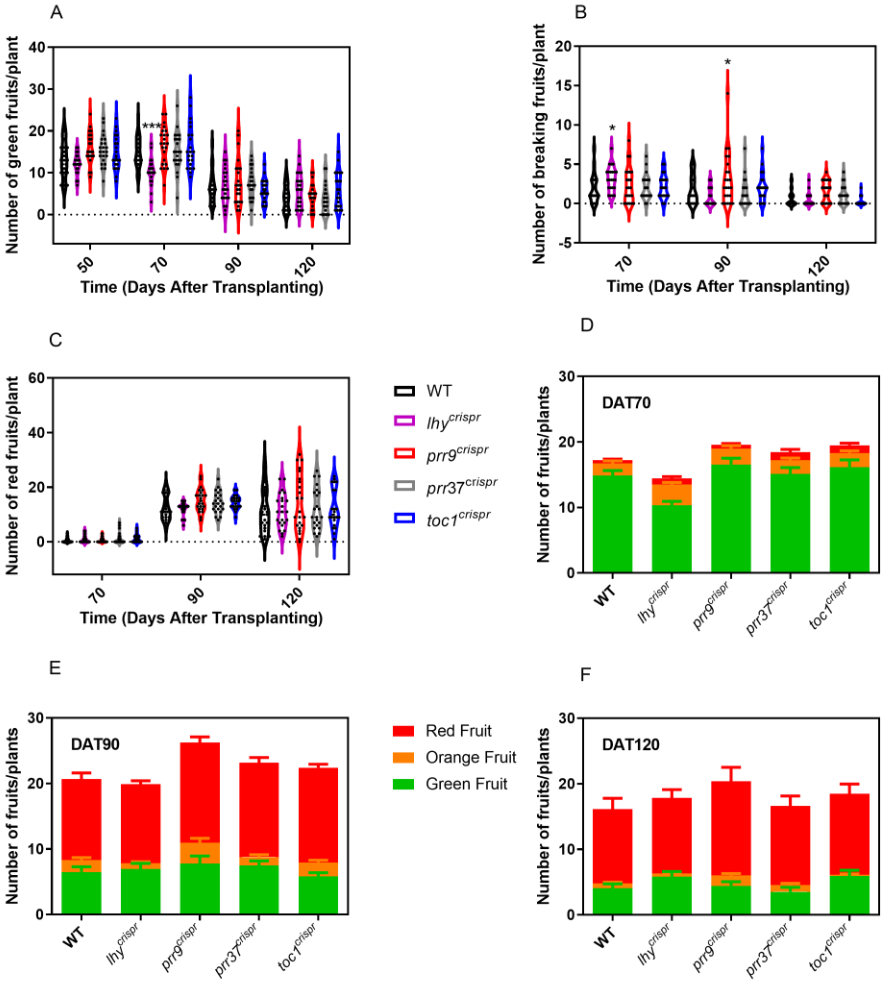


Figure 32: Total number of fruit by plant. Green fruit (A), breaking fruit (B) and red fruit (C) at different times. Proportion of each green, breaking and red fruits at DAT70 (D), DAT90 (E) and DAT120 (F). Data from >22 plants by lines. Statistical analysis done by ANOVA 1 with Dunnett's multiple comparison test. Signif. Codes: <0.0001 '****' ; <0.001 '***' ; <0.01 '**' ; <0.05 '*' . Data are represented as the mean ± SEM.

We next checked plant productivity in the different CRISPR-Cas9 lines generated. To that end, we counted the total number of fruits at different stages (50 DAT, 70 DAT, 90 DAT and 120 DAT). We recorded a) Green fruit (fruits bigger than 3mm of diameter) to breaking stage; b) Breaking fruits (from breaking stage until red fruit); and c) Red fruit (full mature red). Our results showed that the total number of green fruits was similar for all the lines at all the times examined except for *lhy*^{crispr} that showed less green fruits at 70 DAT (Figure 32A). Regarding the total number of fruit at breaking stage, the *lhy*^{crispr} plants contained more breaking fruits than WT at 70 DAT (Figure 32B). At 90 DAT, *prr9*^{crispr} plants also showed more fruits at breaking stage than WT (Figure 32B). For all the CRISPR-Cas9, the total number of red fruits per plant did not show significant differences with WT plants. When we plot together the different type of fruits, green, breaking and red fruits, together, there is a clear tendency for *prr9*^{crispr} to have more fruits by plants, at DAT70 (Figure 32D), at DAT90 (Figure 32E) and also at DAT120 (Figure 32F).

The fact that *lhy*^{crispr} plants showed fewer green fruits than WT at 70 DAT but more fruits at the breaking stage confirmed our previous conclusions of a faster fruit ripening in this line (Figure 31D). The *prr9*^{crispr} showing more fruits at breaking stage can be also related with the observed higher number of flowers in this line (Figure 30B). Together, the results suggest a possible role of LHY in fruit ripening, of TOC1 in flowering time and ripening, and of PRR9 in reproductive inflorescences.

5.3.3 Yield and red fruit characterization

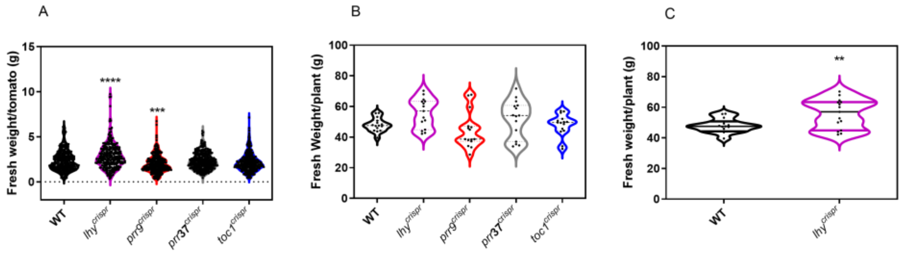


Figure 33: Characterization of red mature tomato fruits. Fresh weight of individual tomato fruits collected (A) and yield by plant (B and C). All red mature fruits of each plant were collected at DAT90 and DAT120 then the data were pooled. Data from at least 291 fruits by line, with at least 22 plants by line. Statistical analysis done by ANOVA 1 with Dunnett's multiple comparison test (A and B). Statistical analysis done by T test (C). Signif. Codes: <0.0001 '****' ; <0.001 '***' ; <0.01 '**' ; <0.05 '*'

Red mature fruits were harvested at 90 DAT and at 120 DAT, and weighted to get the fresh weight (FW). Our results showed that *lhy*^{crispr} red fruits weighed on average more than those of WT plants while the *prr9*^{crispr} red fruits weighed less (Figure 33A). The other lines did not show statistical differences compared to WT. We also weighted the total fruits per plant and found no significant differences among the lines using the test ANVOA 1 (Figure 33B), even though a trend of *lhy*^{crispr} fruits having increased weight was suggested (about 56 g ±9 per plant for *lhy*^{crispr} versus 48 g ±4 per plant for WT). Consistently, T-test analyses comparing WT and *lhy*^{crispr} showed significant differences (accepting the hypothesis H1 at <0.01) (Figure 33C). Analyses of the two bigger red fruits per plant rendered a fresh weight significantly higher of *lhy*^{crispr} compared to WT (about 33% more) with an average of 4.4 g ±1.8 for *lhy*^{crispr} versus 3.3 g ±1.3 for WT (Figure 34A-C). The dry weight was also measured in order to calculate the fruit water content but there were no significant differences among the lines (Figure 34B-C).

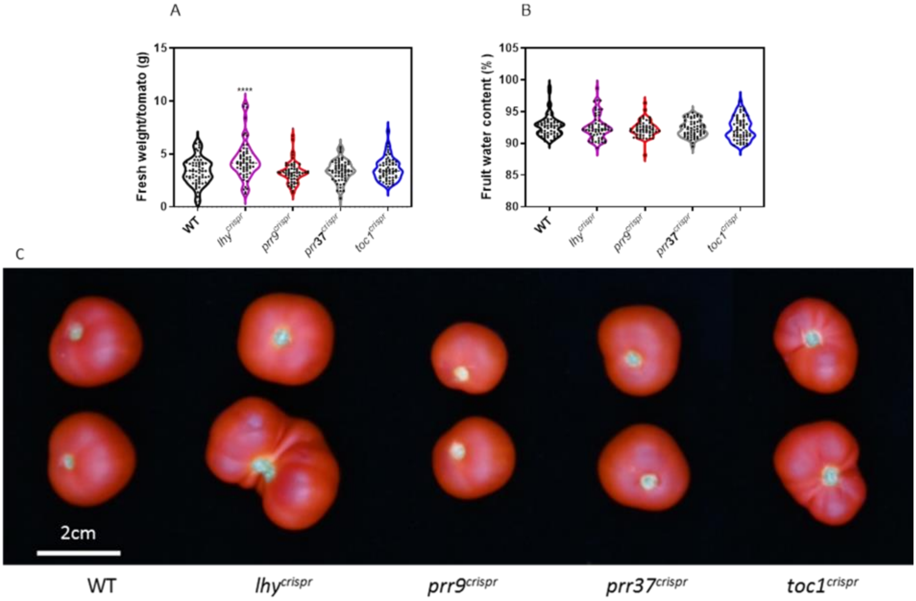
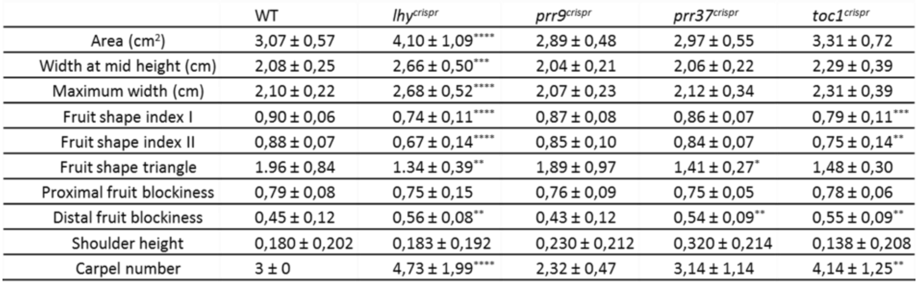


Figure 34: Characterization of the two bigger tomato fruits per plant. Fresh weight (A) and fruit water (B. Representative picture of the two bigger fruit of each line at DAT90 (C). Data from at least 57 fruits, only the two heavier fruit of each plant were taking in account. Statistical analysis done by ANOVA 1 with Dunnett's multiple comparison test. Signif. Codes: <0.0001 '****' ; <0.001 '***' ; <0.01 '**' ; <0.05 '*' .

Longitudinal sections were also made to examine other fruit parameters (Table 2). The results showed that the longitudinal areas of *lhy*^{crispr} fruits were higher than WT (4.10 cm^2 ±1.09 of *lhy*^{crispr} versus 3.07 cm^2 ±0.57 of WT). The width at mid-height and the maximum width were also bigger in *lhy*^{crispr} (2.66 cm ±0.50 and 2.68 cm ±0.52, respectively compared to WT 2.08 cm ±0.25 and 2.10 cm ±0.22 respectively). The other CRISPR-Cas9 lines did not show significant differences compared to

WT. Calculation of the fruit shape index I (see Materials and Methods, Figure 37 and Equation 2) showed that the fruits of *lhy*^{crispr} and *toc1*^{crispr} plants had bigger width than height, with ratios of 0.74 ±0.11 (*lhy*^{crispr}) and 0.79 ±0.11 (*toc1*^{crispr}) versus 0.90 ±0.06 for WT plants (Table 2). The results were confirmed by the calculation of the fruit shape index II giving ratios of 0.67 ±0.14 for *lhy*^{crispr}, 0.75 ±0.14 for *toc1*^{crispr} and 0.88 ±0.07 for WT. The other lines did not show differences compared with WT. The results indicate that WT, *prr37*^{crispr} and *prr9*^{crispr} have fruits with a round, proportionate shape whilst *lhy*^{crispr} and *toc1*^{crispr} have squat fruits.

Table 2: Analyses of red fruit morphology. Statistical analysis done by ANOVA 1 with Dunnett's multiple comparison test. Signif. Codes: <0.0001 '****' ; <0.001 '***' ; <0.01 '**' ; <0.05 '*' . Data are represented as the mean ± SEM.



	WT	*lhy*^{crispr}	*prr9*^{crispr}	*prr37*^{crispr}	*toc1*^{crispr}
Area (cm^2)	3,07 ± 0,57	4,10 ± 1,09****	2,89 ± 0,48	2,97 ± 0,55	3,31 ± 0,72
Width at mid height (cm)	2,08 ± 0,25	2,66 ± 0,50***	2,04 ± 0,21	2,06 ± 0,22	2,29 ± 0,39
Maximum width (cm)	2,10 ± 0,22	2,68 ± 0,52****	2,07 ± 0,23	2,12 ± 0,34	2,31 ± 0,39
Fruit shape index I	0,90 ± 0,06	0,74 ± 0,11****	0,87 ± 0,08	0,86 ± 0,07	0,79 ± 0,11***
Fruit shape index II	0,88 ± 0,07	0,67 ± 0,14****	0,85 ± 0,10	0,84 ± 0,07	0,75 ± 0,14**
Fruit shape triangle	1.96 ± 0,84	1.34 ± 0,39**	1,89 ± 0,97	1,41 ± 0,27*	1,48 ± 0,30
Proximal fruit blockiness	0,79 ± 0,08	0,75 ± 0,15	0,76 ± 0,09	0,75 ± 0,05	0,78 ± 0,06
Distal fruit blockiness	0,45 ± 0,12	0,56 ± 0,08**	0,43 ± 0,12	0,54 ± 0,09**	0,55 ± 0,09**
Shoulder height	0,180 ± 0,202	0,183 ± 0,192	0,230 ± 0,212	0,320 ± 0,214	0,138 ± 0,208
Carpel number	3 ± 0	4,73 ± 1,99****	2,32 ± 0,47	3,14 ± 1,14	4,14 ± 1,25**

Analyses of the fruit shape triangle (see Materials and Methods, Figure 37 and Equations 2) showed that the lines the *lhy*^{crispr} and *prr37*^{crispr} were significantly different compared to WT, with a proximal end around 1.34 times ±0.39 and 1.47 times ±0.27 wider to the distal end, respectively as opposed to WT, which had a proximal end around 1.96 times ±0.84 wider than its distal end (Figure or table). The proximal fruit blockiness was similar in the different lines. But the ratio of the distal fruit blockiness of *lhy*^{crispr}, *prr37*^{crispr} and *toc1*^{crispr} were significantly different to WT, with a ratio of 0.56 ±0.08, 0.54 ±0.09 and 0.55 ±0.09, respectively, compared to the WT ratio of about 0.45 ±0.12. These results show that these three lines are less sharp than WT in their distal end. Analyses of carpel number showed that WT fruits contained a highly reproducible number (3) whereas the carpel number in the CRISPR-Cas9 lines was more variable, with *lhy*^{crispr} and *toc1*^{crispr} having both higher carpel number (4.73 ±1.99 and 4.14 ±1.25, respectively).

Overall, our results show that LHY contributes to fruit size, weight, shape and carpel number as well as fruit yield. Altering *PRR9* expression leads to smaller fruits, but without affecting negatively the yield. PRR37 might be important in the regulation of fruit shape while TOC1 influences fruit shape and carpel number.

DISCUSSION

In this Doctoral Thesis, we have performed an initial characterization of the circadian system in *Solanum lycopersicum*. Our studies have uncovered the conservation of the rhythmic expression of several clock genes in *Arabidopsis thaliana* and *Solanum lycopersicum*. The generation of CRISPR-Cas9 lines has allowed us to identify that proper expression of a subset of circadian clock genes affects the amplitude of clock genes in tomato leaves and fruits. Furthermore, these genes also play a role in the regulation of tomato growth and development.

1. Conservation of clock rhythmic gene expression between *Arabidopsis* and tomato

Our analyses of gene expression in WT leaves show the conservation of the rhythmicity and oscillation of several clock genes between *Arabidopsis* and tomato. In *Arabidopsis,* AtLHY and AtCCA1 heterodimerize (Lu *et al.* 2009) to act as a repressor (Alabadí *et al.* 2001; Li *et al.* 2011; Adams *et al.* 2015; Kamioka *et al.* 2016). The partially redundant function of AtLHY and AtCCA1 (Lu *et al.* 2009) could relate to the presence of only one homolog in tomato (SlLHY). , which suggest the possible homodimerization like in observed in *Arabidopsis* (Lu *et al.* 2009), if the function is conserved between these two species. In fact the pattern of *SlLHY* expression in tomato leaves (Figure 5) is quite conserved compared to *Arabidopsis* (Mizoguchi *et al.* 2002), there is a peak of expression at the sunrise. The analysis of the *lhy*^{crispr} lines generated gives more information about the possible role of SlLHY in tomato. *lhy*^{crispr} shows a clear advanced phase of clock genes (Figure 17-18), like the double mutant in *Arabidopsis cca1/lhy* that also shows an advanced phase with a shorter period (Alabadí *et al.* 2002). Our results also suggest a shorter period for *lhy*^{crispr}, but further experiments are required to verify it. Also, it is interesting to notice that SlLHY seems necessary for the proper expression of *SlPRR37* (Figure 17D). However, in *Arabidopsis*, this phenomenon is not reported for *AtPRR3* neither *AtPRR7*. *cca1/lhy Arabidopsis* plants are smaller than WT and the leaves are also smaller and paler than WT (Alabadí *et al.* 2002). In tomato plants, we observe the leaf a bit paler than WT but the plant and leaves are much bigger than WT (Figure 27 and 28A). The *lhy*^{crispr} tomato plants tend to be early flowering (albeit not significantly) (Figure 27A) similar to the *Arabidopsis* double mutant *cca1/lhy* showing an early flowering phenotype in SD (Mizoguchi *et al.* 2002). Thus, we observe a conserved oscillatory pattern of expression for *LHY* in *Arabidopsis* and tomato, but a different role in the regulation of a subset of outputs.

Regarding the PRRs family, the oscillations and pattern of expression seem also conserved between *Arabidopsis* and tomato. *PRRs* are sequentially expressed during the day in *Arabidopsis AtPRR9* peaks close to dawn, then *AtPRR7*, *AtPRR5*, *AtPRR3* and finally *AtTOC1* (*PRR1*) close to dusk (Mizuno and Nakamachi 2005; Nakamachi *et al.* 2005). In tomato, *SlPRR9* still peaks close to dawn (Figure 6) like in *Arabidopsis*. *SlPRR37* and *SlPRR73* are expressed during the rest of day until dusk where their expression decreases drastically (Figure 7-8). Interestingly, *SlPRR5* is expressed during a short time with a peak at the end of the day (Figure 9). *SlTOC1* peaks at dusk (Figure 14) like in *Arabidopsis*. These results suggest a conservation of the expression patterns for *SlPRR9* and *SlPRR5*, with an overlapping and a longer time of expression for *SlPRR37* and *SlPRR73*, covering the individual expression of *AtPRR7* and *AtPRR3* in *Arabidopsis*.

In several crops, *PRR37* and *PRR73* are reported as the homologs of *PRR3* and *PRR7*, such as rice, sorghum but also in trees like poplar (Hotta 2022).

The *prr9*^{crispr} line generated can give us some information about the role of SlPRR9 in tomato. In *Arabidopsis*, *prr9* mutant lines have longer period compared to WT (Ito *et al.* 2003, Salomé and McClung 2005) Also, the *Arabidopsis prr9* mutant line shows lower amplitude in the expression of *AtCCA1* and higher amplitude for the expression of *AtTOC1* (Eriksson *et al.* 2003). In the tomato *prr9*^{crispr} line, we observed a lower amplitude for *SlLHY* expression (Figure 20A), and a lower o similar amplitude for *SlTOC1*, (Figure 20H). Our results suggest also a possible role of SlPRR9 in the regulation of the evening complex genes, such as *SlLUX*, *SlLUX-like* and *SlELF4* (Figure 20). In *Arabidopsis*, AtPRR9 and AtPRR7 negatively regulate *AtELF3*, *AtELF4* and *AtLUX* in the circadian gating of thermoresponse (Yuan *et al.* 2020). These results suggest a possible conservation in the role of PRR9 between *Arabidopsis* and tomato, about the regulation the evening complex genes. The lack of SlPRR9 increases the number of flower in tomato, but this phenomenon was not reported in *Arabidopsis*.

In tomato, the expression pattern of *SlPRR37* and *SlPRR73* (Figure 7-8) seems to encompass the expression of *AtPRR7* and *AtPRR3* in *Arabidopsis* (Mizuno and Nakamachi 2005; Nakamachi *et al.* 2005). *SlPRR37* expression is higher than its homolog *SlPRR73*, also it seems that *SlPRR37* expression is more robust than *SlPRR73*. In *Arabidopsis*, the *prr3* mutant line sustains the same period than WT, while *prr7* mutant has a longer period compared to WT (Salomé and McClung 2005). Our *prr37*^{crispr} tomato line is delayed in the expression of almost all the clock genes

(Figure 22-23), suggesting a possible longer period compared to WT. This phenotype will be similar to that reported for the *Arabidopsis* mutant *prr7* (Farré *et al.* 2005). Our *prr37*^{crispr} tomato line shows lower amplitude and phase delayed in the expression of *SlLHY* in leaves (Figure 22A). In *Arabidopsis*, *prr3* mutant plants also show lower amplitude of *AtLHY* expression (Salomé and McClung 2005) but interestingly, the phase is advanced (Para *et al.* 2007). On the other hand, in *Arabidopsis prr7* mutant plants, the phase of *AtCCA1* expression is delayed compared to WT (Farré *et al.* 2007). As we saw for SlPRR9, SlPRR37 seems to be a negative regulator of the evening complex genes, *SlLUX*, *SlLUX-like*, *SlELF4* and also *SlELF3* (Figure 23). *SlTOC1* expression is reduced in our *prr37*^{crispr} tomato line (Figure 23M). In *Arabidopsis*, the expression of *AtTOC1* is reduced in the *prr3* mutant (Para *et al.* 2007). Overall, our analyses suggest that SlPRR37 might have a conserved function with the *Arabidopsis* AtPRR3 and AtPRR7 in the regulation of clock gene expression. Additionally, we found an increased germination rate in our *prr37*^{crispr} tomato line (Figure 29), in *Arabidopsis prr7* mutant line seems also to have an higher germination rate compared to WT (Arana *et al.* 2017).

The *toc1*^{crispr} lines generated can give us some information about the role of SlTOC1 in tomato. The phases of the circadian clock genes in *toc1*^{crispr} are clearly advanced in mostly all the genes examined (Figure 25 and 26), suggesting a shorter period compared to WT. In *Arabidopsis*, the *toc1* mutant line shows shorter period compare to WT (Strayer *et al.* 2000). Also, SlTOC1 seems necessary for the proper oscillation of the gene *SlPRR37* in tomato (Figure 25G). In *Arabidopsis*, *toc1* mutant plants have an early flowering phenotype under short-day conditions (Somers *et al.*

1998). Our tomato *toc1*^{crispr} plants show a clear early flowering phenotype under long-day conditions (Figure 30). Interestingly, *toc1*^{crispr} show a longer process of ripening compared to WT (Figure 31E). We have also found an increase in the germination rate for *toc1*^{crispr} (Figure 29). Similar to the other clock components described above, SlTOC1 seems to have a conserved function in the regulation of clock genes. However, the regulation of biological outputs might differ in tomato and *Arabidopsis*.

2. Clock gene expression in tomato fruits

We have examined the expression of several clock genes in leaves, green and red mature fruits. In *Arabidopsis*, the clock runs at different speed in different organs and the phase of several clock genes peaks at different times depending of the organs studied, (Takahashi et al. 2015; Greenwood *et al.* 2019). Not only the period and phase can change, but also the amplitude can be different between different organs (Takahashi et al. 2015; Bordage *et al.* 2016). In the model legume *Medicago truncatula*, the clock gene homologs *MtLHY*, *MtTOC1a*, *MtPRR5/9* and *MtLUX* are rhythmically expressed in shoots and roots, but only *MtLHY* keeps its rhythmicity in nodule (Kong *et al.* 2020). Our results suggest that this phenomenon could happen also in tomato. Comparing the result under LD vs LL (Figure 5-15), the expression in leaves seems more robust than in fruits. Under LL, the expression of some clock genes such as *SlPRR9* and *SlPRR37* tends to have a phase delay in fruits compared to leaves. Also we can notice the loss of oscillation for some gene, particularly in red fruit, such as *SlPRR5*, *SlLUX-like* and *SlELF3*. These results confirmed in tomato, the clock genes are differentially expressed in leaves and fruits.

Transcriptomic studies in fruits of the *clock*^{crispr} lines helped us to understand the implication of each component into each organ. SlLHY seems to play an important role in fruits. In *lhy*^{crispr} green fruits, nearly all the clock genes studied in lost their oscillation or have an antiphasic expression compared to WT (Figure 17 and 18). Only *SlELF3, SlTOC1* and probably *SlELF4*, keep their oscillations with an advanced phase. In *lhy*^{crispr}, *SlPRR9* expression is altered in leaves compared to WT (Figure 17A), than in green fruit, there is almost no expression of *SlPRR9* in *lhy*^{crispr} (Figure 17B). Similar phenomenon was observed in *Arabidopsis*: *AtPRR9* expression was severely reduced in *cca1/lhy RNAi* in pistils (Okada *et al.* 2022).

Transcriptomic analysis of *prr37*^{crispr} fruits showed similar patterns to those observed in leaves (Figure 22 and 23). Nearly all the clock genes show a phase-delay compared to WT. In red fruits, *prr37*^{crispr} shows higher amplitude of several genes such as *SlLHY, SlLUX-like*, *SlELF4* and *SlTOC1*. This phenomenon was not reported in the literature in *Arabidopsis*, whether for *prr3* or *prr7* mutant lines. Transcriptomic analysis of *toc1*^{crispr} in fruits shows the importance of SlTOC1 for the proper expression of several clock genes such as *SlLHY, SlPRR37, SlPRR5, SlLUX* and *SlLUX-like* (Figure 25 and 26). *SlPRR9*, *SlELF3* and *SlELF4* expressions are affected in *toc1*^{crispr} compared to WT, but they keep their oscillations with a change in amplitude or phase. In *Arabidopsis*, AtTOC1 is essential for the proper expression of mostly all the clock genes in pistils (Okada *et al.* 2022). Thus, the comparison of clock gene expression in fruits and leaves suggest a different role of the different clock components (see Table 3 for resume of the transcriptomic phenotype).

3. Improvement of agronomical traits

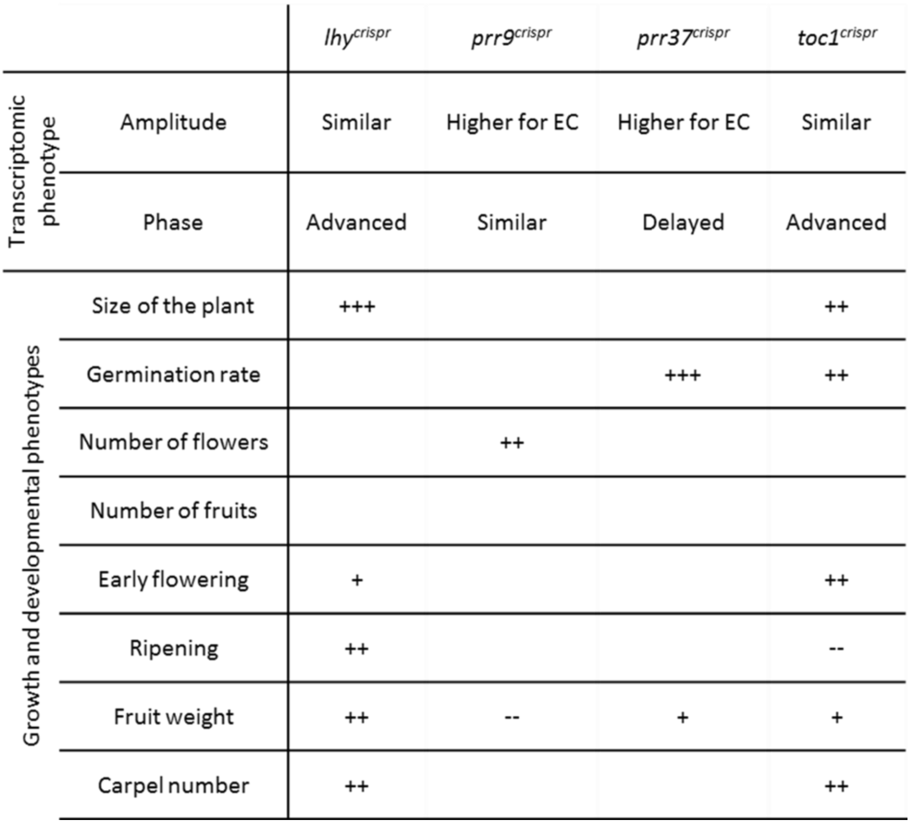
There are several interest to improve agronomical traits of tomato fruits: (1) Yield and productivity: by improving traits like size, weight and number of fruit per plant, it can increase the yield and so the productivity to contributes to the food security and economic prosperity. (2) Market demand: consumers prefer larger, uniform and appealing fruits, controlling the agronomical traits, famer can feat with the market demand to enhance the market competitiveness and profitability for the farmers. (3) Shelf-life and transportation: agronomical traits such as fruit firmness and skin thickness impact the shelf life and post-harvested quality of tomato fruits, improving these traits can extend the storage period, reduce post-harvest losses and make easier the transportation over long distances. (4) Disease and pest resistance: enhancing agronomical traits of pathogen resistance can minimize crop losses and reduce dependence of chemical pesticides. (5) Environmental sustainability: for instance, by developing tomato varieties with higher water-use efficiency, to reduce water consumption of crop production, by addition, traits like drought resistance could enable farming in challenging environment, mitigating the impact of climate change. (6) Agronomical traits influence nutritional composition of tomato fruits, fruits with higher levels of antioxidants, vitamins or any other beneficial compounds could promote consumer well-being. (7) Crop management efficiency: traits like uniform ripening to synchronize harvesting, can facilitate farmer work, reducing labour requirements.

The central oscillator controls the rhythms of many output pathways (see Introduction 3.4). In our studies, we have generated several *clock*^{crispr} lines in tomato not only to understand the function of the clock genes, but also in order to examine the possible improvement of agronomical traits. *lhy*^{crispr} plants are higher compared to WT (Figure 27). *AtCCA1* and *AtLHY* are known to control flowering time and hypocotyl elongation in *Arabidopsis* (Schaffer *et al.* 1998), in fact *cca1/lhy Arabidopsis* plants are smaller and have smaller leaves compared to WT (Mizoguchi *et al.* 2002). *lhy*^{crispr} tomato plants show the opposite with higher plants and bigger leaves compared to WT. On the hand, *lhy*^{crispr} tomato plants have bigger fruits with higher carpel number (Figure 33A and 34, Table 2). There is a direct correlation between these two results: the carpel number affects the size of the fruit (Ariizumi *et al.* 2013; Yuste-Lisbona et al. 2020). *lhy*^{crispr} plants have also higher yield compared to WT (Figure 33C). Regarding the *prr9*^{crispr} line, the plants have more fruits (Figure 32) and are lighter compared to WT (Figure 33A). The negative correlation between these two factors is already known from farmer practice, but almost no studies describe this phenomenon. In Strawberry, Rindom and Hansen (1994) saw this phenomenon: they observed the relationships between fruit size and number of fruits per plant and concluded the existence of a negative correlation between the number of fruits per plants and the weight of the fruit.

prr37^{crispr} and *toc1*^{crispr} tomato plants have higher germination rate compared to WT (Figure 29). In *Arabidopsis*, *toc1* and *prr7* mutant lines seem also to have a higher percentage of germination (Arana *et al.* 2017). *toc1*^{crispr} tomato plants have an early-flowering phenotype (Figure 30A and 31A) similar to *Arabidopsis toc1* mutant plants (Somers *et al.* 1998). Interestingly, we observed in our *toc1*^{crispr} tomato lines, a delay in the ripening of the fruit (Figure 31D), that was not reported before in other species.

Overall, we found that *lhy*^{crispr} has bigger fruit and higher yield; *prr37*^{crispr} and *toc1*^{crispr} have higher germination rate, while *toc1*^{crispr} also, adding an early flowering phenotype with a delay in ripening (see Table 3 for an overview of the main results).

Table 3: Alteration of the agronomical traits of the *clock*^{crispr} lines, compared to WT. Transcriptomic phenotype is about the main clock genes studying in this work. EC: Evening Complex,. “+” or “-”tends to be higher or lower. “++” or “—” significantly higher or lower and “+++” or “---” highly significantly higher or lower.



		lhy^{crispr}	*prr9*^{crispr}	*prr37*^{crispr}	*toc1*^{crispr}
Transcriptomic phenotype	Amplitude	Similar	Higher for EC	Higher for EC	Similar
	Phase	Advanced	Similar	Delayed	Advanced
Growth and developmental phenotypes	Size of the plant	+++			++
	Germination rate			+++	++
	Number of flowers		++		
	Number of fruits				
	Early flowering	+			++
	Ripening	++			--
	Fruit weight	++	--	+	+
	Carpel number	++			++

CONCLUSIONS

CONCLUSIONS

In this Doctoral thesis we have focused on understanding the circadian function in *Solanum lycopersicum* and its role controlling important traits in tomato plants. The main conclusions of this study are summarized below:

1. By analysing the amino acid sequence similarity of the main components of the *Arabidopsis thaliana* circadian oscillator, we have been able to identify the orthologous clock genes in *Solanum lycopersicum*.

2. The identified candidate genes diurnally and circadianly oscillate with a different degree of circadian robustness. Rhythmic circadian expression is weaker in fruits compared to leaves under constant light conditions.

3. Clock gene editing by using CRISPR-cas9 strategies is a useful approach to examine the function of clock genes in *Solanum lycopersicum*.

4. Altering the expression of the clock genes in *Solanum lycopersicum* affects the circadian phase of expression of the other clock genes. Most prominently, affecting *LHY* and also *TOC1* expression leads to an advance phase of most of the clock genes. On the other hand, altering *PRR37* expression results in a delayed circadian phase.

5. Several growth and developmental phenotypes are affected in the CRISPR-Cas9 clock lines. The lhy^{crispr} line displays bigger fruits

and higher yield compared to WT. The *prr9*^{crispr} line produces more flower and fruits per plant albeit with lower weight compared to WT. The *prr37*^{crispr} line has a higher germination rate while the *toc1*^{crispr} line has also higher germination rate, an early flowering phenotype albeit with delayed ripening compared to WT.

SUMMARY

SUMMARY

The circadian clock is a molecular timekeeping mechanism that generates ~24h rhythms in metabolism and physiology in synchronization with the day and night cycle. During the last decade, our understanding of the circadian clock's role in plant biology has gained significant attention due to its potential to improve agronomical traits in the context of the overpopulation and global warming. In this Doctoral Thesis, we have elucidated the pattern of expression of the main clock components in *Solanum lycopersicum*. By using time course analyses by Reverse Transcription – Polymerase Chain Reaction, we found that the circadian oscillation of the main clock components is conserved between *Arabidopsis thaliana* and *Solanum lycopersicum*. We also found that the rhythmic circadian expression is weaker in fruits compared to leaves under constant light conditions. Generation and characterization of tomato mutant lines using the CRISPR-Cas9 technology has provided important information about the clock function in tomato. We found that the lack of LATE ELONGATED HYPOCOTYL (LHY) leads to a circadian advanced phase, plants with bigger size and bigger fruits with higher yield compared to Wild Type. The lack of PSEUDO RESPONSE REGULATOR 9 (PRR9) results in minor circadian defects of gene expression but renders plants, with higher number of flowers and fruits, albeit with smaller size. The lack of PRR37 leads to a circadian phase delay and a higher germination rate. The lack of TIME OF CAB 1 (TOC1) advances the circadian phase, and show higher germination rate. Overall, our results pave the way for further functional studies connecting the circadian clock with improvement of agronomical traits.

RESUMEN

RESUMEN

El reloj circadiano es un mecanismo molecular que genera ritmos biológicos de aproximadamente 24 horas en el metabolismo y la fisiología en sincronización con el ciclo día-noche. Durante la última década, nuestra comprensión del papel del reloj circadiano en la biología de las plantas ha ganado una atención significativa debido a su potencial para mejorar los rasgos agronómicos en el contexto de la superpoblación y el calentamiento global. En esta Tesis Doctoral, hemos dilucidado el patrón de expresión de los principales componentes del reloj en *Solanum lycopersicum*. Mediante el uso de análisis de series temporales mediante la técnica de Transcripción Inversa - Reacción en Cadena de la Polimerasa, encontramos que la oscilación circadiana de los principales componentes del reloj está conservada entre *Arabidopsis thaliana* y *Solanum lycopersicum*. También encontramos que la expresión rítmica circadiana es más débil en los frutos en comparación con las hojas en condiciones de luz constante. La generación y caracterización de líneas mutantes utilizando la tecnología CRISPR-Cas9 ha proporcionado información importante sobre la función del reloj en el tomate. Encontramos que la falta de LATE ELONGATED HYPOCOTYL (LHY) conduce a una fase circadiana avanzada, plantas con mayor tamaño y frutos más grandes, con mayor rendimiento en comparación con el Tipo Salvaje. La falta de PSEUDO RESPONSE REGULATOR 9 (PRR9) resulta en defectos circadianos menores en la expresión génica, pero produce plantas con mayor número de flores y frutos, aunque de menor tamaño. La falta de PRR37 conduce a un retraso de la fase circadiana y a una tasa de germinación más alta. La falta de TIME OF CAB 1 (TOC1) adelanta la fase circadiana y muestra una tasa de germinación más alta. En general, nuestros resultados abren el camino a futuros estudios funcionales que conecten el reloj circadiano con la mejora de los rasgos agronómicos.

MATERIALS AND METHODS

1. Bioinformatics analysis

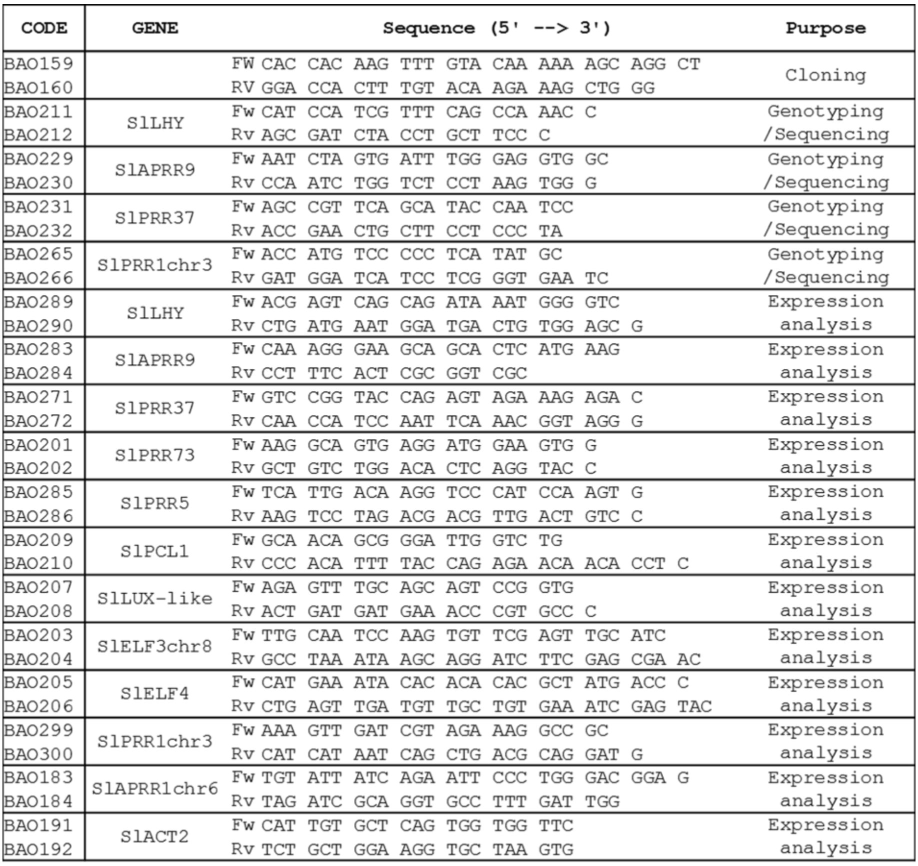
Protein sequences of the *Arabidopsis* genes of interest were obtained from The Arabidopsis Information Resources (TAIR, http://arabidopsis.org) (Berardini *et al.* 2015). Analysis of the *Arabidopsis* clock homologs in *Solanum lycopersicum* was performed by protein-protein Basic Local Alignment Search, using the web-tool (https://blast.ncbi.nlm.nih.gov) (Sayers *et al.* 2010). The protein sequences of the candidates were examined using the current tomato genome (version SL4.0 and annotation ITAG4.0) available in the webpage (http://solgenomics.net) (Fernandez-Pozo *et al.* 2015). The rhythmicity of expression of the candidate genes was examined using the web tool (http://diurnal.sbs.ntu.edu.sg) (Ng *et al.* 2020).

2. Plasmid construction

The CRISPR-Cas9 vectors were kindly provided by Dr. Marianne Mazier from Institute of Agricultural Research of France, INRA (Danilo *et al.* 2018). The guide RNAs (gRNAs) were designed using the web tool http://crispor.tefor.net (Concordet and Haeussler 2018). Two double-strand DNAs including the gRNA fused to the tomato small nuclear RNA (snRNA) U3 or U6 promoters followed by the sgRNA scaffold, both flanked by the attB site, were ordered for each construct (Integrated DNA Technologies IDT). Sl-gRNA-U3 backbones were cloned using the gateway system (BP Clonase II, ThermoFisher) into the pDONR207 vector (Invitrogen) to obtain pDONR207-U3-gRNA. Sl-gRNA-U6 backbones were amplified with Phusion High-Fidelity DNA polymerase (New England BioLabs) using the primers BAO159 (flanked with CACC) and BAO160. Amplification was performed using the following PCR conditions: 98ºC for

30 sec; 25 cycles of 98ºC for 15 sec, 69ºC for 30 sec, 72ºC for 30 sec; and 72ºC for 5 min (see Table 4 for the list of primers and their sequences). The PCR products were cloned into pENTR-D/TOPO by TOPO reaction, following the manufacturer's recommendations (ThermoFisher) to obtain pENTR-U6-gRNA. The pENTR-U6-gRNA was digested using the restriction enzymes XhoI and NsiI (New England BioLabs). After electrophoretic separation, the bands were purified from the gel (QIAquick Gel Extraction kit, Qiagen), and the insert was ligated with the pDONR207-U3 vector digested with SalI and PstI (New England BioLabs) and subsequently ligated using the T4 DNA ligase (New England BioLabs) to obtain pDONR207-U3-U6-doublegRNA. The U3-U6-double gRNA was cloned into the pDE-Cas9 vector (Danilo *et al.* 2018) using the LR reaction following the manufacturer´s recommendations (LR Clonase II, ThermoFisher). We followed this strategy to generate the plasmids: *pDE-CAS9-LHY; pDE-CAS9-PRR9; pDE-CAS9-PRR37;* and *pDE-CAS9-TOC1*, all resistant to Spectinomycin in bacteria and Kanamycin in plants. The plasmids were confirmed by sequencing using a DNA capillary sequencing (ABI3730 DNA analyser, Seqgen). The results were analysed using the software CodonCode Aligner (CodonCode Corporation)

Table 4: List of the primer used in this study.



CODE	GENE	Sequence (5' --> 3')	Purpose
BAO159 BAO160		FW CAC CAC AAG TTT GTA CAA AAA AGC AGG CT RV GGA CCA CTT TGT ACA AGA AAG CTG GG	Cloning
BAO211 BAO212	SlLHY	Fw CAT CCA TCG TTT CAG CCA AAC C Rv AGC GAT CTA CCT GCT TCC C	Genotyping /Sequencing
BAO229 BAO230	SlAPRR9	Fw AAT CTA GTG ATT TGG GAG GTG GC Rv CCA ATC TGG TCT CCT AAG TGG G	Genotyping /Sequencing
BAO231 BAO232	SlPRR37	Fw AGC CGT TCA GCA TAC CAA TCC Rv ACC GAA CTG CTT CCT CCC TA	Genotyping /Sequencing
BAO265 BAO266	SlPRR1chr3	Fw ACC ATG TCC CCC TCA TAT GC Rv GAT GGA TCA TCC TCG GGT GAA TC	Genotyping /Sequencing
BAO289 BAO290	SlLHY	Fw ACG AGT CAG CAG ATA AAT GGG GTC Rv CTG ATG AAT GGA TGA CTG TGG AGC G	Expression analysis
BAO283 BAO284	SlAPRR9	Fw CAA AGG GAA GCA GCA CTC ATG AAG Rv CCT TTC ACT CGC GGT CGC	Expression analysis
BAO271 BAO272	SlPRR37	Fw GTC CGG TAC CAG AGT AGA AAG AGA C Rv CAA CCA TCC AAT TCA AAC GGT AGG G	Expression analysis
BAO201 BAO202	SlPRR73	Fw AAG GCA GTG AGG ATG GAA GTG G Rv GCT GTC TGG ACA CTC AGG TAC C	Expression analysis
BAO285 BAO286	SlPRR5	Fw TCA TTG ACA AGG TCC CAT CCA AGT G Rv AAG TCC TAG ACG ACG TTG ACT GTC C	Expression analysis
BAO209 BAO210	SlPCL1	Fw GCA ACA GCG GGA TTG GTC TG Rv CCC ACA TTT TAC CAG AGA ACA ACA CCT C	Expression analysis
BAO207 BAO208	SlLUX-like	Fw AGA GTT TGC AGC AGT CCG GTG Rv ACT GAT GAT GAA ACC CGT GCC C	Expression analysis
BAO203 BAO204	SlELF3chr8	Fw TTG CAA TCC AAG TGT TCG AGT TGC ATC Rv GCC TAA ATA AGC AGG ATC TTC GAG CGA AC	Expression analysis
BAO205 BAO206	SlELF4	Fw CAT GAA ATA CAC ACA CAC GCT ATG ACC C Rv CTG AGT TGA TGT TGC TGT GAA ATC GAG TAC	Expression analysis
BAO299 BAO300	SlPRR1chr3	Fw AAA GTT GAT CGT AGA AAG GCC GC Rv CAT CAT AAT CAG CTG ACG CAG GAT G	Expression analysis
BAO183 BAO184	SlAPRR1chr6	Fw TGT ATT ATC AGA ATT CCC TGG GAC GGA G Rv TAG ATC GCA GGT GCC TTT GAT TGG	Expression analysis
BAO191 BAO192	SlACT2	Fw CAT TGT GCT CAG TGG TGG TTC Rv TCT GCT GGA AGG TGC TAA GTG	Expression analysis

3. Tomato transformation by *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain GV3101::pMP90 (Larebeke *et al.* 1974) was transformed by electroporation with the plasmids pDE-CAS9-LHY; pDE-CAS9-PRR9; pDE-CAS9-PRR37; and pDE-CAS9-TOC1. Cotyledons of *Solanum lycopersicum* cultivar MicroTOM were transformed following a previously described protocol (Wittman *et al.*

2016). Briefly, tomato seeds were treated with 70% EtOH for 3 min and then sterilized with 1.5% hypochlorite and 0,01% Triton W-100 during 10 min. Seeds were washed three times with sterile water and dried in Petri-dishes. The seeds were kept 2 days in the dark at 4ºC. Ten days before *Agrobacterium* co-incubation, the seeds were sown in germination medium in jars at 22ºC in the dark [GM: 4.3g.L^{-1} Murashige and Skoog Salt; 30g/l sucrose; 100mg/l myo-Inositol; 1ml/l NPT Vitamins stock-solution; pH adjusted to 5.8; with agar (where NPT Vitamins stock-solution: 10mg/ml Thiamine-HCl; 1mg/ml Nicotine acid; 1mg/ml Pyridoxine-HCl)] (Wittman *et al.* 2016). After ten days, small pieces of the cotyledons with about 1 cm long were cut from the centre along the mid-vein on the abaxial surface. Around 20 pieces of cotyledons were placed adaxial surface down in a Petri dish with conditioning medium (CM) and incubated for 2 days at 22ºC in the dark (CM: same as GM media with 0.1mg/l 6-BenzylAminoPurin; 1mg/l α-Naphtalenacetic acid).

An overnight culture of *A. tumefaciens* grown in liquid Luria-Bertani (LB) medium at 28ºC was centrifuged (4000 rpm, 10 min, Room Temperature) (LB: 10g/l peptone; 5g/l yeast extract; 10g/l NaCl). The pellet was resuspended in 10mM of $MgSO_4$ with 150µM of acetosyringone and brought to an OD_{595} of 1. For the co-incubation, two drops of the *Agrobacterium* suspension were applied per cotyledon and co-cultivated during 2 days at 22ºC in dark. The cotyledons were then moved on its abaxial surface into new jars containing selection medium (SM) with of Kanamycin (35mg/l) and Augmentin (150mg/l) to eliminate the *Agrobacterium* (SM: same as germination medium with 1mg/l trans-Zeatin). The jars were moved into plant growth chambers (long days 16 h light: 8 h dark) at 22ºC. After 3 days, the cotyledons were transferred to fresh SM every week. For the next 2 weeks, 50mg/l of Kanamycin was used, and then, from the fourth week, 100mg/l. After 2 months, shoots were cut at the base and transferred to root medium (RM) containing 20mg/l Kanamycin (RM: same as GM with 0.1mg/l Indole-3-Acetic Acid; 500mg/l Vancomycin). When the roots were formed, plants were transferred to soil and grown in the greenhouse with a photoperiod of 14 h light: 10 h dark at 22ºC.

4. DNA extraction and mutation detection

Genomic DNA was extracted from 25mg of fresh frozen leaves. The leaves were ground with a TissueLyser II (Qiagen). Extraction buffer was added (200mM Tris pH7.5; 250mM NaCl; 25mM EDTA; 10% SDS) diluted 10 times in TE buffer (20mM Tris; 25mM EDTA adjusted to pH 8). 1µl of the lysates was used to perform the first PCR screen using the NZYtaq II 2x Green Master mix (Nzytech). Amplification was performed using the following PCR conditions: 95ºC for 3 min; 35 cycles of 95ºC for 30 sec, 52ºC for 30 sec, 72ºC for 1 min; and 72ºC for 5 min. The primers used are BAO211+212 for *lhy*^{crispr}; BAO229+230 for *prr9*^{crispr}; BAO231+232 for *prr37*^{crispr}; BAO265+266 for *toc1*^{crispr} (see Table 4 for the list of primers and their sequences). The PCR products were electrophoretically separated in a 2% agarose gel to detect deletions superior to 20 nucleotides.

Genomic DNA from the positive candidates was extracted from 25 mg of fresh frozen leaves. Extraction buffer was added and samples were centrifuged 5 min at 13000 rpm at Room Temperature (RT). The supernatants were transferred to a new tube containing phenol/chloroform/isoamyalchol (PCI, 25:24:1). After mixing, the samples were centrifuged 10 min at 13000 rpm at RT and the aqueous phases were transferred to a new tube containing an equal volume of isopropanol. After mixing, samples were incubated at RT for 5 min, and then centrifuged for 10 min at 13000 rpm at 4ºC. The supernatant was discarded, and the pellet washed in 70% of EtOH. After another centrifugation of 10 min at 13000 rpm, the pellets were dried under vacuum for 20 min (Concentrator plus, Eppendorf). The DNA was lastly resuspended in 50µL of TE buffer. The extracted genomic DNA was then used as template to amplify the region of the target genes (*LHY*, *PRR9*, *PRR37* or *TOC1*) using primers flanking the target site (see Table 4). Phusion High-Fidelity DNA polymerase (New England BioLabs) was used in standard PCR program: 98ºC for 30 sec; 35 cycles of 98ºC for 10 sec, ##ºC for 20 sec, 72ºC for 30 sec; and 72ºC for 5 min (where ## was adjusted depending of the primers used and following the recommendation of the web tool: https://tmcalculator.neb.com/). PCR products were purified using QIAquick Gel extraction kit (Qiagen) following the manufacture recommendations. The PCR fragments were sequenced using the DNA capillary sequencing (ABI3730 DNA analyser, Seqgen). The results were analysed using the software CodonCode Aligner (CodonCode Corporation).

5. Plant materials, growth conditions and sample collection for transcriptomic assays

Tomato seeds were sown directly in soil and transferred to individual pots after 10 days in a mix of substrate 2:vermiculite:perlite [3:1:1] (substrate 2 from Kasmann Deilmann; perlite expanded y vermiculite exfoliated from Perlindustria). The plants were grown in a Phytotron chamber at constant temperature of 25ºC, with a photoperiod of 14h light : 10h darkness and a light intensity of 150µmol.m^{-2}.s^{-1}. At 10 Days After

Transplanting (DAT10), leaves (25mg) were harvested from the last leaflet of the third true leaf every 4h during a time course analysis over a 24 h cycle. For experiment under Light-Dark conditions (LD), leaves samples were harvested at Zeitgeber Time 3, ZT7, ZT11, ZT15, ZT19 and ZT23. For experiment under continuous light conditions (Light-Light or LL), plants synchronized under light/dark cycles were transferred to LL for 1 day before samples were collected at Circadian Time 27, CT31, CT35, CT39, CT43 and CT47. Other time courses were done using Green Mature Fruits and Red Mature Fruits at DAT60 and DAT88, respectively, under LD and LL (Figure 35). For fruit analyses, the placentas and seeds were removed and the fruits were cut in small pieces and frost in liquid nitrogen. Then, the fruit samples were lyophilised during 3 days for the green mature fruit, and during 7 days for the red mature fruit with vacuum set at -48ºC and 0.05mbar (Lyophiliser Alpha2-4 LD plus from Christ, combined with the Bomb E2M28 from Edwards).

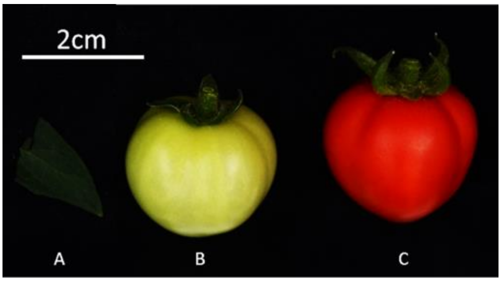


Figure 35: Representative pictures of the leaves and fruit samples harvested. Piece of ultimate leaflet (A), green mature fruit (B) and red mature fruit (C).

6. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from 25mg of fresh frozen leaves and from 12.5 mg of lyophilized fruits using a Maxwell RSC Plant RNA kit (Promega) and following the manufactory recommendations. The concentrations of RNA from red mature fruit were increased by ethanol precipitation. Briefly, 1/10 volume of Sodium acetate (3M, pH5.2) and 3 volume of absolute Ethanol was added. The tubes were incubated overnight at -20ºC and then centrifuged at 13000 rpm for 30 min at 4ºC. The supernatants were removed and the pellet washed with 70% EtOH. After another centrifugation at 13000 rpm for 15 min at 4ºC, the pellets were dried under vacuum (concentrator plus, Eppendorf) and the RNA resuspended in 12µl of DNAse-free water.

The reverse transcription was performed with iScript cDNA synthesis kit (Bio Rad) using 1µg of RNA and following the manufacture recommendations. The cDNA was diluted 3 times before quantitative PCR analyses (QPCR). The standard qPCR mixture consisted of 1 µl of diluted cDNA was mixed to 0.5µL of Forward primer (Fw) and 0.5µl of Reverse primer (Rv) both at 10mM with 5µl of Sybr green mix (Brilliant III ultrafast SYBR qRT-PCR master Mix from Agilent) and water to reach a total reaction volume of 10µl. The RT-qPCRs were performed on a CFX96 Real-time system C1000 touch thermal cycler (Bio-Rad). The qPCR program consisted of a first step at 95ºC during 3min and 40 cycles alternating between 5 sec at 95ºC and 10sec at 60ºC. See Table 4 for the list of primers and their sequences. The tomato *ACTIN2* gene (*SlACT2*, Solyc11g0053300) was used as a reference gene for all the qPCR (González-Aguilera *et al.*

2016). Overall, the qPCR primers were examined with the web tools https://www.ncbi.nlm.nih.gov/tools/primer-blast/ and https://eu.idtdna.com/. To avoid any genomic contamination during the amplification, primers were designed using this priority order: (1) in 3'utr-3'utr; (2) in 3'utr-exon; (3) in exon-exon; (4) in the CDS.

7. Plant materials and growth conditions for phenotypic analysis

Tomato seeds were sown directly in soil and transferred to individual pots (12cm X 12cm) after 10 days in 210 g of soil with a mix of substrate 2:vermiculite:perlite [3:1:1] (substrate 2 from Kasmann Deilmann; perlite expanded y vermiculite exfoliated from Perlindustria). Three biological replicates with at least 7 technical replicates were performed. The plants were grown in a plant growth chamber (ARALAB) at a constant temperature of 22ºC under a long photoperiod of 16h:8h, light intensity of 150 μmol.m-^{2}.s^{-1} and 60% of humidity. The sizes of the plants were measured at DAT20, DAT30, and DAT50.

Equation 1: Hydration (H) and Foliar Mass Area (FMA). FW: Fresh Weight; DW: Dry Weight.

$$H = \frac{FW(g) - DW(g)}{DW\ (g)} \qquad FMA = \frac{DW(mg)}{Area\ (cm^2)}$$

At 3 weeks after transplantation (DAT21), the last leaflet of the 5th true leaf of each plant were photographed to measure the area with the software ImageJ (Schneider *et al.* 2012) and weighted to obtain the Fresh Weight (FW). Samples were dried at 80ºC to obtain the Dry Weight (DW).

Similar analyses were performed at DAT42 (6 weeks after transplantation) with the last leaflet of the 6th true leaf. The Hydration (H) and the Foliar Mass per Area (FMA) were obtained following the equation 1.

Figure 36: Representative pictures of the different stages of tomato fruit ripening. Flower with petal visible (A), Green fruit with diameter of 3mm (B), Green mature fruit (C), Breaking fruit (D) and Red mature fruit (E).

The timing of first flower appearance was recorded (considering the time when the petals appeared in the first flower (see Figure 36). We also examined the timing of the first green fruit appearance, when it reached a diameter of 3mm and breaking (considering breaking when at least 10% of the fruit is turning to yellow) (see Figure 36). Similarly, we also examined the timing of the first fully red fruit appearance (Figure 36). After collection of these data, the delta "Δ" (in days) between the first flower, first green fruit, first breaking and first red fruit for each plant were calculated.

8. Red fruit characterization

Fully red fruits were collected at DAT90 (2 biological replicates; 7 technical replicates) and DAT120 (2 biological replicate; 7 technical replicates). Samples from these two time points were pooled and weighted to get the FW and also the yield of each plant. The two bigger fruits of each plant were selected and performed a longitudinal (Figure 37) or a transversal cut. Images from the transversal cuts were used to count the number of carpels per fruit. Images from the longitudinal cuts were used for measurements like area (A^2), width at mid height (Wm), maximum width (W), height at mid width and maximum height (Figure 37A). The proximal width at 10% from to the top (w1) and the distal width also at 10% from the bottom (w2) were calculated (Figure 37B). The fruit shape index I and II, fruit shape triangle, proximal and distal fruit blockiness, shoulder height were calculated following the equation 2 (Figure 37C) (Brewer *et al.* 2006). The fruit shape index is defined as the ratio of height to width, a value higher of 1, indicates an elongated fruit, lower than 1 a squat fruit, equal to a round fruit. The fruit shape triangle is defined as the ratio of the proximal end width to distal end width (both at 10% from the top and bottom, respectively), a value higher than 1 indicates that the proximal end is wider than the distal end, a value lower than 1, the opposite. The proximal and distal fruit blockiness are the ratio of fruit width at the proximal or distal end (at 10%) to mid width. Shoulder height is calculated to evaluate the indented shape at the proximal end of the fruity. The higher is the value, the more indented is the fruit at the proximal end. The software TomatoAnalyzer was used for all the measurements (Rodríguez *et al.* 2010).

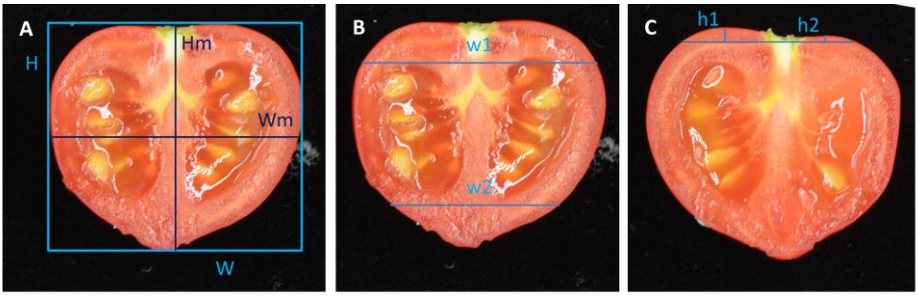


Figure 37: Descriptors of fruit morphology. (A) H and W are maximum Height and Width, Hm and Wm are mid Height and mid Width. (B) w1 and w2 are Width at 10% from each extremity of H. (C) h1 and h2 are the maximal distance to the perpendicular passing by the most indented point at the proximal end.

Equations 2: Fruit parameters. H: maximum Height; W: maximum Width; Hm: mid Height; Wm: mid Width; w1 and w2: Width at 10% from each extremity of H; h1 and h2: maximal distance to the perpendicular passing by the most indented point at the proximal end.

$$Fruit\ shape\ index\ I = H/W$$

$$Fruit\ shape\ index\ II = Hm/Wm$$

$$Fruit\ shape\ triangle = w1/w2$$

$$Proximal\ fruit\ blockiness = w1/Wm$$

$$Distal\ fruit\ blockiness = w2/Wm$$

$$Shoulder\ height = (h1 + h2)/2H$$

9. Germination rate

Seeds were sown in petri dishes containing Murashige and Skoog media in half concentration (½MS) without additional vitamins or sugars (Murashige and Skoog 1962). We used about 15 seeds per plate, 2 plates per line and 2 biological replicates. Seeds were grown in a plant growth chamber (Phytotron) at constant temperature of 25ºC, with a photoperiod of 14h light: 10h darkness and a light intensity of 150μmol.m^{-2}.s^{-1}. The rate of germination was checked every day considering as germinated the visible embryo outside from the seed coat.

10. Statistical analysis and graphic representation

The statistical analyses were done using ANOVA 1 or ANOVA 2. The hypothesis H1 was accepted when the pvalue was below of 0.05. In these cases, Dunnett's multiple comparison test was applied for comparison against WT. The software GraphPad was used to perform these analyses and to make the graphics (Prism, version 6.01, https://www.graphpad.com).

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ACKNOWLEDGEMENTS

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Now I would like to thank the people close to me who have supported me throughout these years. First and foremost, my parents, as it is thanks to them that I am where I am today. My brother and sister, who have always supported me. My friends with whom I lived for many years at WarD. The mother of my daughter, who also supported me in my doctorate. Chema, who has always been there for me. Claudia, who recently gave me the strength to finish, and without whom my thesis would have been in Comic Sans MS with a rather dull cover page. And above all, to my daughter, Meïga, for existing.

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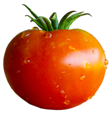

The circadian clock is a molecular timekeeping mechanism that generates ~24h rhythms in metabolism and physiology in synchronization with the day and night cycle. During the last decade, our understanding of the circadian clock's role in plant biology has gained significant attention due to its potential to improve agronomical traits in the context of the overpopulation and global warming. In this Doctoral Thesis, we have elucidated the pattern of expression of the main clock components in Solanum lycopersicum. By using time course analyses by Reverse Transcription – Polymerase Chain Reaction, we found that the circadian oscillation of the main clock components is conserved between Arabidopsis thaliana and Solanum lycopersicum. We also found that the rhythmic circadian expression is weaker in fruits compared to leaves under constant light conditions. Generation and characterization of tomato mutant lines using the CRISPR-Cas9 technology has provided important information about the clock function in tomato. We found that the lack of LATE ELONGATED HYPOCOTYL (LHY) leads to a circadian advanced phase, plants with bigger size and bigger fruits with higher yield compared to Wild Type. The lack of PSEUDO RESPONSE REGULATOR 9 (PRR9) results in minor circadian defects of gene expression but renders plants, with higher number of flowers and fruits, albeit with smaller size. The lack of PRR37 leads to a circadian phase delay and a higher germination rate. The lack of TIME OF CAB 1 (TOC1) advances the circadian phase, and show higher germination rate. Overall, our results pave the way for further functional studies connecting the circadian clock with improvement of agronomical traits.

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