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

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

19

20 Abstract

21

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

38

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

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

49

50 **Introduction**

51 Organisms, from bacteria to human beings, are subjected to periodic oscillations in the
 52 environment due the planet rotation around its axis. Circadian clocks are a complex set of
 53 genes allowing organisms to anticipate and adapt to daily environmental variations. In plants,
 54 the circadian clock is a network of interlocked loops comprising transcriptional, translational
 55 and posttranslational coordination (Harmer, 2009). Circadian processes have been studied in
 56 plants for a long period of time (see McClung for a historical overview, (McClung CR,
 57 2006)). Most molecular studies have been done in *Arabidopsis thaliana*. The Arabidopsis core
 58 clock is formed by several genes. Two MYB transcription factors *CIRCADIAN CLOCK*
 59 *ASSOCIATED 1 (CCA1)*, *LATE ELONGATED HYPOCOTYL (LHY)* and the *PSEUDO*
 60 *RESPONSE REGULATOR TIMING OF CAB EXPRESSION (TOC1)* form the so-called core
 61 clock. Later studies found other clock components including the *PSEUDO-RESPONSE*
 62 *REGULATOR* gene family (*PRR*), out of which *PRR3*, *PRR5*, *PRR7* and *PRR9* are clock
 63 genes, and the Evening Complex (EC), which is formed by the *EARLY FLOWERING 3*
 64 (*ELF3*), *EARLY FLOWERING 4 (ELF4)* and *LUX ARRHYTMO (LUX)* proteins. In addition,
 65 other genes playing a key role and considered part of the clock include the protein with blue
 66 light reception capacity *ZEITLUPE (ZTL)* and the single copy gene *GIGANTEA (GI)*. The
 67 various models developed are based on mutually repressing genes and a set of activating
 68 genes coded by the *REVEILLE* MYB transcription factors (Hsu *et al.*, 2013). Every new
 69 discover has added a level of complexity and new interpretation of the circadian clock model
 70 (Hernando *et al.*, 2017).

71

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

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

88

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

101

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

108

109 Petal development starts with the activation of the so-called B function genes in both
110 gymnosperms and angiosperms (Theissen and Becker, 2004). The initial transcriptional
111 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 *PhGII* 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

144 **Plant materials and experiment design**

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

153 Flowers were marked before opening, and samples were taken at day 2-3 after anthesis. We
154 used the petal limbs for all experimental procedures. We used young leaves with a length of
155 1.5-2.5 cm for all the experiments. Sampling of petal limbs and leaves was made every three
156 hours, starting at ZT0 and tissues were immediately frozen in liquid nitrogen. In the case of
157 12DD experiment, sampling also started at ZT0, during the first 24h under continuous dark.

158

159 **Phylogeny and bioinformatics**

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

175

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

182

183 **Gene expression analysis by qPCR**

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

188 PCR analysis was performed as described before (Mallona *et al.*, 2010), the following
189 protocol was used for 40 cycles: 95 °C for 5 s, 60 °C for 20 s and 72 °C for 15 s (Clontech
190 SYBR Green Master Mix and Mx3000P qPCR Systems, Agilent Technologies). Primers for
191 circadian clock genes were designed using pcrEfficiency (Mallona *et al.*, 2011b)
192 (Supplementary Table S3) and the following protocol was used for 40 cycles: 95 °C for 5 s,
193 60 °C for 20 s (55 °C for *PhGII* and *PhGI2*) and 72 °C for 15 s. Samples were run in
194 duplicate. Primer combinations were tested with genomic DNA from Mitchell and we found
195 that all of them gave a single copy DNA on agarose gels. The endpoint PCR was further
196 verified by melting point analysis where all primer combinations gave a single peak of
197 melting (Supplementary Fig. S1). Normalized expression was calculated as described
198 (Schmittgen and Livak, 2008) and *PhACT* was the internal control gene, a stable gene in
199 circadian studies in petunia leaves and petals (Terry *et al.*, 2019).

200

201 **Results**

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

203 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).

223

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*.

232

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 than 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 *PhGII*, *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 (*PhGII*, *PhGI2*, *PhPRR3* and *PhPRR7b*) or at dusk 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 (*PhGII*, *PhPRR3*, *PhPRR5a* and *PhPRR5b*, *PhFKF*) and those with a maximum expression at the end of the night at ZT21 (*PhELF4* 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 *PhGII*, *PhGI2*, *PhFKF* and the *PRR* genes

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

304

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

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

314

315 We compared the expression between 12LD and 12DD in leaves (Fig. 4). We classified the
316 clock genes in three groups either showing a delay in maximum expression between 1.5 and
317 7.5 hours (*PhPRR9*, *PhPRR5a*, *PhPRR5b*, *PhTOC1*, *PhGII*, *PhGI2*, *PhFKF* and *PhCHL*) an
318 advance: *PhPRR7b*, *PhPRR3* (1.5 hours) and *PhLHY* (18 hours) or a maintained maximum
319 expression regardless of photoperiod (Table 1) (*PhPRR7a* and *PhELF4*).

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

325 Altogether, *PhLHY* showed advanced expression under DD conditions while *PhGII*, *PhGI2*,
326 *PhPRR5b*, *PhPRR9* and *PhCHL* were delayed in both leaves and petals. The only gene that
327 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 *PhGII*, *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 Westermarck, 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*, *PhGII*, *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 *PhGII*, *PhPRR5b* and *PhPRR7b* were differentially expressed in leaf versus petal.
349 When we compared leaves at 12LD versus 12DD, *PhGII*, *PhGI2* and *PhTOC1* showed
350 significant changes whereas in petals this group included *PhGII*, *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.

384

385 Discussion

386 The petunia clock gene show structural evolutionary changes

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

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

400
401 The complete genome paleohexaploidization of petunia, found in the Solanaceae group
402 (Bombarely *et al.*, 2016) is reflected in the retaining of several clock genes as duplications
403 that are found as single copy genes in Arabidopsis and other species. These include *PhPRR5a*,
404 *PhPRR5b*, *PhPRR7a*, *PhPRR7b*, *PhGII* and *PhGI2*. Other genes that are found as single copy
405 include *PhLHY*, *PhPRR9*, *PhPRR3*, *PhTOC1*, *PhFKF* and *PhCHL*. Interestingly genes found
406 as single copy in petunia such as *PhTOC1*, *PhPRR9* and *PhPRR3* are found as single copy in
407 most Solanaceae except for *N. benthamiana* that appears to have two copies of each gene (Fig
408 1). Two of the petunia paralogs *PhPRR7a*, *PinfS6PRR7a* and *PhPRR5a* and *PinfS6PRR5a*
409 cluster between Arabidopsis and the rest of the Solanaceae genes. In contrast the single copy
410 genes *TOC1*, *PRR3* and *PRR9* are found as a subclade for all the Solanaceae together
411 including *Petunia*. This indicates that there has been a loss of *PRR5* and *PRR7* paralogs in the
412 Solanaceae that have a single copy gene, while *Petunia* has retained the older copy closer to
413 the Arabidopsis, *Vitis vinifera* and *Amborella trichopoda* genes. The additional changes
414 observed in the number of exons indicate a specific evolution of one paralog. Indeed, *AtPRR5*
415 has six exons whereas *AtPRR7* presents nine exons (AT5G24470.1 and AT5G02810,
416 consulted in TAIR database) while *PhPRR5a* and *PhPRR7b* present 7 exons whereas
417 *PhPRR5b* and *PhPRR7a* have 8 exons, indicating possible sub or neofunctionalization of
418 these paralogs (see below).

419 We found two domains, REG and CCT in all analyzed *TOC1*, *PRR3*, *PRR5* and *PRR9*
420 sequences. In contrast, the CCT domain was absent in most *PRR7* paralogs in *Capsicum spp.*,
421 *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.*, 2019a). 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).

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

459

460 **The expression of clock genes differs between paralogs.**

461 Changes in gene expression concerning timing, quantity and rhythm may hint at possible
462 subfunctionalization or neofunctionalization of duplicated clock genes. We found that *PhGII*,
463 *PhGI2*, *PhPRR7b* and *PhPRR5b* had similar expression patterns to those previously described
464 in other plants in leaves (Fowler *et al.*, 1999; Matsushika *et al.*, 2000; Marcolino-Gomes *et al.*,
465 2014). In contrast, *PhPRR5a* and *PhPRR7a* that were the closest paralogs to the rest of the
466 species, showed modified expression patterns. *PhPRR5a* and *PhPRR7a* showed an advanced
467 phase, peaking before their respective paralogs, *PhPRR5b* and *PhPRR7b*. Interestingly, in
468 petals, *PhPRR7a* displayed a profile similar to the canonical *AtPRR7*. Moreover, the paralogs
469 *PhGII*, *PhGI2*, *PhPRR5a*, *PhPRR5b* and *PhPRR7b* delayed their maxima to the dark period.

470

471 **Leaves and petals have different clock coordination**

472 In the present work we identified significant oscillations in gene expression using the
473 JTK_CYCLE algorithm, a non-parametric method which also provided measures of phase
474 and period (Hughes *et al.*, 2010). As mentioned above, most analyzed genes displayed a
475 robust rhythm. Second, we performed an HANOVA test and we found genes that displayed a
476 differential expression pattern, comparing tissues and light conditions. The core clock genes
477 *LHY* and *TOC1* are found in basal picoeukaryotes, mosses, *Marchantia polymorpha* and all
478 higher plants (Corellou *et al.*, 2009; Holm *et al.*, 2010; Linde *et al.*, 2017). We found that
479 *PhLHY* and *PhPRR9* did not show any statistical differences regardless the tissue or light
480 cycle. In contrast, *PhTOC1* expression pattern differed between leaves and petals. This
481 indicates a basal change in the clock coordination between both tissues. This scenario maybe
482 further supported by the significant changes found for *PhFKF*, *PhPRR3*, and *PhGII* between
483 tissues. Finally, *PhGII*, 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 al.*,
498 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

542 phylogenetic reconstruction and for the annotation of protein sequences.

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

544 **Table S3.** Primers used for qPCR.

545 **Table S4.** Rhythmic analysis of transcriptional data.

546 **Table S5:** Coefficient of variation, gene expressions.

547

548 **Authors' contributions**

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

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555

556 **Competing interests**

557 The authors declare that they have no competing interests.

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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							
<i>PhPRR9</i>	R	R	Delay	R	Delay	AR	Delay
<i>PhPRR7a</i>	R	AR	Advance	AR	Advance	AR	Advance
<i>PhPRR7b</i>	R	R	Shift D/N	R	Advance	R	Delay
<i>PhPRR5a</i>	R	R	Shift D/N	R	Delay	R	Stable
<i>PhPRR5b</i>	R	R	Shift D/N	R	Delay	R	Delay
<i>PhPRR3</i>	R	R	Shift D/N	R	Advance	R	Stable
Midday loop							
<i>PhTOC1</i>	R	R	Shift D/N	R	Delay	R	Stable
<i>PhLHY</i>	R	R	Stable	AR	Advance	R	Advance
Evening loop							
<i>PhG11</i>	R	R	Shift D/N	R	Delay	R	Delay
<i>PhG12</i>	R	R	Shift D/N	R	Delay	R	Delay
<i>PhELF4</i>	AR	R	Delay	R	Stable	R	Stable
<i>PhCHL</i>	AR	AR	Delay	AR	Delay	AR	Delay
<i>PhFKF</i>	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
<i>PhCHL</i>	0.981	0.697	0.150	0.042
<i>PhELF4</i>	0.154	0.140	0.390	0.479
<i>PhFKF</i>	0.003	0.366	0.468	0.318
<i>PhGII</i>	0.019	0.049	0.011	0.009
<i>PhGI2</i>	0.291	0.298	0.041	0.012
<i>PhLHY</i>	0.675	0.222	0.192	0.137
<i>PhPRR3</i>	0.014	0.084	0.411	0.872
<i>PhPRR5a</i>	0.061	0.109	0.420	0.616
<i>PhPRR5b</i>	0.223	0.021	0.143	0.004
<i>PhPRR7a</i>	0.588	0.785	0.270	0.988
<i>PhPRR7b</i>	0.196	0.043	0.897	0.009
<i>PhPRR9</i>	0.405	0.486	0.508	0.584
<i>PhTOC1</i>	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 $\pm$ 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 $\pm$ 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

Figure 2

Figure 3

Figure 4

Figure 5

Petunia 12LD, ZT0

Petunia 12LD, ZT3

Petunia 12LD, ZT6

Petunia 12LD, ZT9

Petunia 12LD, ZT12

Petunia 12LD, ZT15

Petunia 12LD, ZT18

Petunia 12LD, ZT21

Figure 6

Petunia 12DD, ZT0

Petunia 12DD, ZT3

Petunia 12DD, ZT6

Petunia 12DD, ZT9

Petunia 12DD, ZT12

Petunia 12DD, ZT15

Petunia 12DD, ZT18

Petunia 12DD, ZT21