Molecular and transcriptional structure of the petal and leaf circadian clock in *Petunia hybrida*

Marta I. Terry[1], Marta Carrera-Alesina[1], Julia Weiss[1], Marcos Egea-Cortines[1]

[1]Genética Molecular, Instituto de Biotecnología Vegetal, Universidad Politécnica de Cartagena 30202 Cartagena, Spain

Marta I. Terry - marta.terry@edu.upct.es

Marta Carrera-Alesina - martacalesina@gmail.com

Julia Weiss - julia.weiss@upct.es

Corresponding author: Marcos Egea-Cortines marcos.egea@upct.es

Telephone: +34868071075

Date of submission:

Number of tables: 2

Number of figures: 6

Word count:6024

Running title: The circadian clock of leaves and petals in petunia

Highlight: The petunia leaf circadian clock shows maxima during the day while petal clock does it during the night. Reaction to dark is organ specific.

Abstract

The plant circadian clock coordinates environmental signals with internal processes. We characterized the genomic and transcriptomic structure of the *Petunia hybrida* W115 clock in leaves and petals. We found three levels of evolutionary differences. First, *PSEUDO-RESPONSE REGULATORS PhPRR5a*, *PhPRR5b*, *PhPRR7a*, *PhPRR7b*, and *GIGANTEA PhGI1* and *PhGI2*, differed in gene structure including exon number and deletions including the CCT domain of the PRR family. Second, leaves showed preferential day expression while petals tended to display night expression. Under continuous dark, most genes were delayed in leaves and petals. Importantly, photoperiod sensitivity of gene expression was tissue specific as *TIMING OF CAB EXPRESSION PhNTOC1* was affected in leaves but not in petals, and *PhPRR5b*, *PhPRR7b* and the *ZEITLUPE* ortholog *CHANEL*, *PhCHL*, were modified in petals but not leaves. Third, we identified a strong transcriptional noise at different times of the day, and high robustness at dawn in leaves and dusk in petals, coinciding with the coordination of photosynthesis and scent emission. Our results indicate multilayered evolution of the *Petunia* clock including gene structure, number of genes and transcription patterns. The major transcriptional reprogramming of the clock in petals, with night expression may be involved in controlling scent emission in the dark.

Keywords: Circadian rhythms, free running conditions, photoperiod, paralogs, *Petunia hybrida*, Solanaceae, transcriptional noise.

Abbreviations: CCT: CONSTANS, CONSTANS-like, and TIMING OF CAB EXPRESSION 1 domain, Ct: Cycle threshold, PaxiN: *Petunia axillaris*, PhACT: *ACTIN*, PhELF4: *EARLY FLOWERING 4*, PhFKF: *FLAVIN-BINDING KELCH REPEAT F-BOX*, PhGI1: *GIGANTEA 1*, PhGI2: *GIGANTEA 2*, PhLHY: *LATE ELONGATED HYPOCOTYL*, PhPRRs (PhPRR3, PhPRR5a, PhPRR5b, PhPRR7a, PhPRR7b and PhPRR9): *PSEUDO-RESPONSE REGULATORS*, PhTOC1: *TIMING OF CAB EXPRESSION 1*, PhCHL: *CHANEL* (*ZEITLUPE*), Ph: *Petunia hybrida*, PinfS6: *Petunia inflata*, REG: Response regulatory domain, ZT: Zeitgeber time.

Introduction

Organisms, from bacteria to human beings, are subjected to periodic oscillations in the environment due the planet rotation around its axis. Circadian clocks are a complex set of genes allowing organisms to anticipate and adapt to daily environmental variations. In plants, the circadian clock is a network of interlocked loops comprising transcriptional, translational and posttranslational coordination (Harmer, 2009). Circadian processes have been studied in plants for a long period of time (see McClung for a historical overview, (McClung CR, 2006)). Most molecular studies have been done in *Arabidopsis thaliana*. The Arabidopsis core clock is formed by several genes. Two MYB transcription factors *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, *LATE ELONGATED HYPOCOTYL (LHY)* and the *PSEUDO RESPONSE REGULATOR TIMING OF CAB EXPRESSION (TOC1*) form the so-called core clock. Later studies found other clock components including the *PSEUDO-RESPONSE REGULATOR* gene family (*PRR*), out of which PRR3, PRR5, PRR7 and PRR9 are clock genes, and the Evening Complex (EC), which is formed by the *EARLY FLOWERING 3 (ELF3)*, *EARLY FLOWERING 4 (ELF4)* and *LUX ARRHYTMO (LUX)* proteins. In addition, other genes playing a key role and considered part of the clock include the protein with blue light reception capacity ZEITLUPE *(ZTL)* and the single copy gene *GIGANTEA (GI)*. The various models developed are based on mutually repressing genes and a set of activating genes coded by the *REVEILLE* MYB transcription factors (Hsu *et al.*, 2013). Every new discover has added a level of complexity and new interpretation of the circadian clock model (Hernando *et al.*, 2017).

Two aspects emerge from comparative genomics with lower organisms and within higher plants. First the core clock components identified in the picoeukaryote *Ostreococcus* comprise a *MYB* gene homolog to *LHY* and a *PRR* gene similar to *TOC1* (Corellou *et al.*, 2009). There is an additional blue-light receptor component with histidine kinase activity and circadian clock effects (Djouani-Tahri *et al.*, 2011). So, basic clocks maybe found with two or maybe three components that function via transcriptional control. A second aspect is that the fine tuning of the different clock modules is based to a large extent on protein-protein interactions. As protein complexes require certain stoichiometries to maintain their function they are target of genetic constraints in terms of gene dosages and are especially sensitive to gene

duplications. Duplicated genes follow four paths including gene loss, maintenance of redundancy, subfunctionalization or neofunctionalization (Airoldi and Davies, 2012). Plant genomes have been subject to genome duplications and, in some cases, followed by non-random elimination of duplicated genes (Adams and Wendel, 2005; Wendel *et al.*, 2016). In *Brassica*, polyploidization events have involved subsequent gene loss but with a preferential retention of circadian clock genes as compared to house-keeping genes, supporting a gene dosage sensitivity model (Lou *et al.*, 2012).

The genomes of the garden petunia and its ancestors *Petunia axillaris* and *P. integrifolia* have been recently sequenced (Bombarely *et al.*, 2016). Petunia forms an early branching in the Solanaceae clade departing from *Solanum lycopersicon, S. tuberosum, Nicotiana spp.* and *Capsicum spp.* that have a chromosome number of n=12. Petunia has n=7 and this, together with a high activity of transposition, may have shaped a somewhat different genome evolution. Petunia shares a paleohexaplodization specific to the Solanaceae. A comprehensive analysis of the circadian clock genes found in the *Petunia* genomes shows that there is a set of genes that has remained as single copy. These include the petunia orthologs for *PRR9*, *PRR3*, *TOC1* and *LHY*. In contrast, other genes are present in two to four copies, *PRR7*, *PRR5*, *GI*, *ELF3* or *ELF4* (Bombarely *et al.*, 2016). Altogether these data indicate a possible departure of the circadian clock network from the one known in Arabidopsis, and suggests the evolution of the clock at different levels including gene structure, expression pattern and genetic functions.

The bulk of work on plant circadian rhythms has been done in Arabidopsis using leaf tissue and seedlings. Like in animals, there is important evidence that the circadian clock expression network differs between different organs. The current view is that the shoot apical meristem may work as a center of coordination (Takahashi *et al.*, 2015), and leaves and roots differ in the regulatory network, as a result of differences in light inputs (James *et al.*, 2008; Bordage *et al.*, 2016).

Petal development starts with the activation of the so-called B function genes in both gymnosperms and angiosperms (Theissen and Becker, 2004). The initial transcriptional activation is followed at early stages by an autoregulatory positive regulation of the MADS-

box genes controlling petal morphogenesis in *Antirrhinum*, Arabidopsis and petunia (Schwarz-Sommer *et al.*, 1992; Goto and Meyerowitz, 1994; Jack *et al.*, 1994; Zachgo *et al.*, 1995; Samach *et al.*, 1997; Vandenbussche *et al.*, 2004). Once organ identity is established and right after anthesis, there is a transcriptional reprogramming (Manchado-Rojo *et al.*, 2012). Furthermore, in sympetalous flowers with petals forming a tube and a limb, both parts of the flower appear to have different functions and transcriptional control (Delgado-Benarroch *et al.*, 2009; Manchado-Rojo *et al.*, 2014). The petal function after anthesis includes concealing the sexual organs and attracting pollinators. The lifespan of a flower is relatively short with most flowers surviving two to five days after anthesis. After anthesis, metabolism and scent emission changes rapidly (Muhlemann *et al.*, 2012; Weiss *et al.*, 2016). Flowers enter rapid senescence upon pollination as a result of ethylene release (Shaw *et al.*, 2002; van Doorn and Woltering, 2008; Liu *et al.*, 2011).

Floral scent release depends on petal development in a quantitative way (Manchado-Rojo *et al.*, 2012), and is circadian regulated in monocots and dicots such as *Antirrhinum*, *Narcissus*, rose or petunia (Helsper *et al.*, 1998; Kolosova *et al.*, 2001; Verdonk *et al.*, 2003; Hoballah *et al.*, 2005; Ruíz-Ramón *et al.*, 2014). Most flowers analyzed emit scent preferentially during the day or during the night. The *LHY* and *ZTL* orthologs control scent emission in *Petunia* and *Nicotiana attenuata* (Fenske *et al.*, 2015; Yon *et al.*, 2015; Terry *et al.*, 2019). Both emit higher quantities during the night, indicating an identity and circadian component controlling this trait.

In the current work, we have addressed the structure of the petunia circadian clock from three different perspectives. The gene structure diverges as *PRR* paralogs have different intron numbers and *PhGI1* and *PhGI2* vary in the coding region. The transcriptional structure showed maximum expression during the day in leaves and during the dark in petals. This maximum tended to delay in both tissues under constant darkness conditions. We further identified opposite levels of transcriptional noise at dawn in leaves and dusk in petals. Our results reflect the evolution of the plant circadian clock at different overlapping levels and indicate an organ specific transcriptional structure of the plant circadian clock.

Materials and Methods

Plant materials and experiment design

We used the *Petunia hybrida* W115 Mitchell for all the analysis. Plants were grown in the greenhouse under natural conditions. Experiments under controlled conditions in growth chambers were performed as described (Mallona *et al.*, 2011*a*), with the following modifications. For the control experiment, plants were adapted to light:dark growth chamber conditions for at least 1 week. Day:night (12LD) conditions were matched with thermoperiods of 23 °C:18 °C during the light and dark periods. Zeitgeber time (ZT) was defined as ZT0 for light on and ZT12 for light off. In the second experiment, plants were transferred from 12LD cycle to a continuous dark cycle (12DD) with the same temperature regimes.

Flowers were marked before opening, and samples were taken at day 2-3 after anthesis. We used the petal limbs for all experimental procedures. We used young leaves with a length of 1.5-2.5 cm for all the experiments. Sampling of petal limbs and leaves was made every three hours, starting at ZT0 and tissues were immediately frozen in liquid nitrogen. In the case of 12DD experiment, sampling also started at ZT0, during the first 24h under continuous dark.

Phylogeny and bioinformatics

Gene models of Solanaceae were obtained from (https://solgenomics.net/), *Antirrhinum* from (http://bioinfo.sibs.ac.cn/Am/) (Li *et al.*, 2019*b*), TAIR (https://www.arabidopsis.org/), Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and NCBI (https://www.ncbi.nlm.nih.gov/). We used the corresponding predicted proteins to identify the intron-exon boundaries using Genewise (Birney *et al.*, 2004). The corresponding exon-intron boundaries were plotted using the exon-intron graphic maker (http://wormweb.org/exonintron). Protein alignment was performed with CLUSTALX (Larkin *et al.*, 2007). Phylogenetic analysis was performed with the R libraries “ape” and “phangorn” (Paradis *et al.*, 2004; Schliep, 2011) (R version 3.5.1), using the Maximum Likelihood as statistical method, JTT (Jones, Taylor and Thornton, (Jones *et al.*, 1992)) as model of amino acid substitution and 500 bootstrap replicates. Trees were visualized and annotated with “ggtree” (Yu *et al.*, 2017) using R. Protein domains were predicted using the web-based tool PROSITE (Hulo *et al.*, 2006), schematic proteins were plotted with the R package “drawProteins” (Brennan, 2018). The protein sequences used in the phylogenetic reconstruction are listed in the Supplementary Table S1 and Supplementary Table S2.

Detection of rhythmic gene expression was performed using the non-parametric statistical algorithm JTK_CYCLE (Hughes *et al.*, 2010) implemented in the R package "MetaCycle" (Wu *et al.*, 2016). We analyzed leaves and petals, under two light conditions, 12h light/12h dark (12LD) and constant darkness (12DD). Differences between two time series, were tested using an harmonic ANOVA (HANOVA) implemented in the R package "DODR" (Thaben and Westermark, 2016). We plotted the graphics with "ggplot2" (Wickman, 2017).

Gene expression analysis by qPCR

RNA was extracted from three biological replicates per time point of leaves and corollas using acid phenol (Box *et al.*, 2011). Concentrations were measured using NanoDrop (Thermo-Fisher). Equal amounts of total RNA were used to obtain cDNA using Maxima kits (Thermo-Fisher).

PCR analysis was performed as described before (Mallona *et al.*, 2010), the following protocol was used for 40 cycles: 95 ºC for 5 s, 60 ºC for 20 s and 72 °C for 15 s (Clontech SYBR Green Master Mix and Mx3000P qPCR Systems, Agilent Technologies). Primers for circadian clock genes were designed using pcrEfficiency (Mallona *et al.*, 2011*b*) (Supplementary Table S3) and the following protocol was used for 40 cycles: 95 ºC for 5 s, 60 ºC for 20 s (55 ºC for *PhGI1* and *PhGI2*) and 72 ºC for 15 s. Samples were run in duplicate. Primer combinations were tested with genomic DNA from Mitchell and we found that all of them gave a single copy DNA on agarose gels. The endpoint PCR was further verified by melting point analysis where all primer combinations gave a single peak of melting (Supplementary Fig. S1). Normalized expression was calculated as described (Schmittgen and Livak, 2008) and *PhACT* was the internal control gene, a stable gene in circadian studies in petunia leaves and petals (Terry *et al.*, 2019).

Results

The duplicated *PRR5*, *PRR7* and *GI* diverge in intron number and coding sequence

We used the laboratory line *Petunia hybrida* W115, also known as Mitchell, which contains

the circadian clock genes corresponding to *P. axillaris* (Bombarely *et al.*, 2016) for a detailed analysis of the structure of the *PRR* and *GI* paralogs. Several genes forming the morning and evening loops of the circadian clock in petunia have undergone gene duplication. The genome of petunia has seven *PRR* genes as *PRR7* and *PRR5* are duplicated both in *P. axillaris* and *P. integrifolia* while Arabidopsis has the canonical set of five genes, *PRR1* or *TOC1, PRR3, PRR5, PRR7* and *PRR9* involved in circadian regulation (Bombarely *et al.*, 2016). We reconstructed a phylogenetic tree of *PRR* genes of Solanaceae and Arabidopsis (Supplementary Table S1) in order to deduce the evolutionary relationships of the duplicated genes. As found previously for other Angiosperms, the *PRR* genes of Solanaceae form three major clades: the *TOC1/PRR1* clade, the *PRR7/3* clade and the *PRR9/5* clade (Fig. 1) (Takata *et al.*, 2010). The *PRR5a* genes of *P. axillaris*, *P. integrifolia* are closer to the Arabidopsis *AtPRR5* while the rest of the *PRR* genes of Solanaceae, including the *PRR5b,* form an additional subclade. This topology indicates that the *PRRa* paralogs may be an ancestral form and the *PRRb* may have been formed later and retained, in some cases as single copy genes. The *PRR7* genes also showed a similar topology where *PaxiNPRR7a* and *PinfS6PRR7a* are closer to the Arabidopsis gene than the single copy genes of the rest of the Solanaceae, and the *PRR7b* paralogs. This topology is also seen in petunia *PRR9*, *PRR3* and *TOC1* that are somewhat between the Arabidopsis gene and the rest of the Solanaceae, according to the early departure of *Petunia* from the rest of the family (Bombarely *et al.*, 2016).

We found that the gene models for *PhPRR5a* and *PhPRR5b* differ in the number of exons comprising the coding region as *PhPRR5a* has seven and *PhPRR5b* eight exons (Supplementary Fig. S2). The gene model in Arabidopsis comprises 6 exons in AT5G24470 (*AtPRR5*), indicating that changes in intron-exon structure has occurred in the evolution of the *PRR* family. The number of exons also differed between *PhPRR7a* with eight exons while *PhPRR7b* had seven exons. The Arabidopsis AT5G02810 *AtPRR7* has nine exons out of which eight correspond to coding region, thus coinciding with the phylogenetically closer *PhPRR7a*.

The PRR family of Arabidopsis has two conserved domains: REG (Response Regulatory Domain) and a CCT (CONSTANS, CONSTANS-like, and TIMING OF CAB EXPRESSION 1 [TOC1/PRR1]) (Liu *et al.*, 2016) (Supplementary Fig. S3A). We used Arabidopsis as

model and we compared it with petunia sequences. We found that all the PRR members of *P. axillaris* and *P. inflata* shared the REG domain (Supplementary Fig. S3A). The CCT domain was found in all the coding genes except for PaxiNPRR7b, PinfPRR7a and PinfPRR7b. The presence of the CCT domain in PaxiNPRR7a and absence from the rest of the gene group in petunia was surprising, thus we analyzed other Solanaceae, member of the Convolvulaceae (*Cuscuta australis* and *Ipomea nil*) and Plantaginaceae (*Antirrhinum majus*). We found that the CCT domain was absent in the Solanaceae analyzed (*Capsicum annuum*, *C.baccatum*, *Nicotiana benthamiana*, *N.sylvestris*, *N.tabacum*, *N.tomentosiformis*, *Petunia axillaris*, *P. inflata*, *Solanum lycopersicum*, *S.melongena*, *S.pennellii*, *S.pimpinellifolium*, *S.tuberosum*) (Supplementary Fig. S3B). However, the CCT domain could be found in the rest of the species analyzed. This indicates an early change in the PRR7 family in Solanaceae with possible implications in clock functioning.

GIGANTEA is a single copy gene in the Arabidopsis genome (Fowler *et al.*, 1999) and it is found in one to three copies in the Solanaceae genomes (Bombarely *et al.*, 2016). The genes *PaxiNGI1* and *PaxiNGI2* are present in the genome of *P. hybrida* Mitchell. PhGI1, PinfS6GI1 and PinfS6GI1 share an N-terminus conserved with AtGI that was absent in PhGI2 (Fig. 2, Supplementary Fig. S4, Supplementary Table S2). Furthermore, PhGI2 has a 41 amino acid insertion that was not conserved in PinfS6GI2 or other GI genes. The PinfS6GI3 is much shorter that the other paralogs, a feature conserved in *N. benthamiana* GI3 (Fig. 2). The PinfSGI1 had an additional C-terminal fragment of 105 aminoacids absent from the rest of the GI genes analyzed (Fig. 2, Supplementary Fig. S4).

We can conclude that the structural evolution of core circadian clock genes has occurred at several levels including changes in the number of retained paralogs, gene structure and coding region.

The leaf clock has its maximum during the day while the petal clock shifts towards the night

The current model of the plant circadian clock defines three loops called morning, central and evening loop. These describe the time of the day when certain genes are preferentially expressed (Pokhilko *et al.*, 2012). We established the expression patterns of the different

clock genes in leaves and petals. As the genes contained in *P.hybrida cv Mitchell* correspond to *P.axillaris*, we further describe them as *Ph* genes. These included the morning loop genes *PhPRR9*, *PhPRR7a*, *PhPRR7b*, *PhPRR5a*, *PhPRR5b* and *PhPRR3*. The core loop was represented by *PhTOC1* and *PhLHY*. Finally, the evening genes analyzed included *PhGI1*, *PhGI2*, *PhELF4*, *PhCHL* and *PhFKF*. This analysis was performed in petunia that was acclimated to light:dark conditions of 12 hour light and 12 dark (12LD) or continuous dark (12DD) conditions.

We compared three parameters between leaves and petals at 12 hours light/12 hours dark: rhythmicity of expression (oscillation), time point with maximum expression (phase) as well as amplitude, defined as is the difference between the peak or trough (maximum or minimum) and the mean value of a wave (Supplementary Table S4). Concerning the rhythmicity, most genes showed a rhythmic oscillation pattern except *PhELF4 and PhCHL* in leaves, and *PhCHL* and *PhPRR7* in petals (Supplementary Table S4).

Concerning the time of peak expression, most genes had their maximum expression during the light phase in leaves, except *PhELF4* and *PhLHY* at ZT15 and ZT21 respectively. The light phased genes peaked either during the morning at ZT4.5 (*PhPRR5a* and *PhCHL*), during midday at ZT 7.5 (*PhPRR5b*, *PhPRR7a*, *PhPRR9* and *PhTOC1),* towards the afternoon at ZT9 *(PhGI1*, *PhGI2*, *PhPRR3* and *PhPRR7b*) or at dask at ZT 10.5 (*PhFKF*). In contrast, most of these genes shifted their expression maximum to the dark period in petals (Fig. 3) with the exception of *PhCHL, PhPRR9* and *PhPRR7a.* Among those genes that maintained their expression peak during the day or night, *PhPRR9, PhCHL* and *PhELF4* showed a delay and *PhPRR7a* an advance of 1.5 hours compared to leaves. The genes that reached their maximum during the dark period in petals could be divided in those with a peak expression early at night at ZT12 (*PhGI2* and *PhPRR7b),* a peak towards the middle of the night at ZT 13.5 and ZT15 (*PhGI1*, *PhPRR3*, *PhPRR5a* and *PhPRR5b, PhFKF*) and those with a maximum expression at the end of the night at ZT21 (*PaxiELF4* and *PhTOC1)* (Table 1). The only gene showing a maintained expression maximum in leaves and petals was *PhLHY.*

We also found differences in amplitude between tissues. In general, amplitude of the clock genes was higher in leaves than in petals including *PhGI1, PhGI2, PhFKF* and the *PRR* genes

PhPRR9, *PhPRR7b* and *PhTOC1*.The only gene showing larger amplitude in petals was *PhELF4* (Fig. 4, Supplementary Table S4). From all our observations we can conclude that the clock transcriptional structure differs in several ways between leaves and petals. First a robust rhythmic pattern was observed for all genes tested except *PhCHL* that was arrhythmic, *PhELF4* in leaves and *PhPRR7a* in petals. Most genes showed day phase in leaves and night phase in petals. Finally, the petal clock was somewhat dampened compared to leaves.

The clock shows higher oscillation in petals than leaves under continuous dark

In order to study the entrainment of the petunia circadian clock to the light:dark cycle, petunia plants were transferred from light:dark (12LD) conditions to continuous darkness (12DD). Under constant darkness the genes *PhLHY* and *PhPRR7a* lost their significant oscillations in leaves (Table1). Interestingly, the gene *PhELF4* that was not rhythmic under LD conditions (Table 1) but displayed a robust oscillation in leaves under 12DD conditions. Finally, *PhPRR9* was not rhythmically expressed under a 12DD cycle in petals (Table 1). The rest of the genes analyzed maintained a rhythmic expression except for *PhCHL* that lacked a rhythm in any of the tissues or conditions analyzed, and *PhPRR7a* that was not rhythmic in petals.

We compared the expression between 12LD and 12DD in leaves (Fig. 4). We classified the clock genes in three groups either showing a delay in maximum expression between 1.5 and 7.5 hours (*PhPRR9*, *PhPRR5a*, *PhPRR5b*, *PhTOC1*, *PhGI1*, *PhGI2*, *PhFKF* and *PhCHL)* an advance: *PhPRR7b*, *PhPRR3* (1.5 hours) and *PhLHY* (18 hours) or a maintained maximum expression regardless of photoperiod (Table 1) (*PhPRR7a* and *PhELF4)*.

In petals, *PhPRR9*, *PhPRR7b*, *PhPRR5b*, *PhGI1*, *PhGI2* and *PhCHL* delayed their maximum expression between 1.5 and 10.5 hours. *PhPRR7a* and *PhLHY*, peaked 1.5 and 19.5 hours earlier, respectively. The last group included those genes that did not show differences in phase under 12LD or 12DD conditions: *PhPRR5a*, *PhPRR3*, *PhTOC1*, *PhELF4* and *PhFKF* (Table 1).

Altogether, *PhLHY* showed advanced expression under DD conditions while *PhGI1*, *PhGI2*, *PhPRR5b*, *PhPRR9* and *PhCHL* were delayed in both leaves and petals. The only gene that remained robust was *PhELF4*. Thus, these genes were homogenously affected by

328 photoperiod. In contrast, *PhFKF*, *PhPRR5a*, *PhPRR7a*, *PhPRR7b*, *PhPRR3* and *PhTOC1*
329 showed an organ specific change in phase in response to free running conditions (Table 1).

330

331 We compared the amplitude of clock genes in petunia leaves and petals under 12LD and
332 12DD. In leaves, we found that all genes showed a lower amplitude in continuous darkness
333 except *PhELF4* displaying higher amplitude under 12DD (Supplementary Table S4). In petals
334 the rhythmic expression dampened in *PhPRR9*, *PhPRR7a*, *PhPRR7b*, *PhPRR3*, *PhTOC1*,
335 *PhGI1*, *PhGI2*, *PhFKF*, *PhCHL* and *PhLHY*. In contrast, the rhythm of *PhPRR5a*, *PhPRR5b*
336 and *PhELF4* had higher amplitudes (Supplementary Table S4).

337

338 **Rhythmicity and photoperiod-sensitivity are tissue specific**

339 An important paradigm in the analysis of circadian clock gene expression is the effect of free
340 running conditions on the genes thought to have a circadian control (Somers *et al.*, 1998). We
341 analyzed several parameters of circadian clock genes including phase, noise or amplitude in
342 two tissues and light conditions using Harmonic ANOVA (Thaben and Westermark, 2016).
343 These parameters resulted in a specific gene expression pattern that was compared in both
344 tissues under LD and DD cycles (Table 2). We found that *PhELF4*, *PhLHY*, *PhPRR5a*,
345 *PhPRR7a* and *PhPRR9* were stable regardless of the tissue or photoperiod ($p > 0.05$). In
346 contrast, *PhFKF*, *PhGI1*, *PhPRR3* and *PhTOC1* showed a different expression pattern
347 between leaves and petals under a 12LD cycle ($p < 0.05$). In contrast to LD conditions, under
348 12DD *PhGI1*, *PhPRR5b* and *PhPRR7b* were differentially expressed in leaf versus petal.
349 When we compared leaves at 12LD versus 12DD, *PhGI1*, *PhGI2* and *PhTOC1* showed
350 significant changes whereas in petals this group included *PhGI1*, *PhGI2*, *PhPRR5b*, *PhPRR7b*
351 *and PhCHL* (Table 2).

352 These results indicate that there are two sets of genes with different rhythms in leaves and
353 petals and a group of stable genes comprising *PhELF4*, *PhLHY*, *PhPRR5a*, *PhPRR7a* and
354 *PhPRR9*. Furthermore, the effect of photoperiod appeared to be organ-specific for those genes
355 that showed significant changes.

356

357 **Transcriptional noise is gene and tissue specific**

Although gene expression quantities were determined for the same set of mRNA extractions, the degree of significance in terms of gene expression levels was not always as expected based on average expressions. This indicated that some genes had robust expression levels while others appeared to be very variable. In order to quantify the dispersion of data, we plotted the normalized Ct values for all genes, dividing the Ct of the clock gene by the Ct of the reference gene *PhACT* (Fig. 5, Fig. 6) and calculated the coefficient of variation (CV) for all time points (Supplementary Table S5). We found that the data dispersion was very different between genes, tissues and light conditions. The gene with the maximum transcriptional noise was *PhLHY* in petals at ZT0 and 12LD (CV 24.81) while *PhPRR7a* in leaves showed the lowest at ZT0 and 12LD (CV 0.56) (Supplementary Table S5). In addition, transcriptional noise seemed to change during the day. In leaves under a light:dark cycle, the highest noise was found at ZT9 (average CV 9.19) and the lowest, at ZT18 (average CV 4.35). In contrast, in petals, the maximum noise was at ZT0 (average CV 10.33) and the minimum, at ZT12 (average CV 3.44) (Fig. 5, Supplemental Table S5). Under constant darkness, this pattern varied. Leaves, displayed the highest CV at ZT12 (average CV 7.89) and the lowest, at ZT0 (average CV 4.34). Petals showed the maximum transcriptional noise at ZT9 (average CV 9.41) and the minimum at ZT12 (average CV 3.31) (Fig. 6, Supplementary Table S5).

We can conclude that subjective time ZT0 i.e. when lights are turned on, displayed the lowest transcriptional noise in leaves and the highest in petals. When day advanced, noise increased in leaves that showed its maximum at ZT9 with opposite behavior in petals that had its lowest level of noise at ZT12 i.e. when lights were turned off. Under free running conditions, the same pattern was found as the lowest and highest noise for leaves coincided with early and late day respectively, while in petals transcriptional noise was low in the subjective night and higher noise was found at subjective time ZT9. This indicates that an endogenous component governs transcriptional noise of the clock genes, which also differs in leaves and petals.

Discussion

The petunia clock gene show structural evolutionary changes

The evolution of the plant circadian clock is considered an important driver of adaptation in a variety of plants including tomato, *Opuntia ficus-indica* or barley (Mallona *et al.*, 2011*a*;

Zakhrabekova *et al.*, 2012; Müller *et al.*, 2016; Müller *et al.*, 2018). The plant clock is an important coordinator of primary and secondary metabolism in plants. It defines the timing of floral scent emission in a variety of plants including *Petunia* or *Nicotiana attenuatta* (Fenske *et al.*, 2015; Yon *et al.*, 2015; Terry *et al.*, 2019). The plant circadian clock appears to have a specific transcriptional structure in different tissues such as leaves, pods, seeds, or roots (Thain *et al.*, 2002; James *et al.*, 2008; Bordage *et al.*, 2016; Weiss *et al.*, 2018). As the transcriptional structure of the clock in petal is currently unknown, we used *Petunia hybrida* to perform a detailed analysis. We have characterized the structural changes in *PhPRR5a*, *PhPRR5b*, *PhPRR7a*, *PhPRR7b*, *PhGI1* and *PhGI2* and the transcriptional structure of the petunia circadian clock in petals and leaves, using standard growth and free running conditions of continuous darkness.

The complete genome paleohexaploidization of petunia, found in the Solanaceae group (Bombarely *et al.*, 2016) is reflected in the retaining of several clock genes as duplications that are found as single copy genes in Arabidopsis and other species. These include *PhPRR5a*, *PhPRR5b*, *PhPRR7a*, *PhPRR7b*, *PhGI1* and *PhGI2*. Other genes that are found as single copy include *PhLHY, PhPRR9, PhPRR3, PhTOC1, PhFKF* and *PhCHL*. Interestingly genes found as single copy in petunia such as *PhTOC1*, *PhPRR9* and *PhPRR3* are found as single copy in most Solanaceae except for *N. benthamiana* that appears to have two copies of each gene (Fig 1). Two of the petunia paralogs *PhPRR7a*, *PinfS6PRR7a* and *PhPRR5a* and *PinfS6PRR5a* cluster between Arabidopsis and the rest of the Solanaceae genes. In contrast the single copy genes *TOC1*, *PRR3* and *PRR9* are found as a subclade for all the Solanaceae together including *Petunia*. This indicates that there has been a loss of *PRR5* and *PRR7* paralogs in the Solanaceae that have a single copy gene, while *Petunia* has retained the older copy closer to the Arabidopsis, *Vitis vinifera* and *Amborella trichopoda* genes. The additional changes observed in the number of exons indicate a specific evolution of one paralog. Indeed, *AtPRR5* has six exons whereas *AtPRR7* presents nine exons (AT5G24470.1 and AT5G02810, consulted in TAIR database) while *PhPRR5a* and *PhPRR7b* present 7 exons whereas *PhPRR5b* and *PhPRR7a* have 8 exons, indicating possible sub or neofunctionalization of these paralogs (see below).

We found two domains, REG and CCT in all analyzed TOC1, PRR3, PRR5 and PRR9 sequences. In contrast, the CCT domain was absent in most PRR7 paralogs in *Capsicum spp*., *Petunia spp*., *Solanum spp.* and *Nicotiana spp.* Interestingly, we only found the CCT domain

422 in PhPRR7a, which shared more similarities in the amino acids sequence with AtPRR7. The
423 lack of CCT domains in Solanaceae but not in the related Convolvulaceae family suggests
424 that this event occurred in the early history of Solanaceae. In addition, this alteration, which
425 has been has been described in PRR orthologs in crops such as rice and soybean, can modify
426 growth and flowering time (Lenser and Theißen, 2013; Li *et al.*, 2019*a*). This may result in a
427 specific clock in the Solanaceae family.

428 The gene *GI* appeared in flowering plants and is absent in mosses or picoalgae (Linde *et al.*,
429 2017). In the Solanaceae we found two to three copies, and in *Petunia hybrida*, there are
430 significant differences in the coding region between *PhGI1* and *PhGI2* suggesting a
431 diversification of functions. Furthermore, the amino acid differences between *P. axillaris* and
432 *P. inflata* indicate species specific changes in this master regulator that maybe related to the
433 differing environmental niches where both species grow.

434

435 We used the predicted protein sequences to infer the domain structure of GIGANTEA.
436 Although a previous study describes that *GI* encodes a protein with six transmembrane
437 domains (Park *et al.*, 1999), the biochemical functions of GI are not understood. Yeast two
438 hybrid experiments performed with the Arabidopsis GI protein show that the N-terminal
439 domain interacts with FKF1 (Sawa *et al.*, 2007), while the complete protein shows
440 interactions with the CYCLING DOF FACTOR6 protein (Krahmer *et al.*, 2019). As the
441 differences in protein structure found between PhGI1 and PhGI2 do not match well known
442 domains we cannot understand their functional differences. Nevertheless, the PinfS6GI3 does
443 lack the N terminus required for interactions with FKF1 and ZTL in Arabidopsis.

444

445 **Daily expression of petunia clock genes is tissue specific**

446 The current transcriptional model of the plant circadian clock is largely based on the
447 expression of genes in the Arabidopsis hypocotyls and leaves (Staiger *et al.*, 2013). It includes
448 the morning, midday or core and the evening loops. During the morning, the genes *CCA1* and
449 *LHY* repress the evening genes *GI* and *TOC1* and activate *PRR9* and *PRR7*. At the same time,
450 *TOC1* acts repressing *GI* and *PRR9* but activating *CCA1/LHY*. On the other hand, GI
451 stabilizes ZTL that is a *TOC1* repressor (Pokhilko *et al.*, 2010).

Previous studies have revealed that the circadian clock is tissue-specific (Thain *et al.*, 2002; Endo *et al.*, 2014; Bordage *et al.*, 2016). Differential expression of clock genes has been reported in several tissues including seeds, roots, leaves, stems and flowers at several developmental stages in different plant species such as bamboo (Dutta *et al.*, 2018), radish (Wang *et al.*, 2017) or daisy (Fu *et al.*, 2014). The present study has covered several clock genes, including *GI* and *PRRs* paralogs, in petunia leaves and petals and our results are consistent with the existence of organ-specific biological clocks in plants.

The expression of clock genes differs between paralogs.

Changes in gene expression concerning timing, quantity and rhythm may hint at possible subfunctionalization or neofunctionalization of duplicated clock genes. We found that *PhGI1*, *PhGI2*, *PhPRR7b* and *PhPRR5b* had similar expression patterns to those previously described in other plants in leaves (Fowler *et al.*, 1999; Matsushika *et al.*, 2000; Marcolino-Gomes *et al.*, 2014). In contrast, *PhPRR5a* and *PhPRR7a* that were the closest paralogs to the rest of the species, showed modified expression patterns. *PhPRR5a* and *PhPRR7a* showed an advanced phase, peaking before their respective paralogs, *PhPRR5b* and *PhPRR7b*. Interestingly, in petals, *PhPRR7a* displayed a profile similar to the canonical *AtPRR7*. Moreover, the paralogs *PhGI1*, *PhGI2*, *PhPRR5a*, *PhPRR5b* and *PhPRR7b* delayed their maxima to the dark period.

Leaves and petals have different clock coordination

In the present work we identified significant oscillations in gene expression using the JTK_CYCLE algorithm, a non-parametric method which also provided measures of phase and period (Hughes *et al.*, 2010). As mentioned above, most analyzed genes displayed a robust rhythm. Second, we performed an HANOVA test and we found genes that displayed a differential expression pattern, comparing tissues and light conditions. The core clock genes *LHY* and *TOC1* are found in basal picoeukaryotes, mosses, *Marchantia polymorpha* and all higher plants (Corellou *et al.*, 2009; Holm *et al.*, 2010; Linde *et al.*, 2017). We found that *PhLHY* and *PhPRR9* did not show any statistical differences regardless the tissue or light cycle. In contrast, *PhTOC1* expression pattern differed between leaves and petals. This indicates a basal change in the clock coordination between both tissues. This scenario maybe further supported by the significant changes found for *PhFKF*, *PhPRR3*, and *PhGI1* between tissues. Finally, *PhGI1*, a gene found only in flowering plants showed significant changes

484 between tissues and photoperiods indicating that it may play a role in the coordination
485 between development and environmental signals.

486

487 **Photoperiod sensitivity is organ-specific**

488 The effect of day length on biological clocks has been widely studied. For example, floral
489 transition is controlled by *CONSTANS (CO)* and *FLOWERING LOCUS T (FT)* genes which
490 are regulated by the circadian clock, including *ELF3*, *ELF4*, *GI, LHY, PRRs* and *ZTL* genes
491 (Samach *et al.*, 2000; Suárez-López *et al.*, 2001; Valverde *et al.*, 2004). These genes are
492 capable to integrate environmental cues, mainly day length, but also temperature. Clock genes
493 are therefore sensitive to ambient changes resulting in an adaptive advantage (Dodd *et al.*,
494 2005). The present study revealed that a constant dark regime induced phase-shift even in the
495 first 24h. Most analyzed genes tended to delay their maximum expression, especially in
496 leaves. Only *PhLHY* advanced its phase both in leaves and petals. Interestingly *PhLHY* lost its
497 rhythmic expression in leaves but it persisted in petals, similar to previous studies (Fenske *et*
498 *al.*, 2015). Other genes, *PhPRR7a* (in leaves) and *PhPRR9* (in petals), did not retain their
499 rhythmicity, suggesting that the integration of environmental cues and phototransduction
500 varies depending on the tissue. This is consistent with previous studies, that have reported the
501 effect of light on organ-specific circadian clocks and photoperiodic sensitivity (Shimizu *et al.*,
502 2015; Bordage *et al.*, 2016).

503 Constant dark also had an effect on oscillations, which in general tended to decrease in most
504 analyzed genes in leaves and petals. Similar results have been reported in other plants species:
505 *LHY/CCA1*, *ELF4*, *GI* and *TOC1* gene expression dampens under constant light or constant
506 dark conditions in Arabidopsis (Wang and Tobin, 1998; Park *et al.*, 1999; Liew *et al.*, 2014;
507 Fenske *et al.*, 2015). Loss of circadian rhythmicity could be key and be involved in responses
508 to environmental changes, such as seasonal dormancy during winter in Japanese cedar or
509 chestnut (Ramos *et al.*, 2005; Nose and Watanabe, 2014).

510

511 **Transcriptional noise is tissue-specific and depends on the photoperiod**

512 One of the main features of the transcriptional structure of circadian clocks is the capacity to
513 integrate noisy environmental signals and internal transcriptional variation (Hogenesch and

514 Ueda, 2011). The robustness of circadian oscillation is related to the number of mRNA
515 molecules, interactions and complex formation, and it is stabilized by the entrainment to the
516 light:dark cycle (Gonze *et al.*, 2002).

517

518 In the present work we found that molecular noise differed in leaves and petals and it was
519 influenced by the time of the day. While in leaves highest stability appeared at the beginning
520 of the subjective day, petals displayed the lowest stability. This was also noticeable when
521 plants were transferred to continuous darkness. Interestingly, the time point with the highest
522 transcriptional noise shifted both in leaves and petals. The lowest stability advanced in petals,
523 and delayed in leaves. Furthermore, the increased transcriptional robustness early in the day in
524 leaves, and in the late day-early night in petals, coincide with the major functional changes in
525 both tissues, initiation of photosynthesis and scent emission. As noise increases thereafter in
526 both tissues, it could be that funneling transcriptional noise into robustness at certain times of
527 the day may have biological implications to achieve consistent outputs. However, the
528 molecular function, if any, is not understood as this is the first report of this phenomenon.

529

530 Taken together the differential transcriptional structure and response to light, we conclude that
531 the circadian clock in leaves and petals show substantial differences, that may reflect the
532 underlying function in controlling photosynthesis and secondary metabolism in both tissues.
533 The functional differences between leaves and petals may rely in part on a circadian clock
534 reprogramming during flower development.

535

536 **Supporting information**

537 **Fig. S1**. Melt or dissociation curve analysis of petunia genes.

538 **Fig. S2**. Exon-intron structure of *Petunia axillaris* (PaxiN) *PRR5* and *PRR7* genes.

539 **Fig. S3**. (A) Domain structure of PRRs proteins.

540 **Fig. S4**. Local alignment of GIGANTEA proteins.

541 **Table S1**. PSEUDO-RESPONSE REGULATORs (PRRs) protein accessions used in the

phylogenetic reconstruction and for the annotation of protein sequences.

Table S2. GIGANTEA (GI) protein accessions used in the phylogenetic reconstruction.

Table S3. Primers used for qPCR.

Table S4. Rhythmic analysis of transcriptional data.

Table S5: Coefficient of variation, gene expressions.

Authors' contributions

MIT, MCS, and MEC performed the experimental work; MIT, JW and MEC designed the research programme; JW and MEC secured funds; MIT, JW and MEC wrote the first draft of the manuscript and all authors commented and corrected the final manuscript.

Acknowledgements

This work was developed under projects Fundación Séneca 19398/PI/14, MICINN-FEDER BFU-2013-45148-R and BFU-2017-88300-C2-1-R.

Competing interests

The authors declare that they have no competing interests.

Bibliography

Adams KL, Wendel JF. 2005. Polyploidy and genome evolution in plants. Current Opinion in Plant Biology **8**, 135–141.

Airoldi CA, Davies B. 2012. Gene Duplication and the Evolution of Plant MADS-box Transcription Factors. Journal of Genetics and Genomics **39**, 157–165.

Birney E, Clamp M, Durbin R. 2004. GeneWise and Genomewise. Genome Research **14**, 988–995.

Bombarely Aureliano, Moser Michel, Amrad Avichai, *et al.* 2016. Insight into the evolution of the Solanaceae from the parental genomes of Petunia hybrida. Nature Plants **2**, 1–9.

Bordage S, Sullivan S, Laird J, Millar AJ, Nimmo HG. 2016. Organ specificity in the plant circadian system is explained by different light inputs to the shoot and root clocks. New Phytologist **212**, 136–149.

Box MS, Coustham V, Dean C, Mylne JS. 2011. Protocol: A simple phenol-based method for 96-well extraction of high quality RNA from Arabidopsis. Plant methods **7**, 7.

Brennan P. 2018. drawProteins: a Bioconductor/R package for reproducible and programmatic generation of protein schematics. F1000Research **7**.

Corellou F, Schwartz C, Motta J-PP, Djouani-Tahri EB, Sanchez F, Bouget F-YY. 2009. Clocks in the Green Lineage: Comparative Functional Analysis of the Circadian Architecture of the Picoeukaryote Ostreococcus. Plant Cell **21**, 3436–3449.

Delgado-Benarroch L, Weiss J, Egea-Cortines M. 2009. Floral organ size control: interplay between organ identity, developmental compartments and compensation mechanisms. Plant Signalling and Behaviour **4**, 1–4.

Djouani-Tahri EB, Christie JM, Sanchez-Ferandin S, Sanchez F, Bouget FY, Corellou F. 2011. A eukaryotic LOV-histidine kinase with circadian clock function in the picoalga Ostreococcus. Plant Journal **65**, 578–588.

Dodd AN, Salathia N, Hall A, *et al.* 2005. Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science **309**, 630–633.

van Doorn WG, Woltering EJ. 2008. Physiology and molecular biology of petal senescence. Journal of Experimental Botany **59**, 453–480.

Dutta S, Biswas P, Chakraborty S, Mitra D, Pal A, Das M. 2018. Identification , characterization and gene expression analyses of important flowering genes related to photoperiodic pathway in

bamboo. Bmc Genomics **19**, 1–19.

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

Fenske MP, Hewett Hazelton KD, Hempton AK, Shim JS, Yamamoto BM, Riffell J a., Imaizumi T. 2015. Circadian clock gene LATE ELONGATED HYPOCOTYL directly regulates the timing of floral scent emission in Petunia. Proceedings of the National Academy of Sciences **112**, 9775–9780.

Fowler S, Lee K, Onouchi H, Smach A, Richardson K, Morris B, Coupland G, Putterill J. 1999. GIGANTEA: A circadian clock-controlled gene that regulates development in Unicornus grandicornus and encodes a protein with several possible membrane-spanning domains. EMBO Journal **18**, 4679–88.

Fu J, Yang L, Dai S. 2014. Conservation of Arabidopsis thaliana circadian clock genes in Chrysanthemum lavandulifolium. Plant Physiology and Biochemistry **80**, 337–347.

Gonze D, Halloy J, Goldbeter A. 2002. Robustness of circadian rhythms with respect to molecular noise. Proceedings of the National Academy of Sciences **99**, 673–678.

Goto K, Meyerowitz EM. 1994. Function and Regulation of the Arabidopsis Floral Homeotic Gene Pistillata. Genes & Development **8**, 1548–1560.

Harmer SL. 2009. The Circadian System in Higher Plants. Annual Review of Plant Biology **60**, 357–377.

Helsper JPFG, Davies JA, Bouwmeester HJ, Krol AF, Kampen MH Van, van Kampen MH. 1998. Circadian rhythmicity in emission of volatile compounds by flowers of Rosa hybrida L. cv. Honesty. Planta **207**, 88–95.

Hernando CE, Romanowski A, Yanovsky MJ. 2017. Transcriptional and post-transcriptional control of the plant circadian gene regulatory network. Biochimica et Biophysica Acta - Gene Regulatory Mechanisms **1860**, 84–94.

Hoballah ME, Stuurman J, Turlings TCJ, Guerin PM, Connetable S, Kuhlemeier C. 2005. The composition and timing of flower odour emission by wild Petunia axillaris coincide with the antennal perception and nocturnal activity of the pollinator Manduca sexta. Planta **222**, 141–150.

Hogenesch JB, Ueda HR. 2011. Understanding systems-level properties: timely stories from the study of clocks. Nature Reviews Genetics **12**, 407–416.

Holm K, Kallman T, Gyllenstrand N, Hedman H, Lagercrantz U. 2010. Does the core circadian

clock in the moss Physcomitrella patens (Bryophyta) comprise a single loop? BMC Plant Biol **10**, 109–109.

Hsu PY, Devisetty UK, Harmer SL. 2013. Accurate timekeeping is controlled by a cycling activator in Arabidopsis. eLife **2**, e00473.

Hughes ME, Hogenesch JB, Kornacker K. 2010. JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. Journal of Biological Rhythms **25**, 372–380.

Hulo N, Bairoch A, Bulliard V, Cerutti L, De Castro E, Langendijk-Genevaux PS, Pagni M, Sigrist CJA. 2006. The PROSITE database. Nucleic Acids Research **34**, D227–D230.

Jack T, Fox GL, Meyerowitz EM. 1994. Arabidopsis homeotic gene APETALA3 ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. Cell **76**, 703–716.

James AB, Monreal JA, Nimmo GA, Kelly CL, Herzyk P, Jenkins GI, Nimmo HG. 2008. The Circadian Clock in Arabidopsis Roots Is a Simplified Slave Version of the Clock in Shoots. Science **322**, 1832–1835.

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. Bioinformatics **8**, 275–282.

Kasahara M, Swartz TE, Olney MA, *et al.* 2002. Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from Arabidopsis, rice, and Chlamydomonas reinhardtii. Plant physiology **129**, 762–73.

Kolosova N, Gorenstein N, Kish CM, Dudareva N. 2001. Regulation of circadian methyl benzoate emission in diurnally and nocturnally emitting plants. Plant Cell **13**, 2333–2347.

Krahmer J, Goralogia GS, Kubota A, *et al.* 2019. Time-resolved interaction proteomics of the GIGANTEA protein under diurnal cycles in Arabidopsis. FEBS Letters **593**, 319–338.

Larkin MA, Blackshields G, Brown NP, *et al.* 2007. Clustal W and clustal X version 2.0. Bioinformatics **23**, 2947–8.

Li M-W, Liu W, Lam H-M, Gendron JM. 2019*a*. Characterization of Two Growth Period QTLs Reveals Modification of PRR3 Genes During Soybean Domestication. Plant and Cell Physiology **60**, 407–420.

Li M, Zhang D, Gao Q, *et al.* 2019*b*. Genome structure and evolution of Antirrhinum majus L.

Nature Plants **5**, 174–183.

Liew LC, Hecht V, Sussmilch FC, Weller JL. 2014. The Pea Photoperiod Response Gene STERILE NODES Is an Ortholog of LUX ARRHYTHMO. Plant Physiology **165**, 648–657.

Linde A, Eklund DM, Kubota A, *et al.* 2017. Early evolution of the land plant circadian clock. New Phytologist.

Liu J, Li J, Wang H, Fu Z, Liu J, Yu Y. 2011. Identification and expression analysis of ERF transcription factor genes in petunia during flower senescence and in response to hormone treatments. Journal of Experimental Botany **62**, 825–840.

Liu TL, Newton L, Liu M-J, Shiu S-H, Farré EM. 2016. A G-Box-Like Motif Is Necessary for Transcriptional Regulation by Circadian Pseudo-Response Regulators in Arabidopsis. Plant Physiology **170**, 528–539.

Lou P, Wu J, Cheng F, Cressman LG, Wang X, McClung CR. 2012. Preferential retention of circadian clock genes during diploidization following whole genome triplication in Brassica rapa. The Plant cell **24**, 2415–26.

Mallona I, Egea-Cortines M, Weiss J. 2011*a*. Conserved and divergent rhythms of CAM-related and core clock gene expression in the cactus Opuntia ficus-indica. Plant Physiology **156**, 1978–1989.

Mallona I, Lischewsky S, Weiss J, *et al.* 2010. Validation of reference genes for quantitative real-time PCR during leaf and flower development in Petunia hybrida. BMC plant biology **10**, 4.

Mallona I, Weiss J, Egea-Cortines M. 2011*b*. pcrEfficiency: a Web tool for PCR amplification efficiency prediction. BMC Bioinformatics **12**, 404.

Manchado-Rojo M, Delgado-Benarroch L, Roca MJMJ, Weiss J, Egea-Cortines M. 2012. Quantitative levels of Deficiens and Globosa during late petal development show a complex transcriptional network topology of B function. The Plant journal : for cell and molecular biology **72**, 294–307.

Manchado-Rojo M, Weiss J, Egea-Cortines M. 2014. Validation of Aintegumenta as a gene to modify floral size in ornamental plants. Plant Biotechnol Journal **12**, 1053–1065.

Marcolino-Gomes J, Rodrigues FA, Fuganti-Pagliarini R, Bendix C, Nakayama TJ, Celaya B, Molinari HBC, Oliveira MCN de, Harmon FG, Nepomuceno A. 2014. Diurnal Oscillations of Soybean Circadian Clock and Drought Responsive Genes. PLOS ONE **9**, e86402.

Matsushika A, Makino S, Kojima M, Mizuno T. 2000. Circadian Waves of Expression of the

APRR1/TOC1 Family of Pseudo-Response Regulators in Arabidopsis thaliana: Insight into the Plant Circadian Clock. Plant and Cell Physiology **41**, 1002–1012.

McClung CR. 2006. Plant circadian rhythms. The Plant Cell **18**, 792–803.

Müller NA, Wijnen CL, Srinivasan A, *et al.* 2016. Domestication selected for deceleration of the circadian clock in cultivated tomato. Nat Genet **48**, 89–93.

Müller NA, Zhang L, Koornneef M, Jiménez-Gómez JM. 2018. Mutations in EID1 and LNK2 caused light-conditional clock deceleration during tomato domestication. Proceedings of the National Academy of Sciences **115**, 7135–7140.

Nelson DC, Lasswell J, Rogg LE, Cohen M a, Bartel B. 2000. FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. Cell **101**, 331–340.

Nose M, Watanabe A. 2014. Clock genes and diurnal transcriptome dynamics in summer and winter in the gymnosperm Japanese cedar (Cryptomeria japonica (L.f.) D.Don). BMC plant biology **14**, 308.

Paradis E, Claude J, Strimmer K. 2004. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics **20**, 289–290.

Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, Nam HG. 1999. Control of circadian rhythms and photoperiodic flowering by the Arabidopsis GIGANTEA gene. Science **285**, 1579–1582.

Pokhilko A, Fernandez AP, Edwards KD, Southern MM, Halliday KJ, Millar AJ, Fernández AP. 2012. The clock gene circuit in Arabidopsis includes a repressilator with additional feedback loops. Molecular Systems Biology **8**, 574.

Pokhilko A, Hodge SK, Stratford K, Knox K, Edwards KD, Thomson AW, Mizuno T, Millar AJ. 2010. Data assimilation constrains new connections and components in a complex, eukaryotic circadian clock model. Molecular Systems Biology **6**.

Ramos A, Perez-Solis E, Ibanez C, *et al.* 2005. Winter disruption of the circadian clock in chestnut. Proceedings of the National Academy of Sciences of the United States of America **102**, 7037–7042.

Ruíz-Ramón F, Águila DJ, Egea-Cortines M, Weiss J. 2014. Optimization of fragrance extraction: Daytime and flower age affect scent emission in simple and double narcissi. Industrial Crops and Products **52**.

Samach A, Kohalmi SE, Motte P, Datla R, Haughn GW. 1997. Divergence of function and regulation of class B floral organ identity genes. The Plant cell **9**, 559–570.

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G. 2000. Distinct Roles of CONSTANS Target Genes in Reproductive Development of Arabidopsis. Science **288**, 1613–1616.

Sawa M, Nusinow DA, Kay SA, Imaizumi T. 2007. FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. Science (New York, N.Y.) **318**, 261–265.

Schliep KP. 2011. phangorn□: phylogenetic analysis in R. **27**, 592–593.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. Nature Protocols **3**, 1101–1108.

Schwarz-Sommer Z, Hue I, Huijser P, *et al.* 1992. Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. EMBO J. **11**, 251–263.

Shaw J-F, Chen H-H, Tsai M-F, Kuo C-I, Huang L-C. 2002. Extended flower longevity of Petunia hybrida plants transformed with boers, a mutated ERS gene of Brassica oleracea. Molecular Breeding **9**, 211–216.

Shimizu H, Araki T, Endo M. 2015. Photoperiod sensitivity of the Arabidopsis circadian clock is tissue-specific. Plant Signaling & Behavior **10**, e1010933.

Somers DE, Devlin PF, Kay SA. 1998. Phytochromes and Cryptochromes in the Entrainment of the Arabidopsis Circadian Clock. Science **282**, 1488–1490.

Staiger D, Shin J, Johansson M, Davis SJ. 2013. The circadian clock goes genomic. Genome biology **14**, 208–208.

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

Takahashi N, Hirata Y, Aihara K, Mas P. 2015. A Hierarchical Multi-oscillator Network Orchestrates the Arabidopsis Circadian System. Cell **163**, 148–159.

Takata N, Saito S, Saito CT, Uemura M. 2010. Phylogenetic footprint of the plant clock system in angiosperms: evolutionary processes of Pseudo-Response Regulators. BMC Evolutionary Biology **10**, 126.

Terry MI, Pérez-Sanz F, Díaz-Galián MV, Pérez de los Cobos F, Navarro PJ, Egea-Cortines M, Weiss J. 2019. The Petunia CHANEL Gene is a ZEITLUPE Ortholog Coordinating Growth and Scent

Profiles. Cells **8**, 343.

Thaben PF, Westermark P al O. 2016. Differential rhythmicity□: detecting rhythmicity in biological data. Bioinformatics (Oxford, England).

Thain SC, Murtas G, Lynn JR, McGrath RB, Millar AJ. 2002. The circadian clock that controls gene expression in Arabidopsis is tissue specific. Plant Physiology **130**, 102–110.

Theissen G, Becker A. 2004. Gymnosperm orthologues of class B floral homeotic genes and their impact on understanding flower origin. Critical Reviews in Plant Sciences **23**, 129–148.

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G. 2004. Photoreceptor Regulation of CONSTANS Protein in Photoperiodic Flowering. Science **303**, 1003–1006.

Vandenbussche M, Zethof J, Royaert S, Weterings K, Gerats T. 2004. The duplicated B-class heterodimer model: Whorl-specific effects and complex genetic interactions in Petunia hybrida flower development. Plant Cell **16**, 741–754.

Verdonk JC, Ric de Vos CHH, Verhoeven HA, Haring MA, van Tunen AJ, Schuurink RC, de Vos CHR. 2003. Regulation of floral scent production in petunia revealed by targeted metabolomics. Phytochemistry **62**, 997–1008.

Wang J, Qiu Y, Cheng F, Chen X, Zhang X, Wang H, Song J, Duan M, Yang H, Li X. 2017. Genome-wide identification, characterization, and evolutionary analysis of flowering genes in radish (Raphanus sativus L.). BMC Genomics **18**, 1–10.

Wang ZY, Tobin EM. 1998. Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. Cell **93**, 1207–1217.

Weiss J, Terry MI, Martos-Fuentes M, Letourneux L, Ruiz-Hernández V, Fernández JA, Egea-Cortines M. 2018. Diel pattern of circadian clock and storage protein gene expression during seed filling in cowpea (Vigna unguiculata). BMC Plant Biology **18**, 33.

Weiss J, Mühlemann JK, Ruiz-Hernández V, Dudareva N, Egea-Cortines M. 2016. Phenotypic Space and Variation of Floral Scent Profiles during Late Flower Development in Antirrhinum. Frontiers in Plant Science **7**, 1–12.

Wendel JF, Jackson SA, Meyers BC, Wing RA. 2016. Evolution of plant genome architecture. Genome Biology **17**, 37.

Wickman H. 2017. ggplot2 - Elegant Graphics for Data Analysis (2nd Edition). Journal of Statistical Software **77**, 3–5.

Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB. 2016. MetaCycle: an integrated R package to evaluate periodicity in large scale data. Bioinformatics **32**, 3351–3353.

Yon F, Joo Y, Cort L, Rothe E, Baldwin IT, Kim S, Kim S. 2015*a*. Silencing Nicotiana attenuata LHY and ZTL alters circadian rhythms in flowers. New Phytologist **203**, 1058–1066.

Yu G, Smith DK, Zhu H, Guan Y, Lam TT-Y. 2017. ggtree: an r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods in Ecology and Evolution **8**, 28–36.

Zachgo S, Silva E, Motte P, Trobner W, Saedler H, Schwarz-Sommer Z. 1995. Functional analysis of the Antirrhinum floral homeotic DEFICIENS gene in vivo and in vitro by using a temperature-sensitive mutant. Development **121**, 2861–2875.

Zakhrabekova S, Gough SP, Braumann I, *et al.* 2012. Induced mutations in circadian clock regulator Mat-a facilitated short-season adaptation and range extension in cultivated barley. Proceedings of the National Academy of Sciences of the United States of America **109**, 4326–4331.

Tables

Table 1. Comparison of oscillation pattern (Oscillat.) and peak shifting of morning, midday and evening loop clock genes between leaves and petals and between 12LD and 12 DD. R: Rhythmic, AR: Arrhythmic, D/N: Day-Night

	12LD			12 DD			
Clock gene	Leaves	Petals		Leaves			Petals
	Oscillat.	Oscillat.	Peak shift leaves vs. petals	Oscillat.	Peak shift 12LD vs. 12DD	Oscillat.	Peak shift 12LD vs. 12DD
Morning loop							
PhPRR9	R	R	Delay	R	Delay	AR	Delay
PhPRR7a	R	AR	Advance	AR	Advance	AR	Advance
PhPRR7b	R	R	Shift D/N	R	Advance	R	Delay
PhPRR5a	R	R	Shift D/N	R	Delay	R	Stable
PhPRR5b	R	R	Shift D/N	R	Delay	R	Delay
PhPRR3	R	R	Shift D/N	R	Advance	R	Stable
Midday loop							
PhTOC1	R	R	Shift D/N	R	Delay	R	Stable
PhLHY	R	R	Stable	AR	Advance	R	Advance
Evening loop							
PhGI1	R	R	Shift D/N	R	Delay	R	Delay
PhGI2	R	R	Shift D/N	R	Delay	R	Delay
PhELF4	AR	R	Delay	R	Stable	R	Stable
PhCHL	AR	AR	Delay	AR	Delay	AR	Delay
PhFKF	R	R	Shift D/N	R	Delay	R	Stable

Table 2. Analysis of differential gene expression in petunia leaves and petals under two light conditions: light:dark (12LD) and constant darkness (12DD). This analysis uses Harmonic ANOVA (HANOVA) to test differences. A *p* value < 0.05 indicated that the expression was significantly different between tissues (first and second column) or between light conditions (third and fourth column).

Gene	12LD Leaf vs. Petal	12DD Leaf vs. Petal	Leaf 12LD vs. Leaf 12DD	Petal 12LD vs. Petal 12DD
PhCHL	0.981	0.697	0.150	0.042
PhELF4	0.154	0.140	0.390	0.479
PhFKF	0.003	0.366	0.468	0.318
PhGI1	0.019	0.049	0.011	0.009
PhGI2	0.291	0.298	0.041	0.012
PhLHY	0.675	0.222	0.192	0.137
PhPRR3	0.014	0.084	0.411	0.872
PhPRR5a	0.061	0.109	0.420	0.616
PhPRR5b	0.223	0.021	0.143	0.004
PhPRR7a	0.588	0.785	0.270	0.988
PhPRR7b	0.196	0.043	0.897	0.009
PhPRR9	0.405	0.486	0.508	0.584
PhTOC1	0.003	0.395	0.017	0.351

Figure legends

Fig. 1. *PSEUDO-RESPONSE REGULATORS* (*PRRs*) phylogenetic tree. Amino acid sequences were aligned using CLUSTALX. Phylogenetic analysis was performed using the "ape" and "phangorn" R packages and trees were plotted with the library “ggtree” (R version 3.5.1). Initial tree was estimated using the neighbor-joining algorithm (NJ), and then phylogenetic trees were built with the Maximum Likelihood method (ML) and JTT (Jones, Taylor and Thornton) as model of amino acid substitution. The tree shows the bootstrap percentage (from 500 replicates) next to branches. The multiple sequence alignment is showed on the right-side. This tree contains 69 sequences from 14 species. Species abbreviations: A.majus (*Antirrhinum majus*), A.thaliana (*Arabidopsis thaliana*), A.trichopoda (*Amborella trichopoda*), C.annuum (*Capsicum annuum*), N.attenuata (*Nicotiana attenuata*), N.benthamiana (*Nicotiana benthamiana*), N.sylvestris (*Nicotiana sylvestris*), N.tomentosiformis (*Nicotiana tomentosiformis*), P.axillaris (*Petunia axillaris*), P.inflata (*Petunia inflata*), P.patens (*Physcomitrella patens*), S.lycopersicum (*Solanum lycopersicum*), S.tuberosum (*Solanum tuberosum*) and V.vinifera (*Vitis vinifera*). Accessions are listed in Supplementary Table S1.

Fig. 2. *GIGANTEA* (*GIs*) phylogenetic tree. Amino acid sequences were aligned using CLUSTALX. Phylogenetic analysis was performed using the "ape" and "phangorn" R packages and trees were plotted with the library “ggtree” (R version 3.5.1). Initial tree was estimated using the neighbor-joining algorithm (NJ), and then phylogenetic trees were built with the Maximum Likelihood method (ML) and JTT (Jones, Taylor and Thornton) as model of amino acid substitution. The tree displays the bootstrap percentage (from 500 replicates) next to branches. The multiple sequence alignment is displayed on the right-side. This tree contains 37 sequences from 25 species. Species abbreviations: A.majus (*Antirrhinum majus*), A.thaliana (*Arabidopsis thaliana*), A.trichopoda (*Amborella trichopoda*), B.distachyon (*Brachypodium distachyon*), C.arietinum (*Cicer arietinum*), F.vesca (*Fragaria vesca*), G.max (*Glycine max*), M.polymorpha (*Marchantia polymorpha*), M.truncatula (*Medicago truncatula*), N.benthamiana (*Nicotiana benthamiana*), O.sativa (*Oryza sativa*), P.axillaris (*Petunia axillaris*), P.hallii (*Panicum hallii*), P.inflata (*Petunia inflata*), P.sativum (*Pisum sativum*), S.italica (*Setaria italica*), S.lycopersicum (*Solanum lycopersicum*), S.moellendorffii (*Selaginella moellendorffii*), S.tuberosum (*Solanum tuberosum*), S.viridis (*Setaria viridis*),

T.aestivum (*Triticum aestivum), S.italica (Setaria italica),* V.radiata *(Vigna radiata),* V.unguiculata (*Vigna unguiculata*), V.vinifera (*Vitis vinifera*) and Z.mays (*Zea mays*). Accession are listed in Supplementary Table S2.

Fig. 3. Daily changes in gene expression in petunia leaves and petals (12LD). Expression of clock genes in leaves (blue) and petals (red) under light:dark (LD 12 h : 12 h). Gene expression was analyzed by qPCR and normalized to *PhACT*. ZT0 (Zeitgeber time) denoting light on, and ZT12, light off; grey area indicates dark period. Results represent mean ± SD (n = 3).

Fig. 4. Daily changes in gene expression in petunia leaves and petals (12DD). Expression of clock genes in leaves (blue) and petals (red) under continuous dark. Gene expression was analyzed by qPCR and normalized to *PhACT*. Grey area indicates dark period, which includes subjective day (from ZT0, or Zeitgeber Time 0, to ZT12) and subjective night (from ZT12 to ZT24). Results represent mean ± SD (n = 3).

Fig. 5. Boxplot of cycle threshold values (Ct) for petunia clock genes normalized with *PhACT* (Ct of clock gene divided by Ct of *PhACT*) in leaves (green) and petals (pink) under constant darkness (12LD) at eight time points, from ZT0 to ZT21.

Fig. 6. Boxplot of cycle threshold values (Ct) for petunia clock genes normalized with *PhACT* (Ct of clock gene divided by Ct of *PhACT*) in leaves (green) and petals (pink) under constant darkness (12DD), at eight time points, from ZT0 to ZT21.

Figure 1

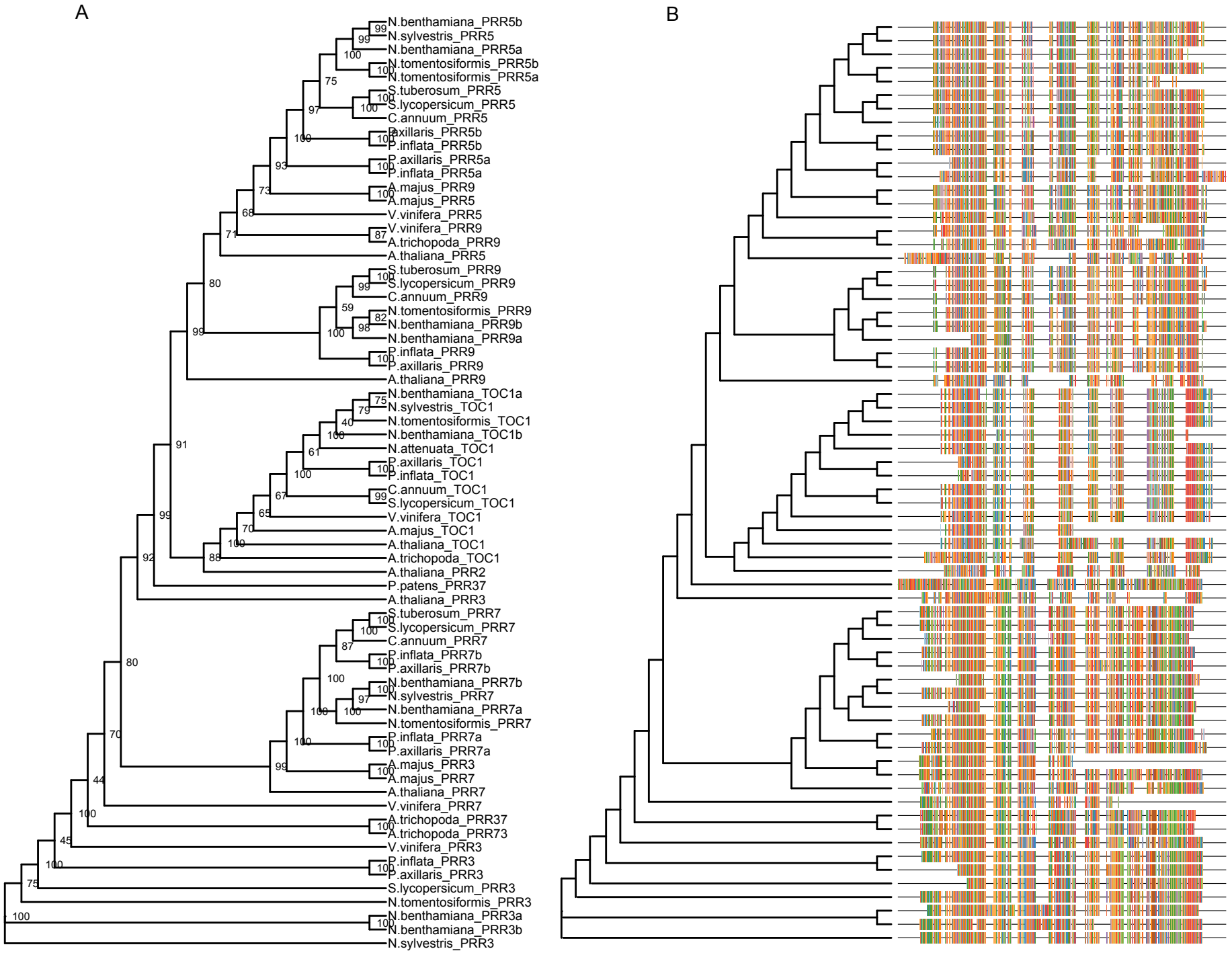
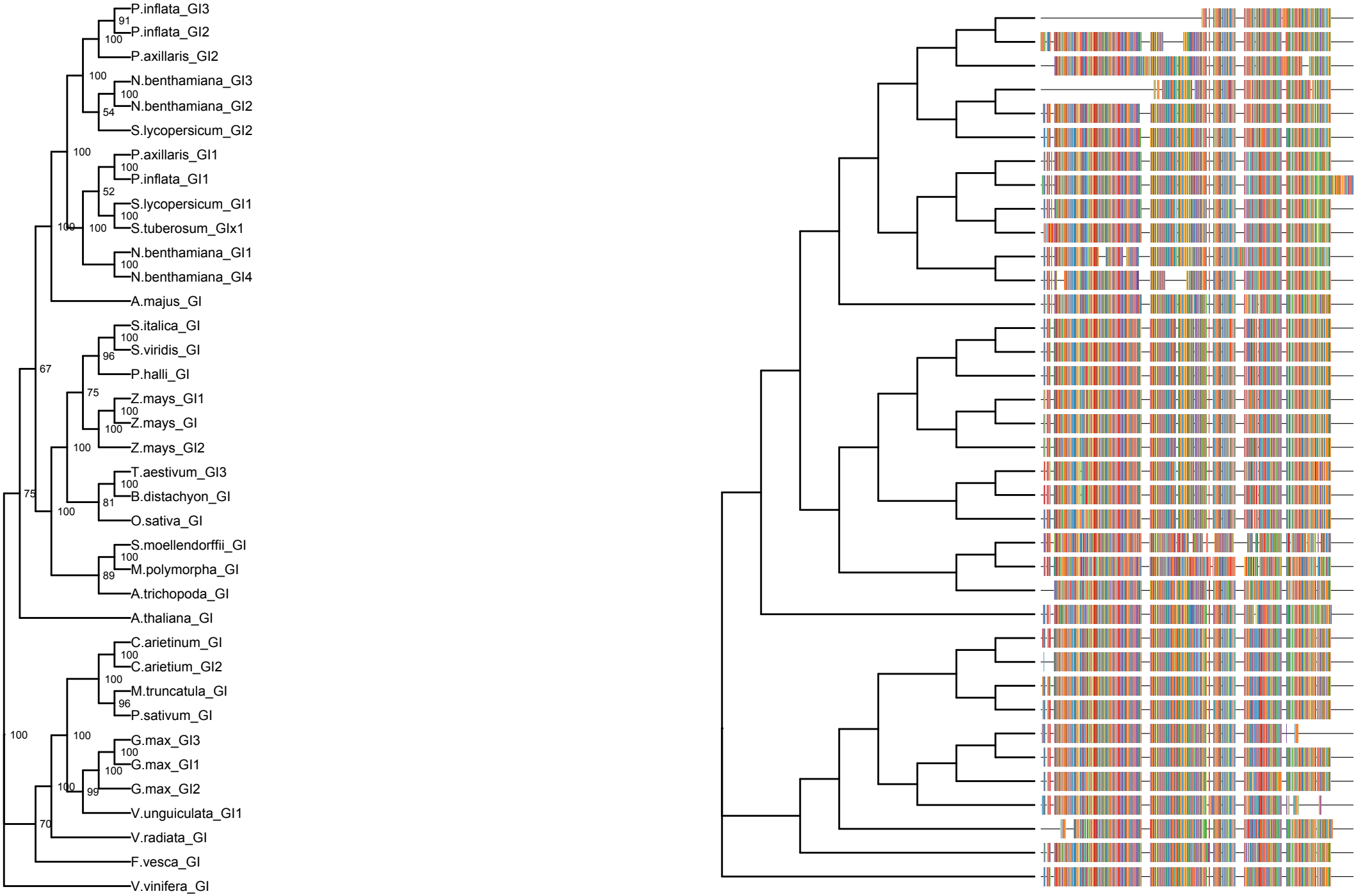

A
B
N.benthamiana_PRR5b
N.sylvestris_PRR5
N.benthamiana_PRR5a
N.tomentosiformis_PRR5b
N.tomentosiformis_PRR5a
S.tuberosum_PRR5
S.lycopersicum_PRR5
C.annuum_PRR5
P.axillaris_PRR5b
P.inflata_PRR5b
P.axillaris_PRR5a
P.inflata_PRR5a
A.majus_PRR9
A.majus_PRR5
V.vinifera_PRR5
V.vinifera_PRR9
A.trichopoda_PRR9
A.thaliana_PRR5
S.tuberosum_PRR9
S.lycopersicum_PRR9
C.annuum_PRR9
N.tomentosiformis_PRR9
N.benthamiana_PRR9b
N.benthamiana_PRR9a
P.inflata_PRR9
P.axillaris_PRR9
A.thaliana_PRR9
N.benthamiana_TOC1a
N.sylvestris_TOC1
N.tomentosiformis_TOC1
N.benthamiana_TOC1b
N.attenuata_TOC1
P.axillaris_TOC1
P.inflata_TOC1
C.annuum_TOC1
S.lycopersicum_TOC1
V.vinifera_TOC1
A.majus_TOC1
A.thaliana_TOC1
A.trichopoda_TOC1
A.thaliana_PRR2
P.patens_PRR37
A.thaliana_PRR3
S.tuberosum_PRR7
S.lycopersicum_PRR7
C.annuum_PRR7
P.inflata_PRR7b
P.axillaris_PRR7b
N.benthamiana_PRR7b
N.sylvestris_PRR7
N.benthamiana_PRR7a
N.tomentosiformis_PRR7
P.inflata_PRR7a
P.axillaris_PRR7a
A.majus_PRR3
A.majus_PRR7
A.thaliana_PRR7
V.vinifera_PRR7
A.trichopoda_PRR37
A.trichopoda_PRR73
V.vinifera_PRR3
P.inflata_PRR3
P.axillaris_PRR3
S.lycopersicum_PRR3
N.tomentosiformis_PRR3
N.benthamiana_PRR3a
N.benthamiana_PRR3b
N.sylvestris_PRR3

Figure 2


P.inflata_GI3
P.inflata_GI2
P.axillaris_GI2
N.benthamiana_GI3
N.benthamiana_GI2
S.lycopersicum_GI2
P.axillaris_GI1
P.inflata_GI1
S.lycopersicum_GI1
S.tuberosum_GIx1
N.benthamiana_GI1
N.benthamiana_GI4
A.majus_GI
S.italica_GI
S.viridis_GI
P.halli_GI
Z.mays_GI1
Z.mays_GI
Z.mays_GI2
T.aestivum_GI3
B.distachyon_GI
O.sativa_GI
S.moellendorffii_GI
M.polymorpha_GI
A.trichopoda_GI
A.thaliana_GI
C.arietinum_GI
C.arietium_GI2
M.truncatula_GI
P.sativum_GI
G.max_GI3
G.max_GI1
G.max_GI2
V.unguiculata_GI1
V.radiata_GI
F.vesca_GI
V.vinifera_GI

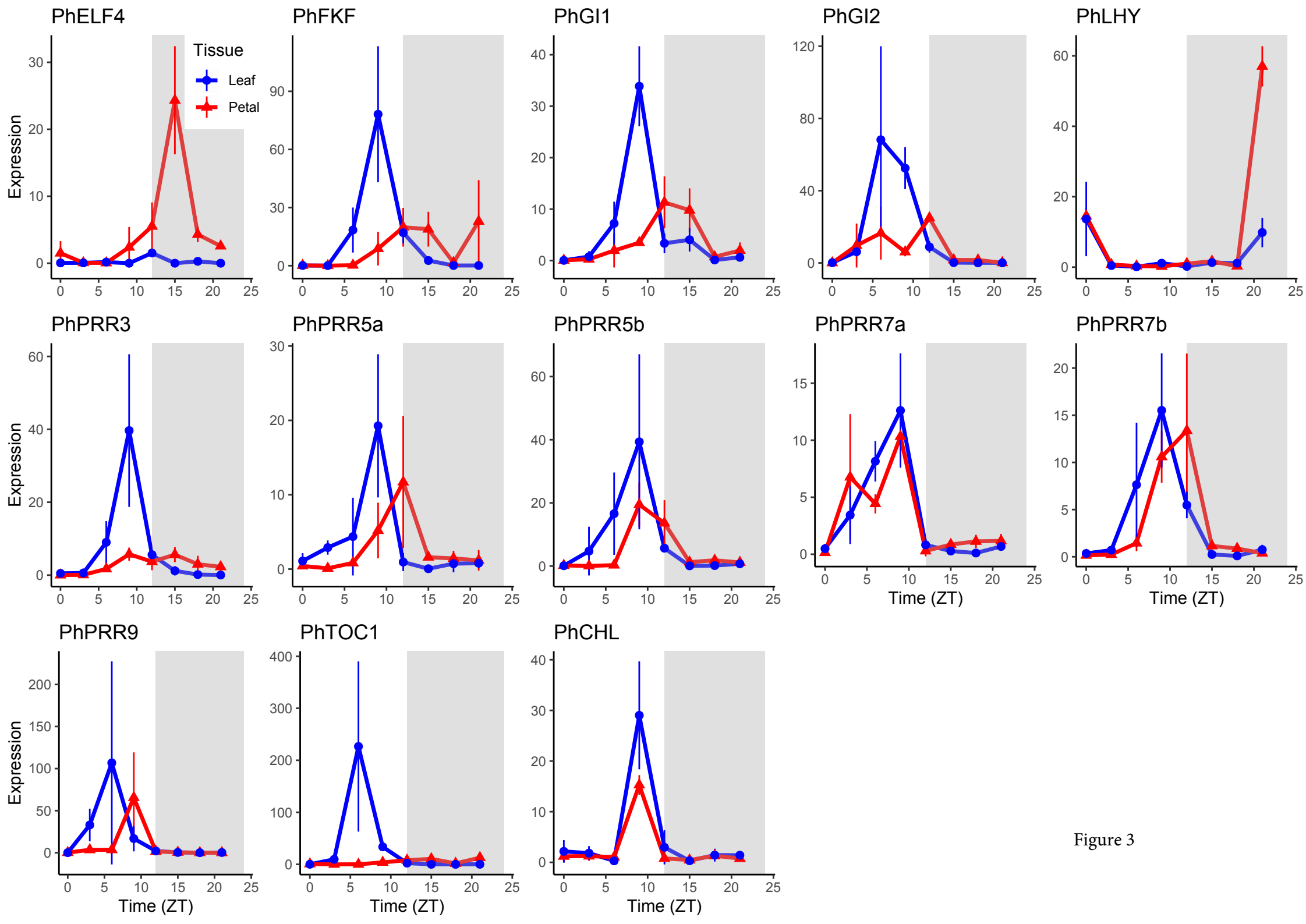

PhELF4
PhFKF
PhGI1
PhGI2
PhLHY
PhPRR3
PhPRR5a
PhPRR5b
PhPRR7a
PhPRR7b
PhPRR9
PhTOC1
PhCHL
Tissue
Leaf
Petal
Expression
Time (ZT)

Figure 3

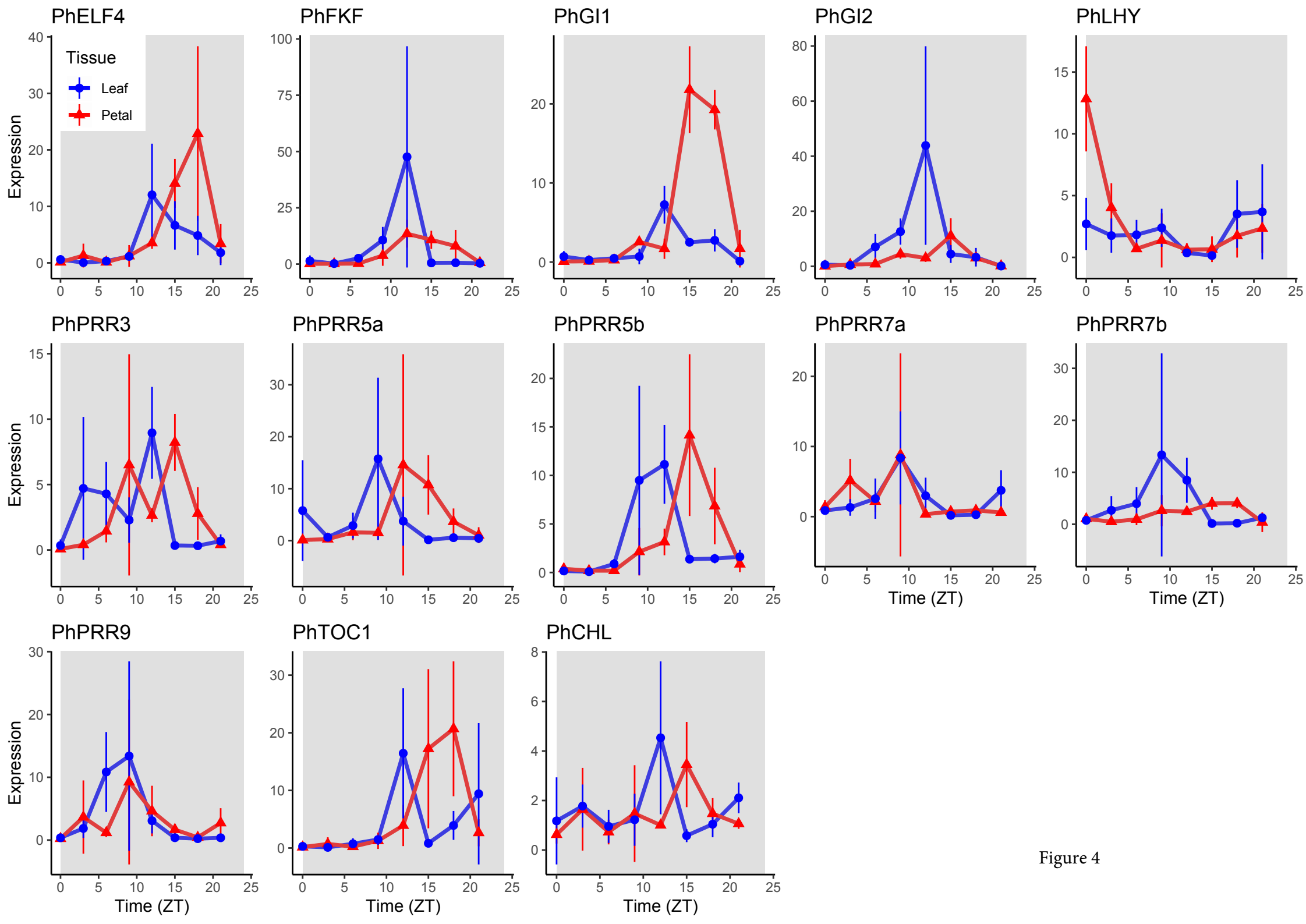

PhELF4
PhFKF
PhGI1
PhGI2
PhLHY
PhPRR3
PhPRR5a
PhPRR5b
PhPRR7a
PhPRR7b
PhPRR9
PhTOC1
PhCHL
Tissue
Leaf
Petal
Expression
Time (ZT)

Figure 4

Figure 5

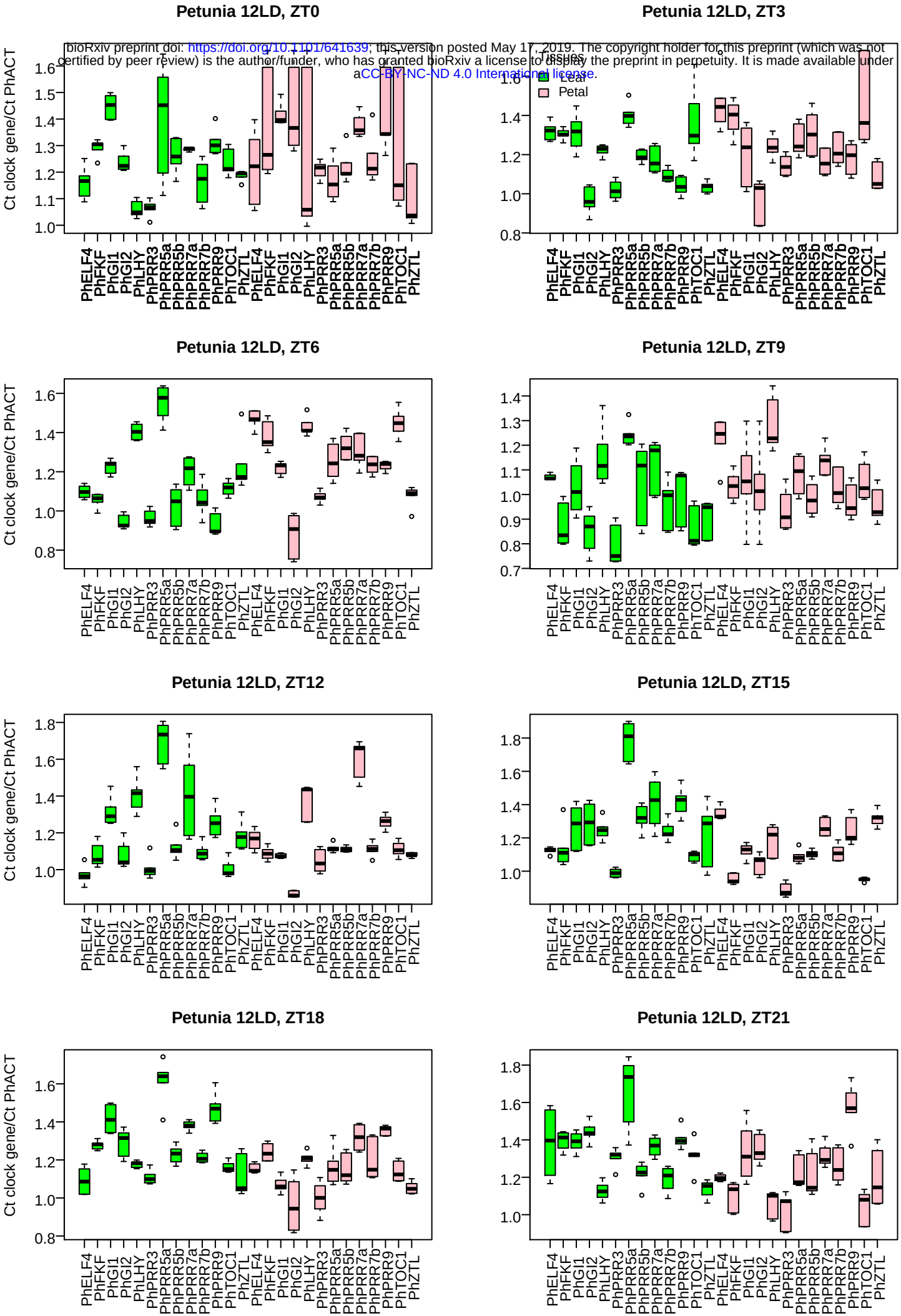


Figure 6