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Analysis of the Circadian Clock and its Role in Scent Emission in *Antirrhinum majus* and *Petunia hybrida*

*Técnicas Avanzadas en Investigación y
Desarrollo Agrario y Alimentario*

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Preface

Preface

This document has been written according to the regulations of the *Universidad Politécnica de Cartagena*. This thesis has the following sections: a general introduction, three chapters which correspond to accepted publications and the conclusions obtained from the present work. Every chapter includes its “material and methods” section.

During my PhD I performed a research stay at the University of Amsterdam in the research group of Dr. Robert Schuurink. This stay was financed by the *Programa de Movilidad de doctorandos en Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario (TAIDA) de la Universidad Politécnica de Cartagena*.

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Publications unrelated to the PhD thesis

Terry, M.I., & Mallona, I. (2015). First record of albinism in salema, *Sarpa salpa* (Perciformes: Sparidae), in the Mediterranean Sea. *Marine Biodiversity Records*, 8.

Weiss, J., Terry, M.I., Martos-Fuentes, M., Letourneux, L., Ruiz-Hernández, V., Fernández, J. A., & Egea-Cortines, M. (2018). Diel pattern of circadian clock and storage protein gene expression in leaves and during seed filling in cowpea (*Vigna unguiculata*). *BMC Plant Biology*, 18(1),33.

Abstract

Abstract

Organisms are adapted to cyclic environmental changes, such as tides, day-night alternance or seasonality. An innate timing device that registers these changes is called biological clock. Circadian clocks are comprised of interlocked regulatory loops of genes and proteins. Anticipation to periodic changes allows organisms to adapt their secondary metabolism, growth or reproduction. In plants, the simplest model found to date is the picoalga *Ostreococcus tauri*, which has only two clock genes: *CIRCANDIAN CLOCK ASSOCIATED 1* and *TIMING OF CAB EXPRESSION 1*. The analysis of species as *Arabidopsis thaliana* revealed the existence of a more complex net of clock elements, including the *PSEUDO-RESPONSE REGULATORS* family, the evening complex and the blue light receptors *ZEITLUPE* and *FLAVIN KELCH F-BOX PROTEIN 1*, among others.

An example of a character controlled by the circadian clock in flowering plants is scent emission, which shows a daily pattern even under constant light or dark conditions, a characteristic feature of processes controlled by the circadian clock. Although the volatile biosynthesis has been widely characterized, the regulation by the integration of external cues, such as light or temperature, is still poorly understood.

In the present work we followed two approaches in order to get a better understanding about circadian clock structure and processes controlled by the circadian clock. First, we characterized and compared the structure of circadian clock genes in leaves and petals of petunia (*Petunia hybrida*) under two different light conditions. We show that the clock genes *PSEUDO RESPONSE REGULATOR 7*, *5* and *GIGANTEA* are duplicated in petunia and that the gene expression patterns differ in leaves and petals with most genes peaking during the light phase in leaves and during the dark phase in petals. One of the petunia duplicated paralogs PaxiNPRR5a from *P. axillaris* and PinfSPRR5a from *P. inflata* have lost the canonical repression domain indicating a departure of their function from a standard transcriptional repression. When plants were transferred to constant darkness, clock genes tended to delay their maximum expression in both tissues. However, *LATE ELONGATED HYPOCOTYL*, which is a highly conserved gene across the green lineage, did not vary its expression pattern, regardless of the tissue or light conditions. We found that gene expression variability in leaves and petals depended on the time of the day. We studied the variability of expression and under light:dark condition, most analyzed genes showed their highest stability during the light

period in leaves, whereas in petals, stability was higher during the dark period. In general, when petunia was kept under constant darkness, the variability increased in both tissues.

In a second approach, we performed a detailed analysis of the effect of silencing two clock genes on growth, flower phenotype and scent emission. We silenced *CHANEL* (*PhCHL*), a *ZEITLUPE* ortholog, in petunia and *LATE ELONGATED HYPOCOTYL* (*AmLHY*) in snapdragon (*Antirrhinum majus*). The expression of *PhCHL* in leaves and petals differed when plants were acclimated to short days, characterized by an earlier expression peak in petals. *RNAi:PhCHL* silenced lines were shorter than wild-type plants and growth analysis revealed that stem and flower growth rate was slower. We also observed differences in the daily scent emission between non-transgenic and *RNAi:PhCHL* lines, both concerning the amount of scent emission, the scent profile and the timing of emission. While the emission of most volatiles, including ethyl benzoate and acetophenone, decreased in the silenced lines, others increased including benzaldehyde and phenylacetaldehyde. This differing effect was also found for timing as ethyl benzoate, displayed an early scent-emission phenotype while benzyl benzoate, displayed a delayed emission.

In case of *RNAi:AmLHY* snapdragon lines, down-regulation of *AmLHY* led to a decrease in the flower growth speed, even so final flower size was not affected. The floral scent profile in silenced lines was characterized-by a higher percentage of linalool and ocimene, whereas other volatiles such as 3,5-dimethoxytoluene, nonanal and decanal decreased. Interestingly, compounds such as the benzenoids methyl benzoate and methyl salicylate and the monoterpenes myrcene and ocimene showed a delayed emission, whereas maximum emission of phenylpropanoid methyl cinnamate was advanced compared to wild-type.

The present work leads to several general conclusion: first, the circadian clock transcriptional structure is tissue specific, and this specificity may be related to specialized functions performed in the different tissues, such as stomata opening in leaves or volatile emission in petals. Second, the variation in gene expression as well as the transcriptional noise depended on the tissue, day time and light:dark conditions. Finally, the floral aroma, in terms of contribution and amount of every single compound as well as number and class of volatiles, is controlled by the circadian clock. Circadian clock genes are involved in the maintenance of composition and timing of emission. Another interesting aspect is that the clock gene *AmLHY* regulated volatile biosynthesis at the biosynthetic pathway level: the

compounds that derive from benzoic acid, such as methyl benzoate and methyl salicylate, displayed a delayed emission whereas the monoterpenes myrcene and ocimene advanced their emission.

Resumen

Resumen

Los organismos están adaptados a cambios cíclicos y ambientales, tales como las mareas, la alternancia día-noche o el paso de las estaciones. Este sistema innato que registra dichos cambios se denominada reloj biológico, consiste en una red de genes y proteínas. Los genes reloj conforman bucles interconectados y mutuamente regulados. La anticipación a los cambios periódicos permite a los organismos ajustar su metabolismo secundario, crecimiento o reproducción. In plantas, el modelo más simple descrito hasta la fecha se encuentra en la picoalga *Ostreococcus tauri*, que solo posee dos genes reloj: *CIRCANDIAN CLOCK ASSOCIATED 1* y *TIMING OF CAB EXPRESSION 1*. El análisis de especies como *Arabidopsis thaliana* reveló la existencia de una red más compleja, incluyendo a la familia de los *PSEUDO-RESPONSE REGULATORS*, el complejo de la tarde y los receptores de luz azul *ZEITLUPE* y *FLAVIN KELCH F-BOX PROTEIN 1* entre otros.

Un ejemplo de un carácter controlado por el reloj circadiano en plantas con flor es la emisión de aromas, la cual muestra un patrón diario, incluso bajo condiciones de luz u oscuridad constante, una característica de los procesos controlado por el reloj circadiano. Aunque la biosíntesis de volátiles ha sido ampliamente estudiada, la regulación por parte del reloj circadiano continúa siendo poco conocida.

En el presente trabajo hemos seguido dos aproximaciones con el objetivo de entender mejor el reloj circadiano y los procesos controlados por el reloj circadiano. Primero, analizamos y caracterizamos la estructura de los genes reloj en hojas y pétalos de petunia (*Petunia hybrida*) bajo dos condiciones de luz diferentes. Mostramos que los genes *PSEUDO RESPONSE REGULATOR 7, 5* y *GIGANTEA* están duplicados en petunia y los patrones de expresión génica variaron entre hojas y pétalos, la mayoría de genes mostraban su máximo durante la fase de luz y durante la fase de oscuridad en pétalos. Cuando las plantas se transfirieron a oscuridad continua, los genes reloj tendieron a retrasar su máximo de expresión en ambos tejidos. Sin embargo, *LATE ELONGATED HYPOCTYL*, un gen altamente conservado en el linaje verde, no varió su patrón de expresión, independientemente del tejido o de las condiciones de luz. Además, encontramos que la variabilidad en la expresión génica en hoja y pétalo dependió de la hora del día. Con referencia a la variabilidad de expresión, en condiciones de luz-oscuridad la mayoría de genes analizados en hoja mostró su mayor estabilidad durante el periodo luminoso mientras que el pétalo mostró su estabilidad en la oscuridad. En general, cuando bajo condiciones de oscuridad constante, la variabilidad aumentó en ambos tejidos.

Hemos realizado un detallado análisis del efecto del silenciamiento de dos genes reloj en el crecimiento, fenotipo de la flor y la emisión de aromas. Silenciamos *CHANEL (PhCHL)*, un ortólogo de *ZEITLUPE*, en petunia y *LATE ELONGATED HYPOCOTYL (AmLHY)* en boca de dragón (*Antirrhinum majus*). La expresión de *PhCHL* en hojas y pétalos difirió cuando las plantas se aclimataron a días cortos, caracterizado por una expresión *PhCHL* avanzada en pétalos. Las líneas silenciadas *RNAi:PhCHL* eran más pequeñas que las silvestres y el análisis del crecimiento reveló que la tasa de crecimiento de tallo y flor era más lenta en plantas *RNAi:PhCHL*. Observamos también diferencias en la emisión de aromas diaria entre líneas no transgénicas y *RNAi:PhCHL*, en el perfil de aromas y en el tiempo de emisión. Mientras que la emisión de la mayoría de los volátiles, incluyendo el benzoato de etilo o acetofenona, disminuyó en las líneas transgénicas, otros aumentaron incluyendo benzaldehído y fenilacetaldehído aumentó en las plantas *RNAi:PhCHL*. Además en las líneas *RNAi*, algunos compuestos como el benzoato de etilo mostraron un fenotipo de emisión adelantada y el benzoato de bencilo, una emisión tardía.

En el caso de las líneas *RNAi:AmLHY* de boca de dragón, el silenciamiento de *AmLHY* disminuyó la velocidad de crecimiento de la flor, pero no afectó al tamaño final de la flor. El perfil aromático en las líneas silenciadas estuvo caracterizado por un porcentaje mayor de linalool y ocimeno, mientras que otros volátiles como el 3,5-dimetoxitolueno, nonanal y decanal disminuyeron. Curiosamente, encontramos compuestos tales como los bencenoides benzoato de metilo y salicilato de metilo y los monoterpenos mirceno y ocimeno que mostraron una emisión tardía, mientras que la emisión máxima del fenilpropanoide cinnamato de metilo fue avanzada en comparación con el silvestre.

De la presente tesis, podemos esbozar unas conclusiones generales. Primero, la transcripción del reloj circadiano es tejido específico, lo cual podría estar relacionado con funciones especializadas como la apertura de estomas en hojas o la emisión de volátiles en pétalos. Segundo, la variación en expresión génica o ruido transcripcional dependió del tejido, hora del día y condiciones de luz-oscuridad. Finalmente, el aroma floral depende de la contribución o cantidad de cada volátil y del número y clase de volátiles. Este perfil aromático varió a lo largo del día y está controlado por el reloj circadiano. Los genes reloj son necesarios para mantener la composición y tiempo de emisión. Otro aspecto interesante es que el gene *AmLHY* reguló la biosíntesis de volátiles en diferentes puntos: compuestos que derivan del ácido benzoico como benzoato de metilo y el salicilato de metilo mostraron una

emisión tardía mientras que los monoterpenos mirceno y ocimeno adelantaron su emisión.

Introduction

Introduction

Adaptation and anticipation to periodic oscillations: Biological clocks

Organisms are exposed to a changing environment: tides, day and night or season alternances are some examples. These changes are periodic, allowing organisms to anticipate and coordinate several internal processes such as metabolism, growth or reproduction, which typically show a rhythmic pattern. For example, the seasonal migrations in birds and mammals, sleep patterns or daily patterns of plasma hormones are well known rhythmic events. This innate timing control is known as biological clock has been widely studied in several models, including bacteria, plants and animals [1].

Most Biological clocks consist in a set of genes that establish interlocked feedback loops. At the same time, the integration of external and regulatory factors form key elements that maintain and set this clock. External cues can be classified in abiotic, such as temperature and light, and biotic, as interactions with relatives or other species, as pests [2], [3]. Internal cues include redox state, sugar availability and nutrients [4]. Circadian clocks share three characteristics: endogenous rhythmicity (persists in absence of external cues), entrainment (biological clocks are set by external signals, such as light or temperature) and temperature compensation (rhythm periodicity is maintained close to 24-h in a range of temperatures).[5]–[7]

In absence of these stimuli during a certain time (e.g. days), the periodicity may change, either becoming shorter or longer. A classical experiment consists in acclimating an organism to constant darkness or light and constant temperature. In this situation, called free-running conditions, cyclic processes or behavior of organisms, such as locomotor activity, hormone metabolism or clock gene

expression, can be irregular or damped. The study of organisms under natural and/or free-running conditions is useful in order to identify clock genes, analyze their functions and possible regulation, using mutants or genetically modified organisms (GMOs) in comparison to wild-type. Interestingly, when an organism presents, for example, a disrupted locomotor activity as a result of exposure to constant light or darkness, this disruption can be “fixed”. If the organism is acclimated or reentrained to a light:dark cycle, it will recover its rhythmic behavior [8]. As most analyzed cycles have a periodicity (or duration) of approximately 24-h, biological clocks are also called circadian clocks.

A simplified model of the circadian clock in plants: The picoalga *Ostreococcus tauri*

Picophytoplankton, which covers organisms with a maximum size of 2 μm , include several cyanobacteria and algae species such as *Synechococcus spp.*, *Prochlorococcus spp.* and *Ostreococcus spp.* In the last years, phytoplankton, especially *O. tauri*, has become important in studies related to ecology and genomics [9], [10].

Ostreococcus has been proposed as a model to study the biological clock in the green lineage. Recent works have identified and described the simplest clock model in this species comprised of the genes *TIMING OF CAB EXPRESSION 1* (*TOC1*), also called *PSEUDO-RESPONSE REGULATOR 1* (*PRR1*), and *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*). They constitute a simple but effective loop [11]. The expression of *TOC1* increases during the day, peaking before dusk, while *CCA1* increases its expression levels towards the end of the light phase, reaching its maximum at night. Interestingly, these oscillations are maintained under continuous light (LL). *TOC1* encodes a protein that contains two domains: CONSTANS, CONSTANS-like and TIMING OF CAB EXPRESSION1 (CCT) and RESPONSE REGULATORY (REG) whereas *CCA1* protein has a MYB-domain. *CCA1* represses

TOC1 via direct binding to the promoter. *TOC1* activates the expression of *CCA1*, conforming a simple but effective biological clock [11] (Figure 1A). Other clock related genes such as *EARLY FLOWERING (ELF)* or *GIGANTEA (GI)* are absent in *O. tauri* [11].

Figure 1: Circadian clock model

Ostreococcus tauri model (A), *PRRs* loop in *Arabidopsis thaliana* (B), the role of the evening complex (EC) (C) and the role of *GI* and *ZTL* (D). Every panel updates the previous scheme. Green arrow indicates activation, red line indicates inhibition. The transcriptional regulation of flowering is not shown here. Schematic representation based on Corellou *et al* 2009 (A),

Ito *et al.*, 2008 and Nakamichi *et al.*, 2010 (B), Kolmos *et al.*, 2011; Onai and Ishiura, 2005 and Helfer *et al.*, 2011 (C), Más *et al.*, 2003 and Kim *et al.*, 2007 (D).

Biological clocks in plants: The model derived from thale cress (*Arabidopsis thaliana*)

As mentioned above, the core loop of the circadian clock in plants consists of two genes, *TIMING OF CAB EXPRESSION1* (*TOC1*) and *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*) (Figure 1) [11]. *TOC1* and *CCA1* or the related gene *LATE ELONGATED HYPOCOTYL* (*LHY*), which also encodes a MYB protein, are highly conserved along the green lineage including the earthmoss (*Physcomitrella patens*), umbrella liverwort (*Marchantia polymorpha*), gymnosperms as the Japanese cedar (*Cryptomeria japonica*), grass species as rice (*Oryza sativa*) and flowering plants such as thale cress (*Arabidopsis thaliana*), garden petunia (*Petunia x hybrida*), wild tobacco (*Nicotiana attenuata*) and snapdragon (*Antirrhinum majus*) [12]–[17].

In *Arabidopsis*, lines overexpressing *LHY* and *CCA1* display a similar phenotype, which consists in altered flowering time and elongated hypocotyls, suggesting that their functions may be similar [15], [18]. The possible effect of *CCA1* or *LHY* on growth rate has not been reported, but they coordinate hypocotyl elongation with other genes such as *ELF3*, *MLK1* and *MLK2*. *CCA1* and *LHY* are partially redundant and necessary to maintain the circadian rhythmicity [19], [20]. In addition, *LHY/CCA1* repress the expression of *TIMING OF CAB EXPRESSION 1* (*TOC1*) whereas *TOC1* activates *LHY/CCA1* [21].

Other clock genes described include members of the *PSEUDO-RESPONSE REGULATOR* (*PRR*) family, the evening complex (*EC*), which includes the genes *EARLY FLOWERING3*, *EARLY FLOWERING4* (*ELF3* and *ELF4*) and *LUX ARRHYTHMO* (*LUX*), and the related genes *ZEITLUPE* (*ZTL*), *FLAVING-BINDING KELCH REPEAT F-BOX1* (*FKF1*) and *LOV KELH PROTEIN2* (*LKP2*); which are also involved in flowering signaling. The addition of these genes to the circadian clock

model resulted in a complex network established for the well know model plant *Arabidopsis thaliana*.

PSEUDO-RESPONSE REGULATORS

In *Arabidopsis*, five *PSEUDO-RESPONSE REGULATORS* (*PRRs*) members have been identified and widely studied -based on their role in the circadian clock: *PRR9*, *PRR7*, *PRR5*, *PRR3* and *TOC1/PRR1* [22], [23]. The mentioned order denotes the sequential expression maxima during the day. *PRRs* genes encode a protein that contain two principal domains: pseudo-receiver (PR) and CONSTANS, CONSTANS-LIKE and TOC1 (CCT) [24], [25]. Loss of function phenotypes for different *PRR* diverge slightly: *prp9* and *prp7* mutants display a longer period [26] whereas *prp5* and *toc1* shown a shorter period [27]. *LHY/CCA1* proteins activate the expression of *PRR7* and *PRR9* but repress *TOC1*. At the same time, *TOC1* acts as a positive regulator of *LHY/CCA1*. Moreover, the *PRRs* *PRR5*, *PRR7* and *PRR9* repress the expression of *LHY/CCA1* during the day until midnight (from ZT2 to ZT16). *PRR5*, *PRR7* and *PRR9* have a repression motif which consists in two regions, $L^{(E/D)}(L/I)S^{(L/I)}(R/K)R$ and $SXXSAF^{(S/T)}(R/Q)(Y/F)$, located between the PR and CCT domains [28] (Figure 1B).

The evening complex

The evening complex (EC) comprises the clock genes *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4* (*ELF4*) and *LUX ARRHYTHMO* (*LUX*, also known as *PHYTOCLOCK 1*, *PCL1*). *ELF3* and *ELF4* were identified in *Arabidopsis* as genes involved in flowering time [29], [30]. The *elf3* and *elf4* mutants display an early flowering phenotype compared to wild-type plants, as well as longer hypocotyls,

indicating that these genes play a role in photoreception. The EC is repressed by the core clock genes *CCA1/LHY* (Figure 1C) and by itself. In addition, *ELF3* has been proposed as a repressor of *PRR7* and *PRR9* [31]. On the other hand, *LUX* is a MYB transcription factor necessary to maintain a robust oscillation in higher plants [32]. *LUX* binds to *PSEUDO-RESPONSE REGULATOR 9* (*PRR9*) promoter, acting as a repressor [33] (Figure 1C).

***GIGANTEA* and *ZEITLUPE/FLAVIN-BINDING KELCH REPEAT F-BOX 1/LOV-KELCH PROTEIN 2* family**

GIGANTEA (*GI*) plays a critical role in flowering time but is also needed to maintain the amplitude and period length of clock genes [34], [35]. On the other hand, *ZEITLUPE* (*ZTL* or *ADAGIO1*, *ADO1*), *FLAVIN-BINDING KELCH REPEAT F-BOX1* (*FKF1*, also known as *ADAGIO3*, *ADO3*) and *LOV-KELCH PROTEIN2* (*LKP2* or *ADAGIO2*, *ADO2*) are paralogs. This family shares a PAS domain, kelch motifs and a LOV (Light-Oxygen-Voltage) domain, acting as a blue-light receptor. *GI*, *ZTL*, *FKF1* and *LKP2* are regulated by the circadian clock and are involved in flowering [36]–[38].

FKF1 encodes a protein that is 62% identical to its paralog *ZEITLUPE* [36]. In *Arabidopsis*, flowering is accelerated under long days. Under this condition, *GI* and *FKF1* proteins reach their maximum at the same time, before dusk, allowing the formation of the *FKF1-GI* complex [39]. The *FKF1-GI* protein complex plays a role in the transition from the vegetative to reproductive phase-by degrading *CYCLING DOF FACTOR 1* (*CDF1*), a flowering repressor.

ZTL is a gene related to the circadian clock but it is also involved in flowering and growth: *ztl* mutants display shorter hypocotyls and delayed flowering [40], while its

effect on growth speed has not been reported. This gene is not rhythmically expressed but its protein production shows a daily pattern. mRNA accumulation of *ZTL* is light independent, indicating that this gene does not participate in a feedback loop although it is necessary to maintain the circadian clock [37]. *ZTL* mediates the degradation of *TOC1* via its F-box domain [41] whereas *GI*, which is evening expressed, stabilizes *ZTL*, preventing *TOC1* from degradation [42] (Figure 1D). *ZTL* and *LKP2* delay flowering as these proteins avoid the interaction *FKF1*-*CDF1* [43], [44].

REVEILLE* and *PHYTOCHROME INTERACTION FACTORS

REVEILLES and *PHYTOCHROME-INTERACTION FACTORS* are involved in several processes such as growth and light transduction. These genes also play a role on the regulation of the circadian clock.

REVEILLES (*RVEs*) are MYB-like transcription factors, homologs to *CCA1/LHY*. In *Arabidopsis*, there are at least eight *RVEs*. *RVE8* displays a robust oscillation, reaching its maximum at dusk. This gene binds directly to the evening element EE, a regulatory element present in some clock genes such as *TOC1* or *PRR5*, activating the expression of *PRR5* [45]. *RVE4* and *RVE6* seem to act similar to their close homolog *RVE8*, binding to EE sites. *RVE4*, *RVE6* and *RVE8* may be partially redundant [46]. In contrast, *RVE1* connects the circadian clock and growth by promoting the activation of auxin synthesis during the light period [47].

The *PHYTOCHROMES* (*PHYs*) and *PHYTOCHROME-INTERACTION FACTORS* (*PIFs*) are bHLH transcription factors and regulate the light/dark and temperature transduction to the circadian clock and growth [48]. *PHYs* and the evening complex repress *PIF4* and *PIF5*, which causes the inhibition of growth at night [49], [50].

Gene and genome duplications

Gene and genome duplication events have been documented in animals and plants. Gene duplication may be followed by a mutation that causes a loss of function or an inactivation of one copy. Alternatively, both copies (paralogs) may retain part of the original function (subfunctionalization) or one of the duplicate genes may acquire a new function as a result of one or more new mutations (neofunctionalization) (see [51]–[53]).

Interestingly, duplicated genes seem to be retained in plants. Whole genome duplications (WGD, also referred as polyploidy) have occurred during plant evolution, contributing to speciation and diversification [54], [55]. The first recorded plant genome duplication occurred 319 million years ago (Mya) followed by another duplication event 192 Mya. These two events affected common ancestors of extant seed plants and extant angiosperms, respectively [56]. Then, genome triplication occurred associated with early diversification of Eudicots (gamma or γ -event) [57], which has been found in several genus including *Solanum*, *Glycine* or *Brassica* [16], [58], [59]. These species differ in the copy number of clock genes. As mentioned above, *Arabidopsis* presents one *GIGANTEA* copy gene whereas soybean (*Glycine max*) has three copies and show differences in daily expression (temporal) and among tissues (spatial) [60]. *Brassica rapa* shows a genome triplication event that occurred after the *Arabidopsis-Brassica* speciation and while *Arabidopsis* has five *PSEUDO-RESPONSE REGULATOR* members (*PRRs*), *B. rapa* has eleven copies which differ in structure, including partial deletions and partial fusions [61]. Structural changes, temporal and spatial expression patterns are congruent with subfunctionalization and neofunctionalization after duplication events, and can elucidate the evolution and specialization of the circadian clock across the green lineage.

Going further in plant models: Snapdragon (*Antirrhinum majus*) and petunia (*Petunia hybrida*)

The well-known plant model *Arabidopsis thaliana*, also known as thale cress, was the first plant to be completely sequenced [62]. This species has been widely used for studying plant development, genetics and evolution. There are other species useful to investigate other aspects related to plant biology, including dormancy in cherry trees (*Prunus avium*) and poplars (*Populus spp.*) [63], anthocyanin accumulation and synthesis in petunia (*Petunia spp.*) [64] or volatile organic compound (VOCs) biosynthesis [65].

Volatile organic compounds comprise a complex group of metabolites which plays a critical role in plant defense, acting as pest repellents or as bactericides and fungicides, in plant-plant interaction and in attracting pollinators. Two flowering plants, garden petunia (*Petunia spp.*) and snapdragon (*Antirrhinum majus*), have been used to study the synthesis and emission of floral volatiles, which are mainly emitted by petals [66]–[69]. In addition, the petunia and snapdragon genomes have been recently sequenced [16], [70], which allows us, combined with other techniques including gene silencing and CRISPR editing, to understand the molecular basis of biological processes.

A general description: Snapdragon and petunia

The snapdragon (*Antirrhinum majus*) is an herbaceous and annual plant, characterized by a flower of bilateral symmetry with five petals, partially fused resulting in a tube that ends in two lobes. *Antirrhinum* was included in the Scrophulariaceae family until 2005, when it was reassigned to the Plantaginaceae family [71]. The number of valid species seems uncertain. The European Environment Agency (EEA) lists 35 species (without including subspecies) but only 21 are valid names, i.e. *Antirrhinum orontium* is a synonym of *Misopates orontium* (consulted online September 2019). In contrast, other studies considered at least 25 species, which are widely distributed in the Mediterranean basin [72].

Petunia is a genus in the Solanaceae family, which includes the crops aubergine, tomato, pepper and potato, widely distributed in South America. Typically, *Petunia* spp. are herbaceous and annual plants, with flowers composed of five petals with actinomorphic or zygomorphic symmetry. *Petunia* species are pollinated by diurnal or nocturnal insects [73], except *P. exserta*, that is pollinated by hummingbirds. Thus, flower shape and size, color and floral fragrance vary widely among the genus *Petunia*. Several studies focused on these differences among species, such as *Petunia axillaris*, *P. exserta* and *P. integrifolia*. Furthermore, their hybrids, such as *Petunia x hybrida*, a cross between *P. axillaris* and *P. integrifolia*, are commonly used lines. Interestingly, most of the garden petunias correspond to *P. hybrida*.

Volatile organic compounds

Volatile organic compounds (VOCs) are involved in several processes including stress responses, plant defense, plant-plant interaction and pollinator attraction. Plant volatiles are classified in four principal groups based on their metabolic

pathway: lipoxygenase (LOX), mevalonic acid (MVA), methylerythritol (MEP) and shikimate/phenylalanine (Figure 2) [74].

Figure 2: Volatile organic compounds: Metabolic pathways.

Pathways: LOX: lipoxygenase, MVA: mevalonic acid, MEP: methylerythritol and shikimate/phenylalanine. (From Dudareva et al., 2013).

There is another classification of plant volatiles, based on the type of molecule:

- Phenylpropanoid/benzenoids are compounds derived from the aromatic aminoacid phenylalanine (Phe) through the shikimic acid pathway. These volatiles are characterized by an aromatic ring. Phenylpropanoids and benzenoids are commonly found in the floral fragrance of petunia, wild tobacco or snapdragon [75], [76], [77], [78].
- Terpenes. This group of compounds is derived from the five carbons (C_5) unit isopentenyl diphosphate (IPP). The addition of C_5 units results in monoterpenes (C_{10}) that included the volatiles linalool, myrcene and ocimene, sesquiterpenes (C_{15}) such as the VOCs farnesene or caryophyllene and diterpenes (C_{20}) as gibberellins, among other terpenoids [74], [79], [80].

- Fatty acid derivatives. Several volatiles, including the aldehydes *n*-hexenal and *n*-nonenal and methyl jasmonate (MeJA) derive from the fatty acid linolenic acid [74]. These volatile organic compounds are commonly found in leaves and are known as green leaf volatiles (GLVs). GLVs are involved in plant defense acting as repellents [81], conferring fungal resistance or acting as fungicides [82], bactericides [83] or in the attraction of predators and parasites of pests (parasitoids) [84], [85]. In addition, MeJA also is involved in the stress signaling network [86], can induce a defensive response in unharmed plants and is related with germination and flowering among other pathways.
- Nitrogen containing compounds (NCC), which include benzoxazinoids and oximes, are involved in plant defense due to their toxicity and antifeedant activity among other functions [87], [88]. The NCC group includes volatile organic compounds such as indole and methyl nicotinate. When a plant is damaged by an herbivore, the plant activates a defense mechanism consisting on volatile release, which is enhanced by the NCC indole [89].

Scent profiles and biological functions

The complexity of the floral fragrance depends on the contribution (or amount) of every single VOC, but also on the class (e.g. monoterpene, phenylpropanoid) and number of emitted compounds [90]. The combination of these factors results in a specific aroma blend. The aroma of *Petunia* is mainly composed of benzenoids [91], while in the carnation flower (*Caralluma europaea*), monoterpenes are the most abundant compounds [92]. Interestingly, this particular aroma varies among species and accessions [77], [93], [94]. Pollinators are capable to discriminate and identify the floral fragrance of different plant species [95], [96] or even cultivars, differing in their scent profile [97]. In addition, the emission of certain species is positively

correlated with pollinator activity, such as bumblebees and snapdragon or moths and petunia [66], [98]. The daily scent emission is maintained under constant light or dark conditions, indicating that this trait is controlled by the circadian clock. Besides, the flower scent, in qualitative and quantitative terms, can vary depending on the flower age or developmental stage, environmental conditions and after pollination [77], [78], [99], [100]. For example, the number of detected VOCs in *Lilium* flowers increases during flower development while the amount decreases towards the last stage [99]. The synthesis of volatile compounds and their emission is therefore a complex process influenced by the development stage as well as abiotic and biotic factors.

Several studies have questioned whether a floral fragrance, or a single volatile, is attractant or repellent. There are different approaches such as counting and/or capturing insect visitors per plant or flower for a period of time [101], [102] or analyzing electroantennogram responses [103]. However, it can be difficult to determine if a single volatile acts as attractant or deterrent: an insect can be exposed to a pure compound but the response may vary depending on the volatile concentration [104]. The relationship between insects and other animals, both pollinators and harmful species, and flowers suggest an interesting viewpoint: the scent profile may attract non-desired herbivores or repel potential pollinators. This possibility leads to the conclusion that flower fragrance may be under selective pressure. Kessler et al. [105] demonstrate by using transgenic petunias that a lower VOC emission or the lack of emission of certain VOCs, leads to a reduction in damage by herbivores.

Scent emission: Importance of the circadian clock

Several studies describing the daily fragrance emission in flowering plants including *Antirrhinum* [66], *Silene* [106], *Petunia* [98], *Nicotiana* [107] or *Rosa* [108], show that VOC emission follows a rhythmic pattern. Although the biosynthesis of volatile

compounds has been described in detail, the regulation of scent emission by the circadian clock genes is still poorly known. The analysis of natural variation, mutant lines or genetically modified organism (GMOs) are the key to understand the relationship between scent synthesis, its release and the integration of environmental cues. Recently, the knock-down regulation of the clock genes *LATE ELONGATED HYPOCOTYL* (*LHY*) in garden petunia [109], and *LHY*, *ZEITLUPE* (*ZTL*) and *TIMING OF CAB EXPRESSION1* (*TOC1*) in wild tobacco [110], [111] has revealed that these genes are involved in the regulation of scent emission. In petunia, *LHY* represses the expression of *ODORANT1* (*ODO1*) [109], which regulates the phenylpropanoid/benzenoid pathway [112]. As result, the emitted levels of the benzenoids methyl benzoate, benzyl benzoate and benzaldehyde and the phenylpropanoid isoeugenol are in general lower and shifted, showing an advanced phenotype. These results are similar to observations in wild tobacco, where the down regulation of *LHY* decreases the emission of the benzenoid benzyl acetone and also advances its maximum emission. Similarly, silencing of *ZTL* decreases drastically the emission of this volatile [110]. In contrast, the silenced *TOC1* tobacco lines show a slight delay in the emission time of benzyl acetone and an increase in the emitted amount [111].

Petunia and wild tobacco belong to the Solanaceae family and are considered nocturnally emitting plants [98], [113]. So, the regulation of floral fragrance emission by the circadian clock may vary in daytime emitting species, such as snapdragon or orchids [66], [114]. Furthermore, the regulation of compounds other than phenylpropanoid/ benzenoids, such as terpenes and fatty acid derivatives, may also differ. Another important aspect is the biological effect of the altered floral aroma on pests or pollinators. Yon et al. [111] found that flowers with early emission and flower angle movement (*irLHY* lines) were preferred by moths and as a result, these plants presented more mature capsules than control plants. The advanced phenotype does not explain this preference, as moth activity occurs later, but authors

suggest an alternative hypothesis based on changes in minor volatiles with special attraction to moths [111]. In conclusion, the analysis of clock gene effects on volatile organic compounds should include major and minor compounds in order to describe changes in the scent profile.

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Objectives of the Thesis

Objectives of the Thesis

The general aim of this Thesis was to characterize the transcriptional structure of the circadian clock in *Petunia hybrida* leaves and petals and uncover the role of clock genes in the regulation of scent emission in the diurnally emitting plant *Antirrhinum majus* and the nocturnally emitting *P. hybrida*. The present work contributes to understand the integration of external cues and volatile organic compound emission.

Chapter I

- Identification and characterization of the transcriptional structure of circadian clock genes in leaves and petals of petunia (*Petunia hybrida*) under light:dark and constant darkness.
- Characterization of gene expression variability (transcriptional noise) across tissues and light conditions.

Chapter II

- Characterization of daily expression by qPCR of *CHANEL* (*PhCHL*), a *ZEITLUPE* ortholog, in petunia (*Petunia hybrida*) leaves and petals. We also validated the reference genes in leaves and petals.
- Analysis of the effect of knocking down *PhCHL* on scent profile and daily scent emission.

Chapter III

- Characterization of daily expression by qPCR of *LATE ELONGATED HYPOCOTYL* (*AmLHY*), in snapdragon (*Antirrhinum majus*) leaves.
- Analysis of the effect of knocking down *PhCHL* on flower phenotype, scent profile and daily flower fragrance emission.

Chapter I: Transcriptional structure of petunia clock in leaves and petals.

Graphical abstract

- Genes and proteins identification (Arabidopsis as **query sequence**)
- Primer design

*P. axillaris*_GI1 THYNRPVYKAVHRDGGADRSSRGS
*P. inflata*_GI3 THYNRPVYKAVHRDGGADRSSRGS
*P. inflata*_GI1 THYNRPVYKAVHRDGGADRSSRGS
*A. thaliana*_GI THYNRPVYKTEQNGDTERN-CLSP
*P. inflata*_GI2 THYNRPLFKVDRODKGADRSTSGSC
*P. axillaris*_GI2 THYNRPLFKVDRODKGADRSTSGSC
160.....170.....

- Phylogeny
- Domain identification

Petunia: growth and acclimation

- Light:dark
- Constant darkness

- Sampling: leaves and petals (time course)
- RNA extraction for qPCR
- Data analysis

Article

Transcriptional structure of petunia clock in leaves and petals.

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Abstract: The plant circadian clock coordinates environmental signals with internal processes including secondary metabolism, growth, flowering and volatile emission. Plant tissues are specialized in different functions, and petals conceal the sexual organs while attracting pollinators. Here we have analyzed the transcriptional structure of the petunia (*Petunia x hybrida*) circadian clock in leaves and petals. We recorded the expression of 13 clock genes in petunia under light:dark (LD) and constant darkness (DD). Under light:dark conditions, clock genes reached maximum expression during the light phase in leaves and the dark period in petals. Under free running conditions of constant darkness, maximum expression was delayed, especially in petals. Interestingly, the rhythmic expression pattern of *PhLHY* persisted in leaves and petals in LD and DD. Gene expression variability differed among leaves and petals, day time and photoperiod. The transcriptional noise was higher especially in leaves under constant darkness. We found that *PhPRR7*, *PhPRR5* and *PhGI* paralogs showed changes in gene structure including exon number and deletions of CCT domain of the PRR family. Our results revealed that petunia petals presented a specialized clock.

Keywords: Flower development; circadian clock; entrainment of circadian rhythm; petal; Solanaceae; tissue specific; transcriptional noise

1. 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 [1]. Circadian processes have been studied in plants for a long period of time (see McClung for a historical overview [2]). 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 ARRHYTHMO (*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 [3]. Every new discovery has added a level of complexity and new interpretation of the circadian clock model [4].

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* [5]. There is an additional blue-light receptor component with histidine kinase activity and circadian clock effects [6]. Therefore, basic clocks may be 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 [7]. Plant genomes have been subject to genome duplications and in some cases, followed by non-random elimination of duplicated genes [8,9]. 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 [10].

The genomes of the garden petunia and its ancestors *Petunia axillaris* and *P. integrifolia* have been recently sequenced [11]. *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 paleohexaploidization 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* [11]. 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 [12], and leaves and roots differ in the regulatory network, as a result of differences in light inputs [13,14].

Petal development starts with the activation of the so-called B function genes in both gymnosperms and angiosperms [15]. 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 [16–21]. Once organ identity is established and right after anthesis, there is a transcriptional reprogramming [22]. 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 [23,24]. 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 [25,26]. Flowers enter rapid senescence upon pollination as a result of ethylene release [27–29].

Floral scent release depends on petal development in a quantitative way [22], and is circadian regulated in monocots and dicots such as *Antirrhinum*, *Narcissus*, rose, or garden petunia [30–34]. Most flowers analyzed emit scent preferentially during the day or during the night. The *LHY* and *ZTL* orthologs control scent emission in *Petunia*, *Nicotiana attenuata*, and *Antirrhinum majus* [35–38].

The Solanaceae *Petunia* and *Nicotiana* emit higher quantities during the night, indicating an identity and circadian component controlling this trait [34,39].

In the current work, we have addressed the structure of the *petunia* circadian clock from three different perspectives: gene structure, transcript abundance, and gene expression variability. 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 the structural and expression level and suggests an organ specific transcriptional structure of the plant circadian clock.

2. Materials and Methods

2.1. Plant materials and experiment design

We used the *Petunia hybrida* W115 or Mitchell for all the analyses. Plants were grown under greenhouse conditions until further use. Experiments under controlled conditions in growth chambers were performed as described [40], with the following modifications. For the control experiments, plants were adapted to light:dark growth chamber conditions for at least 1 week. Day:night (LD) 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. Plants were transferred from LD cycle to a continuous dark cycle (DD) 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 3 h, starting at ZT0 and tissues were immediately frozen in liquid nitrogen. In the case of DD experiment, sampling also started at ZT0, during the first 24 h under continuous dark.

2.2. Phylogeny, bioinformatics and data analysis

Previous studies showed that most genes of *Petunia* W115 can be assigned to *Petunia axillaris* [11]. We used clock genes sequences from *Arabidopsis thaliana* (<https://www.arabidopsis.org/>) to identify *P. axillaris* orthologs by blast in Sol Genomics Network (<https://solgenomics.net/>). Protein sequences from *Petunia* and other species were obtained from several databases including Sol Genomics Network (<https://solgenomics.net/>, Solanaceae), Snapdragon Genome Database (<http://bioinfo.sibs.ac.cn/Am/>, *Antirrhinum majus*) [41], Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) and GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) (Tables S1 and S2 for protein accessions and details). We used the corresponding predicted proteins to identify the intron-exon boundaries using Genewise [42]. The corresponding exon-intron boundaries were plotted using the exon-intron graphic maker (<http://wormweb.org/exonintron>). Protein alignment was performed with CLUSTALX [43]. Phylogenetic analysis was performed with MEGA X [44] using neighbor-joining method (NJ), JTT (Jones, Taylor and Thornton, [45]) as model of amino acid substitution and 500 bootstrap replicates. Phylogenetic tree of PSEUDO-RESPONSE REGULATORS (PRR) proteins was conducted by using the highly conserved domain pseudo-receiver (PR) [46] whereas phylogenetic tree of GIGANTEA (GI) proteins was performed by using full length proteins. Trees were visualized and annotated with “ggtree” [47] using R (R version 3.5.1), as well as the multiple sequence alignment. Protein domains were predicted using the web-based tool PROSITE [48], schematic proteins were plotted with the R package “drawProteins” [49].

Detection of rhythmic gene expression was performed using the non-parametric statistical algorithm “RAIN” [50]. We analyzed leaves and petals, under two light conditions, 12 h light/12 h dark (LD) and constant darkness (DD). To determine if the gene expression pattern of certain gene differed between tissues (leaves and petals) or light conditions (light:dark and constant darkness),

we used an harmonic ANOVA (HANOVA) and to detect noise changes we used a scale test (HarmScaleTest) implemented in the R package “DODR” [51]. The HANOVA detects changes in amplitude and phases and HarmScaleTest uses a F-Test for variances to determine if the biological noise levels differed or not in two time series. We plotted the graphics with “ggplot2” [52].

2.3. Gene expression analysis by qPCR

RNA was extracted from three biological replicates per time point of leaves and petals (flower tubes were excluded) using acid phenol [53]. 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 [54], the following protocol was used for 40 cycles: 95 °C for 5 s, 60 °C 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 [55] (Table S3) and the following protocol was used for 40 cycles: 95 °C for 5 s, 60 °C for 20 s (55 °C for *PhGI1* and *PhGI2*) and 72 °C 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 (Figure S1). Normalized expression was calculated as described [56] and *ACTIN* (*PhACT*) was the internal control gene, a stable gene in circadian studies in petunia leaves and petals [37].

We determined the mean normalized expression. For each gene, tissue, and light condition, normalized expression was calculated as described previously [56], then the expression level for each time point was divided by the average expression across the time-course [57]. In addition, to analyze the gene expression variability, we calculated the coefficient of variation (CV) of every gene per time point, tissue and light:dark conditions. We also plotted the normalized expression of every biological replicate.

3. Results

3.1. The duplicated *PRR7*, *PRR5* 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* [11] 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 [11]. We reconstructed a phylogenetic tree of *PRR* genes, by using the conserved domain pseudo-receiver (PR), of Solanaceae and Arabidopsis (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 (Figure 1) [58]. 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 [11].

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 (Figure S2). The gene model in *Arabidopsis* comprises six 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: PR (Pseudo-receiver Domain) and a CCT (CONSTANS, CONSTANS-like, and TIMING OF CAB EXPRESSION 1 (TOC1/PRR1)) [59] (Figure S3A). PRR5, PRR7, and PRR9 have a repression motif which consists of two regions, $L^{(E/D)}(L/I)S^{(L/I)}(R/K)R$ and $SXXSAF^{(S/T)}(R/Q)(Y/F)$, located between the PR and CCT domains. They interact with TOPLESS (TPL) and TOPLESS-RELATED PROTEINS (TPRs) [59,60], creating a transcriptional repression complex. This complex represses the transcription of the core genes CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*) and LATE ELONGATED HYPOCOTYL (*LHY*) [59,61]. We used *Arabidopsis* as the model, and we compared it with petunia sequences. We found that all the PRR members of *P. axillaris* and *P. inflata* shared the PR domain (Figure 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 from the PRR7 protein 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*, and *S. tuberosum*) (Figure S3B). However, the CCT domain was 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.

We also identified the repression motif in *Petunia axillaris* and *P. inflata*, which shared a high homology with *Arabidopsis* (Figure 2). These motifs are present in PRR5, PRR7, and PRR9 but absent in PRR3 and TOC1 (Figure 2). We found that the paralog PRR5a of *P. axillaris* and *P. inflata* had two major differences. The $L^{(E/D)}(L/I)S^{(L/I)}(R/K)R$ motif present in the PRR9, 7, and 5 was not canonical as the glutamic/aspartic amino acid of the second position had been replaced by an alanine. Furthermore, both PRR5a had lost the $SXXSAF^{(S/T)}(R/Q)(Y/F)$ motif. We compared the PRR5 proteins of several Solanaceae and *Arabidopsis* and found that all share the aspartic acid in the second position of the first motif and the complete repression motif absent in *PaxiNPRR5a* and *PinfSPRR5a* (Figure S4). This indicates that the D->A amino acid substitution and motif deletion are specific to the *Petunia* lineage.

The $SXXSAF^{(S/T)}(R/Q)(Y/F)$ of *PaxiNPRR7b* was interrupted by a sequence insertion of 26 amino acids. The sequence of both regions varied among paralogs, as SELSAFSRY was found in the PRR7a lineage and SHLSAFSRY in the PRR7b lineage (Figure 2). Altogether, these changes indicate that PRR duplication occurred before *Petunia* speciation. The absence of the transcriptional repression motif in PRR5a and the interruption of PRR7b in *Petunia inflata* suggest that these paralogs have lost their repression activity.

GIGANTEA (*GI*) plays a role in flowering time but is needed to maintain the period and amplitude of clock genes [62,63]. *GI* is a single copy gene in the *Arabidopsis* genome [62] and it is found in one to three copies in the Solanaceae genomes. *Petunia axillaris* has two copies *PaxiNGI1*, *PaxiNGI2*, whereas *P. inflata* has three copies *PinfS6GI1*, *PinfS6GI2*, and *PinfS6GI3* [11]. Interestingly, the genes *PaxiNGI1* and *PaxiNGI2* are present in the genome of *P. hybrida* Mitchell. We will refer to *GIGANTEA* genes in *P. hybrida* as *PhGI1* and *PhGI2*. The proteins *PinfS6GI1* and *PaxiNGI1* share an N-terminus conserved with *AtGI* that was absent in *PaxiNGI2* (Figure 3, Figure S5). Furthermore, *PaxiNGI2* showed a 41 amino acid insertion that was not conserved in *PinfS6GI2* or other *GI* genes. The *PinfS6GI3* was much shorter than the other paralogs, a feature conserved in *Nicotiana benthamiana*

NbGI3 (Figure 3). The PinfSGI1 had an additional C-terminal fragment of 105 amino acids absent from the rest of the GI genes analyzed (Figure 3, Figure S5).

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

Figure 3. *GIGANTEA* (*GI*s) phylogenetic tree. Amino acid sequences were aligned using CLUSTALX. Phylogenetic analysis was performed using MEGA X and trees were plotted with the library “ggtree” (R version 3.5.1). The phylogenetic trees were built with the neighbor-joining method (NJ) and JTT (Jones, Taylor and Thornton) as model of amino acid substitution. The tree displays the bootstrap percentage (from 500 replicates) next to branches (A). The multiple sequence alignment is displayed on the right-side (B). This tree contains 35 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*). Accessions are listed in Table S2.

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

Several clock models have been described in the last years; one of the most popular plant clocks defines three loops called morning, central, and evening loops. These describe the time of the day when certain genes are preferentially expressed [64]. In *Arabidopsis*, during the morning *CIRCADIAN CLOCK-ASSOCIATED 1* and *LATE ELONGATED HYPOCOTYL* (*CCA1/LHY*) repress *PSEUDO-RESPONSE REGULATOR* members (*PRRs*), which are sequentially expressed (*PRR9*, *PRR7*, *PRR5* and finally *TIMING OF CAB EXPRESSION 1*, *TOC1* or *PRR1*, at dusk) [59]. The *PRR* family represses *CCA1/LHY*. *CCA1/LHY* also inhibits *GIGANTEA* (*GI*) and the evening complex (*EC*) expression during the morning [65]. The *EC* is comprised by *LUX ARRHYTHMO* (*LUX*), *EARLY FLOWERING 4* and *3*, (*ELF4* and *ELF3*). At dusk, *TOC1* represses the evening genes *GI*, *ELF4*, and *LUX*. *TOC1* interacts with *CCA1 HIKING EXPEDITION* (*CHE*) and mediates the repression of *CCA1* [66]. Several activators have been described, including *REVEILLE 8* (*RVE8*) and *LIGHT-INDUCIBLE AND CLOCK-REGULATED* (*LNKs*) that positively regulate the transcription of *PRR5* and *TOC1* or the complex *LIGHT-REGULATED WD-TEOSINTE BRANCHED 1-CYCLOIDEA-PCF* (*LWD-TCP*), which activates the transcription of *CCA1* [67–69]. 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* [11], 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 and clock-associated genes analyzed included *PhGI1*, *PhGI2*, *PhELF4*, *PhCHL* (the *ZEITLUPE* ortholog), and *PhFKF* (*FLAVIN-BINDING KELCH REPEAT FBOX1*). This analysis was performed in petunia that was acclimated to light:dark conditions of 12 h light and 12 h dark (LD) or continuous dark (DD) conditions.

The non-parametric algorithm RAIN groups gene expression data by their sampling time point e.g., ZT0, ZT3. These groups are assigned to the falling and to the rising part of the oscillation or wave. Then each part, falling and rising, is analyzed by a test against umbrella alternatives [70]. The peak of the umbrella varied, allowing RAIN to test different wave forms. Regardless, if a gene is rhythmically expressed (significant *p*-value) or not, RAIN provides the time point of a peak, if found. We compared two parameters between leaves and petals under a LD cycle: rhythmicity of expression (oscillation) and time point with maximum expression (phase) (Table 1). Concerning rhythmicity, most genes showed a robust oscillation pattern except *PhELF4* and *PhCHL* in leaves, and *PhCHL* in petals, that showed a peak but no significant rhythm (Table 1).

In leaves, most genes had their maximum expression during the light phase, except *PhELF4* that reached its maximum level at ZT12. Petunia clock genes peaked at early morning (*PhLHY*, at ZT0), midday (*PhPRR9* and *PhGI2*, at ZT6), and before dusk (*PhPRR3*, *PhPRR5a*, *PhPRR5b*, *PhPRR7a*,

PhPRR7b, *PhTOC1*, *PhCHL*, *PhGI1*, and *PhFKF*, at ZT9) (Table 1). In contrast in petals, whereas the clock genes *PhPRR5b*, *PhPRR7a*, *PhPRR7b*, *PhCHL*, *PhELF4*, and *PhLHY* peaked at the same time as observed in leaves, the remaining genes delayed their expression. *PhPRR9* peaked at ZT9 meanwhile *PhPRR5a*, *PhPRR3*, *PhTOC1*, *PhGI1*, *PhGI2*, and *PhFKF* shifted their expression maximum to the dark period. These genes peaked at ZT12 (*PhPRR5a*, *PhGI1*, *PhGI2* and *PhELF4*) and ZT15 (*PhPRR3*, *PhTOC1*, and *PhFKF*) (Table 1).

Table 1. Rhythmic analysis of transcriptional data. Detection of rhythmicity in petunia clock genes in leaves and petals under a light:dark cycle (LD) and constant darkness (DD). This analysis was performed with a non-parametric method implemented in the Bioconductor package “RAIN”. Significant *p*-values ($p < 0.05$) indicate a robust oscillation. Phase denotes the time point with the maximum gene expression or peak.

Gene	LD				DD				
	Leaf		Petal		Leaf		Petal		
	<i>p</i> -value	Phase	<i>p</i> -value	Phase	<i>p</i> -value	Phase	<i>p</i> -value	<i>p</i> -value	Phase
<i>PhPRR9</i>	0.000	6	0.000	9	0.001	9	0.007	12	0.000
<i>PhPRR7a</i>	0.000	9	0.037	9	0.007	9	0.094	3	0.000
<i>PhPRR7b</i>	0.000	9	0.000	9	0.330	9	0.006	18	0.000
<i>PhPRR5a</i>	0.008	9	0.000	12	0.001	9	0.000	15	0.008
<i>PhPRR5b</i>	0.000	9	0.000	9	0.016	12	0.000	15	0.000
<i>PhPRR3</i>	0.000	9	0.000	15	0.143	12	0.000	15	0.000
<i>PhTOC1</i>	0.000	9	0.001	15	0.017	21	0.000	18	0.000
<i>PhGI1</i>	0.000	9	0.007	12	0.000	12	0.000	18	0.000
<i>PhGI2</i>	0.000	6	0.000	12	0.000	12	0.000	15	0.000
<i>PhELF4</i>	0.074	12	0.011	12	0.000	15	0.000	18	0.074
<i>PhCHL</i>	0.251	9	0.059	9	0.380	0	0.075	15	0.251
<i>PhFKF</i>	0.000	9	0.004	15	0.001	9	0.000	12	0.000
<i>PhLHY</i>	0.000	0	0.030	0	0.005	0	0.000	0	0.000

3.3. Continuous darkness shifted the expression patterns of clock genes

In order to study the entrainment of the petunia circadian clock to the light:dark cycle, petunia plants were transferred from 12 h light:dark (LD) conditions to continuous darkness (DD). We found that *PhPRR7b* and *PhPRR7a* lost their robust oscillation ($p > 0.05$) in leaves and petals, respectively; and as described under a LD cycle, *PhCHL* did not oscillate significantly ($p > 0.05$). In addition, we observed that DD tended to shift the expression patterns of some genes (Table 1).

In leaves, we divided the clock genes into three groups. The first group included *PhPRR5a*, *PhPRR7a*, *PhPRR7b*, *PhFKF*, and *PhLHY*, which maintained their time of maximum expression. The second group included *PhCHL*, advanced by 9 h. Finally, the last group comprised *PhPRR3*, *PhPRR5b*, *PhPRR9*, *PhGI1*, and *PhELF4* delayed by 3 h, *PhGI2* by 6 h, and *PhTOC1* by 15 h (Figure 4, Table 1).

The effect of continuous darkness on petunia petals differed from leaves. Only two genes, *PhPRR3* and *PhLHY* kept their peak or time with maximum expression. *PhPRR7a* and *PhFKF* showed an advanced pattern, peaking 3 h early. Finally, the maximum expression was delayed 3 h for *PhPRR5a*, *PhTOC1*, *PhGI1*, *PhGI2*, 6 h for *PhPRR5b*, *PhPRR9*, *PhCHL*, and *PhELF4* and 9 h *PhPRR7b* (Figure 4, Table 1).

Altogether, *PhLHY* showed a rhythmic oscillation, peaking at early morning regardless of light conditions and tissue. On the other hand, *PhCHL* did not show a rhythmic expression in leaves or petals under light:dark or constant darkness. Besides, we observed that continuous darkness had a

different effect on clock genes in leaves and petals, as *PhPRR7b*, *PhPRR5a*, or *PhPRR3* that showed an organ specific change in phase in response to constant darkness (Table 1).

Figure 4. Daily changes in gene expression in petunia. Expression of clock genes in leaves (red) and petals (blue) under a 12 light:dark cycle (LD) and constant darkness (DD). LD cycle is represented by a white and grey area whereas DD cycle is represented by a dark area. Gene expression is represented as mean normalized expression (NE), first every clock gene was normalized to *PhACT* and then, for each time point the expression was divided by the mean expression level. ZT 0 (*zeitgeber* time) denoting light on, and ZT12, light off. Results represent mean $\pm$ SD (n = 3).

3.4. Rhythmicity and photoperiod sensitivity are tissue specific

An important paradigm in the analysis of circadian clock gene expression is the effect of continuous darkness on the genes thought to have a circadian control [71]. We performed an analysis to detect changes in amplitude and phase, in two time series by using Harmonic ANOVA [51]. First, we analyzed differences between tissues by comparing daily gene expression in leaf and petals under light:dark (LD) and under constant darkness (DD). Second, in order to identify the effect on light conditions, we compared LD and DD cycles for leaves and for petals (Table 2). We found that *PhPRR5a*, *PhPRR7a*, *PhPRR9*, and *PhLHY* showed a robust expression pattern regardless of the tissue or photoperiod ($p < 0.05$). In contrast, under light:dark conditions, *PhGI1*, *PhPRR3*, and *PhTOC1* had higher amplitude and were delayed in petals compared to leaves (Table 2). *PhFKF* showed higher amplitude in leaves and was delayed in petals (Table 2). When we compared leaves and petals at LD versus DD, *PhTOC1*, *PhCHL*, and *PhGI2* in leaves, and *PhPRR7b*, *PhPRR5b*, *PhCHL*, *PhGI1*, and *PhGI2* in petals, showed a different expression pattern. Interestingly, all these genes were delayed under constant darkness, but differed in their highest amplitude. In leaves, *PhTOC1* displayed the highest amplitude in LD whereas *PhGI2* and *PhCHL*, in DD. In petals, *PhPRR7b* and *PhGI1* amplitude was higher under a light:dark cycle and *PhPRR5b*, *PhGI2*, and *PhCHL* showed their maximum amplitude in DD (Table 2).

These results indicate that there are two sets of genes with changes in rhythmicity, both in phase and amplitude, in leaves and petals and a group of stable genes comprising *PhPRR5a*, *PhPRR7a*, *PhPRR9*, and *PhLHY*. Furthermore, the effect of photoperiod appeared to be organ-specific for those genes that showed significant changes. From all the different genes analyzed, *PhPRR7b* appeared to be specifically affected by DD in petals, indicating a modified control in this organ.

Table 2. Analysis of differential gene expression in petunia leaves and petals under two light conditions: light:dark (LD) and constant darkness (DD). We compared the daily gene expression of both tissues (Petal vs. leaf), under light:dark and constant darkness. We also compared every single tissue under two light conditions (LD vs. DD). This analysis uses Harmonic ANOVA (HANOVA) to test differences in amplitude and phase. A p value < 0.05 indicated that the amplitude and/or phase were significantly different between tissues (Petal vs. leaf) or between light conditions (LD vs. DD). Amp displays the tissue with the higher amplitude and Phase symbols + and – denotes delayed and advanced, respectively, per tissue or experimental condition.

Gene	Petal vs. leaf				LD vs. DD							
	LD	Amp	Phase	DD	Amp	Phase	Leaf	Amp	Phase	Petal	Amp	Phase
<i>PhPRR9</i>	0.364	Leaf	+3 h petal	0.626	Leaf	+3 h petal	0.588	DD	+3 h DD	0.459	DD	+3 h DD
<i>PhPRR7a</i>	0.588	Leaf	No changes	0.700	Leaf	-6 h petal	0.161	LD	No changes	0.988	LD	-6 h DD
<i>PhPRR7b</i>	0.196	Leaf	No changes	0.094	Leaf	+9 h petal	0.876	LD	No changes	0.035	LD	+9 h DD
<i>PhPRR5a</i>	0.061	Petal	+3 h petal	0.175	Petal	+6 h petal	0.464	LD	No changes	0.616	LD	+3 h DD
<i>PhPRR5b</i>	0.223	Leaf	No changes	0.169	Petal	+3 h petal	0.064	LD	+3 h DD	0.004	DD	+6 h DD
<i>PhPRR3</i>	0.014	Petal	+6 h petal	0.151	Petal	+3 h petal	0.330	LD	+12 h DD	0.872	LD	No changes
<i>PhTOC1</i>	0.012	Petal	+6 h petal	0.638	Petal	-3 h petal	0.033	LD	+3 h DD	0.399	LD	+3 h DD
<i>PhGI1</i>	0.019	Petal	+3 h petal	0.109	Petal	+6 h petal	0.080	LD	+3 h DD	0.009	LD	+6 h DD
<i>PhGI2</i>	0.291	Petal	+6 h petal	0.298	Petal	+3 h petal	0.041	DD	+6 h DD	0.012	DD	+3 h DD
<i>PhELF4</i>	0.049	Leaf	No changes	0.131	Petal	+3 h petal	0.135	DD	+3 h DD	0.739	DD	+6 h DD
<i>PhCHL</i>	0.981	Petal	No changes	0.803	Leaf	+15 h petal	0.037	DD	+3 h DD	0.042	DD	+6 h DD
<i>PhFKF</i>	0.003	Leaf	+6 h petal	0.437	Petal	+3 h petal	0.479	LD	No changes	0.318	DD	-3 h DD
<i>PhLHY</i>	0.675	Leaf	No changes	0.205	Petal	No changes	0.254	LD	No changes	0.137	DD	No changes

3.5. 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. We asked if the variability of gene expression depended on tissue, time, and/or light conditions. When we analyzed the variability under a light:dark cycle (LD) we found that the clock genes *PhPRR7b* and *PhPRR3* showed the lowest variation in leaves and petals whereas *PhELF4* was a highly variable gene in both tissues. The stability of remaining genes varied among tissues, such as *PhPRR9* or *PhTOC1*, showing lower transcriptional noise in leaves than petals. In addition, we observed that the lowest and the highest variability for all analyzed genes varied along the day, suggesting that transcriptional noise may be time-dependent (Figure 5, Figure S6).

When plants were transferred to constant darkness (DD), we found that the variability of clock genes tended to increase, except for *PhLHY* in leaves and *PhGI2* in petals that were the most stable genes. Interestingly, the increase in CV was higher in leaves (Figure 5, Figure S6). The genes *PhPRR7a*, *PhPRR7b*, *PhPRR3*, and *PhFKF* showed the highest variability under constant darkness. In petals, the transcriptional noise of *PhPRR5a* and *PhTOC1* increased under a DD cycle (Figure 5). As previously mentioned, under a light:dark cycle, the coefficient of variation changed depending on the time point. The highest and lowest 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.

We used a scale test (HarmScaleTest) implemented in the DODR package [51] in order to detect significant changes in noise when petunia was transferred to constant darkness (Table 3). The CV was in general higher under constant darkness, suggesting that the transcriptional noise tended to increase in DD. The exceptions were *PhGI1* and *PhELF4* in leaves and *PhCHL*, *PhFKF*, *PhGI1*, and *PhGI2* in petals, displaying slightly higher CV under a LD cycle. We confirmed that the effect was tissue dependent, as changes in CV were significant ($p < 0.05$) in leaves for *PhPRR7b*, *PhFKF*, and *PhGI1* and in petals, for *PhPRR7a*, *PhPRR3*, and *PhELF4* (Table 3). The only gene affected in leaves and petals was *PhCHL*, but it also displayed opposite trends of increased CV (Table 3). This indicates that an endogenous component governs transcriptional noise of the clock genes, which also differs in leaves and petal

Figure 5. Variability in clock gene expression in petunia leaves (red) and petals (blue) under a light:dark cycle. Coefficient of variation (CV) was calculated for every clock gene and time point for leaves (red) and petals (blue). ZT 0 (zeitgeber time) indicates light on, ZT12 indicates light off. White area represents the light period and grey area, the dark period. The black triangle denotes a gene with low variation in both tissues and the white triangle, a gene with high variation. The white square indicates a gene with high CV in petals whereas the black square denotes a gene with high variation in leaves.

Table 3. Detection of changes in noise in petunia clock genes in two tissues, leaves and petals, comparing light:dark (LD) and constant darkness (DD) cycles. This analysis was performed by using the function HarmScaleTest provided by the R package DODR. A p value < 0.05 indicates significant changes between LD and DD, the phase with higher variability is specified in Noise columns.

Gene	Leaf, LD vs. DD	Noise	Petal, LD vs. DD	Noise
<i>PhPRR9</i>	0.341	DD	0.508	DD
<i>PhPRR7a</i>	0.228	DD	0.002	DD
<i>PhPRR7b</i>	0.014	DD	0.958	DD
<i>PhPRR5a</i>	0.137	DD	0.312	DD
<i>PhPRR5b</i>	0.839	DD	0.805	DD
<i>PhPRR3</i>	0.385	DD	0.004	DD
<i>PhTOC1</i>	0.575	DD	0.137	DD
<i>PhGI1</i>	0.000	LD	0.580	LD
<i>PhGI2</i>	0.301	DD	0.860	LD
<i>PhELF4</i>	0.082	LD	0.005	DD
<i>PhCHL</i>	0.016	DD	0.007	LD
<i>PhFKF</i>	0.013	DD	0.124	LD
<i>PhLHY</i>	0.140	DD	0.084	DD

4. Discussion

4.1. The petunia clock genes 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 [40,72–74]. The plant clock is an important coordinator of primary and secondary metabolism in plants. It defines the timing of floral scent emission and floral blend in a variety of plants including *Antirrhinum majus*, *Nicotiana attenuata*, or *Petunia* [35–38]. The plant circadian clock appears to have a specific transcriptional structure in different tissues such as leaves, pods, seeds, or roots [13,14,75,76]. As the transcriptional structure of the clock in petals is currently unknown, we used *Petunia hybrida* to perform a detailed analysis. We characterized the structural changes in *PhPRR5a*, *PhPRR5b*, *PhPRR7a*, *PhPRR7b*, *PhGI1*, and *PhGI2* and the transcriptional structure of the petunia circadian clock and associated genes in petals and leaves, using standard growth and continuous darkness.

The complete genome paleohexaploidization of petunia, found in the Solanaceae group [11] 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* the ortholog of *ZTL*. Interestingly, genes found as single copy in petunia such as *PhTOC1*, *PhPRR9*, and *PhPRR3* are found as single copy in most Solanaceae except for *Nicotiana benthamiana* that appears to have two copies of each gene (Figure 1). Two of the petunia paralogs of *AtPRR7*, *PhPRR7a*, *PinfS6PRR7a* and the ones corresponding to *AtPRR5* *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 (NP_568446.2 and AT5G02810, consulted in NCIB and TAIR database) while *PhPRR5a* and *PhPRR7b* present seven exons whereas *PhPRR5b* and *PhPRR7a* have eight exons, indicating possible sub or neofunctionalization of these paralogs (see below).

We found two domains, PR 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 in PhPRR7a, which shared more similarities in the amino acids sequence with AtPRR7. The lack of CCT domains in Solanaceae but not in the related Convolvulaceae family suggests that this event occurred in the early history of Solanaceae. In addition, this alteration, which has been described in PRR orthologs in crops such as rice and soybean, can modify growth and flowering time [77,78]. This may result in a specific clock in the Solanaceae family.

The current hypothesis about the transcriptional structure of the plant circadian clock includes a three component repressilator structure [64]. The PRR9, 7, and 5 genes function as transcriptional repressors in Arabidopsis [59]. The interaction between PRR9, 7, and 5 with TOPLESS occurs via two motifs. We found that the repression motifs were lost in the PhPRR5a and PinfS6PRR5a but was present in the rest of the paralogs of the PRR9, 5, and 3 genes. In addition, in *P. axillaris* PRR7b repression motif was interrupted by a sequence of 26 amino acids. This may result in the loss of gene repression activity of PRR5a and PRR7b [79]. Our results thus indicate that one of the repressilator loops in Petunia is maintained by one of the paralogs, while the second one PRR5a and PRR7b missing the repression domain may have undergone neofunctionalization.

The gene *GI* appeared in land plants and is absent in mosses or picoalgae [80]. In the Solanaceae we found two to three copies, and in *Petunia hybrida*, there are significant differences in the coding region between *PhGI1* and *PhGI2* suggesting a diversification of functions. Furthermore, the amino acid differences between *P. axillaris* and *P. inflata* indicate species specific changes in this master regulator that may be related to the differing environmental niches where both species grow.

We used the predicted protein sequences to infer the domain structure of GIGANTEA. Although a previous study describes that *GI* encodes a protein with six transmembrane domains [63], the biochemical functions of *GI* are not understood. In yeast, two hybrid experiments performed with the Arabidopsis *GI* protein show that the N-terminal domain interacts with FKF1 [81], while the complete protein shows interactions with the CYCLING DOF FACTOR6 and DELLA protein [82,83]. As the differences in protein structure found between PhGI1 and PhGI2 do not match well known domains we cannot understand their functional differences. Nevertheless, the PinfS6GI3 does lack the N terminus required for interactions with FKF1 and ZTL in Arabidopsis.

4.2. Daily expression of petunia clock genes is tissue specific

The current transcriptional model of the plant circadian clock is largely based on the expression of genes in the Arabidopsis hypocotyls and leaves [84]. It includes the morning, midday or core, and the evening loops. During the morning, the genes *CCA1* and *LHY* repress the evening genes *GI* and *TOC1* and activate *PRR9* and *PRR7*. At the same time, *TOC1* acts repressing *GI* and *PRR9* but activating *CCA1/LHY*. On the other hand, *GI* stabilizes ZTL that is a *TOC1* repressor [85].

Previous studies have revealed that the circadian clock is tissue-specific [14,75,76]. 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 [86], radish [87], or daisy [88].

Changes in gene expression of timing, quantity, and rhythm may hint at possible subfunctionalization or neofunctionalization of duplicated clock genes. We found that gene expression patterns of *PhPRR5* and *PhGI* paralogs differed in petals and leaves respectively, under a light:dark cycle. *PhPRR5b* and *PhGI2* displayed an advanced phase, peaking before *PhPRR5a* and *PhGI1*. Under DD, *PhPRR5b* was delayed 3 h in leaves. In petals, *PhPRR7a* advanced 6 h while *PhPRR7b* delayed 9 h. Given the differences in protein structure observed between

PhPRR5a, PhPRR5b, PhPRR7a, and PhPRR7b, and the drastic changes in expression pattern we can conclude that these paralogs have undergone important changes in biological function. As changes are tissue specific, it appears that the coordination of the clock in petals differs from the canonical expression in leaves.

4.3. *Leaves and petals have different clock coordination*

The current hypothesis is that several independent inputs such as light, temperature, or sugar act as signals helping the plant clock to remain stable. One of the signals considered differing between tissues is light [89]. In the present work we explored oscillations in gene expression in leaves and petals that do not differ in their exposure to light or temperature. We used the RAIN algorithm, a non-parametric method which also provided measures of phase [50]. We also performed an HANOVA test comparing tissues and light conditions. The core clock genes *LHY* and *TOC1* are found in basal picoeukaryotes, mosses, *Marchantia polymorpha*, and all higher plants [5,80,90]. 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 in LD, i.e., standard growing conditions. This indicates a basal change in the clock coordination between both tissues. This scenario may be further supported by the significant changes found for *PhFKF*, *PhPRR3*, *PhGI1*, and *PhGI2* between tissues. Finally, *PhGI1*, a gene found only in flowering plants showed significant changes between tissues and photoperiods indicating that it may play a role in the coordination between development and environmental signals. An initial hypothesis proposed different clock structures in leaves and roots of *Arabidopsis* based on the light reception by the organs [14]. However work performed in cowpea shows that aerial organs exposed to light such as leaves, pods, or developing seeds show different transcriptional clock structures [76]. Our results indeed indicate a differential coordination of the leaf and petal clock that may be the result of the establishment of the petal identity by the floral organ identity genes.

4.4. *Photoperiod sensitivity is organ-specific*

The effect of day length on biological clocks has been widely studied. For example, floral transition is controlled by *CONSTANS* (CO) and *FLOWERING LOCUS T* (FT) genes which are regulated by the circadian clock, including *ELF3*, *ELF4*, *GI*, *LHY*, *PRRs*, and *ZTL* genes [91–93]. These genes are capable to integrate environmental cues, mainly day length, but also temperature. Clock genes are therefore sensitive to ambient changes resulting in an adaptive advantage [85]. The present study revealed that a constant dark regime induced phase-shift even in the first 24 h. Most analyzed genes tended to delay their maximum expression, especially in leaves. Only *PhLHY* did not change its expression and displayed a robust oscillation in leaves and petals. Other genes, *PhPRR7a* and *PhELF4* (in leaves), did not retain their rhythmicity, suggesting that the integration of environmental cues and phototransduction varies depending on the tissue. This is consistent with previous studies, that have reported the effect of light on organ-specific circadian clocks and photoperiodic sensitivity [14,94].

Constant dark also had an effect on oscillations, which in general tended to decrease in most analyzed genes in leaves and petals. Similar results have been reported in other plant species: *LHY/CCA1*, *ELF4*, *GI*, and *TOC1* gene expression dampens under constant light or constant dark conditions in *Arabidopsis* [35,63,95,96]. Loss of circadian rhythmicity could be key and be involved in responses to environmental changes, such as seasonal dormancy during winter in Japanese cedar or chestnut [97,98].

4.5 *Transcriptional noise is tissue-specific and depends on photoperiod*

One of the main features of the transcriptional structure of circadian clocks is the capacity to integrate noisy environmental signals and internal transcriptional variation [99]. The robustness

of circadian oscillation is related to the number of mRNA molecules, interactions, and complex formation, and it is stabilized by the entrainment to the light:dark cycle [100].

In the present work we used a set of biological samples to analyze 13 genes under two light conditions and two tissues. We found that for the same set of samples some genes showed very high noise at a given time of the day, while others were very stable (Figure 5). We also found that molecular noise differed in leaves and petals and it was influenced by the time of the day. While in leaves highest stability appeared at the beginning of the subjective day, petals displayed the lowest stability. This was also noticeable when plants were transferred to continuous darkness. Interestingly, the time point with the highest transcriptional noise shifted both in leaves and petals. The lowest stability advanced in petals, and delayed in leaves. Furthermore, the increased transcriptional robustness early in the day in leaves, and in the late day-early night in petals, coincide with the major functional changes in both tissues, initiation of photosynthesis, and scent emission. As noise increases thereafter in both tissues, it could be that funneling transcriptional noise into robustness at certain times of the day may have biological implications to achieve consistent outputs. However, the molecular function, if any, is not understood as this is the first report of this phenomenon.

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

Supplementary Materials: The following are available online at <https://www.mdpi.com/2073-4425/10/11/860/s1>, Figure S1: Metl or dissociation curve analysis of petunia genes, Figure S2: Exon-intron structure of *Petunia axillaris* (PaxiN) *PRR5* and *PRR7* genes, Figure S3: Domain structure of PSEUDO-RESPONSE REGULATORS (PRRs), Figure S4: Local alignment of repression motifs of *PRR5*, Figure S5: Local alignment of GIGANTEA (GIs) proteins, Figure S6: Normalized expression (NE) for the biological replicates, 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,

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Supporting information

Figure S1: Melt or dissociation curve analysis of petunia genes.

Figure S2: Exon-intron structure of *Petunia axillaris* (PaxiN) *PRR5* and *PRR7* genes. The exon-intron boundary was calculated using the predicted mRNA molecules from petunia and aligning them to the corresponding genomic sequences.

Figure S3: (A) Domain structure of PRRs proteins in *Arabidopsis thaliana* (At), *Petunia axillaris* (Pa) and *P. inflata* (Pi). (B) Domain structure of PRR7s proteins in *Arabidopsis thaliana* (At), *Capsicum annuum* (Ca), *C. baccatum* (Cb), *Nicotiana benthamiana* (Nb), *N. sylvestris* (Ns), *N. tabacum* (Nta), *N. tomentosiformis* (Nto), *Petunia axillaris* (Pa), *P. inflata* (Pi), *Solanum lycopersicum* (Sl), *S. melongena* (Sm), *S. pennellii* (Spe), *S. pimpinellifolium* (Spi), *S. tuberosum* (St), *Cuscuta australis* (Cuas), *Ipomea nil* (In) and *Antirrhinum majus* (Am).

Figure S4: Local alignment of repression motifs of PRR5 proteins. *Arabidopsis thaliana* (A.thaliana), *Capsicum annuum* (C.annuum), *Nicotiana benthamiana* (N.benthamiana), *N. tomentosiformis* (N.tomentosiformis), *N. sylvestris* (N.sylvestris), *Petunia axillaris* (P.axillaris), *P. inflata* (P.inflata), *Solanum lycopersicum* (S.lycopersicum) and *S. tuberosum* (S.tuberosum).

Figure S5: Local alignment of GIGANTEA proteins.

Figure S6. Normalized expression (NE) for biological replicates. Expression of clock genes and associated-genes in leaves (red dots) and petals (blue dots) under a light:dark (LD) and constant darkness (DD). Gene expression was normalized to *PhACT*, the value of every single biological replicate is shown here. Grey areas indicate dark periods. ZT denotes zeitgeber time.

Table S1: PSEUDO-RESPONSE REGULATORS (PRRs) accessions.

Species	Protein	Accession
<i>Antirrhinum majus</i>	PRR5	Am07g14220
	PRR7	Am07g35120
	TOC1	Am01g23590
<i>Arabidopsis thaliana</i>	PRR3	AT5G60100.2
	PRR5	NP_568446.2
	PRR7	AT5G02810.1
	PRR9	AT2G46790
	TOC1	AT5G61380.1
	TOC1	AT5G61380.1
<i>Amborella trichopoda</i>	PRR37	XP_006828277.1
	PRR73	XP_020529326.1
	PRR9	XP_011629028.1
	TOC1	XP_011625651.1
<i>Capsicum annuum</i>	PRR5	CA03g21230
	PRR9	CA10g05550
	PRR7	CA10g11870
	TOC1	CA03g29830
<i>Nicotiana attenuata</i>	TOC1	XP19229967
<i>N. benthamiana</i>	PRR3a	Niben101Scf05009g04012.1
	PRR5a	Niben101Scf00225g05002.1
	PRR5b	Niben101Scf01109g04021.1
	PRR7a	Niben101Scf00294g03009.1
	PRR7b	Niben101Scf01994g01007.1
	PRR9a	Niben101Scf11670g02004.1
	PRR9b	Niben101Scf02026g04007.1
	TOC1a	Niben101Scf11469g00017.1
	TOC1b	Niben101Scf00063g06009.1
<i>N. sylvestris</i>	PRR3	gi 698444565
	PRR5	gi 698515802
	PRR7	gi 698585162
	TOC1	XP_009791525.1
<i>N. tomentosiformis</i>	PRR3a	gi 697159824
	PRR5a	XP_018627531.1
	PRR5b	gi 697192415
	PRR7	gi 697100515
	PRR9	mRNA_36525_cds
<i>Petunia axillaris</i>	TOC1	gi 697126282
	PRR37	Peaxi162Scf01024g00009.1
	PRR5b	Peaxi162Scf00912g00022.1
	PRR5a	Peaxi162Scf00542g03011.1
	PRR7a	Peaxi162Scf01517g00133.1
	PRR7b	Peaxi162Scf00146g01328.1
	PRR9	Peaxi162Scf00313g00210.1
<i>P. inflata</i>	TOC1	Peaxi12Scf00274g00351.1
	PRR3	Peinf101Scf02768g00025.1
	PRR5a	Peinf101Scf00223g03002.1
	PRR5b	Peinf101Scf01482g12029.1
	PRR7a	Peinf101Scf00931g05033.1
	PRR7b	Peinf101Scf00756g02019.1
	PRR9	Peinf101Scf00559g10011.1

	TOC1	Peinf101Scf01556g09043.1
<i>Physcomitrella patens</i>	PRR37	XP_024364994.1
<i>Solanum lycopersicum</i>	PRR3	Solyc04g049670.2
	PRR5	Solyc03g081240.2
	PRR7	Solyc10g086000.1
	PRR9	Solyc10g005030.2
	TOC1	Solyc03g115770.2
<i>S. tuberosum</i>	PRR5	PGSC0003DMP400001175
	PRR7	PGSC0003DMP400001175
	PRR9	XP_006352628
<i>Vitis vinifera</i>	TOC1	GSVIVG01007965001
	PRR3	GSVIVT01025088001
	PRR5	GSVIVG01027443001
	PRR7	GSVIVG01032644001
	PRR9	GSVIVT01038647001

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

Species	Protein	Accession
<i>Arabidopsis thaliana</i>	GI	AAT80910.1
<i>Amborella trichopoda</i>	GI	PRGDB24499
<i>Antirrhinum majus</i>	GI	Am06g07460.P03
<i>Brachypodium distachyon</i>	GI	XP_003564357.1
<i>Cicer arietinum</i>	GI1	XP_004496435.1
	GI2	XP_004496436.1
<i>Fragaria vesca</i>	GI	XP_004290483.1
<i>Glycine max</i>	GI1	BAJ22595.1
	GI2	BAN82589.1
	GI3	ACA24490.1
<i>Marchantia polymorpha</i>	GI	Mapoly0019s0145.1
<i>Medicago truncatula</i>	GI	XP_003592048.2
<i>Nicotiana benthamiana</i>	GI1	NbS00010804g0011.1
	GI2	NbS00006512g0008.1
	GI3	Niben101Scf09649g01002.1
	GI4	NbS00010805g0006.1
<i>Oryza sativa</i>	GI	XP_015649578.1
<i>Panicum hallii</i>	GI	Pahal.E04224.2
<i>Petunia axillaris</i>	GI1	Peaxi162Scf00360g00418.1
	GI2	Peaxi162Scf00160g01744.1
<i>P.inflata</i>	GI1	Peinf101Scf00386g02019.1
	GI2	Peinf101Scf02877g01057.1
	GI3	Peinf101Scf02652g00046.1
<i>Pisum sativum</i>	GI	ABP81863.1
<i>Selaginella moellendorffii</i>	GI	XP_024528715.1
<i>Setaria italica</i>	GI	Seita.5G129500.1
<i>S.viridis</i>	GI	Sevir.5G127000.1
<i>Solanum lycopersicum</i>	GI1	Solyc04g071990.2.1
	GI2	Solyc12g056650.1.1
<i>S.tuberosum</i>	GI	XP_006359039.1
<i>Triticum aestivum</i>	GI3	AAT79487.1
<i>Vigna radiata</i>	GI	XP_014514153.1
<i>V.unguiculata</i>	GI	Vun_T01130.1_6
<i>Vitis vinifera</i>	GI	XP_010665061.1
<i>Zea mays</i>	GI1	GRMZM2G107101_T03
	GI2	GRMZM5G844173_T01

Table S3: Primers used for qPCR.

Gene	Forward primer	Reverse primer
<i>PhCHL</i>	TGCATCTGTTGGCTCTGTTT	CCCCAACCCAATCTCTTAGC
<i>PhFKF</i>	CTGGGCAACCTCCAAAGTT	CATGGATCAGAATCTTGTTTGCT
<i>PhGI1</i>	TGGAGAAAGGGCAGAGACAT	GTGGAGCCACCCTTACGTT
<i>PhGI2</i>	TTTAGAGTCCTTTCACTCATCCATC	AATACAGCATTTGTTACATGGAGGT
<i>PhLHY</i>	CGACGTGGTAGGAATTGCATC	GCAAGAAATGGTCATCAAAGGAC
<i>PhPRR3</i>	GTGGAAGTGGTGATCGCTCT	TCCATCCTGACGCACAAAT
<i>PhPRR5a</i>	CCAATGATGCCCATCTTCAT	ACGCTGCTCTGCAAGTTGTT
<i>PhPRR5b</i>	GGAAGTCCCATCGATCTCAA	GGAGCCTCACTAGGTAATTGACG
<i>PhPRR7a</i>	CAGAAGAGGAAGGAGCGATG	AGGGAAAGTAGGAAGGAAAGCA
<i>PhPRR7b</i>	AGGTGCAAATGCGGAAAGT	AGCATCTATCTTTCTTCTTGACG
<i>PhPRR9</i>	GAGCTCATGTTTCCGAGAGC	GGTCGCTGTTCTGCAAGTCT
<i>PhTOC1</i>	TGATGGTAAGGGGAGCAAAG	CTGAAGCAGGATGCCCATTA
<i>PhACT</i>	TGCACTCCCACATGCTATCCT	TCAGCCGAAGTGGTGAAAGAG

Chapter II: The Petunia *CHANEL* Gene is a *ZEITLUPE* Ortholog Coordinating Growth and Scent Profiles.

Graphical abstract

Article

The Petunia CHANEL Gene is a ZEITLUPE Ortholog Coordinating Growth and Scent Profiles

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Abstract:

The floral perianth, comprising sepals and petals, conceals the sexual organs and attracts pollinators. The coordination of growth and scent emission is not fully understood. We have analyzed the effect of knocking down *CHANEL* (*PhCHL*), the *ZEITLUPE* ortholog in petunia (*PhCHL*) by hairpin RNAs. Plants with low *PhCHL* mRNA had overall decreased size. Growth evaluation using time lapse image analysis showed that early leaf movement was not affected by *RNAi:PhCHL*, but flower angle movement was modified, moving earlier during the day in knockdown plants than in wild types. Despite differences in stem length, growth rate was not significantly affected by loss of *PhCHL*. In contrast, petal growth displayed lower growth rate in *RNAi:PhCHL*. Decreased levels of *PhCHL* caused strongly modified scent profiles, including changes in composition and timing of emission resulting in volatile profiles highly divergent from the wild type. Our results show a role of *PhCHL* in controlling growth and development of vegetative and reproductive organs in petunia. The different effects of *PhCHL* on organ development indicate an organ-specific interpretation of the down regulation of *PhCHL*. Through the control of both timing and quantitative volatile emissions, *PhCHL* appears to be a major coordinator of scent profiles.

Keywords: floral scent; petal development; growth rate; phenomics; circadian clock; *ZEITLUPE*; image analysis; hairpin RNA

Introduction

Plant aerial organs grow from lateral primordia that form in the shoot apical meristem [1]. The type of organs produced, i.e., leaves or flowers, are the result of a vegetative or reproductive developmental program. The formation of flowers is the result of the activation of the so-called floral organ identity genes. They comprise a set of MADS-BOX proteins that in a combinatorial fashion allow the formation of the different organs [2]. The interaction of different MADS-box proteins occur via formation of protein complexes that activate the different organ identity programs leading to the formation of sepals and petals in the perianth and stamens and carpels [3,4].

Floral organs play an important role in reproductive success in many plants. Proper floral size is a key component of flower-pollinator interaction [5]. Floral size, like in leaves, is controlled by coordinated cell division and expansion processes [6]. The genes controlling lateral organ size appear to be conserved. Indeed, general regulators of lateral growth such as *AINTEGUMENTA* control floral size in *Arabidopsis*, *petunia*, *Antirrhinum* and tobacco [7,8,9].

During late flower development and maturation there is a major transcriptional reprogramming and scent emission starts with flower opening [10,11]. Scent emission takes place when the floral organ identity genes are not fully active, indicating a quantitative effect of the *DEFICIENS/GLOBOSA* MADS box proteins and downstream factors on scent emission [11].

There are a large number of plants that emit floral volatiles with significantly larger outputs during the day or during the night [12,13,14,15,16], suggesting a circadian regulation of scent emission. The circadian clock genes *LATE ELONGATED HYPOCOTYL* in *petunia* (*PhLHY*) and *NaLHY* and *ZEITLUPE* (*NaZTL*) in *Nicotiana attenuata* have been investigated and control the timing of emission of methyl benzoate and benzyl benzoate in *petunia* and benzyl acetone in *N. attenuata* [17,18]. Thus, a default pathway controlled by petal identity may activate floral scent emission, and the fine tuning in terms of emission timing should be regulated by the clock. However, this emerging hypothesis requires further experimental support; the studies mentioned have analyzed a small number of volatile organic compounds (VOCs), but the effect, if any, of the circadian clock on scent profiles is not known.

In this work, we have analyzed the down-regulation of the *petunia* gene ortholog of *ZTL* and its effect on growth and scent emission. We found that *PhZTL* plays a differential role in stem and floral size and is a major coordinator of floral scent profiles in *petunia*. As a result, we named the gene *CHANEL* (*PhCHL*).

Materials and Methods

Gene Identification and Phylogenetic Analysis

We identified *ZTL* orthologs and paralogs in the *Petunia* genome using BLAST [19]. The identified scaffolds and cDNAs were used to confirm the genome annotation using Genewise [20]. Protein alignment was performed with CLUSTALX [21]. Phylogenetic analysis was performed with the R libraries *ape* and *phangorn* [22,23], using the Maximum Likelihood as statistical method, JTT (Jones, Taylor and Thornton) as model of amino acid substitution, and 1000 bootstrap replicates. Trees were visualized and annotated with *ggtree* [24] using R, (R version 3.5.1). Protein accessions are listed in Table S1.

Silencing of *PhCHL*

We obtained sequence information from the genomic clone of *PhCHL* (Peaxi162Scf01124g00126.1) found in W115 (or Mitchell) and amplified 255 bp encompassing the last nine coding codons and 3'untranslated region. As the major effect of silencing of *ZTL* in petunia was a major disruption of scent profiles, we called it *CHANEL*, a famous perfume. The specific sequence of *PhCHL* was:

>*PhCHL*

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TGAACTATCTTTAGCAAGCTCTGTCATTTGAATAAAGAAAAAAGTAATGATGAAGAGAAGGTGTTGTGC
AGTATTCATAATGAAAATTTGCCTCAAGAATAAAGAGAGTCCCGAGCAAACCTATTGCAGTGCGGTTTT
TGCATTGCACCAATGCATAAATGACTAGCAAGTACCTGTGAGTTAGTGGCTGTCTTGTTTATTCTTGTC
TGGCTCATATGCCATGGTGAGCAAATGGTCCTATTGAGCAGATGGTC
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We used primers, introducing a partial attb1 and attb2 recombination site for GATEWAY cloning using *PhCHL*rnaiattb1 and *PhCHL*rnaiattb2 (Table S2). We performed a second PCR using attb1 and attb2 to add the corresponding sequences for recombination. The amplified fragment was recombined into pDONR221 and into the GATEWAY vector pHELLSGATE12 to obtain a hairpin construct [25]. The pHELLSGATE12 drives the hairpin construct using a standard 35S promoter. The W115 Mitchell double haploid was transformed as described previously [7].

Transformed plants were identified by PCR with primers amplifying *NPTII*. The completeness of the construct was tested with the Agri 51 and Agri 56 primers [26] (see Table S2). The PCR conditions were 3 min at 95 °C followed by 35 cycles of 15 s at 95 °C, 15 s at 55 °C and 15 s at 72 °C, and terminated by 5 min at 72 °C (Kapa Biosystems). The PCR reactions were loaded on 1% agarose gel containing ethidium bromide. PCR products from T2 plants were purified and sequenced.

Plant Growth Conditions and Sampling

Petunia plants were grown using a commercial substrate in a greenhouse under natural conditions. All experiments were conducted with at least three biological replicates. To study the expression of *PhCHL*, non-transgenic siblings and two independent *RNAi:PhCHL* T2 lines (*RNAi:PhCHL3* and *RNAi:PhCHL10*) were transferred to a climate chamber with 12 h of light and 12 h of dark (12LD) or 8L:16D and 23 °C and 18 °C for day and night, respectively. Plants were acclimated for 4–5 days. Young leaves and petals from 2–3 day-old flowers were sampled every three h for 24 h; tissues were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

To analyze the floral scent of non-transgenic and transgenic petunia flowers, we took at least three biological replicates of 2–3 day-old flowers (one flower per plant) every three h.

Housekeeping Genes and Gene Expression Analysis by qPCR

RNA was extracted from three biological replicates per time point of leaves and corollas using acid phenol [27]. Concentrations were measured using NanoDrop (Thermo-Fisher). Equal amounts of total RNA were used to obtain cDNA using Maxima kits (Thermo-Fisher) according to the user manual.

Previously, we performed a study to validate the housekeeping gene or genes, in two tissue samples, petal and leaf, for time course studies according to [28]. The candidate genes were *ACTIN 11* (*ACT*), *CYCLOPHILIN* (*CYP*), *ELONGATION FACTOR 1 α* (*EF1 α*), *GLYCALDEHYDE-*

3-PHOSPHATE DEHYDROGENASE (GADPH), RIBOSOMAL PROTEIN S13 (RPS13), GTP-NUCLEAR BINDING PROTEIN (RAN1) and POLYUBIQUITIN (UBQ) (Table S2). PCR analysis was performed as described previously [28]. The following protocol was used for 40 cycles: 95 °C for 5 s, 60 °C for 20 s and 72 °C for 15 s (Clontech SYBR Green Master Mix and Mx3000P qPCR Systems, Agilent Technologies), samples were run in duplicate. Cycle threshold (Ct) values were analyzed by BestKeeper [29], NormFinder [30], geNorm [31] and comparative Δ Ct methods [32] implemented in the web-based tool RefFinder [33] at different time points (Figure S1).

Analyzing all tissues, the most stable genes were *PhCYP* and *PhEF1 α* (Figure S2A). Moreover, for individual tissues, results were slightly different: the best genes for normalization in leaves were *PhACT* and *PhEF1 α* , and for petals, *PhACT* and *PhCYP* (Figure S2B,C).

We used *PhACT* to normalize the expression of clock genes in petunia leaves and petals as described [34]. Normalized expression was calculated as described [34] using the REST program [29] and *PhACT* as internal control gene.

Primers for circadian clock genes were designed using pcrEfficiency [35] (Table S2) and the following protocol was used for 40 cycles: 95 °C for 5 s, 60 °C for 20 s and 72 °C 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 (Figure S3).

Image Acquisition

We used an image acquisition system described previously [36]. Plants were grown inside a growth chamber comprising LED lights covering from UV to red light. Day and night images were taken by activating an Infrared light at 840 nm wavelength during short intervals of time (3 s). Images were acquired every ten minutes with an artificial vision camera comprising two CCD sensors, a multichannel 24-bit RGB absorbing at 610 nm, 540 nm and 460 nm, and a monochromatic sensor capturing at 800 nm. The acquired images have a resolution of 1296 × 966 pixels.

We obtained data using transgenic lines and compared them to the segregating siblings. Leaf growth and movement in seedlings was recorded for a period of 12 days with a total of 1728 images. Stem and flower growth was recorded for a period of 5 days and 16 h for line 3 and 3 days for line 10, for a total of 822 images and 432 images respectively. Using a semi-automatic procedure, we measured the length and angle of the longitudinal axis as referred to the horizontal plane of the flowers, as well as the length of the stem. The acquisition intervals were 1 hour for line 3 and 2 h for line 10, to explore differences in flower and stem growth patterns between wild-type and transgenic individuals. Analysis of growth was performed using the R package grofit [37]. Graphics were done using the color-blind friendly palette.

Scent Analysis

Flowers were placed in a glass beaker with a solution 4% of glucose inside a desiccator. Emitted volatiles were collected with twisters from the headspace every three h and analyzed by GM/CS as described [16]. Total and relative amounts were calculated using total integrated area divided by fresh weight as described before [38]. Detection of rhythmic scent emission was performed

using the JTK_CYCLE algorithm [39] implemented in the R package MetaCycle [40]. Scent profile figures were plotted using the R library ggplot2 [41], using color palettes providing by the package viridis [42].

Results

The Petunia Genomes Have a Single ZTL Gene

We mined the petunia genomes to identify *ZEITLUPE/FLAVIN-BINDING KELCH REPEAT F-BOX* (*ZTL/FKF*) orthologs and paralogs using BLAST. We found two genes with homology to the Arabidopsis genome in *P. axillaris*: Peaxi162Scf01124g00126.1 and Peaxi162Scf00655g00114.1; and three in *P. inflata*: Peinf101Scf01230g02037.1, Peinf101Scf02808g00015.1 and Peinf101Scf04186g00007.1 (Table S3).

We performed a phylogenetic reconstruction of the genes found in petunia and compared them to the LOV-F-box-KELCH proteins from several monocots, dicots and the basal plant Marchantia polymorpha (Figure S4). As previously mentioned, the silencing of *PhCHL* caused a major change in the composition of floral scent. Thus, we named the gene *CHANEL* (*PhCHL*) (see below).

The phylogenetic reconstruction shows two major clades, one containing all the *FKF*-like genes and a second one comprising the *ZTL* and the Arabidopsis *LOV KELCH PROTEIN2* (*LKP2*). In both clades, two subclades separate monocots from dicots. The Marchantia *FKF* like gene [43] falls somewhere between the *FKF* group of coding genes and the *ZTL* coding set of genes. The genomes of *P. axillaris* and *P. inflata* appear to have one gene corresponding to *PhCHL*, but differ in the copy number of *FKF* that is found in two copies in *P. inflata*. According to the ancestral region by gene analysis of *Petunia hybrida* W115, the *ZTL*-*FKF* genes present in *P. hybrida* correspond to *P. axillaris* [19]. Thus, based on the phylogenetic reconstruction, we identified Peaxi162Scf01124g00126.1 as *PhCHL* and Peaxi162Scf00655g00114.1 as *PhFKF*.

The Expression of PhCHL is Organ Specific and Affected by Day Length

The *PhCHL* expression has been studied in leaves and seedlings of *Nicotiana attenuata* and *Arabidopsis* [17,44,45]. In these plants and tissues, *PhCHL* does not show rhythmic expression. We determined the set of reference genes for circadian expression in leaves and petals in order to establish the circadian gene expression patterns in petunia (see Materials and Methods). We analyzed the expression of *PhCHL* in petunia corolla using a 12:12 LD light regime and found that the expression had its maximum at ZT9 (Figure 1A). We found that the expression at ZT9 was significantly higher, and the lowest expression occurred at ZT15. Although the expression of *PhCHL* in corollas showed a peak at ZT9, the mathematical analysis for circadian oscillation using the JTK_CYCLE algorithm indicated that the expression of *PhCHL* in petunia corollas was not rhythmic ($p = 1$). We also analyzed the expression of *PhCHL* in leaves and the pattern was similar to corollas: *PhCHL* increased at ZT9 (Figure 1A), and its expression was not rhythmic ($p = 1$). Under short days (8L:16D), the expression of *PhCHL* in leaves reached its maximum at midnight (ZT17) while the maximum expression in petals was detected during the afternoon (ZT5) (Figure 1B). As observed under 12L:12D, *PhCHL* did not display a rhythmic expression either in leaves or petals ($p > 0.05$).

Figure 1: *PhCHL* expression in W115 petunia under 12LD (A) and 8L16D cycle (B) in leaves (blue line) and petals (red line) revealed a different pattern depending on light conditions. The maximum expression occurred before the dark period (ZT9) in leaves and petals under a 12LD cycle (A). This maximum was delayed in leaves under short days. *PhCHL* maintained its peak before dusk in petals (B). Results represent average $\pm$ standard deviation from three replicates. White bar indicates the light period and black bar, the dark period.

Silencing *PhCHL* Does Not Affect *PhFKF*

We knocked down *PhCHL* using a RNAi hairpin construct and found significant down regulation of *ZTL* in several lines. We used two lines for further analysis. *RNAi:PhCHL10* showed a down regulation of 80% ($p = 0.032$), while *RNAi:PhCHL3* showed a downregulation of 68% ($p = 0.047$) (Figure 2A). We also analyzed the expression of *FLAVIN-BINDING KELCH REPEAT F-BOX 1* (*PhFKF*), to discard co-silencing of the paralogous genes (*ZT5*). The expression of *PhFKF* did not differ significantly between transgenic and non-transgenic petunias (Figure 2B).

Figure 2: *PhCHL* (A) and *PhFKF* (B) expression in wild-type (blue bar) and RNAi:PhCHL plants (red bar and green bar) petals. *PhCHL* expression was significantly down-regulated in RNAi:PhCHL3 ($p = 0.047$) and RNAi:PhCHL10 ($p = 0.032$) compared to the wild-type (A). Silencing *PhCHL* did not affect *PhFKF* expression RNAi:PhCHL3 (p value = 0.6), RNAi:PhCHL10 (p value = 0.06) (B).

PhCHL Is a Positive Regulator of Lateral Organ Growth

We quantified the effect of *RNAi:PhCHL* on plant development and found that while not all parameters analyzed showed statistically significant changes, in general terms, *PhCHL* appears to play a role in the production of above ground biomass affecting organ size (Table 1). Thus, *RNAi:PhCHL* plants were significantly shorter than the non-transgenic siblings by roughly 23%. We analyzed floral size by measuring the length of the tube and the maximum expansion of the limb and found that, while the tube length was not always affected, the limb expansion was significantly reduced by *RNAi:PhCHL*, with size changes in the range of 12–18% (Table 1). Finally, we did not find differences in chlorophyll content (Table 1) or in the general canopy architecture i.e., number of branches (data not shown).

Table 1: Phenotypic analysis of *RNAi:PhCHL* lines 3 and 10 and comparison to non-transgenic siblings in T2 generation. Plant height, floral limb and floral tube length were measured from *RNAi:PhCHL* lines (3, 10) and the wild-type W115. Results, in centimeters, represent average $\pm$ standard deviation. p-value < 0.05 indicates a significant difference between non-transgenic and transgenic plants, significance levels are indicated with one asterisks (* for $p < 0.05$), two asterisks (** for $p < 0.001$) and three asterisks (***) for $p < 0.0001$). NS indicates not significant differences.

Measure	RNAi:PhCHL		W115	p-value	
	Line 3	Line 10		RNAi:PhCHL3	RNAi:PhCHL10
Plant height	25.1 $\pm$ 1.89	21.7 $\pm$ 4.18	37.33 $\pm$ 3.0	0.07	0.022 *
Floral limb	4.37 $\pm$ 0.44	4.76 $\pm$ 0.55	5.38 $\pm$ 0.29	0.00009 ***	0.0038 **
Floral tube	4.07 $\pm$ 0.37	4.81 $\pm$ 0.51	4.82 $\pm$ 0.35	0.00055 ***	NS
Chlorophyll	39.43 $\pm$ 6.32	36.65 $\pm$ 9.77	39.30 $\pm$ 9.80	NS	NS

We can conclude that *RNAi:PhCHL* is a positive regulator of lateral organ size with a pleiotropic effect causing a decrease in shoot and flower size.

PhCHL Is Involved in Flower Angle Changes but Not Leaf Movement

As size was negatively affected by *RNAi:PhCHL* (Table 1), we used a phenomics approach with time lapse image acquisition to identify the effects on growth kinetics [46]. We tried to analyze leaf growth in seedlings; however, leaf movement, a trait under light and circadian control, was so extreme that we could not obtain reliable data for growth (Movie S1). Changes in leaf position appeared to correspond to day/night changes. We measured the changes in leaf position every hour for a period of four days. We found that both non-transgenic and transgenic lines had open leaves during the day and closed leaves during the night (Movie S1). The changes in leaf opening and closing, in terms of speed or timing were not affected by the *PhCHL* expression levels (Figure 3A). Indeed, a mathematical analysis of the period, lag phase and amplitude did not show significant differences between *RNAi:PhCHL* and wild type (Table S4).

Figure 3: Down regulation of *PhCHL* does not affect leaf movement (A). The distance between the center of the plant and the end of each leaf is shown. Furthermore, these data correspond to the distance in the X axis. Negative values correspond to leaf positions where the tip of the leaf passed the center of the plant. Down regulation of *PhCHL* causes increased flower angles and advanced daily changes (green line, B). Orange line represents WT plants, green line represents *RNAi:PhCHL* plants.

We analyzed flower angle against the horizontal axis, as it is a parameter related to pollination that is regulated by *NaZTL* in *Nicotiana attenuata* [17]. Both wild-type and transgenic lines had a similar daily pattern of flower movement; however, RNAi line 3 flowers had always higher

angles than the non-transgenic siblings. A decomposed time series showed that their daily changes in angle were slightly advanced compared to wild type (Figure 3B).

Differential Effect of PhCHL on Stem and Flower Growth Rate

The difference in overall plant size indicated a role of *PhCHL* in plant growth. We analyzed stem growth and found that growth curves (Table 2) were similar in wild-type and transgenic plants (Figure 4A; Table 2). Still, maximal growth speed in transgenic line 3 was 80.7% compared to wild type and area under the curve was 82%. The data indicates that stems are shorter because growth occurs during shorter periods of time, but growth speed appears to be less affected in the stem (see discussion).

Table 2: Growth rate of petunia stems and flowers. Maximal growth is expressed in mm. Stems grew from day 0 and total growth refers to size achieved after 5 days and 16 h taking as reference the stem at time zero. Maximum growth for flowers refers to the same period but comprises the overall floral size. The maximum slope depicts growth rate while the area under the model (integral) gives an estimation of the overall difference in accumulated growth.

Group	Rate	Max.Growth/ Std.Error	Max.Slope/ Std.Error	Area under Model
WT	Stem growth	16.084/0.142	0.121/0.005	1778.3
<i>RNAi:PhCHL</i>	Stem growth	12.984/0.096	0.118/0.006	1471.53
WT	Flower growth	57.698/0.403	0.798/0.013	3598.88
<i>RNAi:PhCHL</i>	Flower growth	39.677/0.344	0.566/0.011	2328.95

Figure 4: Down regulation of *PhCHL* affects growth length in stems (A) and growth rate in flowers (B,C). Graphics show the raw data (points), the adjusted curve and confidence interval (grey shade). Orange line represents WT plants, green line represents *RNAi:PhCHL* plants. Yellow and blue areas indicated light and dark period, respectively. (D) Flower growth rate by period, day versus night in wild-type and *RNAi:PhCHL* transgenic lines.

We analyzed the increase in flower length. Down regulation of *PhCHL* caused a strong decrease in flower growth speed in line 3 (Movie S2). The wild-type flowers had an overall growth speed that was always higher than in transgenic lines (Figure 4B). We calculated the differences in growth speed and found that the maximal slope indicating growth speed was 1.4-fold higher in wild type compared to transgenic line 3 (Table 2). We analyzed line 10, but due to flowering time differences, we were able to obtain data only from older flowers. These flowers also grew at a lower speed than wild type, but differences were not so pronounced (Movie S3) (Figure 4C).

Growth of hypocotyls in *Arabidopsis* is gated by the plant circadian clock [47]. We tested the hypothesis that petunia flowers may grow at different speeds during day and night. Indeed, we found that overall flower growth speed was higher during the day than during the night, coming to a sharp decrease in growth speed when flowers open (Figure 4D). The flowers corresponding to *RNAi:PhCHL* always grew slower than the wild types, but the time required to become fully developed was not affected by *PhCHL* (Figure 4D). Altogether, our data shows that *PhCHL* plays a role in organ growth speed and duration that is organ-specific.

***PhCHL* Coordinates Daily Changes in Scent Profiles**

Petunia flowers emit mostly benzenoids/phenylpropanoids, including methyl benzoate, benzaldehyde and benzyl benzoate [48]. We also identified emission of terpenoids, cadinene and limonene. We selected 17 volatile organic compounds (VOCs) to analyze the effect of *PhCHL* silencing in volatile emission for a 24-hour period (Table 3). The major VOC emitted by *P. hybrida* was methyl benzoate (74.90% of selected volatiles) whereas cadinene was the volatiles with the lowest emission (0.02% of selected compounds). Methyl benzoate was also the principal emitted volatile in transgenic lines: 79.1% in *RNAi:PhCHL3* and 58.51% in *RNAi:PhCHL10* petunia flowers. The volatile with the lowest emission was acetophenone for *RNAi:PhCHL3* (0.01%) and cadinene for *RNAi:PhCHL10* (0.018%). Due to the high emission of methyl benzoate in all petunia lines, we analyzed the volatile profile excluding this compound (Figure 5, Figure S5A).

Table 3: Retention time (RT) expressed in minutes, name and CAS number (assigned by the Chemical Abstract Services) of selected volatiles.

RT	Name	CAS
4.873	Benzaldehyde	100-52-7
6.435	Limonene	138-86-3
6.539	Benzyl alcohol	100-51-6
6.735	Phenylacetaldehyde	122-78-1
7.230	Acetophenone	98-86-2
7.825	Methyl benzoate	93-58-3
8.149	Phenylethyl alcohol	60-12-8
9.125	Benzyl acetate	140-11-4
9.244	Ethyl benzoate	93-89-0
9.668	Methyl salicylate	119-36-8
10.720	Phenylethyl acetate	103-45-7
12.287	Eugenol	97-53-0
12.711	Benzyl 2-methylbutyrate	56423-40-6
13.587	Isoeugenol (isomers)	97-45-1; 5932-68-3
14.254	Benzyl tiglate	37526-88-8
14.625	Cadinene	483-76-1
17.525	Benzyl benzoate	120-51-4

Figure 5: Petunia scent profiles expressed as percentage. Due to its high emission, methyl benzoate was excluded to visualize the remaining volatiles. Profiles were determined for 24 h emission under a 8L:16D cycle for the wild-type (first bar) and the transgenic lines (second and third bar) (A) and in a time-course (B–D), where each bar represent one sampling point (in ZT h) for wild-type (B), *RNAi:PhCHL3* (C) and *RNAi:PhCHL10* (D) petunia flowers.

Excluding methyl benzoate (Figure S5A), the second-highest emitted volatile was ethyl benzoate in wild-type petunia and *RNAi:PhCHL10* line (45.72% and 48.61% of selected compounds, respectively). In contrast, the emission of ethyl benzoate was lower in *RNAi:PhCHL3* (10.23%) and the second major volatile in this transgenic line was isoeugenol (24.38%). Isoeugenol represented 15.84% in wild type and 10.92% in *RNAi:PhCHL10* (Figure 5A). We found that certain volatiles increased their emission in both *RNAi:PhCHL* lines: benzaldehyde, phenylacetaldehyde, benzyl tiglate and benzyl 2-methylbutyrate (Figure 5A). The volatiles limonene, benzyl benzoate and acetophenone decreased their emission in *RNAi:PhCHL3* and *RNAi:PhCHL10* petunia flowers (Figure 5A) Finally, the quantities of some emitted volatiles varied among lines; the emission of benzyl acetate, cadinene, methyl salicylate, phenethyl acetate and phenethyl alcohol was higher

in *RNAi:PhCHL3*, whereas the quantities of these compounds decreased in the *RNAi:PhCHL10* line (Figure 5A).

As the quantitative changes in scent could occur at different times of the day, we collected scent every three h in an 8L:16D cycle. The profile analysis of floral compounds throughout 24 h revealed modifications in the contribution of single compounds to the scent profile.

Methyl benzoate was the major emitted compound by non-transgenic and transgenic petunia flowers. Its contribution to the floral scent varied from 69 to 79% in wild-type petunias, (Figure S5B), from 73 to 87% in *RNAi* line 3 (Figure S5C) and from 46% to 71% in *RNAi* line 10 (Figure S5D). Methyl benzoate tended to decrease during the light period in wild-type and *RNAi:PhCHL10* plants, in contrast in *RNAi:PhCHL3* the highest contribution to the floral scent occurred at ZT5 (Figure S5B–D).

As mentioned above, we excluded methyl benzoate to analyze the temporal variation in scent emission profiles of those volatiles with lower emission. Briefly, in the wild-type plants, benzyl 2-methylbutyrate contribution to the scent profile increased during the light period (ZT2, ZT5). Cadinene, had the highest contribution at dawn and transition to day light (ZT23–ZT2). In contrast, the VOCs benzaldehyde, ethyl benzoate, limonene and phenylacetaldehyde showed their major contribution to the scent composition at dusk and early night (ZT8, ZT11). Acetophenone and benzyl benzoate displayed their highest contribution to the flower aroma at midnight (ZT14, ZT17). Finally, benzyl alcohol, benzyl acetate, eugenol, isoeugenol, methyl salicylate, phenethyl alcohol and phenethyl acetate percent composition was higher at late night (ZT20, ZT23) (Figure 5B).

When we analyzed the floral scent composition in *RNAi* petunias, three types of changes in the scent profile were observed. A set of volatiles showed similar profiles to the wild type (Figure 5B–D). These included of benzaldehyde, benzyl 2-methylbutyrate, cadinene, eugenol, isoeugenol and phenethyl alcohol. A second set of volatiles showed increased production during the late night and dawn (ZT23) as in wild type but did not decay during the light period (ZT2). Finally, acetophenone that had its maximal emission at midnight (ZT14–ZT17) and methyl salicylate (ZT20–ZT23) shifted their maxima to the light period at ZT2–ZT5.

These changes in timing of emission are reflected in the different quantitative combinations of VOCs found between wild-type and transgenic lines. Indeed, at the time when lights went off at ZT8, ethyl benzoate was the second important emitted volatile in W115 (70% of selected volatiles), decreasing in *RNAi:PhCHL10* to 57% and *RNAi:PhCHL3* to 8.7%. The VOCs acetophenone and phenethyl alcohol also decreased their emission in both transgenic lines. In contrast, benzaldehyde emission increased in both transgenic lines from 6.85% W115 (6.85%) to 41.8% in *RNAi:PhCHL3* and 22.1% in *RNAi:PhCHL10*. Phenylacetaldehyde also increased its emission in both *RNAi:PhCHL3* (18.7%) and *RNAi:PhCHL10* (4.3%) as petunia this volatile only represented a 1.4% of the profile in the wild-type (Figure 5B–D). When the lights went on at ZT0, scent also differed in transgenic and non-transgenic petunia flowers. The predominant compounds in the wild-type scent profile were phenethyl alcohol (30.5%), benzyl alcohol (21.9%) and isoeugenol (19.3%). In contrast, the major compounds in *RNAi:PhCHL3* were isoeugenol (33%), phenethyl

alcohol (21.8%) ethyl benzoate (12.7%) meanwhile in *RNAi:PhCHL10* were ethyl benzoate (62.7%), phenethyl alcohol (11.4%) and isoeugenol (9.6%) (Figure 5B–D).

***PhCHL* Is Required for Timing of Scent Profiles**

Petunia is considered a nocturnal plant; its scent emission is synchronized with pollinator activity. We analyzed the circadian rhythmicity of scent emission and the effect of silencing the gene *PhCHL*. In wild-type plants, benzaldehyde, benzyl acetate, benzyl alcohol, benzyl benzoate, eugenol, isoeugenol, methyl benzoate, phenylacetaldehyde, phenethyl acetate and phenethyl alcohol were rhythmically emitted ($p < 0.05$), while acetophenone, benzyl 2-methylbutyrate, benzyl tiglate, cadinene, limonene, ethyl benzoate and methyl salicylate turned out to be arrhythmic ($p > 0.05$) (Table 4). In the *RNAi* plants, most compounds emitted in a rhythmic fashion in wild type were emitted in a rhythmic manner (Table 4). Moreover, benzyl tiglate, benzyl 2-methylbutyrate and methyl salicylate were not rhythmically emitted both in W115 and *RNAi:PhCHL10* line oscillated significantly in *RNAi:PhCHL3* petunias (Table 4).

Table 4: Analysis of emitted volatiles with MetaCycle. The JTK_CYCLE algorithm implemented in the R library “MetaCycle” was used to detect rhythms in emitted volatiles of petunia wild-type (W115) and transgenic *RNAi:PhCHL* lines (*PhCHL3*, *PhCHL10*). Volatiles with significant p value (p value < 0.05) showed a rhythmic emission. Phase is defined as the time point, in ZT h, with the highest emission.

Volatile	W115		PhCHL3		PhCHL10	
	p value	Phase	p value	Phase	p value	Phase
Benzyl tiglate	1.000	2	0.014	0.5	0.476	5
Cadinene	0.445	5	0.246	23	1.000	9.5
Ethyl benzoate	1.000	11	1.000	20	1.000	20
Acetophenone	1.000	12.5	1.000	8	0.210	11
Benzaldehyde	0.000	15.5	0.000	12.5	0.000	15.5
Benzyl benzoate	0.001	15.5	0.023	17	0.001	17
Limonene	0.165	15.5	0.280	17	1.000	11
Phenylacetaldehyde	0.001	15.5	0.000	12.5	0.000	20
Methyl benzoate	0.017	17	0.003	15.5	0.011	17
Isoeugenol	0.001	18.5	0.007	18.5	0.014	17
Benzyl acetate	0.003	20	0.001	20	0.005	20
Eugenol	0.001	20	0.000	20	0.019	20
Methyl salicylate	1.000	20	0.009	20	1.000	15.5
Phenylethyl acetate	0.048	20	0.002	20	0.000	20
Phenylethyl alcohol	0.000	20	0.000	18.5	0.000	15.5
Benzyl alcohol	0.000	21.5	0.007	20	0.000	20
Benzyl 2-methylbutyrate	1.000	23	0.011	23	0.194	2

We also analyzed the phase or time point with the highest emission of selected volatiles. Most compounds increased their emission during the dark period, and we classified the volatiles according to their maximum emission. In the wild-type petunia, first group comprised benzyl tiglate and cadinene, which showed their peaks during the subjective day (ZT2 and ZT5, respectively). The second group included the volatiles ethyl benzoate and acetophenone, that reached its maximum emission at early night (ZT11 and ZT12.5, respectively). The third group consisted of those VOCs which peaked at midnight, from ZT15 to ZT18.5: benzaldehyde, benzyl acetate, limonene, isoeugenol, methyl benzoate and phenylacetaldehyde. Finally, the fourth

group included benzyl 2-methylbutyrate, benzyl acetate, benzyl alcohol, eugenol, methyl salicylate, phenethyl acetate and phenethyl alcohol, that showed their maximum emission at late night (ZT20 to ZT23) (Table 4).

Analyzing the phase of emitted volatiles in transgenic lines revealed important differences. We found four types of behavior. First, the volatiles benzyl acetate, eugenol and phenethyl acetate did not change their peak in wild-type and transgenic petunias (Table 4). Second, benzyl benzoate, cadinene and ethyl benzoate delayed their maximum emission in both transgenic lines (Table 4). Third, acetophenone, benzyl alcohol and phenethyl alcohol displayed an advanced phase in *RNAi:PhCHL* plants (Table 4). Finally, a group of volatiles did not follow the same pattern in *RNAi:PhCHL3* and *RNAi:PhCHL10* lines; benzyl 2-methylbutyrate and isoeugenol did not change their peak in *RNAi:PhCHL3* compared to W115 petunias whereas these compounds were emitted in advanced in the line *RNAi:PhCHL10*; on the other hand, the compounds benzaldehyde and methyl benzoate peaked at the same time in non-transgenic and *RNAi:PhCHL10* petunias but in the *RNAi:PhCHL3* line this peak was advanced (Table 4).

Altogether, *PhCHL* plays a fundamental role in coordinating floral scent profile of petunia that could be related to the timing of maximal production of different VOCs. The effect on single compounds appears to be opposite in some cases, suggesting a control of scent pathway at one of several points that we do not understand yet.

Discussion

The *ZTL* gene in *Arabidopsis* plays a central role in the circadian clock regulation and affects several traits such as flowering time [45], and in *Nicotiana* it also controls flower daily movement and the emission of benzyl acetone [17]. Here we have performed a systematic study of *PhCHL*, the ortholog of *ZTL*, and its outputs in *Petunia hybrida*, uncovering several unreported functions.

The *ZTL*/*FKF*/*LKP2* proteins are blue light receptors in *Arabidopsis*. The expression of *ZTL* is not rhythmic in *Arabidopsis* or *Nicotiana* [44,45]. While *PhCHL* showed a clear peak of expression in 12L:12D, we could not find a significant rhythm, indicating a conserved expression pattern with other plants where it has been analyzed. This is the first time that *PhCHL*, a *ZTL* ortholog, has been analyzed in petals. The similarity of expression of *PhCHL* in leaves and petals indicates that the leaf and petal clock may be partly conserved. However, this needs further analysis with more genes, as clock transcriptional structure has been shown to change in roots, pods or seeds [49,50].

One of the classical experiments in Chronobiology in plants was performed by d'Ortous de Mairan. He demonstrated that leaf movement was rhythmic and endogenous [51]. Leaf angle and leaf movement have been widely studied in crops, including soybean and maize [52,53] and in the canonical model *Arabidopsis*. The analysis of circadian clock mutants in *Arabidopsis* revealed that the clock genes *GIGANTEA* (*GI*) and *EARLY FLOWERING 4* (*ELF4*) are required to maintain leaf movement rhythmicity [54,55]. In petunia, we detected periodic changes in leaf position but diel changes in position were not affected by the down-regulation of *PhCHL*. This result suggests that *PhCHL* did not play a critical role in leaf rhythms at early stages of development.

The first apparent effect of down-regulating *PhCHL* was a decrease in plant body size. A detailed analysis using time lapse images showed that the stem of wild-type plants and siblings knocked down for *PhCHL* grew at similar speed. This suggests that the identified decrease in shoot length

maybe the result of smaller primordia, decreased growth duration or a combination of both phenomena. In contrast, petal development appeared to be affected in growth rate. Furthermore, petal growth occurred at higher rates during the day than during the night both in wild-type and transgenic plants, suggesting that gated growth of flowers was independent of *PhCHL*. Work in *Arabidopsis* and maize has shown that the process of growth rate and duration maybe differentially affected by mutations [56,57,58]. Our results show that measuring two different organs may yield different results related to growth rate and duration. The plant circadian clock appears to have an organ specific resetting in roots, pods and seeds [50,59,60,61]. In this context, shoot growth is driven by the shoot apical meristem while flowers have undergone major organ identity reprogramming. As both cell division and expansion are under direct control of the clock [62,63], our results indicate a somewhat different interpretation of loss of *PhCHL* in stems and flowers.

We performed a complete scent profile analysis in wild-type and knockdown lines. Floral scent comprised 98.66% benzenoids/phenylpropanoids and 1.34% terpenoids in wild-type petunias. This composition was somewhat similar in transgenic lines, indicating that the effects of *PhCHL* are not specific for a single volatile family of compounds. The emission of several phenylpropanoids/benzenoids compounds, such as methyl benzoate, benzyl alcohol, benzyl benzoate or benzaldehyde, displayed a rhythmic oscillation whereas acetophenone or ethyl benzoate (phenylpropanoids/benzenoids) and the terpenoids cadinene and limonene did not. In addition, the amount of emitted volatiles tended to increase and peak during the dark phase, coinciding with petunia pollinators activity [64]. These results are similar to those described previously for whole flowers, indicating that petal volatiles and those emitted by other organs have similar control mechanism [14]. This suggests that the circadian clock plays a key role in the regulation of biosynthesis and emission of certain compounds that interacts and/or attracts pollinators. In contrast, volatiles which did not oscillate such as acetophenone, or terpenoids may play a role in defense [65,66].

The disruption of *PhCHL* shifted the maximum emission of benzyl alcohol was advanced whereas the peaks of benzyl benzoate and ethyl benzoate were delayed. Other volatiles, such as benzyl alcohol, eugenol and phenethyl acetate, were not affected by the knockdown of *PhCHL*. These results suggest that *PhCHL* was involved, directly or indirectly, in the emission pattern of certain volatiles. However complete knockouts for *PhCHL* may show a stronger effect in the volatiles affected and/or additional effects on those that appear to remain stable.

Flower fragrances play a complex biological role. The dominant nocturnal emitted volatiles of *Petunia axillaris*, benzaldehyde, benzyl alcohol and methyl benzoate, act as attractant of its nocturnal pollinator [64] whereas benzaldehyde has been described as a mild repellent [67]. On the other hand, the highest emitted compound by *P. integrifolia* is benzaldehyde [64]. In *Petunia hybrida*, the three major compounds were methyl benzoate, ethyl benzoate and isoeugenol. In contrast, the three principal released VOCs in *RNAi:PhCHL3* were methyl benzoate, isoeugenol and benzaldehyde, while in *RNAi:PhCHL10*, they were methyl benzoate, ethyl benzoate and benzaldehyde. In addition, in the present work, we described how the proportion of the compounds emitted by wild-type and *RNAi:PhCHL* petunias changed throughout a 24 h period. Previous studies that covered the down-regulation of the clock genes *LHY* and *ZTL* in petunia and wild tobacco, have report a reduction in volatile emission and production [17,18]. Our results

showed that silencing *PhCHL* modified blend ratios, resulting in a different scent profile. Changes in emission pattern and fragrance composition may have an effect in pollinator attraction, plant defense against herbivores and pathogens or plant-plant signaling [68,69,70]. Our work shows that *PhCHL* plays a major role in the quantities and timing of VOC emission, thus coordinating the proper composition and daily changes of scent blends.

Supplementary Materials: Supplementary materials are available online.

Author Contributions: M.I.T. designed experiments, performed experiments, performed formal data analysis, wrote, revised and edited the paper. F.P.S. designed experiments, developed the software, performed experiments, performed formal data analysis and revised and edited the paper. M.V.D.G. performed formal data analysis and revised and edited the paper. F.P.C. performed formal data analysis and revised and edited the paper.

P.J.N. conceived and coordinated the study, designed experiments, developed the software, performed formal data analysis, revised and edited the paper, supervised this work and acquired funding. M.E.C. conceived and coordinated the study, designed experiments, performed formal data analysis, wrote, revised and edited the paper, supervised this work and acquired funding. J.W. conceived and coordinated the study, designed experiments, performed experiments, performed formal data analysis, revised and edited the paper, supervised this work and acquired funding. All authors revised and approved the final draft.

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Supplementary material

Figure S1: Expression levels of candidate housekeeping genes (*PhACT*, *PhCYP*, *PhEF1α*, *PhGAPDH*, *PhRPS13*, *PhRAN1* and *PhUBQ*), represented as cycle threshold values (Ct) for petunia leaves (green boxes) and petals (pink boxes) from qPCR. Box represents first and third quartile, the line across the box is the median and whiskers indicate minimum and maximum.

Figure S2: Gene stability for both tissues (leaves and petals) (A), leaves (B) and petals (C) by Delta CT (Δ Ct), BestKeeper, NormFinder and geNorm method for candidate reference genes *PhACT*, *PhCYP*, *PhEF1 α* , *PhGADPH*, *PhRAN1*, *PhRPS13* and *PhUBI*. Furthermore, comprehensive gene stability offers the average expression stability values for all genes. Most stable genes displayed the lowest values and least stable genes showed the highest values.

Figure S3: Melt or dissociation curve analysis of petunia genes *PhACT* (A), *PhCHL* (B) and *PhFKF* (C).

Figure S4: Phylogenetic tree of CHL, FKF, LKP2 and ZTL genes in *Antirrhinum majus* (A.majus), *Arabidopsis thaliana* (A.thaliana), *Cryptomeria japonica* (C.japonica), *Hordeum vulgare* (H.vulgare), *Marchantia polymorpha* (M.polymorpha), *Mesembryanthemum crystallinum* (M.crystallinum), *Nicotiana attenuata* (N.attenuata), *Oryza sativa* (O.sativa), *Petunia axillaris* (P.axillaris), *Petunia inflata* (P.inflata), *Picea abies* (P.abies), *Solanum lycopersicum* (S.lycopersicum), *Triticum aestivum* (T.aestivum) and *Zea mays* (Z.mays). Amino acid sequences

were aligned using CLUSTALX. Phylogenetic analysis was performed with the R libraries ape and phangorn using the Maximum Likelihood as statistical method, JTT as model of amino acid substitution and 1000 bootstrap replicates, indicated (as percentage) with the numbers next to the branches (A). Protein alignment is shown in B.

Figure S5: Petunia scent profiles expressed as percentage, including methyl benzoate. Profiles were determined for 24h emission under a 8L:16D cycle for the wild-type (first bar) and the transgenic lines (second and third bar) (A) and in a time-course (B-D), where each bar represent one sampling time point (in ZT hours) for wild-type (B), *RNAi:PhCHL3* (C) and *RNAi:PhCHL10* (D) petunia flowers.

Table S1: GenBank, Snapdragon Genome Sol Genomics Network Database accession numbers of proteins used in phylogenetic reconstruction.

Species	Protein	Accession	Database
<i>Antirrhinum majus</i>	ZTL	Am01g33620	Snapdragon
<i>Arabidopsis thaliana</i>	FKF1	OAP14729.1	GenBank
	LKP2	NP_849983.1	GenBank
	ZTL	OAO90691.1	GenBank
<i>Cryptomeria japonica</i>	ZTL	BAP76060.1	GenBank
<i>Hordeum vulgare</i>	ZTL	ACR15149.1	GenBank
<i>Marchantia polymorpha</i>	FKF	BAO66508.1	GenBank
<i>Mesembryanthemum crystallinum</i>	ZTL	AAQ73527.1	GenBank
<i>Nicotiana attenuata</i>	ZTL	AFA35963.1	GenBank
<i>Oryza sativa</i>	ZTL	XP_015643178.1	GenBank
<i>Panicum hallii</i>	ZTL	XP_025810803.1	GenBank
<i>Petunia axillaris</i>	CHL	Peaxi162Scf01124g00126.1	Sol Genomics
	FKF	Peaxi162Scf00655g00114.1	Sol Genomics
<i>Petunia inflata</i>	CHL	Peinf101Scf01230g02037.1	Sol Genomics
	FKF1	Peinf101Scf04186g00007.1	Sol Genomics
	FKF2	Peinf101Scf02808g00015.1	Sol Genomics
<i>Picea abies</i>	ZTL	AGH20050.1	GenBank
<i>Solanum lycopersicum</i>	FKF	Solyc01g005300.3	Sol Genomics
<i>Triticum aestivum</i>	ZTL	ABR14627.1	GenBank
<i>Zea mays</i>	ZTL	PWZ17109.1	GenBank

Table S2: Primers for PCR (attb1, attb2, PhCHLrnaiaattb1, PhCHLrnaiaattb2, Agri51, Agri 56, NPTII and specific fragment of PhCHL for transformation, RNAi:PhCHL) and qPCR (PhACT, PhCYP, PhEF1 α , PhGADPH, PhFKF, PhRAN1, PhRPS13, PhUBQ and PhCHL).

Gene	Forward	Reverse
attb1, attb2	GGGGACAAGTTTGTACAAAAAAGCAGGCT	GGGGACCACTTTGTACAAGAAAGCTGGGTA
PhCHLrnaiaattb1	TACAAAAAAGCAGGCTCTGCATGAACTATCTTTAGCAAGC	
PhCHLrnaiaattb2	CAAGAAAGCTGGGTAATTCAGACCATCTGCTCAATAGG	
Agri51, Agri56	CAACCACGTCTTCAAAGCAA	CTGGGGTACCGAATTCCTC
NPTII	CCTGCTTGCCGAATATCATGGTGG	CGAAATCTCGTGATGGCAGGTTGG
RNAi:PhCHL	TGCATCTGTTGGCTCTGTTT	CCCCAACCCAATCTCTTAGC
PhACT	TGCACTCCACATGCTATCCT	TCAGCCGAAGTGGTGAAAGAG
PhCHL	TGCATCTGTTGGCTCTGTTT	CCCCAACCCAATCTCTTAGC
PhCYP	AGGCTCATCATTCACCGTGT	TCATCTGCGAACTTAGCACCG
PhEF1 α	CCTGGTCAAATTGGAAACGG	CAGATCGCCTGTCAATCTTGG
PhGADPH	AACAAC TCACTCCTACACCGG	GGTAGCACTAGAGACACAGCCTT
PhFKF	CTGGGCAACCTCCAAAGTT	CATGGATCAGAATCTTGTT
PhRAN1	AAGCTCCACCTGTCTGGAAA	TGCTAACAGATTGCCGGAAGCC
PhRPS13	CAGGCAGGTTAAGGCAAAGC	ACTAGCAAGGTACAGAAACGGC
PhUBQ	TGGAGGATGGAAGGACTTTGG	CAGGACGACAACAAGCAACAG

Table S3: Results from BLAST of the *Petunia* genomes and major features of homology of the CHL/FKF/ZTL protein family. Comparisons are made against the Arabidopsis ortholog. The homology refers to the number of identical annealed amino acids in relation to the total coded protein.

Petunia gene	BLAST values	Predicted protein length	Homology to Arabidopsis
PhCHL	0.0	616	489/610
Peaxi162Scf01124g00126.1			
PhFKF	0.0	617	478/582
Peaxi162Scf00655g00114.1			
PinfZTL	0.0	623	502/610
Peinf101Scf01230g02037.1			
PinfFKF1	0.0	593	479/582
Peinf101Scf04186g00007.1			
PinfFKF2	0.0	618	461/577
Peinf101Scf02808g00015.1			

Table S4: Analysis of leaf movement in *RNAi:PhCHL* and non-transgenic segregating siblings (PhWT). Images were analyzed using the JTK algorithm in the Metacycle package. Significant rhythms were tested using the Bejamini-Hochberg critical value (BH.Q). Adjusted P-value (ADJ.P) was used as a cut-off to identify clock-regulated transcripts. When both of them are less than 0.05, the circadian rhythm is accepted. Period is the time lapse between to peaks. LAG is the time of the first peak. Amplitude is the distance between the average of an oscillation and the peak.

Group	Tissue	BH.Q	ADJ.P	Period (h)	LAG (phase)	Amplitude
PhWT	Leaf 1	6.38e ⁻¹⁴	3.19e ⁻¹⁴	25	11	0.1160
	Leaf 2	4.30e ⁻¹³	4.30e ⁻¹³	24	12.5	0.2106
RNAi:CHL	Leaf 1	9.663e ⁻⁰⁵	9.66e ⁻⁰⁵	25	12.5	0.1615
	Leaf 2	1.64e ⁻¹⁴	8.21e ⁻⁰⁵	24	11.5	0.1283

Chapter III: The snapdragon *LATE
ELONGATED HYPOCOTYL* plays a dual
role in activating floral growth and
scent emission.

Graphical abstract

The Snapdragon LATE ELONGATED HYPOCOTYL Plays A Dual Role in Activating Floral Growth and Scent Emission

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Abstract:

The plant circadian clock controls a large number of internal processes, including growth and metabolism. Scent emission displays a circadian pattern in many species such as the snapdragon. Here we show that knocking down LATE ELONGATED HYPOCOTYL in *Antirrhinum majus* affects growth and scent emission. In order to gain an understanding of the growth kinetics, we took a phenomic approach using in-house artificial vision systems, obtaining time-lapse videos. Wild type flowers showed a higher growth speed than knockdown plants. The maximal growth rate was decreased by 22% in plants with lower LHY expression. Floral volatiles were differentially affected as RNAi plants showed advanced emission of compounds synthesized from cinnamic acid and delayed emission of metabolites of benzoic acid. The monoterpenes myrcene and ocimene were delayed, whereas the sesquiterpene farnesene was advanced. Overall, transgenic lines showed an altered volatile emission pattern and displayed a modified scent profile. Our results show that AmLHY plays an important role in the quantitative and qualitative control of floral growth and scent emission.

Keywords: antirrhinum; aroma; artificial vision; biological rhythm; flower development; growth rate; phenomics; RNA interference; volatile organic compounds

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Introduction

Floral organs are arranged in whorls of sepals, petals, stamens, and carpels. They acquire their identities when a set of genes called floral organ identity genes are activated. These organ identity genes activate the morphogenetic program in combinations [1]. The MADS-BOX genes *DEFICIENS* and *GLOBOSA* form protein complexes that drive petal and stamen development [2,3]. The so-called C-function genes are involved in the organ identity of the stamens and carpels [4] and cause the termination of the floral meristem development [5,6].

Floral organ size is primarily controlled by the organ identity genes in a quantitative way [7,8]. Changes in floral organ size occur by changes in the time and/or rate of cell division and expansion [9,10]. Lateral organs grow by a combination of cell division and expansion. Several genes controlling these processes also play a role in the final size of the flower. Amongst them are the transcription factor *AINTEGUMENTA* [1–4], affecting both cell division and expansion and *EXPANSINS*, which control primarily cell expansion [11,12].

The emission of floral scent occurs in many cases from petal tissues [13] and has several functions including the attraction and guidance of pollinators and the repulsion of non-beneficial insects [14,15]. Apart of the important role of Volatile Organic Compounds (VOCs) emission in plant reproduction and protection, the general floral bouquet, which is perceived by the human olfactory system, is also of aesthetic value [16,17]. Floral volatiles are emitted in a circadian fashion and this fact characterizes some plants as day emitters, like *Antirrhinum* or roses, or night emitters, like *Petunia* or *Silene* [18–20], indicating that the majority of the volatiles are released during a given timeframe.

The circadian clock has been extensively studied in *Arabidopsis* [21]. Research on circadian clock in other species is increasingly contributing to the understanding of the genetics of the circadian clock in plants and the importance of its restructuring for optimal outputs in adaptation to the environment [22–24]. Cell expansion is controlled by the circadian clock through the coordination of DELLA (aspartic acid-glutamic acid-leucine-leucine-alanine) proteins and the PHYTOCHROME INTERACTING FACTOR (PIF) transcription factors [25]. Cell division is also under the direct control of the clock via binding of TIMING OF CAB 1 to the promoter of *CDC6* in *Arabidopsis* [26]. Scent emission is also directly controlled by the clock. Knocking down the *LATE ELONGATED HYPOCOTYL* (*LHY*) in *Petunia* causes the down regulation of the benzenoid/phenylpropanoid pathway [27], limiting the timing of floral volatile emission to the evening. Additional evidence for a direct role of the circadian clock on the regulation of floral scent emission has been found in *Nicotiana attenuate*, where knockdown of *LHY* or *ZEITLUPE* cause changes in the emission of the attractant benzyl acetone [28]. Recent work has shown that the ortholog of *ZEITLUPE* in *Petunia*, *PhCHANEL*, coordinates scent profiles [29].

In this work, we have addressed the function of the core clock gene *LHY* on flower development in *Antirrhinum majus* using RNA interference. Using a phenomics approach we analyzed the effect of knocking down *AmLHY* on growth. We also analyzed in detail the function of *AmLHY* on scent emission. Our results indicate a non-linear and complex control of the floral scent synthesis and emission process by *AmLHY*.

Material and methods

Phylogenetic Analysis

We obtained the *AmLHY* gene by BLAST (Basic Local Alignment Search Tool) against a set of genomic scaffolds using the Arabidopsis *LHY* as a query. We used the close relative gene from *Mimulus guttatus* (Migut.N01518.2.p) downloaded from Phytozome to obtain a cDNA model using Genewise [30]. The EMBL-EBI accession number was PRJEB24602.

LHY/CCA1 protein sequences were obtained from National for Biotechnology Information (NCBI) using BLASTp (Table S1). REVEILLE (RVE) Arabidopsis protein sequences were used as a query to identify these proteins in *Antirrhinum majus*, using the program tblastn and the genome version 3 in the Snapdragon Genome Database (<http://bioinfo.sibs.ac.cn/Am/index.php>), that had been recently released [31]. Protein alignment was performed by CLUSTAL X, a Windows interface for the ClustalW multiple sequence alignment software [32]. Phylogenetic trees were built with the R libraries “ape” and “phangorn” [33,34], Maximum Likelihood was used as a statistical method and Jones, Taylor, and Thornton (JTT) as a model of amino acids substitution [35] with 1000 bootstrap replicates. Trees were visualized with the R package “ggtree” [36]. Protein domains and functional sites were inferred using the web-based tool “PROSITE” (<https://prosite.expasy.org/>) [37] and the schematic structure of proteins was plotted with the Bioconductor package “drawProteins” [38]. All libraries were built under R version 3.5.1.

Plant Material and Transformation

We used the pHELLSGATE12 plasmid [39] to obtain a construct encompassing 156 bp, starting 45 bp before the predicted ATG. *Antirrhinum majus* hypocotyls of the Sippe 50 (Max-Planck-Institut für Pflanzenzüchtungsforschung, Köln, Germany) line were transformed using the *Agrobacterium tumefaciens* strain EHA105 as described previously [8]. Sippe 50 was an inbred line that had been used for genetic analysis since 1910 [40,41]. As it has been selfed since then it can be considered completely homozygous. Transgenic plants were confirmed by polymerase chain reaction (PCR) based on the amplification of the gene *NPTII*. Genomic DNA was isolated from leaves using a kit (NucleoSpin® Plant II, Düren, Germany). PCR was conducted with OneTaq® DNA Polymerase (see Table S3 for primers details). The PCR conditions were 30 s at 94 °C followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C and terminated by 5 min at 72 °C.

A. majus Sippe 50 and transgenic snapdragon seeds were germinated in vermiculite and were placed in a growth chamber with 16 h light/8 h dark cycle conditions at 23/18 °C during day and night, respectively. Transgenic plants were detected by PCR (NEB, One Taq DNA Polymerase, Ipswich, MA, USA) using *NPTII* primers (Table S3). We selected 3 independent lines: *RNAi:AmLHY14*, *RNAi:AmLHY26*, and *RNAi:AmLHY27*. Later, seedlings were transferred to pots with a mixture of peat, vermiculite, and perlite 1.1:1 in a greenhouse under natural conditions. Finally, seven days before sampling, plants were placed in a growth chamber with 12 h light/12 h dark cycles with 23/18 °C temperature cycles. Zeitgeber time 0 (ZT 0) was defined by the time when the lights turned on.

Gene Expression Analysis

Fully expanded leaves were collected from 3 to 4 plants at 6 h intervals for a complete day (24 h). Leaves were immediately frozen in liquid N₂ and stored at –80 °C until use. Total RNA from leaves was extracted using a phenol-based method as described in reference [42] and treated with DNase I (Fermentas-ThermoFisher, Waltham, MA, USA). Equal amounts of total RNA (ThermoFisher, NanoDrop 2000, Waltham, MA, USA) were used for cDNA synthesis (ThermoFisher, Maxima First Strand cDNA Synthesis Kit, Waltham, MA, USA).

AmLHY expression levels were analyzed by quantitative polymerase chain reaction (qPCR) (Agilent Mx3000P QPCR System, Santa Clara, CA, USA) with SYBR Green (TaKaRa, SYBR Green Master Mix, Kusatsu, Shiga, Japan) according to manufacture manuals, and the following protocol was used for 40 cycles: 95 °C for 4 min and then cycling at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. *UBIQUITIN* (*AmUBQ*) was used as an internal control and samples were run in duplicate (Table S3). Normalized expression was expressed as $2^{-\Delta\Delta C_t}$, thus we used an efficiency of 2 for data analysis [43]. Daily gene expression of *AmLHY* was normalized by the average expression level across the time-course; *AmLHY* expression in RNAi lines was normalized to WT. To test differences in expression between wild-type and transgenic plants we performed a Student's *t*-test using the package "stats" in R (R version 3.5.1).

Flower Measurements

Flowers from *A. majus* Sippe 50 non-transgenic siblings and T2 generations were measured 2 to 3 days post-anthesis according to reference [44]. Flowers were sampled from 3 to 4 different plants per group. In total, we measured 8 flowers from non-transgenic siblings, 9 flowers from *RNAi:AmLHY14*, 4 flowers from *RNAi:AmLHY26*, and 7 flowers from *RNAi:AmLHY27* line. Floral parameters were compared between non-transgenic and transgenic siblings with the Student's *t*-test performed in R (R version 3.5.1).

Growth Analysis and Artificial Vision

We used an image acquisition system described previously [45,46]. Plants were grown inside a growth chamber comprising LED day lights covering from UV to red light. Night images were taken by activating an infrared light at 840 nm wavelength during short intervals of time (3 s). Images were acquired with an artificial vision camera comprising two CCD sensors, a multichannel 24-bits RGB absorbing at 610 nm, 540 nm, and 460 nm, and a monochromatic sensor capturing at 800 nm. The acquired images have a resolution of 1296 × 966 pixels.

We obtained data using a light/dark 12 h/12 h period of *A. majus* Sippe 50 and the segregating siblings of *RNAi:AmLHY* for a period of 8 days and 7 h. Images were acquired every 10 min for a total of 1196 images comprising 3 wild type and 3 transgenic flowers. Using a semiautomatic process, we measured the length every two hours. A curve was fitted to the raw data using a GENERAL ADDITIVE MODEL (GAM) using the package "mgcv" from R. The maximum growth corresponded to the maximal size of the flowers, corresponding to the maximal value of the curve. We obtained the maximal slope that would correspond to the maximum growth rate by deriving 200 points of the curve and obtaining the highest number. The area under the curve was obtained by integrating the fitted curve using the integrative function from the "stats" package.

Volatiles Collection, Gas Chromatography Mass Spectrometry, and Scent Analysis

Non-transgenic plants and plants belonging to T2 generations were sampled. Three or four flowers per line from 2 to 3 days post anthesis were used as described previously [47]. We collected volatiles starting at ZT3, for a period of 6 h during 24 h. All plants were acclimated to 12 h light/12 h dark (12LD) and 18/23 °C cycle. The volatiles captured were analyzed by gas chromatography mass spectrometry (GC/MS) as described in reference [8]. Flower scent was analyzed by the R package “gcProfileMakeR” (unpublished results), selecting a 60% frequency and default quality. Selected volatiles amount was expressed as an integrated area divided by flower fresh weight [48]. The phase was calculated using the JTK-CYCLE implemented in “MetaCycle” [49] (R version 3.5.1). Graphs were plotted with “ggplot2” [50] using a color blind-friendly palette provided by “viridis” [51]. Volatile pathways were represented using the software “PathVisio” (version 3.3.0) [52].

Results

The *Antirrhinum majus* *LHY*

We obtained a single scaffold that had high similarity to the *LHY* gene from *Arabidopsis*. A multiple sequence alignment of the translated proteins showed a high level of homology of snapdragon *LHY* (*AmLHY*) to other *LHY* and *REVEILLE* (*RVE*) coding genes (Figure S1, Table S1). In total, we identified seven *RVEs* genes in snapdragon and one *LHY* by BLAST, with high similarity to *Arabidopsis* (Table S2). A phylogenetic reconstruction using predicted proteins showed that the *AmLHY* clusters with other *LHY* proteins and they were clearly separated from the *RVE* clade (Figure S1). Moreover, *CCA1a* and *CCA1b* from *Physcomitrella patens* clustered between the *RVE1/2/7* and *RVE4/8* clades. *LHY/CCA1/RVE* (*AtLHY*, *AtCCA1*, and *AtRVE* in *Arabidopsis*) are MYB proteins, which are characterized by a conserved DNA-binding domain [53–55].

We identified the MYB domain and the HTH DNA-binding domain (helix-turn-helix motif) in *AmLHY*, which were highly conserved in size and position, as observed in other species such as *Petunia* or *Vigna* (Figure S1C). The coding region of *AmLHY* differed compared to *AtLHY*. *AmLHY* showed additional aminoacids that resulted in a longer protein (Figure S2). As mentioned above, we identified seven genes related to the *RVE* family in snapdragon, which shared a high homology with *Arabidopsis* except for three genes. We could not identify the MYB domain in *AmRVE1/2/7A* and *AmRVE1/2/7B*, while *AmRVE1/2/7D* showed two MYB domains (Figure S1C), suggesting an internal gene duplication.

The *AmLHY* Shows A Diurnal Expression Pattern

We analyzed the expression of *AmLHY* in leaves of wild-type plants under a 12LD cycle. We found that *AmLHY* expression increased at the end of the subjective night, peaking in the early morning. *AmLHY* showed a robust and significant oscillation (Figure 1a, p value = 3.3×10^{-06}), as described in other species including *Arabidopsis*, cowpea, or wild tobacco [54,56,57]. We selected T1 plants from three independent lines (*RNAi:AmLHY14*, *RNAi:AmLHY26* and *RNAi:AmLHY27*). In T2, all selected lines displayed a significant lower transcript level of *AmLHY* (Figure 1b, Table 1). *RNAi:AmLHY14* showed an average of 90% reduction in *AmLHY* expression levels, *RNAi:AmLHY26* of 65% and finally, *RNAi:AmLHY27* of 74%.

Figure 1: *AmLHY* expression (a) Daily expression in wild-type plants under a 12 h light/12 h dark (12LD) cycle; (b) *AmLHY* expression at ZT1.5 in wild-type (red) and transgenic lines *RNAi:AmLHY14* (green), *RNAi:AmLHY26* (blue) and *RNAi:AmLHY27* (purple). Expression was determined by the $\Delta\Delta C_t$ method using the wild-type as a control. Reference gene was *AmUBI*. Each bar represents the average $\pm$ standard deviation from 3 to 4 biological replicates.

Table 1: *AmLHY* expression in wild-type and RNAi lines at ZT 1.5. Expression was normalized by WT. Average and standard deviation (SD) represents 3 to 4 biological replicates. The knock down percentage in RNAi lines is shown in the % KD column. Differences were tested by a Student's *t*-test; a *p*-value <0.05 indicates a significant down-regulation.

Group	Average	SD	% KD	<i>p</i> -Value
WT	1	0.263		
<i>RNAi:AmLHY14</i>	0.093	0.063	90.66	0.001
<i>RNAi:AmLHY26</i>	0.348	0.306	65.23	0.006
<i>RNAi:AmLHY27</i>	0.256	0.17	74.44	0.003

***AmLHY* Does Not Affect Flower Morphology and Size**

The typical snapdragon flower has five sepals and five petals, these petals are fused resulting in a tubular and bilaterally symmetrical flower. We did not observe changes in organ identity or aberrant growth. All plants showed fully opened flowers.

We characterized snapdragon flowers analyzing 11 floral parameters, comparing the wild-type Sippe 50 and *RNAi:AmLHY* lines. These measurements included sepals, petals, and reproductive organs. The down regulation of *LHY* did not significantly affect the flower size, with the exception of petal height, lower petal expansion, and palate, which decreased in *RNAi:AmLHY14* line (Figure S3, TableS4).

***AmLHY* Enhances Growth Speed**

The study of mutants affecting floral size in *Arabidopsis*, *Antirrhinum*, and other species have relied mostly on endpoint analysis of mutants [10,58]. There is very little information about the growth kinetics that causes the final changes in terms of size. Modifications in growth leading to differences in final size may occur as a result of the duration of the growth period, changes in growth speed, or a combination of both parameters. We used a previously described in-house artificial vision system to obtain time lapse pictures of line *RNAi:AmLHY27* and an azygote segregating sibling (Movie S1). We obtained growth curves for wildtype and transgenic siblings and results showed a very strong effect of the down regulation of *AmLHY* on flower growth rate (Figure 2). The maximal growth speed achieved by the *RNAi:AmLHY* line was roughly 78% of the non-transgenic sibling, and the area under the growth curve model was 73% of the non-transgenic sibling (Table 2). The maximal slope used to calculate growth rate [59] suggested that the overall growth rate was only 51 % as compared to wild type. As far as we could analyze, the length of the flower growth period was similar in WT and in *RNAi:AmLHY27*, indicating that modifying the plant circadian clock affected growth speed in the petals.

Figure 2: Growth curve expressed in mm, of flowers of non-transgenic (WT, purple) and *RNAi:AmLHY27* (green). Dots represent the raw data, solid lines indicate the adjusted curve and dashed lines denote the maximum slope, that corresponds to the maximum growth rate.

Table 2: Parameters obtained from the phenomic analysis. Maximum growth corresponds to final maximum of the curve in mm. The maximum slope indicates the growth rate. The area under the model is an estimation of the difference in accumulated growth.

Group	Max. Growth/Std.	Max. Slope/Std.	Area
WT	34.301/0.191	0.344/0.021	4892.457
<i>RNAi:AmLHY27</i>	27.014/0.531	0.176/0.0078	3604.565

***AmLHY* is Required for Major Volatile Production**

We analyzed the floral scent emitted by wild-type and *RNAi:AmLHY* flowers using a time course with sampling six hours apart. A collection of volatiles was performed under a 12LD cycle. We detected 15 compounds during the time-course sampling (Table S5). Most detected volatiles belonged to the phenylpropanoid/benzenoid pathway but we also found terpenes as described previously [60].

We divided the volatile organic compounds into two groups. The first group comprised those VOCs, which contributed to the scent profile above 2% (major volatiles) and the second group contained the remaining compounds (minor volatiles). The major group comprised five compounds: Acetophenone, methyl benzoate, 3,5-dimethoxytoluene, ocimene, and linalool. We found major changes in the overall profile (24 h) in *RNAi:AmLHY*. The monoterpenes ocimene and linalool increased their levels in transgenic lines by 34.5% and 46.16%, respectively. In contrast, the volatile 3,5-dimethoxytoluene contribution to the scent profile was reduced by 23.6% in RNAi plants. Acetophenone and methyl benzoate, the two major compounds, decreased but not significantly in RNAi lines (2.6 and 7.5% respectively) (Figure S4). These five compounds comprised 98.35% of the profile in wild-type plants and an average of 97.81% in *RNAi:AmLHY* lines. Interestingly, the minor volatiles, whose contribution to the fragrance aroma was 1.65% in WT and 2.19% in transgenic lines, revealed the highest complexity of the snapdragon aroma. We classified the volatiles into two groups. The first group comprised compounds which decreased in the profile: The aldehydes nonanal and decanal. The second group included the benzenoids/phenylpropanoids 2-hydroxyacetophenone, benzaldehyde, cinnamyl alcohol, ethyl benzoate, methyl cinnamate, and methyl salicylate, and the terpenes farnesene and myrcene, which increased their contribution to the odour (Figure S4).

Daily emission also revealed changes in the scent profile. In major compounds, we found that the contribution of the benzenoids acetophenone and 3,5-dimethoxytoluene to the scent profile was lower in RNAi lines along all the time points analyzed. Methyl benzoate level was lower in all time points, except at ZT15, which increased its contribution slightly. In contrast, the monoterpenes linalool and ocimene were higher in transgenic lines (Figure 3).

Figure 3: Daily scent emission of major compounds in wild type (a), and transgenic lines *RNAi:AmLHY14* (b), *RNAi:AmLHY26* (c), *RNAi:AmLHY27* (d). An average of RNAi lines is represented in (e). ZT indicates zeitgeber time.

We also observed changes in minor volatiles. The aldehydes nonanal and decanal contribution were lower in transgenic lines. Furthermore, the benzenoid/phenylpropanoids benzaldehyde, cinnamyl alcohol, and methyl salicylate levels were higher in *RNAi:AmLHY* lines. The remaining compounds, 2-hydroxyacetophenone, ethyl benzoate, methyl cinnamate, and myrcene did not show a robust pattern as myrcene, that showed the highest increment by 668% at ZT9, but decreased by 33% at ZT15 and by 37% at ZT21 (Figure 4).

Figure 4: Daily scent emission of minor compounds in wild type (a), and transgenic lines *RNAi:AmLHY14* (b), *RNAi:AmLHY26* (c), *RNAi:AmLHY27* (d). An average of *RNAi* lines is represented in (e). ZT indicates zeitgeber time.

Altogether these results suggest a complex regulation of synthesis and/or emission of volatile organic compounds by *AmLHY*, acting at different levels over the phenylpropanoid/benzenoid and terpene pathways. All these changes resulted in a different and new flower-odour blend.

***AmLHY* Controls the Timing of Scent Emission**

We detected 15 volatile organic compounds (Table S5; Figures 3 and 4). In wild-type flowers, most selected volatiles showed a diurnal pattern. The release of VOCs started in the early morning, reaching their maximum emission at midday or before dusk. We classified the emitted VOCs in three groups based on their time of maximum emission. The first group comprises myrcene, ocimene, linalool,

decanal, nonanal, 3,5-dimethoxytoluene, 2-hydroxyacetophenone, and methyl cinnamate, that reached their maximum emission at early day and midday. A second group included cinnamyl alcohol and farnesene that peaked at the end of the light period. Finally, the third group covered acetophenone, methyl benzoate, and methyl salicylate, which increased their emission at midnight (Table S6).

In contrast, we found that the maximum emission of some VOCs shifted in RNAi lines, and we divided the volatiles into three groups. First, the monoterpene linalool, the aldehydes nonanal and decanal and the benzenoids/phenylpropanoids cinnamyl alcohol, 3,5-dimethoxytoluene, and 2-hydroxyacetophenone did not present any differences with wild-type flowers, showing its maximum emission at the same time. The second group comprised the derivatives of benzoic acid methyl benzoate and methyl salicylate, and the monoterpenes myrcene and ocimene, which delayed their maximum emission. Finally, acetophenone, benzaldehyde, methyl cinnamate (except in line *RNAi:AmLHY26*, which did not show any difference) and the sesquiterpene farnesene peaked early in transgenic lines (Table S6). We could not determine the phase of the ethyl benzoate, due to their irregular emission (Table S6).

Based on the known phenylpropanoid/benzenoid pathway we divided it into two principal branches, which have a common precursor, L-phenylalanine. The first branch included those compounds that derived from trans-cinnamic acid, as methyl cinnamate. The second branch covered benzoic acid derivatives, methyl salicylate, and methyl benzoate (Figure 5). Our results showed that the down-regulation of *AmLHY* advanced the maximum emission of acetophenone, benzaldehyde, and methyl cinnamate, while it caused a delayed peak of methyl salicylate and methyl benzoate.

Figure 5: Schematic view of some phenylpropanoids/benzenoids (a), monoterpenes (b) and sesquiterpenes (c) pathway. Non-volatile compounds are represented in a dashed and blue rectangle and volatile compounds with a solid line and a rounded rectangle, the green color indicates a volatile that displays a delayed emission and the red color, an advanced emission. Enzymes and the C5 unit IPP (isopentenyl diphosphate) are shown over the arrows. Enzyme abbreviations: BA2H (benzoic acid 2-hydroxylase), BALDH (benzaldehyde dehydrogenase), BAMT (benzoic acid carboxyl methyl transferase), CCMT (cinnamic acid carboxyl methyl transferase), FS (farnesene synthase), LS (linalool synthase), MYS (myrcene synthase), OCS (ocimene synthase), PAL (phenylalanine ammonia lyase) and SAMT (salicylic acid carboxyl methyl transferase).

We found a similar pattern in terpene emission. Geranyl pyrophosphate (GPP) is the precursor of the monoterpenes linalool, myrcene, and ocimene [61], whose reactions are catalyzed by linalool synthase (LS), myrcene synthase (MYS) and ocimene synthase (OCS), respectively. RNAi flowers displayed a delayed emission of myrcene and ocimene, peaking at midday (ZT6).

In contrast, linalool was not affected. The emission of farnesene, a sesquiterpene that derives from farnesyl pyrophosphate (FPP) by addition of isopentyl diphosphate (IPP) to GPP [62], was advanced in transgenic snapdragons (Figure 5, Table S6).

Altogether, our results suggest that *AmLHY* regulates the synthesis and/or emission of volatile compounds at different levels. First, principal changes were found in minor compounds contribution, which comprises the highest diversity fraction of snapdragon aroma. Second, the emission of those VOCs derived from trans-cinnamic acid (benzaldehyde and methyl cinnamate) started earlier, whereas the compounds that derived from benzoic acid (methyl benzoate and methyl salicylate) started later. The terpenes myrcene and ocimene, which shared the precursor GPP, also displayed a delayed emission phenotype, while farnesene peaked early in RNAi lines.

Discussion

In this work, we have performed a functional analysis of the *Antirrhinum majus* *LHY* gene. The *LHY* gene is found as a single copy gene in many plants such as *Petunia*, *Arabidopsis*, tomato, or *Nicotiana attenuata* [23,57]. Our results indicate that in *Antirrhinum* there is a single gene that may have similar functions to the ones described in *Arabidopsis*, *Petunia*, or *Nicotiana*, including control of growth and scent emission. In this work, we have uncovered a function in floral development where it enhances growth speed.

We found that loss of function of *LHY* in *Antirrhinum* does not affect organ identity. Despite the importance of the plant circadian clock on the maintenance of the photosynthetic apparatus [63,64], our results indicate that the plant circadian clock is not involved in the degradation of the chlorophyll machinery during petal development. Sepaloid petals result from decreased activity of B function genes and their targets [65], but we could not find evidence for an effect of *AmLHY* on petal identity. The transcriptional structure of the plant circadian clock varies between tissues and possible changes of the plant clock in petals versus other tissues or organs remains to be determined.

The size of plant lateral organs is the result of a combination of cell division and expansion. A variety of mutants affecting these processes in *Antirrhinum* show that petal growth can be differentially affected by genes indicating an intricate coordination of cell division, cell expansion, and compensation mechanisms [66]. The plant circadian clock is known to control cell expansion, our results demonstrate that *AmLHY* did not affect equally all the floral parameters analyzed. Our results did not differ from the findings in nightshades as down regulation of *LHY* does not affect floral size, petal junction distance, and corolla length, in *Nicotiana attenuata* or *Petunia* [27,28].

The temporal timing of cell division and expansion plays a key role in plant development. Both the length of the growth period and growth rate determine the final size of an organ. Our phenomic analysis of the *Antirrhinum* flower shows that the growth period is not controlled by *AmLHY* whereas the growth rate is substantially reduced. We may conclude that the period between cell divisions could be elongated in *RNAi:AmLHY* plants, resulting in organs with fewer and/or smaller cells. This hypothesis may require additional testing. Recent studies indicate that *LHY* represses abscisic acid (ABA) synthesis and signaling pathways [61], indicating that the newly identified effect of *AmLHY* on growth may occur via modulation of ABA. Our results are congruent due to the slow growth rate observed in RNAi plants highlighting the role of the circadian clock in flower development. The fact that floral organ size is not significantly reduced but growth speed is slowed in *AmLHY* knockdown lines indicates that compensation in terms of growth time may be occurring in order to achieve wild type organ size.

Floral scent profiles play a key role in the interaction of plants with both pollinators and pests [14]. The volatile blend is thought to be a complex interphase of interaction, and it is specific for a species [60]. The *A. majus* scent profile is highly complex as it comprises phenylpropanoids/benzenoids, and terpenes amongst the major VOCs. Previous studies showed that silencing *ZTL* in *Nicotiana attenuata* causes a major reduction of benzyl acetone while *LHY* causes an early emission. The down regulation of *PhLHY* causes an earlier emission and reduction of methyl benzoate and benzyl benzoate [27,28].

Interestingly, bumblebees, one of the *A. majus* pollinators, are capable of detecting variations in aroma blends among snapdragon species, and wild *Antirrhinum* emitting different scent blends appear to have specialized pollinators [67,68]. As silencing *AmLHY* resulted in new aroma ratios, it may alter pollinators behaviour. Future studies may address the effect of silencing *AmLHY* and the interaction with pollinators as well as pests and/or pathogens.

We found that *AmLHY* plays a complex and central role in the coordination of floral scent emission, affecting timing and scent profile. While it does not have a major effect on the total emission, it has a profound effect on the emission of single volatiles. An important difference between *Antirrhinum* and the Solanaceae *Nicotiana* and *Petunia* is that it emits floral volatiles preferentially during the day. In contrast to previously published work in *Petunia* [27], we found that the down regulation of *AmLHY* caused a delayed emission of methyl benzoate (Figure 5). We can conclude that as the expression patterns of *AmLHY* and *PhLHY* are identical, the opposite effect on individual scent components emission timing is probably coordinated by a second layer of transcription factors. As the emission of single volatiles from the phenylpropanoids/benzenoids and monoterpene/sesquiterpenes is advanced or delayed in a consistent manner it appears that there might be either a difference at the transcriptional level or at the actual metabolic flux, giving rise to the observed differences. These two scenarios are not mutually exclusive and future research should help us understand the fine tuning of scent emission by *AmLHY*.

Conclusions

Our work shows that *AmLHY* plays a role in growth and scent emission. The combination of floral organ identity and circadian control are main factors required for proper flower development. Our results also revealed that the complexity of floral aroma depends on timing emission, which is under circadian clock regulation, and the contribution (or amount) of every single volatile. Interestingly, the down-regulation of *AmLHY* affected volatiles biosynthesis pathways at different branches. Timing of emission is an additional variable that may be well worth testing in plant-insect interactions.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4409/8/8/920/s1>, Movie S1: Snapdragon flowers growth, Figure S1: Phylogenetic reconstruction of *AmLHY*, *LHY*, and *RVE* predicted proteins, Figure S2: *AtLHY* and *AmLHY* protein alignment, Figure S3: Floral parameters in non-transgenic (WT) and transgenic lines (*RNAi:AmLHY*), Figure S4: 24 h scent profile, Figure S5: Total volatile amounts, Table S1: Accession numbers of proteins used in phylogenetic reconstruction, Table S2: BLAST analysis, Table S3: PCR primers used in the current study, Table S4: Analysis of floral parameters in non-transgenic and transgenic lines, Table S5: Retention time in minutes Table S6: Analysis of daily emitted volatiles with the algorithm JTK_CYCLE.

Author Contributions:

Conceptualization, M.I.T., F.P.-S., P.J.N., J.W. and M.E.-C.; data curation, M.I.T., F.P.-S., J.W., and M.E.-C.; formal analysis, M.I.T., F.P.-S., P.J.N., J.W., and M.E.-C.; funding acquisition, P.J.N., J.W. and M.E.-C.; investigation, M.I.T., F.P.-S., P.J.N., J.W., and M.E.-C.; Methodology, M.I.T., F.P.-S., P.J.N., and M.E.-C.; project administration, P.J.N., J.W., and M.E.-C.; resources, J.W. and M.E.-C.; software, F.P.-S. and P.J.N.; supervision, P.J.N., J.W., and M.E.-C.; validation, M.E.-C.; writing—original draft, M.I.T., J.W. and M.E.-C.; writing—review and editing, M.I.T., F.P.-S., P.J.N., J.W., and M.E.-C.

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Supplementary files

Supplementary Figure S1. Multiple sequence alignment of the predicted AmLHY and other proteins belonging to the LHY/CCA1 family. Vu (*Vigna unguiculata*), Vr (*Vigna radiata*), Pv (*Pisum vulgaris*), Gm (*Glycine max*), At (*Arabidopsis thaliana*), Na (*Nicotiana attenuata*), Ns (*Nicotiana sylvestris*), Ph (*Petunia hybrida*), Am (*Antirrhinum majus*), Amt (*Amborella trichopoda*), Zm (*Zea mays*), Os (*Oryza sativa*), Hv (*Hordeum vulgare*), Pp (*Physcomitrella patens*), Ot (*Ostreococcus tauri*).

Supplementary Figure S2. AtLHY and AmLHY protein alignment.

Supplementary Figure S3. Floral parameters in non-transgenic (WT) and transgenic lines (RNAi:AmLHY). Asterisk (*) denotes significant differences among wild type and transgenic flowers (see Supplementary Table S4).

Supplementary Figure S4. 24-h scent profile. Major (A) and minor compounds (B) of wild-type, RNAi lines and its average are represented in bars.

Supplementary Figure S5. Total volatile amounts, expressed as area divided by flower fresh weight, of wild type (WT) and transgenic lines (RNAi:AmLHY).

Figure S1

Figure S1: Multiple sequence alignment

Figure S3: Floral parameters in non-transgenic (WT) and transgenic lines (*RNAi:AmLHY*).

Figure S4: 24-h scent profile.

Figure S5: Total volatile amounts.

Table S1: Accession numbers of proteins used in phylogenetic reconstruction.

Specie	Accession	Protein
<i>Arabidopsis thaliana</i> (At)	AT1G01060	LATE ELONGATED HYPOCOTYL
	OAP07470.1	CIRCADIAN CLOCK ASSOCIATED
	OAO92169.1	REVEILLE 1 (RVE1)
	OAO90963.1	REVEILLE 2 (RVE2)
	AT1G01520	REVEILLE 3 (RVE3)
	gi 75324470	REVEILLE 4 (RVE4)
	gi 576017836	REVEILLE 5 (RVE5)
	AT5G52660	REVEILLE 6 (RVE6)
	gi 576017949	REVEILLE 7 (RVE7)
	OAP05994.1	REVEILLE 8 (RVE8)
<i>Amborella trichopoda</i> (Amt)	XP_00682921	LATE ELONGATED HYPOCOTYL
<i>Antirrhinum majus</i> (Am)	Am03g44570	LATE ELONGATED HYPOCOTYL (LHY)
	LT978479.1	
	Am04g07010	REVEILLE 1A-like (RVE1A-like)
	Am06g21890	REVEILLE 1B-like (RVE1B-like)
	Am04g18800	REVEILLE 2A-like (RVE2A-like)
	Am06g21900	REVEILLE 2B-like (RVE2B-like)
	Am02g37040	REVEILLE 3/5A-like (RVE3/5A-
	Am03g44150	REVEILLE 3/5B-like (RVE3/5B-like)
	Am08g25850	REVEILLE 4/8-like (RVE4/8-like)
<i>Glycine soja</i> (Gs)	KHN11366.1	LATE ELONGATED HYPOCOTYL
<i>Hordeum vulgare</i> (Hv)	AEW48245.1	CIRCADIAN CLOCK ASSOCIATED
<i>Nicotiana attenuata</i> (Na)	OIS99379.1	LATE ELONGATED HYPOCOTYL
<i>Nicotiana sylvestris</i> (Ns)	XP_00979479	PREDICTED: protein LHY
<i>Oryza sativa</i> (Japonica Group)	XP_01564984	LHY isoform X4
<i>Ostreococcus tauri</i> (Ot)	AAU14271.1	LHY-like protein, partial
<i>Petunia hybrida</i> (Ph)	AKL88450.1	LATE ELONGATED HYPOCOTYL
<i>Phaseolus vulgaris</i> (Pv)	CAD12767.2	LATE ELONGATED HYPOCOTYL
<i>Physcomitrella patens</i> (Pp)	BAI39991.1	CIRCADIAN CLOCK ASSOCIATED
	BAI39992.1	CIRCADIAN CLOCK ASSOCIATED
<i>Vigna radiata</i> (Vr)	XP_01452159	LATE ELONGATED HYPOCOTYL
<i>Vigna unguiculata</i> (Vu)	XP_02790607	LATE ELONGATED HYPOCOTYL-
<i>Zea mays</i> (Zm)	NP_00113152	LATE ELONGATED HYPOCOTYL
	ADU60099.1	CIRCADIAN CLOCK ASSOCIATED
	AQK41466.1	CIRCADIAN CLOCK

Table S2: BLAST analysis. Arabidopsis accessions (Query ID) were used to identify REVEILLE (RVE, Protein column) sequences in the snapdragon genome database (<http://bioinfo.sibs.ac.cn/Am/index.php>). RVE names were assigned based on a tree generated by simple phylogeny (EMBL-EBI, <https://www.ebi.ac.uk/>).

Query ID	Protein	Snapdragon accession	P identity	e value	Assigned name
OAO92169.1	AtRVE1	Am04g07010	86.49	9e ⁻²³	AmRVE1/2/7A
		Am06g21890	47.15	5e ⁻²⁰	AmRVE1/2/7B
OAO90963.1	AtRVE2	Am04g18800	80.56	2e ⁻²³	AmRVE1/2/7C
		<u>Am06g21900</u>	51.00	2e ⁻²⁰	AmRVE1/2/7D
AT1G01520	AtRVE3	Am08g25850	76.79	1e ⁻¹⁸	AmRVE4/8
		Am02g37040	88.37	4e ⁻¹⁷	AmRVE3/5B
gi 75324470	AtRVE4	Am04g07010	72.97	8e ⁻¹⁴	AmRVE1/2/7A
		Am04g18800	70.27	1e ⁻¹³	AmRVE1/2/7C
gi 576017836	AtRVE5	Am03g44150	76.60	1e ⁻¹⁵	AmRVE3/5A
		Am02g37040	79.55	4e ⁻¹⁵	AmRVE3/5B
AT5G52660	AtRVE6	Am04g07010	72.97	3e ⁻¹⁴	AmRVE1/2/7A
		Am04g18800	70.27	5e ⁻¹⁴	AmRVE1/2/7C
gi 576017949	AtRVE7	Am04g18800	67.57	2e ⁻²¹	AmRVE1/2/7C
		Am06g21890	44.86	6e ⁻²⁰	AmRVE1/2/7B
OAP05994.1	AtRVE8	Am04g07010	78.38	2e ⁻¹⁵	AmRVE1/2/7A
		Am04g18800	72.97	5e ⁻¹⁵	AmRVE1/2/7C

Table S3: PCR primers used in the current study

Gene	Forward (5' – 3')	Reverse (5' – 3')
RNAi:AmLH	TCTGGATGAAACAAGTGTCC	TAAAAAGTTTCTGGGCATGA
NPTII	CCTGCTTGCCGAATATCATGGTG	CGAAATCTCGTGATGGCAGGTTGG
AmUBI	CCGATGGAAGTATATGTTTG	CCAGTCTTACAAGACCTAGAAGCT
AmLHY	TTACTTGCTCTCCGGCAACT	TTGAAGTCACAAGCTGATTGC

Table S4: Analysis of floral parameters in non-transgenic and transgenic lines. Parameters were analyzed using a Student's t test. *P* value < 0.05 indicates a significant difference between non-transgenic and transgenic snapdragon flowers, significance levels are indicated with one asterisks (* for $p < 0.05$), two asterisks (** for $p < 0.001$) and three asterisks (***) for $p < 0.0001$).

Parameter	RNAi:AmLHY14	RNAi:AmLHY26	RNAi:AmLHY27
Total length	0.037*	0.858	0.069
Lower length	0.014*	0.486	0.210
Tube width	0.927	0.927	0.001*
Sepal length	0.337	0.646	0.762
Petal height	0.000***	0.060	0.004*
Lower petal expansion	0.005*	0.885	0.885
Upper petal expansion	0.137	0.419	0.000***
Tube length	0.404	0.881	0.404
Pallate	0.000***	0.258	0.160
Stamen length	0.974	0.974	0.974
Gynoecium length	0.048*	0.278	0.278

Table S5: Retention time in minutes (RT), CAS number of detected volatiles.

RT	CAS	Volatile
4.61	100-52-7	Benzaldehyde
5.23	123-35-3	Myrcene
6.29	3779-61-1	Ocimene
7.70	124-19-6	Nonanal
7.84	98-86-2	Acetophenone
8.41	93-58-3	Methyl benzoate
8.51	78-70-6	Linalool
8.77	118-93-4	2-Hydroxyacetophenone
9.38	119-36-8	Methyl salicylate
9.54	112-31-2	Decanal
9.83	93-89-0	Ethyl benzoate
10.5	4179-19-5	3,5-Dimethoxytoluene
11.1	104-54-1	Cinnamyl alcohol
12.3	19713-73-6, 103-26-	Methyl cinnamate
14.0	502-61-4	Farnesene

Table S6: Analysis of daily emitted volatiles with the algorithm JTK_CYCLE in wild type (WT) and RNAi lines. Phase indicates the time point (ZT hours) with maximum emission. Shift indicates if the maximum emission of a VOC was advanced (A), delayed (D) or did not changed (NC) compared to the wild type. No data indicates that a parameter could not be determined.

	WT	RNAi:AmLHY14		RNAi:AmLHY26		RNAi:AmLHY27	
Volatile	Phase	Phase	Shift	Phase	Shift	Phase	Shift
Myrcene	0	6	D	6	D	6	D
Decanal	3	3	NC	3	NC	3	NC
Ocimene	3	6	D	6	D	6	D
2-Hydroxyacetophenone	6	6	NC	6	NC	6	NC
3,5-Dimethoxytoluene	6	6	NC	6	NC	6	NC
Linalool	6	6	NC	6	NC	6	NC
Nonanal	6	6	NC	6	NC	6	NC
Methyl cinnamate	6	3	A	6	NC	3	A
Cinnamyl alcohol	9	9	NC	9	NC	9	NC
Farnesene	9	6	A	6	A	6	A
Methyl benzoate	15	18	D	18	D	18	D
Acetophenone	15	12	A	12	A	12	A
Methyl salicylate	15	21	D	21	D	21	D
Benzaldehyde	18	12	A	12	A	12	A
Ethyl benzoate	No data	No data		No data		No data	

Conclusions

Conclusions

The analysis of the circadian clock in different species contributes to the understanding about evolution and diversification across the green lineage. We have described the transcriptional structure of circadian clock and related genes in petunia leaves and petals, revealing a tissue-specific clock that may set specialized processes in both tissues. Growth speed in stems and flowers is controlled by the circadian clock. In the present work we also examined the complexity of the scent profile in the diurnally emitting snapdragon and the nocturnally emitting petunia. The flower scent proved to be controlled by the circadian clock, which affected both composition and timing emission.

Chapter I

- The genes *PSEUDO-RESPONSE REGULATOR 5*, *7* and *GIGANTEA* are duplicated in petunia. The differences in aminoacid sequence and gene expression, including phase and amplitude, in leaves and petals suggest a specialization of paralogs.
- Expression of circadian clock genes and clock related genes differed among petunia leaves and petals.
- Constant darkness caused, in general, a delayed expression in clock genes as well as clock related genes in leaves and petals.
- The variability in gene expression differed among tissues, daytime and light cycles.

Chapter II

- The expression pattern of *CHANEL* (*PhCHL*) is dependent on day length.
- *PhCHL* plays a role growth speed in stems and flowers.
- Petunia fragrance is composed mainly of phenylpropanoid and benzonoid volatiles. The main volatile, methyl benzoate, is rhythmically emitted.
- The contribution of every single compound to the scent varied along the day. *PhCHL* is required to maintain the aroma composition and timing of scent emission.

Chapter III

- *LATE ELONGATED HYPOCOTYL* (*AmLHY*) shows the canonical expression pattern described in other plants.

- *AmLHY* regulates positively flower growth speed but not its size or morphology.
- The floral aroma of snapdragon is mainly constituted by phenylpropanoid and benzenoid compounds, but also monoterpenes, sesquiterpenes and aldehydes. The most abundant compound is acetophenone, which is constitutively emitted.
- The proportion of every volatile and timing of scent emission are maintained by *AmLHY*.
- *AmLHY* regulates volatile biosynthesis at different branches.

Conferences

Poster
13/SIV
P13

Organ-specific evolution and subfunctionalization of circadian clock in *Petunia*

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The plant circadian clock is comprised by a set of genes that form the so-called loops. These loops are molecular and genetic interactions of activation and repression, and are classified as morning, central and evening loops based on the expression of their components in *Arabidopsis* leaves. We have performed a comprehensive analysis of the clock components in *Petunia*¹ and found that *PRR7*, *PRR5*, *G*, *ELF3* and *ELF4* show gene copy numbers ranging from 2 to 4. In contrast, *PRR9*, *PRR3*, *TOC1*, *ZTL* and *LHY* are single copy genes while *CCA1* is absent. This suggests possible subfunctionalizations of different paralogs. We investigated the transcription of circadian clock genes in leaves and petals of *Petunia* and found highly significant differences in gene expression in single copy genes such as *PRR9* and *ZTL*. The differences identified included changes in their circadian expression pattern and level of expression. The paralogs *PRR7a* and *PRR7b* showed differences of expression between leaves and flowers in terms of daily expression profiles and expression levels. A similar effect was seen in *PRR5a* and *PRR5b* as *PRR5a* was 159 fold higher in petals than in leaves. Our results show that the plant circadian clock is different in petals and leaves, and there is a possible subfunctionalization of paralogs that become specific for the leaf or petal.

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1. Bombarely, A. *et al. Nat. Plants* In press, (2016).

S-V. Póster 5

EFFECT OF SILENCING *PHZTL* ON GROWTH AND SCENT EMISSION IN PETUNIA

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The circadian clock is an adaptation that allows organisms to anticipate periodic changes. In plants, this biological clock is comprised by a set of genes which form feedback interlocked loops. *Zeitlupe* (*ZTL*) codifies an F-box protein that interacts with other two clock components, TIMING OF CAB EXPRESSION 1 (*TOC1*) and PSEUDO-RESPONSE REGULATOR 5 (*PRR5*), playing a role in *TOC1* and *PRR5* degradation (Más et al., 2003; Kiba et al., 2007); additionally *ZTL* has a PAS domain, present in blue-light receptor molecules (Somers et al., 2000). In *Arabidopsis*, *ztl* mutants displayed a longer period, for example, flowering was delayed (Somers et al., 2004). Moreover, the role of circadian clock genes in several processes such as organ development, movement or scent emission is still poorly known (Fenske et al., 2015; Yon et al., 2016). In the present work, we silenced the expression of *ZTL* by RNAi in *Petunia* (*PhZTL*) and we analysed growth and scent emission. Growth was significantly shorter or slower depending on the organ analysed. Most volatile organic compounds showed a rhythmic emission. Time of maximum emission was delayed or advanced in RNAi plants depending on the volatile. The relative contribution of every compound to scent varied between non-transgenic and transgenic *Petunia* flowers resulting in quantitative changes in scent blends at different times of the day. These results suggest that *PhZTL* plays a role, directly or indirectly, in biological rhythms in plants, affecting flowering time, stem and flower growth and scent emission.

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RNAi:Ph2TL line versus wild type Petunia

Conclusion

- Our results show that Ph2TL plays a central role in the regulation of growth. The difference in growth rate between vegetative and generative stages appears to be the result of decreased growth duration or growth rate. Furthermore, daily pattern of flowering is slightly advanced in RNAi:Ph2TL plants.
- Concerning volatile production, time of maximum production depends on the volatile. The relative difference in production between non-transgenic and transgenic Petunia scent blends at different times of the day.
- Results suggest that Ph2TL plays a role, directly or indirectly, in the regulation of growth and volatile production.

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Other communications

Title: Identification of nana as circadian clock mutant in *Antirrhinum majus*

Authors: Marta Terry López; Fabiola Ruíz-Ramón; Julia Weiss; Marcos Egea Gutiérrez-Cortines.

Poster.

Conference: XII Reunión de Biología Molecular de Plantas.

Venue: Cartagena (Spain). Date: 11-13th June, 2014.

Title: Análisis genético del reloj circadiano de *Antirrhinum majus*

Authors: Marta Terry López; Julia Weiss y Marcos Egea Gutiérrez-Cortines.

Poster.

Conference: 4º Workshop de Investigación Agroalimentaria – WiA.15

Venue: Cartagena (Spain). Date: 11-12th May, 2015.

<http://repositorio.upct.es/handle/10317/5290>

Title: Genetic and Environmental Factors Affecting Scent and Volatile Production

Authors: Egea Gutiérrez-Cortines; Julia Weiss; Victoria Ruiz-Hernández; Marta Terry-López.

Conference: BIT's 6th World Gene Convention.

Venue: Qingdao (China). Date: 13-15th November, 2015.

Title: Influencia de la condiciones ambientales en la emisión de volátiles en boca de dragón (*Antirrhinum majus*).

Authors: Marta Terry López; Julia Weiss; Marcos Egea Gutiérrez-Cortines.

Oral communication.

Conference: 5º Workshop de Investigación Agroalimentaria – WiA.16

Venue: Cartagena (Spain). Date: 9-10th May, 2016.

<http://repositorio.upct.es/handle/10317/5558>

Title: Estructura del reloj circadiano en hoja y pétalo de petunia

Authors: Marta Terry López; Marcos Egea Gutiérrez-Cortines; Julia Weiss.

Oral communication.

Conference: 6º Workshop de Investigación Agroalimentaria – WiA.17

Venue: Cartagena (Spain). Date: 8-9th May, 2017.

<http://repositorio.upct.es/handle/10317/6706>

Title: Circadian regulation of lateral organ development.

Authors: Marcos Egea-Cortines; Julia Weiss; Marta I. Terry; Fernando Pérez-Sanz; Victoria Ruiz-Hernández; María Victoria Díaz-Galián; Pedro Javier Navarro

Conference: X Jornadas de Genética e Biotecnología

Venue: Vila Real (Portugal). Date: 6-8th March, 2018.

Title: Análisis genético del reloj circadiano de *Antirrhinum majus*.

Authors: Marta Terry López; Julia Weiss; Marcos Egea Gutiérrez-Cortines.

Conference: I Jornadas Doctorales de la UPCT.

Poster.

Venue: Cartagena (Spain). Date: 16th April, 2018.

Title: Efecto del silenciamiento de *PhZTL* en la emisión de aromas en petunia (*Petunia x hybrida*).

Authors: Marta Terry López; Marcos Egea Gutiérrez-Cortines; Julia Weiss.

Oral communication.

Conference: 7º Workshop de Investigación Agroalimentaria – WiA.18

Venue: Cartagena (Spain). Date: 7-8th May, 2018.

<http://repositorio.upct.es/handle/10317/7652>

Title: El silenciamiento de *LATE ELONGATED HYPOCOTYL* en boca de dragón modifica la emisión de aromas.

Authors: Marta Terry López; Marcos Egea Gutiérrez-Cortines; Julia Weiss.

Oral communication.

Conference: 8º Workshop de Investigación Agroalimentaria – WiA.19

Venue: Cartagena (Spain). Date: 27-28th May, 2019.

Contributions unrelated to the PhD thesis

THE CRUSTACEAN SOCIETY MID-YEAR MEETING

22 de junio de 2017

P-060

Alien brachyurans in eastern Iberian Peninsula coast: the case of *Callinectes sapidus* and *Percnon gibbesi***Marta Isabel Terry¹, Diego Castejón²**

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The portunid *Callinectes sapidus* (Rathbun, 1896) and the grapsoid *Percnon gibbesi* (H. Milne-Edwards, 1853) are considered among the 100 'worst invasive' species in the Mediterranean Sea.

The presence of *C. sapidus* was studied in the Ebro Delta (Tarragona, North-Eastern Iberian Peninsula) from 2012 to 2016 using specimens provided by local fishermen. The captures occurred in the coastal waters of Ebro Delta and associated brackish lagoons and channels, being obtained 12 males and 19 females with a mean size of 147 ± 32 mm considering the tips of the lateral spines. The specimens included a "berried" female with mature eggs in June 2016. In August 2016 was officially established the local fishery of *C. sapidus* in Ebro Delta, the monthly catches varied from 20 Kg in December to 229 Kg in September. The presence of *P. gibbesi* was studied in Cabo Palos (Murcia, South-Eastern Iberian Peninsula) during the years 2012, 2015 and 2016 by snorkel surveys performed at a depth from 0 to 3 m. *P. gibbesi* was found in 17 of 37 replicate trials in four sampling localities (53.1 %). In total, 59 individuals were counted.

The higher density was observed in rocky bottoms between 0.5 and 1.5 m depth with abundance of crevices and rock walls as shelters.

Fig. 1
 captured in 2016 had a small
 The minimal reproductive
 have been captured from the
 Mediterranean Sea 3 miles
 In 2017 were captured (j
 fishermen). This suggest a n

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