Circadian clock regulation of chemical communication between plants and pollinators: a case study of *Petunia* flowers and *Manduca* hawkmoths.

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Abstract

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Pollination services from animals ensure successful reproduction and outcrossing for many flowering plants. Recruitment of pollinators most often involves a combination of signals which are deployed from floral tissues. The shape of a flower, its color, smell, opening time, nectar secretion, floral angle, and other factors have evolved in a variety of ways to attract pollinators, while also restricting visitation from unsuitable guests. In particular, floral scent is a "double-edged sword" which can allow for long-distance recruitment of pollinators, while potentially risking the attention of herbivores and ineffective pollinators. A daily rhythm of advertisement may help to ensure that only the most efficient pollinators are recruited, while mitigating advertisement to potential predators. In Chapter 1, I begin by reviewing the behavioral, physiological, and molecular evidence for circadian rhythms in floral scent emission. I cover the early empirical observations of floral scent rhythms, and discuss the progress of the field after technological advances in chemical analysis and molecular biology. In Chapter 2, I then investigate the molecular mechanism regulating floral scent emission in the nocturnally fragrant *Petunia hybrida*. I show that the clock component LATE ELONGATED HYPOCOTYL (LHY) directly represses the expression of *ODORANT1* (*ODO1*) and other scent-related genes during the morning, restricting their expression to the evening: which leads to the emission of scent during the night. Chapter 3 then investigates the importance

of the circadian clock to *P. axillaris* floral visitation by the nocturnally active hawkmoth *Manduca sexta*. I show that manipulation of the plant clock allows for disruption of floral visitation. I also show that *M. sexta*'s ability to sense and respond to floral scent signals is clock-regulated but light-repressed. In addition, male hawkmoths show a time-dependent sensitivity and responsiveness to floral scent. Finally, I give a summary of the research topics here considering recent advances in the field and specific areas requiring further study.

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Circadian Rhythms in Floral Scent Emission

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To successfully recruit pollinators, plants often release attractive floral scents at specific times of day to coincide with pollinator foraging. This timing of scent emission is thought to be evolutionarily beneficial to maximize resource efficiency while attracting only useful pollinators. Temporal regulation of scent emission is tied to the activity of the specific metabolic pathways responsible for scent production. Although floral volatile profiling in various plants indicated a contribution by the circadian clock, the mechanisms by which the circadian clock regulates timing of floral scent emission remained elusive. Recent studies using two species in the *Solanaceae* family provided initial insight into molecular clock regulation of scent emission timing. In *Petunia hybrida*, the floral volatile benzenoid/phenylpropanoid (FVBP) pathway is the major metabolic pathway that produces floral volatiles. Three MYB-type transcription factors, ODORANT 1 (ODO1), EMISSION OF BENZENOIDS I (EOBI), and EOBII, all of which show diurnal rhythms in mRNA expression, act as positive regulators for several enzyme genes in the FVBP pathway. Recently, in *P. hybrida* and *Nicotiana attenuata,* homologs of the *Arabidopsis* clock gene *LATE ELONGATED HYPOCOTYL* (*LHY*) have been shown to have a similar role in the circadian clock in these plants, and to also determine the timing of scent emission. In addition, in *P. hybrida,* PhLHY directly represses *ODO1* and several enzyme genes in the FVBP pathway during the morning as an important negative regulator of scent emission. These findings facilitate our understanding of the relationship between a molecular timekeeper and the timing of scent emission, which may influence reproductive success.

Keywords: floral volatile, circadian clock, pollinator, metabolic pathway, petunia, tobacco, *Solanaceae*

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INTRODUCTION

Pollinator attraction is a critical event for many flowering plants that require out-crossing. Flowers and related structures (e.g., bracts) are primarily responsible for attracting pollinators, through variations in shape, color, and scent. In addition, the traits that determine the volume of nectar (which rewards pollinator visits) and the position of reproductive organs (which influence the transfer of pollen to other plants) also affect the success of pollination (Sheehan et al., 2012). These floral traits are temporally regulated in many species. As captured by Linnaeus' widely known flower clock, many flowers open and close at particular times of the day (Rose, 1775).

Among the traits related to pollinator syndrome, floral scent is a critical component of ensuring successful pollination for many plant species (Dudareva et al., 2006). Over 90% of global angiosperms, and about a third of crop species, require pollination by animals (mostly insects; McGregor, 1976; Kearns et al., 1998; Klein et al., 2007). Pollinating insects have well

developed sensory mechanisms for detecting floral odors, and like most organisms, insects exhibit daily fluctuations in their activities (Saunders, 1997). Plants maintain their side of this temporal relationship by emitting scent only during specific times of day, which corresponds with the activity periods of the pollinators. This timing of scent release likely allows for efficient resource utilization, as well as limiting the visibility of the plant to herbivores. In this review, we discuss the brief history of the findings that elucidate the influence of the circadian clock on scent emission as well as recent findings of the molecular links in between.

OBSERVATIONS OF RHYTHMIC SCENT RELEASE

An early scientific documentation of scent rhythmicity comes from Lillian Overland's research at the Missouri Botanical Garden, where she measured the presence of floral fragrance throughout the day (Overland, 1960). She used *Cestrum nocturnum,* commonly known as "night blooming jasmine," a member of the *Solanaceae* family. *C. nocturnum*'s small tube-shaped flowers open and emit a strong, pleasant scent at night. At the time, it was assumed that the emission from *C. nocturnum* was the result of a nighttime-dependent mechanism, rather than circadian control of the rhythm. Until the mid-1950s, the presence of an endogenous oscillator (i.e., the circadian clock) and the importance of its influence on biological responses was not widely accepted. Since then, a significant percentage of physiology has been attributed to circadian clock regulation.

Overland hypothesized that *C. nocturnum's* nocturnal scent emissions were under the regulation of this endogenous timekeeper. Flowering plants were placed into rooms with continuous light and temperature, and the scent presence was recorded over 3 days. Under the conditions, a clear 24-h rhythm was observed, with scent occurring during the time periods corresponding with what would be night (subjective night). This work clearly demonstrated that *C. nocturnum* plants utilized the circadian clock to time floral scent emission. In addition, the cell autonomous nature of the clock was captured by demonstrating that detached corolla lobes emitted scent in a circadian fashion in the same way as that of the intact flower.

TECHNICAL IMPROVEMENT IN FLORAL SCENT MEASUREMENT

Gas chromatography–mass spectrometry (GC–MS) would prove to be a powerful and more quantitative tool for scientists who study flower fragrance, perhaps first used to measure scent periodicity in orchids in 1978 (Nilsson, 1978). Altenburger and Matile (1988, 1990) and Matile and Altenburger (1988) released a series of three papers between 1988 and 1990 that provided thorough analyses of scent rhythmicity using modern chemical analysis and time course scent collection techniques. An automated collection device was created to facilitate the consecutive harvest of scent samples during extended time courses, and GC–MS was used to analyze the sequential changes of scent release.

In their first paper, diurnal rhythms of different volatiles were observed in four species: *Odontoglossum constrictum*, *Citrus medica*, *Hoya carnosa*, and *Stephanotis floribunda* (Matile and Altenburger, 1988). *O. constrictum* and *C. medica* exhibited emission peaks during the day, while *H. carnosa* emitted scent only at night. The timing of the five most abundant compounds emitted from the flowers of *H. carnosa* were notably synchronized, indicating a similar mechanism of regulatory timing. The emission profile of *S. floribunda* was even more intriguing, as its flower emitted separate compounds at different times of the day. The emission of methyl benzoate and linalool peaked at midnight, but the third compound, 1-nitro-2-phenylethane, peaked at noon – directly antiphasic to the first two. This phenomenon raised several interesting points. First, separate mechanisms must set the phase of each emission. In addition, while the primary purpose of methyl benzoate and linalool is attracting nocturnal pollinators, the objective of 1-nitro-2-phenylethane release is unknown. Is 1-nitro-2-phenylethane involved in attracting a separate pollinator during the day, or does it fill an entirely different purpose, perhaps as a repellent? It should be mentioned that methyl benzoate and 1-nitro-2-phenylethane are both components of the benzenoid metabolic pathway, while linalool is a member of the terpenoid pathway (Silva et al., 2006; Muhlemann et al., 2014).

Their second paper provided evidence of circadian clock involvement, as floral scent rhythms were maintained even under continuous light conditions in *H. carnosa* (Altenburger and Matile, 1988). After shifting the photoperiod by 12 h, the plants were able to quickly entrain to the new light/dark cycles. Another critical finding was that flowers separately entrained to 12-h shifted (antiphasic) light/dark cycles from the vegetative body (which they were still attached to) properly entrained to their own surrounding photoperiods, demonstrating that flowers are able to generate their own circadian rhythms independent to the rest of the plant. Similar to the previously described example of detached *C. nocturnum* corolla lobes, this implied the presence of tissue/cell autonomous circadian timekeeper mechanisms in flowers. Cut flowers of *H. carnosa* exhibited nearly undetectable levels of scent emission (Matile and Altenburger, 1988), indicating that, in some plants, inter-tissue connection (i.e., between leaves and flowers) is necessary for floral scent emission.

The third paper elaborated upon the rhythmic scent emissions of the species introduced in the first paper (Altenburger and Matile, 1990). *H. carnosa*'s emissions exhibited temperature compensation in the free-running period, and were able to maintain a circadian rhythm in continuous dark. The emission of *S. floribunda* volatiles in continuous light was found to be circadian in nature, yet they quickly fade into arrhythmicity in just 2 or 3 days. Cut flowers of *O. constrictum* and *C. medica* were unable to maintain a rhythmic emission under both continuous light and dark conditions, but were able to resume a rhythm when returned to diurnal light/dark cycles.

A separate group completed similar experiments using GC–MS analysis. Loughrin et al. (1991) analyzed the daily

emission of volatiles in tobacco flowers (*Nicotiana sylvestris* and *N. suaveolens*). They analyzed the volatile emission patterns under continuous light conditions, and found that the emission of benzyl alcohol and methyl benzoate, the most abundant volatiles in their flowers, occurred in a clear circadian fashion. Interestingly, under the same conditions, emission of the sesquiterpene hydrocarbon, caryophyllene, in *N. sylvestris* did not show any oscillation. This result showed that, even in the same flower, the influences of circadian timing are different depending on the metabolic pathways/products.

These four examples tell us that, even though all of these plants show robust daily scent emission rhythms, the contribution of their circadian clocks to scent emission under continuous environmental conditions varies depending on the species.

The scent model *P. hybrida* exhibits a distinct scent emission at night, but its robust oscillation does not persist in continuous light or dark (Underwood et al., 2005; Fenske et al., 2015). Interestingly, *P. axillaris* (one parent of *P. hybrida*) maintains a robust oscillation of scent release in continuous light, whereas *P. integrifolia* (the other parent) does not (Hoballah et al., 2007). Hybridization between *P. axillaris* and *P. integrifolia*'s light-dependent scent patterns may attribute to *P. hybrida*'s non-robust scent oscillation under continuous light conditions.

MODELS OF SCENT EMISSION

By the 21st century, several model plant species had arisen in the scent biology field, the most prolific of which were rose, snapdragon, tobacco, and petunia. Choosing models to study scent was important to develop a comprehensive understanding of scent production. A focused study of the metabolic pathways that produce scent was a necessary prerequisite to understanding the regulation of scent emission. In this regard, *P. hybrida* has emerged as perhaps the most complete model in scent emission, as the majority of the metabolic pathway has been mapped, and transcriptional regulators of floral scent have been characterized (Dudareva and Pichersky, 2006; Colquhoun and Clark, 2011).

FLORAL VOLATILE GENES HAVE DAILY OSCILLATORY EXPRESSION PATTERNS

An analysis of gene expression within the floral volatile benzenoid/phenylpropanoid (FVBP) pathway provided key insights into the mechanism of scent regulation (Dudareva et al., 2000). Emission of the major volatile methyl benzoate diurnally oscillates over the course of several days in snapdragon, in both continuous light and darkness (Kolosova et al., 2001). The daily changes in supply of the substrate of methyl benzoate (benzoic acid) largely accounts for the circadian oscillation (Kolosova et al., 2001). Kolosova et al. (2001) analyzed the expression of benzoic acid carboxyl methyltransferase (*BAMT*) and phenylalanine ammonia-lyase (*PAL*) genes. BAMT catalyzes the final step for methyl benzoate synthesis by transferring a methyl group to benzoic acid (Dudareva et al., 2000). PAL controls benzoic acid synthesis by producing *trans*-cinnamic acid from the amino acid phenylalanine (Colquhoun and Clark, 2011). The snapdragon *BAMT* and its petunia homologs *BSMT1* and *BSMT2,* all exhibit diurnal oscillations, peaking in the afternoon. However, the *BSMT1* and *BSMT2* expression patterns do not directly correlate with the volatile expression patterns (Kolosova et al., 2001; Fenske et al., 2015). *PAL* mRNA expression (and enzymatic activities) also oscillates in diurnal conditions in both snapdragon and petunia; however, interestingly, it peaks at different times (around dawn in snapdragon, dusk in petunia). *PAL* mRNA expression oscillates in a similar pattern to that of benzoic acid synthesis in diurnal conditions (Kolosova et al., 2001; Fenske et al., 2015). These results support the notion that diurnal emission patterns of scent largely derive from the oscillation of upstream metabolite synthesis, rather than controlling the final reaction timing.

Among the other FVBP enzyme genes in petunia, genes encoding 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), arogenate dehydratase (ADT), chorismate mutase 1 (CM1), eugenol synthase (EGS), isoeugenol synthase (IGS), 3-ketoacyl-CoA thiolase (KAT1), benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase (BPBT), and *S*-adenosylmethionine synthetase (SAMS) also exhibit robust diurnal oscillations in their expression (Verdonk et al., 2005; Fenske et al., 2015) (**Figure 1**), though most show only weak oscillations in continuous dark (none do in continuous light). At least in *P. hybrida*, it appears likely that robust scent emission cycles are derived from the cumulative effect of the oscillatory expression of many FVBP genes in concert.

REGULATORY MECHANISMS OF THE SCENT METABOLISM PATHWAY

The first regulatory component of floral scent metabolism was identified as ODORANT1 (ODO1), an R2R3-type MYB transcription factor in *P. hybrida* (Verdonk et al., 2005). mRNA expression of *ODO1* peaks in the evening, just prior to scent release. RNAi suppression of *ODO1* transcript caused a significant reduction in emission of nearly all detectable volatile benzenoids. In addition, *ODO1* suppression drastically reduced the transcript levels of many key FVBP genes, including *DAHPS*, *EPSPS*, *PAL*, *CM*, and *SAMS* (Verdonk et al., 2005). As the authors note, many of these enzymes are upstream in the FVBP metabolic pathway, which poses the question: does *ODO1* affect more than just scent emission? *ODO1* suppression did not affect accumulation of anthocyanin, which is produced through the FVBP pathway in flower petals (Verdonk et al., 2005). This indicated that while ODO1 controls this pathway broadly, its influence may be more specific to volatile production.

EMISSION OF BENZENOIDS II (EOBII) was the second regulatory component found to be involved in FVBP synthesis (Spitzer-Rimon et al., 2010). Like *ODO1*, *EOBII* encodes an R2R3-type MYB transcription factor. Suppression of *EOBII* significantly decreased the emission of most floral volatiles.

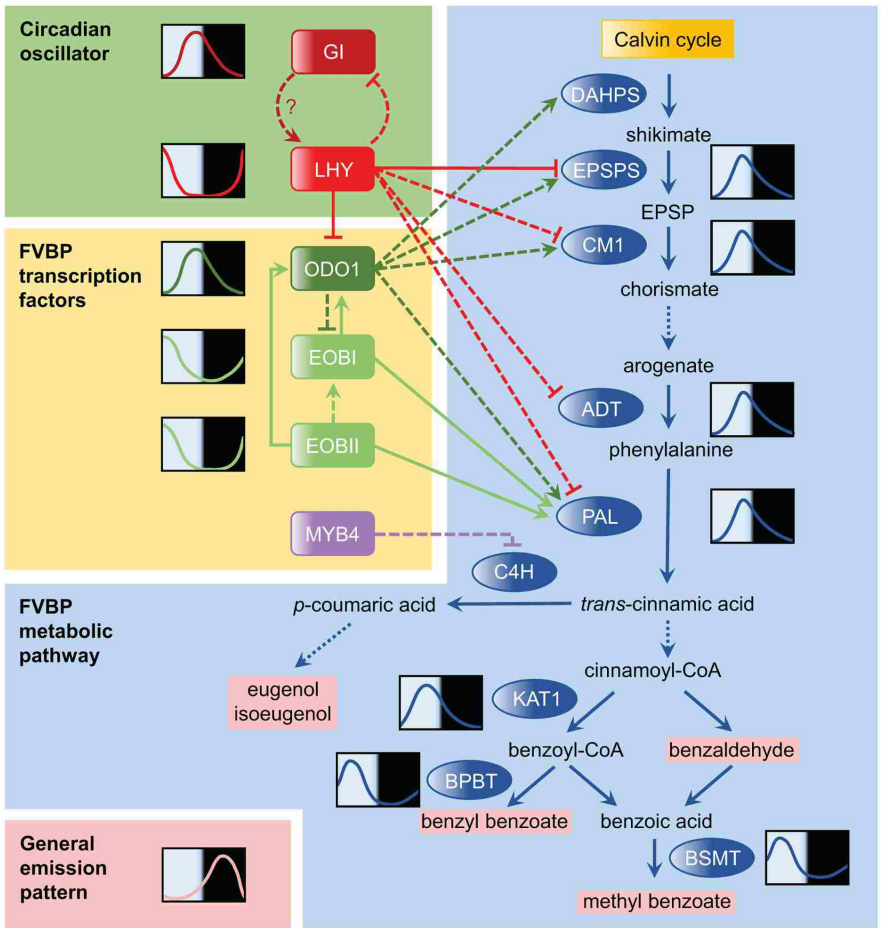

FIGURE 1 | Temporal regulation of the floral volatile benzenoid/phenylpropanoid (FVBP) pathway in petunia. The FVBP pathway is the primary metabolic pathway of petunia for scent production. The FVBP pathway is composed of a series of enzymes (shown in blue ovals), which includes 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), chorismate mutase 1 (CM1), arogenate dehydratase (ADT), phenylalanine ammonia-lyase (PAL), 3-ketoacyl-CoA thiolase (KAT1), benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase (BPBT), *S*-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase (BSMT), and cinnamate 4-hydroxylase (C4H). These enzymes modify products of the Calvin cycle into volatile scents that are emitted by the flowers (volatile products shown in pink). Solid lines in the FVBP pathway represent single enzymatic steps, while dotted lines represent multiple steps. Representative daily mRNA expression patterns are drawn for selected genes. The relationship between LATE ELONGATED HYPOCOTYL (LHY) and GIGANTEA (GI) in the circadian oscillator was conceptualized based on knowledge obtained from *Arabidopsis* research and their expression patterns in petunia. Transcriptional regulators in the FVBP pathway are highlighted by yellow backgrounds, including ODORANT 1 (ODO1), EMISSION OF BENZENOIDS I (EOBI), EOBII, and MYB4. Solid arrows connecting transcription factors to FVBP genes denote confirmation of direct molecular binding demonstrated by electrophoretic mobility shift assay (EMSA), yeast one-hybrid, and/or transient reporter assay; dashed lines lack this confirmation. EOBI may regulate the FVBP gene expression more directly (not through the regulation of *ODO1*). The general emission pattern of benzenoid compounds is shown in the bottom left-hand corner; this profile is synthesized based on daily scent emission profiles of four major benzenoids in Fenske et al. (2015).

Up-regulation and down-regulation of *EOBII* transcription increases and decreases the transcripts of many key enzymes in the FVBP pathway. As with *ODO1*, *EOBII* does not affect floral anthocyanin production. EOBII also directly up-regulated the expression of *EOBI*, a transcription factor gene closely related to *EOBII* (Spitzer-Rimon et al., 2012). *EOBI* suppression also reduces floral scent emission. Mechanistically, EOBI directly binds and activates the *ODO1*, *PAL*, and *IGS* promoters to regulate scent production (**Figure 1**).

A transcriptional enhancer region in the *ODO1* promoter was identified (Van Moerkercke et al., 2011). Mutations in putative MYB binding sites of this region decreased the overall promoter activity by 50%. *EOBII* bound to the MYB binding sites and enhanced *ODO1* promoter activity (Van Moerkercke et al., 2011). Polymorphisms that exist in the MYB binding sites in the *ODO1* promoter between the highly fragrant cultivar Mitchell and the non-fragrant cultivar R27 account for differential *ODO1* expression levels and scent emission levels in these cultivars. In addition, a hint of a potential negative element in the pathway was indicated, as a conserved *cis*-regulatory element known as Evening Element (EE) was present in the *ODO1* promoter. EE is a binding site for the clock transcriptional repressors *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) in *Arabidopsis thaliana* (Harmer, 2009).

These three transcription factors function as activators that broadly regulate the enzyme gene expression in the FVBP pathway. There is also a specific repressor, *PhMYB4* (Colquhoun et al., 2011). Repression of *PhMYB4* increased expression of both *CINNAMATE 4-HYDOXYLASE* (*C4H1* and *C4H2*) genes, which encode enzymes that modify *t*-cinnamic acid into *p*-coumaric acid. The increase in *C4H* expression in *PhMYB4* silenced lines likely accounts for the corresponding increase in the emission of eugenol and isoeugenol, which are derived from *p*-coumaric acid. Thus, *PhMYB4* is a negative regulator of eugenol and isoeugenol emission. A diurnal rhythm, similar to that of *ODO1*, was observed in *PhMYB4* expression.

CLOCK MECHANISMS REGULATING FLORAL SCENT METABOLISM

Directly following the identification of putative binding sites of the clock components in the promoter of *ODO1* (Van Moerkercke et al., 2011), the first petunia clock gene, *Petunia hybrida LHY* (*PhLHY*), was identified (Fenske et al., 2015). Similar to its homologs in other plants, *PhLHY* peaks around dusk (**Figure 1**). Constitutive expression of *PhLHY* leads to an almost complete loss of scent emission, as well as a corresponding decrease in the expression of many FVBP genes (*ODO1, EPSPS, CM1, ADT, PAL,* etc.). Reducing the expression of *PhLHY* advanced the phase of scent emission from night to afternoon as well as FVBP gene expression. In addition, the direct binding of PhLHY to the EEs in the *ODO1* promoter was demonstrated. PhLHY also bound to the EE sequences of *EPSPS* and *IGS* promoters. Based on the results of the expression analysis, it seems possible that PhLHY can bind to other genes in the FVBP pathway and control the expression phase of these genes. As our current understanding of the transcriptional regulation of the FVBP pathway is still incomplete (**Figure 1**), a further investigation into the relationship of PhLHY with the FVBP transcription factors is awaited.

At the same time, similar results were observed in tobacco (*Nicotiana attenuata*; Yon et al., 2015). Repression of the *N. attenuata* homolog for *LHY*, *NaLHY,* caused a shift in scent release to an earlier phase, similar to the case in petunia, and repression of *N. attenuata ZEITLUPE* (*NaZTL*) led to a dramatic decrease in floral emissions. In addition, the same study explored clock effects on other important aspects of pollinator attraction: floral opening and the angle of flower position against the horizon. In long-day conditions, *NaLHY*-silenced lines showed a 2 h advance in floral opening time and flower angle changes, while *NaZTL*-silenced lines showed no clear change in timing, but a significant reduction in overall flower scent emission, limited opening and a change in position. In continuous light, *NaLHY*-silenced flowers started opening 4 h earlier than wild-type flowers, and the circadian period length of opening was approximately 1 h shorter than that of wild-type flowers, indicating that the role of LHY in the tobacco circadian clock is similar to that in the *Arabidopsis* clock.

While the latest findings on *LHY* and *ZTL* provide additional insight into scent regulation, further inquiry into the relationship that *LHY*/*ZTL* and other clock genes share with scent metabolism is called for, once more complete genomic information is available for these plants.

CONCLUSION

Temporal expression of scent appears to be primarily regulated through manipulation of the timing of transcriptional regulators in the metabolic pathway. Regulating the expression of *ODO1* has a significant effect on floral scent emission, establishing its position as a master regulator of FVBP synthesis. Most recently, two independent research groups showed that the clock gene *LHY* set the pace of floral scent emission in two *Solanaceae* species (Fenske et al., 2015; Yon et al., 2015). LHY directly represses *ODO1* and likely other FVBP genes in the morning, restricting the expression of FVBP genes to the evening in petunia. With these findings, we are beginning to understand how the molecular clock regulates the timing of scent emission.

Many questions remain unanswered. For example, what is the role of other possible clock gene homologs on the regulation of scent emission? Are the mechanisms found in these two *Solanaceae* species conserved in other plants? Could changes in clock gene function/activity contribute to changes in pollinator choice during evolution? Finding the answers to these questions would bring us a more comprehensive and broader view of the regulatory networks for daily scent emission.

Most of the research into the regulation of scent emission has focused on transcription and volatile analyses. Thus, any circadian analysis of enzyme abundance and/or activity, together with metabolomic profiling, within the FVBP pathway would also provide further understanding of floral scent regulation. Understanding the mechanisms of floral scent release will hopefully allow for manipulation of the timing of floral scent release. In addition to applications in the floriculture industry, knowledge obtained from this type of work may allow us to generate "designer crops" which could facilitate the utilization of new pollinator populations to increase the success of reproduction under wider ranges of environmental conditions.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Circadian clock gene *LATE ELONGATED HYPOCOTYL* directly regulates the timing of floral scent emission in *Petunia*

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Flowers present a complex display of signals to attract pollinators, including the emission of floral volatiles. Volatile emission is highly regulated, and many species restrict emissions to specific times of the day. This rhythmic emission of scent is regulated by the circadian clock; however, the mechanisms have remained unknown. In *Petunia hybrida*, volatile emissions are dominated by products of the floral volatile benzenoid/phenylpropanoid (FVBP) metabolic pathway. Here we demonstrate that the circadian clock gene *P. hybrida LATE ELONGATED HYPOCOTYL* (*LHY*; *PhLHY*) regulates the daily expression patterns of the FVBP pathway genes and floral volatile production. *PhLHY* expression peaks in the morning, antiphasic to the expression of *P. hybrida GIGANTEA* (*PhGI*), the master scent regulator *ODORANT1* (*ODO1*), and many other evening-expressed FVBP genes. Overexpression phenotypes of *PhLHY* in *Arabidopsis* caused an arrhythmic clock phenotype, which resembles those of *LHY* overexpressors. In *Petunia*, constitutive expression of *PhLHY* depressed the expression levels of *PhGI*, *ODO1*, evening-expressed FVBP pathway genes, and FVBP emission in flowers. Additionally, in the *Petunia* lines in which *PhLHY* expression was reduced, the timing of peak expression of *PhGI*, *ODO1*, and the FVBP pathway genes advanced to the morning. Moreover, PhLHY protein binds to *cis*-regulatory elements called evening elements that exist in promoters of *ODO1* and other FVBP genes. Thus, our results imply that PhLHY directly sets the timing of floral volatile emission by restricting the expression of *ODO1* and other FVBP genes to the evening in *Petunia*.

circadian rhythm | floral volatile | benzenoids | *Petunia hybrida* | LHY

Plant development and physiology are extensively influenced by the circadian clock (1). The precise timing of a single plant behavioral output often requires a suite of internal mechanisms to occur in coincidence or in quick succession before the behavior taking place. Transcriptome analysis revealed that the circadian clock controls transcription of one third of genes in *Arabidopsis* (2). In this way, the clock can exert a holistic effect on a complex mechanism at a precise moment in time. The effectiveness of the clock's ability to coordinate complex behaviors has been used by many aspects of plant physiology, such as photosynthesis, stem and leaf growth, and flowering (3, 4).

The precise timing of sexual reproductive events is critical, as plants are sessile and individuals are often spread over large distances. In addition to regulating the timing of flower formation, when they have opened, many flowers emit floral scents to lure pollinators. Attractive floral volatiles are often emitted in a rhythmic fashion, with peaks of emission coinciding with the primary pollinator's period of activity (5). Although studies have shown that rhythmic emission of scent requires the influence of a circadian clock (6–8), no study of which we are aware has shown a mechanistic link between clock function and floral volatile production.

Research on floral volatile synthesis has often used the common garden petunia, *Petunia hybrida* cv. Mitchell, which exhibits white flowers that peak in scent emission in the middle of night (9). Floral scent in *Petunia* is dominated by volatile benzenoid/phenylpropanoids (FVBP), a group of organic compounds originally derived from phenylalanine (5). FVBP emission relies on the availability of precursor compounds that flow from enzymatic reactions in the shikimate pathway and later throughout the FVBP metabolic pathways (Fig. 1*A*). *ODORANT1* (*ODO1*), a transcriptional activator gene of FVBP gene expression, exhibits a daily oscillatory expression with an evening peak (10). By binding to the promoters of several key enzymes, such as 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*), ODO1 facilitates the introduction of precursor molecules to the FVBP synthesis pathway, suggesting that ODO1 is a master regulator of FVBP emissions in *Petunia*. Two transcription factors, EMISSION OF BENZENOIDS I (EOB I) and EOBII, up-regulate the expression of *ODO1* and other FVBP-related genes (11, 12).

Analysis of the *ODO1* promoter (12), as well as the promoters of several other regulatory genes in the FVBP pathway, revealed the presence of specific *cis*-elements referred to as evening elements (EEs). The EE is a binding site for two similar circadian-clock transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and a related factor, REVEILLE 8, in *Arabidopsis* (13). CCA1 and LHY, which are highly expressed during the morning, function as repressors for evening expressed genes (14, 15) and activators for morning expressed genes (16). Thus, we hypothesized that the *Petunia* ortholog of *CCA1*/*LHY*, through the repression of *ODO1* and several

Significance

Flowering plants attract pollinators in part by emitting volatile scents from their petals. This emission of scent is highly regulated, and is often restricted to a specific portion of the day. Although the biochemical pathways of scent production are well characterized, little is known of their transcriptional regulation. Here we describe a direct molecular link between the circadian clock and floral volatile emissions. We find that a clock transcription factor regulates the timing of multiple genes involved in the production of floral volatiles in *Petunia*. This work provides key insights into the complex yet relatively unexplored transcriptional regulation of scent production, and also sheds light on how the circadian clock can regulate the timing of large metabolic pathways.

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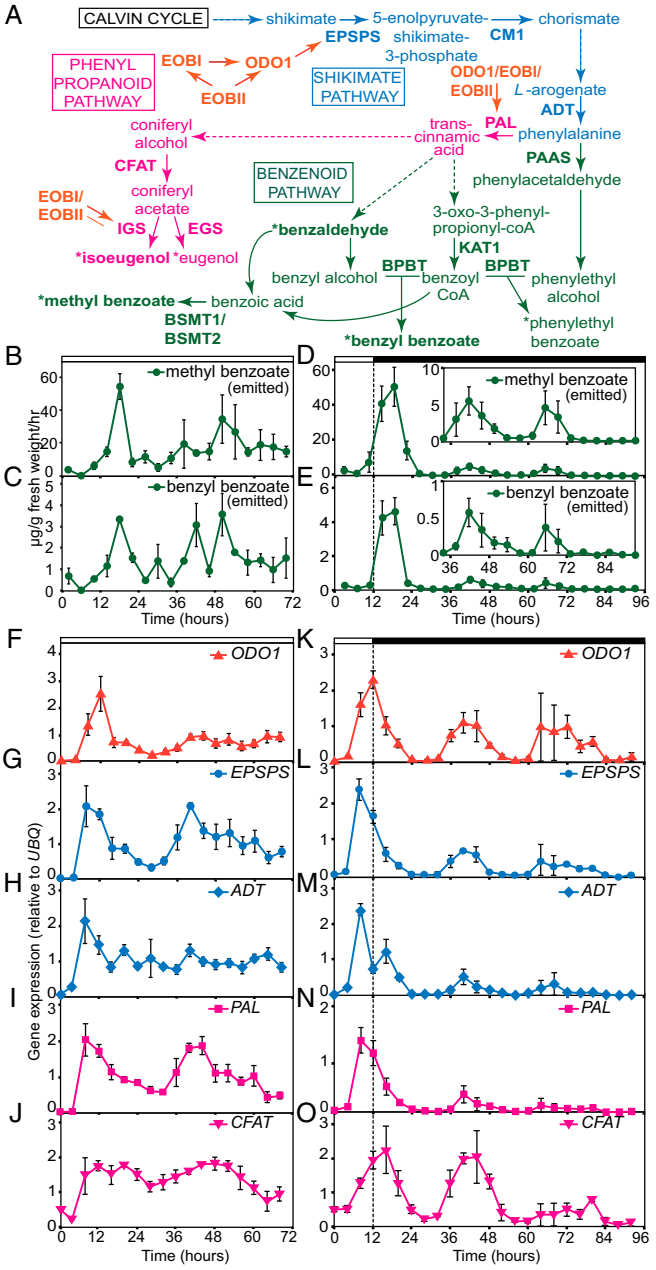

Fig. 1. The floral volatile emission and expression profiles of the genes in the FVBP pathway. (*A*) An overview of selected parts of the FVBP pathway. Different colors indicate steps and products that are categorized in shikimate, benzenoid, and phenyl propanoid pathways. Arrows with dashed lines in the pathway are representative of multiple steps between products. Volatile products are presented with asterisks. We analyzed expression patterns of enzyme genes and products in bold. Transcription factors are in orange, and enzymes shown are EPSPS, CM1, ADT, phenylacetaldehyde synthase (PAAS), BPBT, KAT1, S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase (BSMT) 1, BSMT2, PAL, CFAT, IGS, and eugenol synthase 1 (EGS). (*B–E*) Volatile emission data of methyl benzoate (*B* and *D*) and benzyl benzoate (*C* and *E*) in *Petunia* in continuous light (*B* and *C*) and dark (*D* and *E*). (*Insets*, *D* and *E*) Graphs with enlarged *y*-axes showing the same 32–96 time point results. (*F–O*) Gene expression patterns of transcription factors and enzymes associated with the FVBP pathway in *Petunia* in continuous light (*F–J*) and dark (*K–O*). The line and symbol color of the graphs corresponds to its placement within the FVBP pathway shown in A. Values are relative to *UBIQUITIN* (*UBQ*), and normalized by the average expression values of hours 0–12. Results represent means ± SEM from three biological replicates. White and black bars at the top indicate periods of light and dark, respectively.

other regulatory genes, might restrict the emission of floral volatiles to the evening. Increasing levels of CCA1/LHY in the morning represses the expression of genes necessary for volatile synthesis, and as the levels of CCA1/LHY decrease in the evening, it facilitates the induction of *ODO1* and downstream FVBP enzyme gene expression. Here we report that the circadian clock regulates scent emission timing through the function of *LHY* ortholog in *Petunia*.

Results

The Circadian Clock Regulates Volatile Emission and the Expression of Enzyme Genes in the FVBP Pathway in the Dark. Previous work has shown that daily light/dark transitions are the predominant cues mediating temporal control of the major floral volatile, methyl benzoate, in *Petunia* (17). However, emission of the terpenoid compound β-ionone and the expression of its synthase enzyme showed circadian oscillations, particularly in the dark (8), indicating that the influence of the circadian clock on scent emission can be conditional. To more comprehensively assess the involvement of the circadian clock in scent emission, we measured the emission of four major floral volatiles (methyl benzoate, benzyl benzoate, benzaldehyde, and isoeugenol) generated through the FVBP pathway (Fig. 1*A*) under continuous light and dark conditions (Fig. 1 *B–E* and Fig. S1 *A–D*). In continuous light, volatile emissions ceased to oscillate, and moderate levels of emission were maintained for all four analyzed volatiles for 3 d (Fig. 1 *B* and *C* and Fig. S1 *A* and *B*). In continuous dark, volatile peak strength diminished rapidly (Fig. 1 *D* and *E* and Fig. S1 *C* and *D*); however, smaller peaks (of approximately one tenth of the size of the peaks that occurred in the light/dark conditions) were seen for all volatiles examined in the two following subjective nights, indicating the presence of circadian timing mechanisms in the dark.

Our current understanding of the transcriptional regulation of the FVBP pathway is limited to a few transcription factors: ODO1, EOBI, and EOBII (10–12). *ODO1* exhibits an evening peak (Fig. 1*K*), whereas *EOBI* and *EOBII* exhibit morning peaks (Fig. S1 *O* and *P*). To investigate the molecular link between the circadian clock and the FVBP pathway, we next analyzed the expression patterns of these three transcriptional regulators and 12 enzyme-encoding genes in the FVBP pathway under continuous light and dark conditions (Fig. 1 and Fig. S1). All FVBP synthesis genes examined showed strong daily oscillations in light/dark cycles (see the first 24-h pattern in Fig. 1 *K–O* and Fig. S1 *O–X*). In continuous light, the expression profiles of almost all genes did not sustain daily oscillatory patterns, and often the expression levels became higher than the trough levels observed in 24-h light/dark conditions (Fig. 1 *F–J* and Fig. S1 *E–N*). An exception was the benzoyl-CoA: benzyl alcohol/phenylethanol benzoyltransferase (*BPBT*) gene, which is expressed in a circadian fashion even in continuous light (Fig. S1*L*). Overall, their expression patterns resembled the emission profiles of the four volatiles examined under the same conditions (Fig. 1 *B* and *C* and Fig. S1 *A* and *B*). These results suggest that the circadian clock does not have much influence on the timing expression of the FVBP pathway genes as well as scent emission in continuous light in *P. hybrida* flowers.

Conversely, in continuous dark, *ODO1*, direct target genes of ODO1 [*EPSPS* and phenylalanine ammonia-lyase 1 (*PAL*) genes (10)], and several other enzyme-coding genes in the shikimate, benzenoid, and phenylpropanoid pathways [e.g., arogenate dehydratase 1, coniferyl alcohol acyltransferase (*CFAT*), and 3-ketoacyl-CoA thiolase 1 (*KAT1*) genes] displayed dampened but oscillatory mRNA expression patterns for 3 d (Fig. 1 *K–O* and Fig. S1 *O–X*), indicating the contribution of the circadian clock to the expression timing of these genes. *EOBI* and *EOBII* did not show any rhythmic expression patterns under these conditions, suggesting that daily rhythmic expression of *EOBI* and *EOBII* could largely be regulated by light/dark transitions. Also, this indicates that the daily rhythmic expression of *EOBI* and *EOBII* does not control the daily *ODO1* expression, even

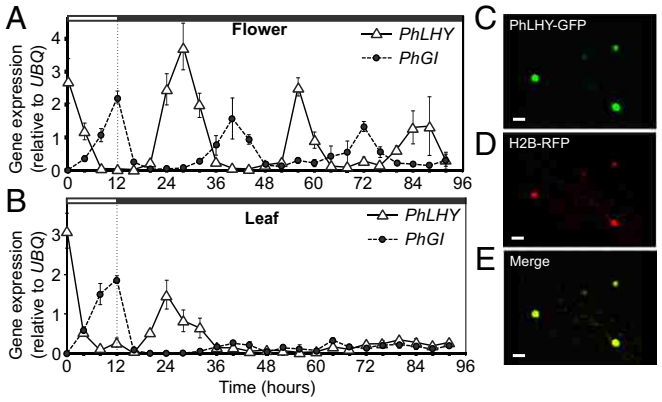

Fig. 2. Circadian expression pattern of *PhLHY* and intracellular localization of PhLHY protein. (*A* and *B*) Expression profiles of *PhLHY* and *PhGI* in continuous dark over 92 h in *Petunia* petals (*A*) and leaves (*B*). Results represent means ± SEM from three biological replicates. (*C*–*E*) PhLHY-GFP is a nuclear localized protein. PhLHY-GFP protein (*C*) and H2B-RFP protein (reference for nuclei) (*D*) were expressed in epidermal cells of *Petunia* petals. (*E*) Merged image of *C* and *D*. (Scale bar: 10 μm.)

though EOBs are direct activators of *ODO1* (11, 12). These results indicate that the circadian clock may contribute in the timing regulation of these genes in the dark in *Petunia* flowers. In addition, the present work and previous findings led us to hypothesize that the clock's regulation of volatile production may be modulated by light conditions. Another interesting finding is that *ODO1*, which directly up-regulates *EPSPS* and *PAL* expression (18), is expressed at the same time as *EPSPS* and *PAL*, especially in the dark (Fig. 1 *K*, *L*, and *N*). This suggests that additional factors are responsible for controlling the timing of the expression of these genes. We found that the *EPSPS* promoter possesses the EE *cis*-element (see Fig. 5*B*). As the *ODO1* (12) and the *EPSPS* promoters contain EEs, we hypothesized that CCA1/LHY homologs in *Petunia* may be negative factors that set the expression timing of these genes.

Identification of *LHY* Homolog in *Petunia*. To identify the *CCA1*/*LHY* homologous gene in *Petunia*, we first identified partial sequences that showed homology to *Arabidopsis CCA1*/*LHY* from publically available EST databases. After cloning the entire coding region of the cDNA based on the sequences of the EST clones, the entire sequence showed a higher homology to *LHY* than *CCA1*; therefore, we named it *P. hybrida LHY* (*PhLHY*). By using deduced amino acid sequences, Bayesian posterior probability and maximum-likelihood phylogenetic analyses placed PhLHY nested within the clade of core eudicots—clustered with *Nicotiana attenuata* LHY (NaLHY) and *Solanum lycopersicum* (*SlLHY*) (Fig. S2*A*). In addition, quantitative PCR (qPCR) analysis of *PhLHY* expression in *Petunia* leaves and flowers revealed peak expression at dawn under light/dark conditions (Fig. 2 *A* and *B* and Fig. S3 *A* and *B*), which is similar to *Arabidopsis CCA1*/*LHY* expression patterns (14, 15). To monitor the status of the *Petunia* circadian clock, the expression patterns of the *Petunia* homolog of *GIGANTEA* (*PhGI*), a clock gene directly regulated by CCA1 (19–21), was also analyzed (Fig. S2*C*). Similar to the *Arabidopsis* counterpart, *PhGI* expression peaks in the evening, demonstrating an antiphasic pattern to *PhLHY* expression. Interestingly, circadian oscillation of *PhLHY* and *PhGI* expression showed distinct patterns in tissue- and light-dependent manners. In *Petunia* flowers kept in continuous dark, robust oscillation of *PhLHY* and *PhGI* was observed for 3 d, whereas the circadian oscillation of *PhLHY* and *PhGI* in leaves ceased in the first day in the dark (Fig. 2 *A* and *B*). In continuous light, the amplitude of *PhLHY* expression levels was severely reduced in flowers and leaves, whereas the expression levels of *PhGI* remained similar to that in the light/dark cycle (Fig. S3 *A* and *B*). In addition, *PhGI* expression levels showed circadian oscillation in leaves, but not in flowers under continuous light conditions. These results indicate the presence of tissue-specific clocks between flowers and leaves in *Petunia*. Although the circadian oscillation patterns of *PhLHY* vary depending on tissues and light conditions, the daily expression patterns of *PhLHY* resemble those of *LHY*/*CCA1*.

Next we examined the intracellular localization of PhLHY protein, as its homologs CCA1 and LHY are nuclear-localized proteins (22, 23). In comparing the intracellular localization patterns of the PhLHY-GFP protein with that of RFP-tagged histone 2B (H2B), PhLHY-GFP localized in the nuclei of *Petunia* flower and leaf cells (Fig. 2 *C*–*E* and Fig. S3 *C*–*E*), indicating that PhLHY is a nuclear protein. Based on the sequence similarity, and the temporal and intracellular expression patterns, *PhLHY* is likely an orthologous gene of *Arabidopsis LHY*.

PhLHY Maintains Functional Relevance in *Arabidopsis*. To assess the function of *PhLHY*, we first tested whether it maintains a similar role with *Arabidopsis LHY*. *PhLHY* was overexpressed in *Arabidopsis* by using the cauliflower mosaic virus (CaMV) *35S* promoter (Fig. 3*A*). *PhLHY*-overexpressing lines (*35S*:*PhLHY*) displayed distinct developmental phenotypes typically observed in *Arabidopsis CCA1*/*LHY* overexpressors: a significantly delayed flowering time, and a longer hypocotyl length in comparison with WT plants (Col-0; Fig. 3 *B* and *C*) (14, 24). The *35S*:*PhLHY* lines also exhibited a similar clock arrhythmia phenotype to the *CCA1*/*LHY* overexpressors under continuous light conditions (14, 24), as measured by a *CCA1*:luciferase (*LUC*) reporter (Fig. 3*D*). In addition, the *CCA1*:*LUC* expression in *35S*:*PhLHY* barely responded to the dark-to-light transitions in the morning (Fig. 3*E*), indicating that the oscillation of circadian clock genes was severely attenuated even under light/dark conditions. Last, we analyzed the expression profiles of the circadian clock genes *LHY*, *CCA1*, *PSEUDO RESPONSE REGULATOR 9* (*PRR9*), *PRR7*, and *TIMING OF CAB EXPRESSION 1* (*TOC1*) in *35S*:*PhLHY* lines. The daily oscillation patterns of these genes became relatively constant in *35S*:*PhLHY* lines throughout the day (Fig. S4). Together, these results imply that *PhLHY* may possess a similar clock function to *CCA1*/*LHY* in *Arabidopsis*.

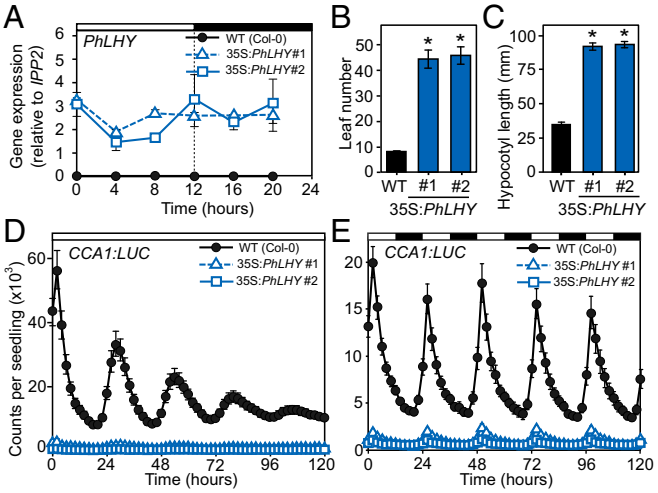

Fig. 3. *PhLHY* functionally resembles *CCA1*/*LHY* in *Arabidopsis*. (*A*) Expression of *PhLHY* in *35S*:*PhLHY* plants under light/dark conditions. (*B*) Flowering time of *35S*:*PhLHY* lines and WT plants is shown. (*Significant difference vs. WT at $P < 0.05$, Student *t* test; $n = 16$.) (*C*) Hypocotyl length of *35S*:*PhLHY* lines and WT plants (*$P < 0.05$; $n = 30$). (*D* and *E*) *CCA1*:*LUC* activity as measured by luminescence counts per seedling over 5 d of continuous light (*D*) and light/dark (*E*) conditions in a comparison between *35S*:*PhLHY* lines and WT. Results represent means ± SEM ($n = 16$).

Constitutive Expression of *PhLHY* Eliminates Floral Volatile Emission in *Petunia*. As *PhLHY* is likely an orthologous gene of *Arabidopsis LHY*, we next examined *PhLHY*'s influence on the daily production of floral scent in *Petunia*. Constitutive expression of *PhLHY* in *Petunia* (*35S*:*PhLHY* no. 37 line) altered expression patterns of two putative clock gene homologs [*PhGI* and *P. hybrida PSEUDO RESPONSE REGULATOR 5* (*PhPRR5*; Fig. S2*D*)] and 15 genes involved in the FVBP pathway in flowers (Fig. 4 *A*–*E* and Fig. S5 *A*–*M*). The expression levels of the genes [*PhGI*, *PhPRR5*, *ODO1*, *EPSPS*, chorismate mutase 1 (*CM1*), arogenate dehydratase 1 (*ADT*), and *PAL*], which peak at approximately 8–12 h from the morning in WT plants, became severely reduced and constitutive throughout the day. We further investigated that overexpression of *PhLHY* severely represses *ODO1* promoter activity in vivo. Transient coinfiltration of *ODO1* promoter-controlled firefly *LUC* gene reporter (*pODO1*:*LUC*) with *35S*:*PhLHY* (but not with *35S*:*GFP*) suppresses diurnal oscillation patterns of the *ODO1* promoter activities in multiple independent *Petunia* flowers (Fig. S6). Taken together, these expression patterns of evening-expressed genes in *35S*:*PhLHY* no. 37 and in the transient assay were similar to those in *CCA1*/*LHY* overexpressors in *Arabidopsis* (14, 24). Additionally, constitutive expression of *PhLHY* in *Petunia* resulted in a near-total decrease in floral volatile production and emission (Fig. 4 *P* and *Q*, and Fig. S5 *N* and *O*). These results indicate that the *PhLHY* expression pattern controls the expression patterns of other clock gene homologs and FVBP pathway genes in addition to the scent emission patterns in flowers.

Repression of *PhLHY* Leads to the Early Production of Scent. *Petunia* is well known for gene silencing resulting from attempts to express transgenes (25, 26). Our attempt to constitutively express *PhLHY* also resulted in transgenic plants that exhibited silencing of *PhLHY*. In the plants with reduced expression levels of *PhLHY*, the phases of peak expression of *PhGI* and *PhPRR5* as well as most of the FVBP pathway genes responsible for volatile precursor synthesis (*EOBI*, *EOBII*, *ODO1*, *EPSPS*, *CM1*, *ADT*, and *PAL*), shifted approximately 4–8 h toward the morning (Fig. 4 *F*–*O* and Fig. S5 *U*–*AG* and *AO*–*BA*). This phase-advance phenotype is also observed in *cca1 lhy* double mutants in *Arabidopsis* (15, 27). Interestingly, the volatile emission peaks in these plants also occurred at the end of the light period (approximately 8–12 h from the morning) instead of the middle of the night, although the total scent emission levels were slightly lowered in line 46 (Fig. 4 *R*–*U* and Fig. S5 *AH*–*HI*, *BB*, and *BC*). These results imply that PhLHY sets the expression timing of evening-expressed FVBP pathway genes.

PhLHY Binds to the EEs in *ODO1*, *EPSPS*, and Isoeugenal Synthase 1 Promoters. Our results indicated that PhLHY regulates the timing of FVBP pathway genes in *Petunia*. The *ODO1* promoter contains several EEs (12), and we found that the promoter sequences of the evening-expressing *EPSPS* and morning-expressing isoeugenol synthase 1 (*IGS*) also contain EEs. Thus, we hypothesized that PhLHY directly binds to these EEs to regulate the temporal expression of these genes. To analyze the direct binding of PhLHY to the EEs, we performed an EMSA of PhLHY (Fig. 5 *A* and *B*). The glutathione S-transferase (GST)-fused PhLHY protein specifically bound to the two EEs (EE1 and EE2) and the CCA1-binding site (CBS) (28) derived from the *ODO1* promoter, but not to the mutated EE (mEE1; Fig. 5*A*). In addition, PhLHY also bound to the EEs in *EPSPS* and *IGS* promoters (Fig. 5*B*). We also tested the functional interaction of PhLHY on the EEs and CBS in the *ODO1* promoter in vivo by using transient expression assay (Fig. 5*C*). We found that coinfiltration with *35S*:*PhLHY* specifically reduced luminescence from *pODO1*:*LUC* in flowers while having no effect on an activity of the *ODO1* promoter with its EE and CBS sites mutated (*pODO1*:*LUC* mEEs + mCBS; Fig. 5*D*), further confirming that PhLHY represses *ODO1* by directly binding to its promoter.

Discussion

Identification of the Circadian Clock Gene *PhLHY* in *Petunia*. Our investigation into the clock's regulation of scent emission implicated a *P. hybrida* ortholog of *CCA1*/*LHY*, which we named

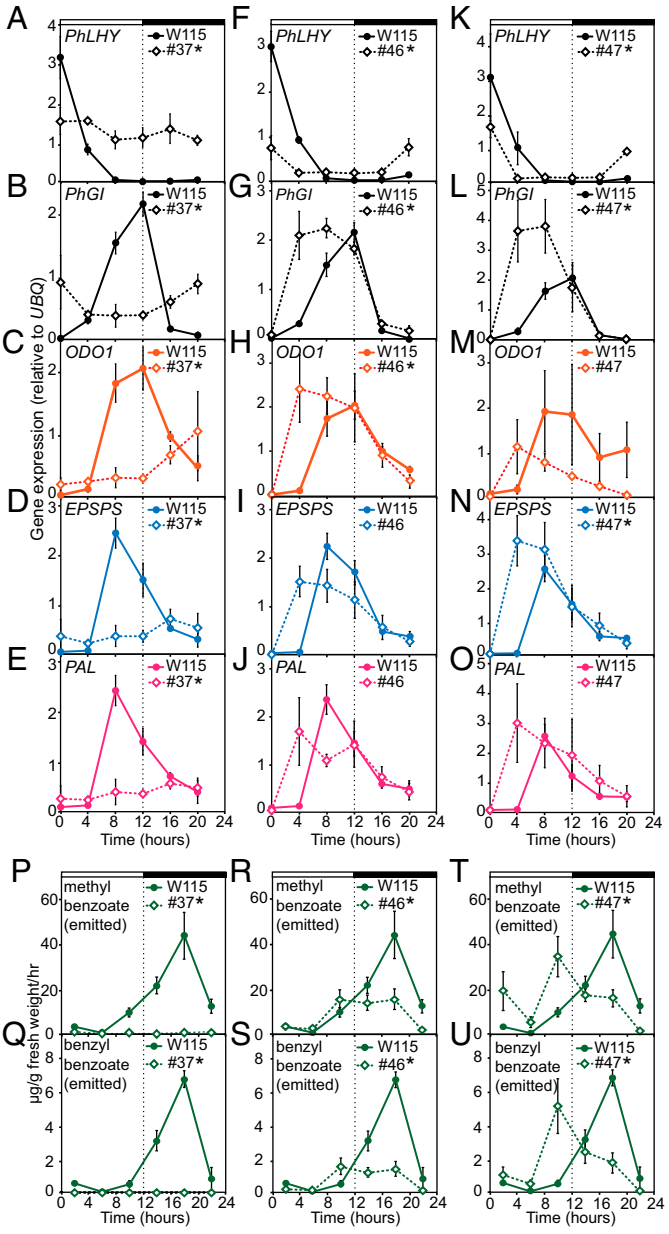

Fig. 4. *PhLHY* regulates daily timing of gene expression and volatile emission in the FVBP pathway. (*A*–*O*) Daily expression patterns of clock genes and genes encoding proteins in the FVBP pathway in the transgenic line (line 37) with constitutive *PhLHY* expression (*A*–*E*) and in the transgenic lines (lines 46 and 47) with altered *PhLHY* expression (*F*–*O*). Gene expression values were normalized by the average expression values of hours 0–12. (*P*–*U*) Daily scent emission patterns of methyl benzoate (*P*, *R*, and *T*) and benzyl benzoate (*Q*, *S*, and *U*) in transgenic line 37 (*P* and *Q*) and in transgenic lines 46 and 47 (*R*–*U*). Results represent means ± SEM from three biological replicates. The line color of the graphs corresponds to its placement within the FVBP pathway (Fig. 1*A*; $*P < 0.05$, daily expression and scent emission patterns of transgenic lines differ significantly from WT *Petunia*; two-way ANOVA).

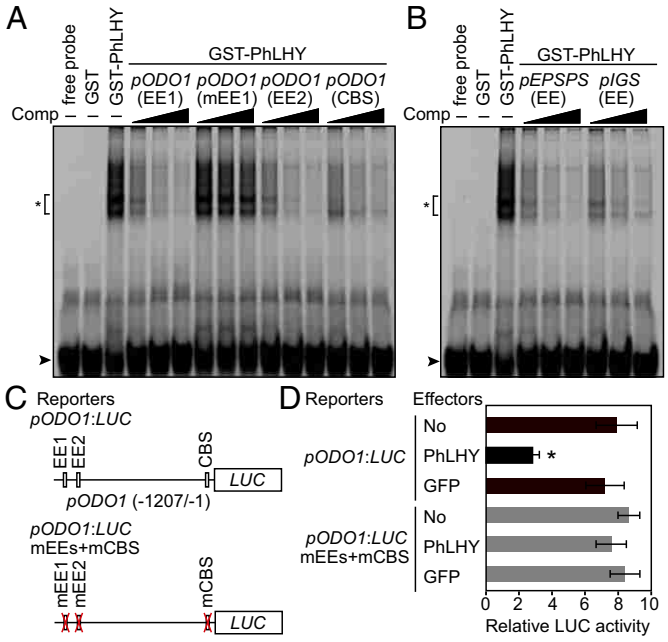

Fig. 5. PhLHY binds to EEs. (*A*) An EMSA shows direct interaction of GST-PhLHY with EEs in the *ODO1* promoter. The EE1 in the *ODO1* promoter (*pODO1*) was used as a labeled probe. Competition with different concentrations of unlabeled EE1, EE2, and CBS fragments and the mutated EE1 are shown along the top. (*B*) EMSA of GST-PhLHY with EEs in the *EPSPS* (*pEPSPS*) and *IGS* (*pIGS*) promoters. For *A* and *B*, GST served as a negative control. Asterisks and arrowheads indicate GST-PhLHY/DNA complexes and free probes, respectively. (*C*) Schematic of reporters used in transient assay. (*D*) The effect of PhLHY protein on the *ODO1* promoter activities. The activities of firefly LUC were normalized by the activities of *35S*:Renilla LUC. Results represent means ± SEM of nine independent samples (*$P < 0.01$ vs. no effector, Student *t* test).

PhLHY, as a mechanistic component. Our phylogenetic analysis placed PhLHY as the closest homolog of other Solanaceae LHY homologs (Fig. S2). The structure of this phylogenetic tree also resembled independent tree structures found in separate analyses (29, 30). qPCR analysis revealed rhythmic oscillation in *PhLHY* expression in *Petunia* floral tissues in continuous dark (Fig. 2). The daily expression patterns of *PhLHY* transcripts and the nuclear localization of the PhLHY protein (Fig. 2) provided additional support to the notion that *PhLHY* acts within *Petunia* in a manner consistent with its homologs (29, 30).

Arabidopsis PhLHY-overexpressing lines displayed several phenotypes similar to established clock-disrupting *CCA1*/*LHY*-overexpressing phenotypes. These lines showed delayed flowering times and elongated hypocotyls compared with WT plants (Fig. 3) (29, 30). In these lines, the disruption of the rhythmic expression of many circadian-clock genes was observed (Fig. 3 and Fig. S4). The disruption of these rhythms by *PhLHY* overexpression suggests that the function of *PhLHY* is similar to *LHY* and *CCA1*, in which overexpression caused general impairment of clock function (29, 30). As seen in numerous examples in animals and plants, clock components are often a part of a negative autoregulatory feedback loop in which the protein products of clock component genes suppress the expression of their own genes (29, 30). By comparing the *CCA1*:*LUC* luminescence and *CCA1* and *LHY* expression patterns of *Arabidopsis PhLHY* overexpressors and WT plants under continuous light and light/dark conditions, *PhLHY* overexpression depressed the rhythmic circadian oscillation of *LHY* and *CCA1* (Fig. 3 and Fig. S4). This same pattern of feedback regulation on *LHY* and *CCA1* was observed under conditions in which *LHY* and *CCA1* were overexpressed separately (29, 30), further supporting that the function of *PhLHY* resembles that of *LHY* and *CCA1*, and that *PhLHY* encodes a core clock component in *Petunia*.

PhLHY Regulates the Daily Timing of the FVBP Pathway. ODO1 is a key transcriptional regulator for many steps in the FVBP pathway (10). ODO1 regulates fragrance biosynthesis by administering the flux of precursor molecules through the shikimate pathway (5). Although *ODO1* exhibits rhythmic expression that peaks in the evening, its peak of expression closely mirrors that of the known ODO1 target genes (Fig. 1). This coincidence of expression suggests that *ODO1* is not wholly responsible for the precise timing of FVBP biosynthesis.

Under light/dark conditions, *PhLHY* morning expression is antiphasic to the evening expression of many FVBP-related genes including *ODO1*, *EPSPS*, and *PAL* (Fig. 4). Constitutive expression of *PhLHY* resulted in the abolishment of nearly all FVBP emission (Fig. 4 and Fig. S5) and suppressed most of the known genes responsible for the synthesis of FVBPs (Fig. 4 and Figs. S5 and S6). Under constitutive expression of *PhLHY*, *BPBT* and *BSMT* expression levels were increased (Fig. S5). As *ODO1* RNAi knockdown lines also showed increased levels of *BPBT* and *BSMT* expression (10), this phenotype might be induced by the low expression level of *ODO1*, which was caused by constitutive *PhLHY* expression. In *PhLHY*-suppressed lines, the peak expression of many FVBP genes examined shifted from late afternoon to earlier in the day (Fig. 4 and Fig. S5). This shift in peaks was seen most clearly in the precursor level of FVBP synthesis, with distinct shifts occurring in *EOBI*, *EOBII*, *ODO1*, *EPSPS*, *CM1*, *ADT*, and *PAL*. These data clearly suggest that PhLHY regulates the timing of FVBP emission by temporally controlling the expression profiles of enzyme-encoding genes that affect the synthesis of FVBP precursors. Furthermore, PhLHY directly bound to the EE and/or CBS *cis*-elements in the promoters of *ODO1*, *EPSPS*, and *IGS* (Fig. 5), indicating that PhLHY is likely setting the phase of *ODO1* and *EPSPS* expression by inhibition (and *IGS* through activation) during the early portion of the day. Based on the similarities of the expression patterns, our expression analyses also suggest that PhLHY may interact with the promoters of other FVBP genes (in perhaps both suppression and activation).

Our analysis of endogenous FVBP compound concentrations (Fig. S5) revealed that the temporal availability of endogenous compounds mirror the external emission levels of those same compounds. These findings support previous evidence that emission of floral volatiles is simply based on diffusion, and regulated primarily at the synthesis level (31, 32).

***Petunia* Likely Possesses a Tissue-Specific Clock that Regulates the FVBP Pathway.** Another interesting result was the apparent tissue specificity of the clock homolog expression in *Petunia*. *PhLHY* and *PhGI* show circadian rhythmic expression in flower tissue during continuous dark conditions, but not in continuous light. Correspondingly, a small but significant oscillation is observed in many FVBP genes also only during continuous dark. In leaf tissue, no circadian rhythm was present in continuous dark, but the expression profile of *PhGI* (but not that of *PhLHY*) shows a strong daily oscillation in continuous light. To regulate the robust oscillation of *PhGI* in leaves, clock components other than PhLHY may be involved. These results clearly indicate that *P. hybrida* plants likely possess circadian clocks that have different properties in flowers and leaves. Although tissue specificity of the circadian clock has been shown in *Arabidopsis* previously (33–35), current examples in other plant species are still limited (36). Of note is the fact that the common study organism for FVBP research, *P. hybrida*, is a hybrid of *Petunia axillaris* and *Petunia integrifolia*. *P. axillaris* shows robust oscillation of floral scent in continuous light conditions, but *P. integrifolia* does not (37). This indicates that the light- and tissue-specific clock phenotypes

observed in *P. hybrida* might be caused by mix of features of *P. axillaris* and *P. integrifolia* circadian clocks. This also suggests that there may be different characteristics in the molecular clocks even among *Petunia* species. Although robust circadian rhythms do not seem to persist in *P. hybrida*'s volatile release under continuous light conditions, the strong effect PhLHY has on FVBP synthesis under light/dark cycle conditions clearly indicates the importance of the clock to the timing of floral emissions in a real world in this species. Together with the light-dependent tissue specificity of rhythmic gene expression, it appears that light and the circadian clock work in concert to regulate floral volatile emissions in *Petunia*.

In summary, we describe what is, to our knowledge, the first molecular-level evidence that the clock component, PhLHY, plays an important role in controlling the timing of volatile synthesis and emission in *Petunia.* During an early part of the day, PhLHY represses the expression of many enzyme-encoding genes in the FVBP biosynthesis pathway, primarily in the upstream area of the pathway responsible for precursor availability. It is also possible that PhLHY is up-regulating certain morning-peaked FVBP genes, such as *IGS*. Further identification and analysis of FVBP regulatory components is required to provide a more precise level of control within the FVBP metabolic pathway. A fuller comprehension of the regulatory network surrounding the FVBP pathway will allow us to not only regulate the abundance of floral volatiles, but the period of emission as well. Such manipulation would allow us to create "designer crops" whose emissions could be adjusted to suit the temporal availability of local pollinators.

Materials and Methods

A full description of the materials and methods is provided in *SI Materials and Methods*. In brief, we created transgenic *Petunia* plants through tissue culture transformation by using *Agrobacterium tumefaciens*. Gene expression was measured through qPCR analysis, and all primer information for qPCR in this research is provided in Table S1. Volatile emissions were collected from live plants in a custom-designed apparatus (Fig. S7) and analyzed by GC/MS. Physical binding of PhLHY to EEs was confirmed by using EMSA and transient LUC assays.

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Supporting Information

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SI Materials and Methods

Plant Materials and Growth Conditions. *Petunia hybrida* cv. Mitchell (W115) and transgenic *Petunia* seeds were first sown on soil suitable for seedling growth (Sunshine 3 Mix; Sun Gro Horticulture) and, after 1–2 wk, the seedlings were transferred to a soil containing a higher amount of nutrients (Sunshine 4 Mix; Sun Gro Horticulture). *Petunia* plants were kept in a growth room with 12-h light/12-h dark cycling conditions at 25 °C. Occasionally, plants were kept in plant growth incubators (Conviron), which have similar growth conditions. At least 1 wk before experiments, all plants were grown in the growth room and entrained to the same 12-h light/12-h dark conditions. Full-spectrum white fluorescent lamps (Octron F032/950/48; Osram-Sylvania) were set to deliver an approximate fluence rate of 80 μmol/m^2/s. Transgenic *Arabidopsis* plants were grown under 12-h light/12-h dark conditions at 22 °C for the gene expression and LUC imaging assays, under continuous light conditions at 22 °C for the hypocotyl length analysis, and under long-day conditions (16 h of light/8 h of dark) at 22 °C for flowering time experiments.

To clone *PhLHY* cDNA, *Petunia* total RNA was isolated from young leaves approximately 2 wk old harvested in the morning by using TRIzol (Life Technologies) (1), and cDNA was synthesized as previously described (2). The *PhLHY* cDNA was amplified by using the primer set 5′-CACCTTGATGGACCCTTACTC-3′ and 5′-GTTCCCTCGTAGAATTGCACA-3′, which was designed based on the sequence information of EST clones FN031564 and FN003047 and showed high sequence homologies to *Arabidopsis CCA1* and *LHY* cDNAs. The amplified cDNA was cloned into the pENTR/D-TOPO vector (Life Technologies) and the complete cDNA sequences were determined. These sequences matched to EST clone sequences, and the deduced amino acid sequences of PhLHY showed high homology to LHY orthologs (Fig. 2*A* and Fig. S2). The determined *PhLHY* cDNA sequences were deposited into GenBank under the accession no. KP017483. *PhLHY* cDNA was transferred to the pK7WG2 (3) plasmid by a Gateway LR recombination reaction (Life Technologies) to make a *35S:PhLHY* binary vector (pK7WG2-PhLHY). Generation of *Petunia* stable *35S:PhLHY* transformants was achieved by transformation of excised leaf tissue with an *Agrobacterium tumefaciens* GV3101 strain containing pK7WG2-PhLHY. Segments (1 cm × 1.5 cm) were excised from 2–4-wk-old leaves from plants that were between 5 and 10 wk old, and inoculated with *Agrobacterium* with an OD_{600} of 0.4–0.6. The leaf tissue excisions and *Agrobacterium* were coincubated for 1 d on plates containing coincubation media [1× Linsmaier and Skoog (LS) salts, pH 5.7 (Caisson Laboratories), 3% (wt/vol) sucrose, 1 mg/L 1-naphthaleneacetic acid (NAA), 0.5 mg/L 6-benzylaminopurine (BAP), 100 mg/L acetosyringone]. The explants were then transferred to regeneration/selection plates [1× LS salts, pH 5.7, 3% (wt/vol) sucrose, 1 mg/L NAA, 0.5 mg/L BAP, 100 mg/L ticarcillin, and 100 mg/L kanamycin] for regeneration and selection. After 2–3 wk of selection, regenerated shoots were cut and placed in rooting media [1× LS salts, pH 5.7, 3% (wt/vol) sucrose, 1 mg/L NAA, 100 mg/L ticarcillin, and 100 mg/L kanamycin]. T_1 and T_2 generations were selected by their expression patterns of *PhLHY*, and T_3 and T_4 generations were used for analyses. To generate *Arabidopsis 35S:PhLHY* transgenic lines, *Arabidopsis* WT plants (Col-0) that possess a *CCA1:LUC* reporter gene (4) were transformed with pK7WG2-PhLHY using the floral dip method (2). Transformants were selected on kanamycin-containing plates as described previously (2). Homozygote T_3 and T_4 plants were used for all analyses.

Collection and GC/MS Analysis of Volatile Compounds. For all FVBP concentration analysis, 2–3 d-old flowers were grown under 12-h light/12-h dark, continuous light, or continuous dark conditions. For analysis of emitted volatiles, flowers were inserted into a three-necked flask collection chamber (inside volume, 250 mL; Fig. S7). Traps were constructed by using Pasteur pipettes with tips cut to a final length of 4.5 inches, loaded with 100 mg of Poropak type Q 80–100 polymer (Waters), held in place by 15 mg of glass wool. Inflow to the collection chamber was purified by Pasteur pipettes loaded with activated charcoal. Flow through the traps was set to 2 L/min, with each time point consisting of 4 h of collection. A detailed diagram of the scent collection system is shown in Fig. S7. The fresh weight of the sampled flowers was taken immediately after scent collection to control for mass effects. The volatiles captured in the traps were eluted with 500 μL of hexane. For endogenous analysis, flowers were harvested at each time point and flash-frozen in liquid nitrogen. Samples were then ground in a Retsch Mixer Mill 400 for 4 × 1 min at 25 frequencies per second, then vortexed with 1 mL hexane for 1 h. Samples were spun down for 10 min at 9,400 × *g*, and 100 μL was then pipetted from the top for analysis. For emitted and endogenous analysis, 1 μL of the hexane elution was injected into a GC/MS device (model 7890A GC system coupled to 5975C inert XL MSD; Agilent Technologies) for quantification of the floral volatiles at each time point (5). Briefly, samples were injected into the inlet and held at 220 °C, and helium was used as the carrier gas at a constant flow of 1 cm^3/min. The initial oven temperature was 45 °C for 4 min, followed by a heating gradient of 10 °C/min to 240 °C, which was held isothermally for 10 min. Chromatogram peaks were identified tentatively with the aid of the NIST mass spectral library (approximately 120,000 spectra) and verified by chromatography with authentic standards. Peak areas for each compound were integrated by using ChemStation software (Agilent Technologies) and are presented in terms of micrograms per gram of fresh floral tissue per hour.

RNA Preparation and Gene Expression Analysis (Quantitative PCR). *Petunia* plants were grown under 12-h light/12-h dark, continuous light, or continuous dark conditions for tissue collection. Young leaf (<2 wk old) and flower tissue (2–3 d postopening corollas, pistil and stamens removed) was collected at the designated time points, then immediately immersed in liquid N_2 for storage at −80 °C. After collection of all samples, total RNA was extracted by TRIzol-based method as described (1). cDNA synthesis and qPCR analysis were performed as previously described (2) with the following differences: 4 μg of total RNA was used to create cDNA, and the following protocol was used for 40–45 cycles: 95 °C for 3 min, and then cycling at 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s. *UBQ* was used as an internal control for normalization (6). Samples were run at least in triplicate, and gene expression values were normalized by the average expression values of hours 0–12. The qPCR primers sequences used in this study are listed in Table S1. RNA isolation, cDNA synthesis, and qPCR of *Arabidopsis* seedlings were performed as previously described (2). Values represent means ± SEM from at least three biological replicates for all gene expression analyses in *Petunia* and *Arabidopsis*. To test for differences in expression patterns over time between transgenic and WT lines, a two-way ANOVA was conducted by using R (www.r-project.org). The categories compared were relative patterns of expression of a gene of interest (*PhPRR5*, *PAL*, *EOBI*, *EOBII*, *CM1*, *ADT*, *CFAT*, *BPBT*, *BSMT1*, *BSMT2*, *EGS*, *EPSPS*, *IGS*, *KAT1*, *ODO1*, *PAAS*, *PhGI*, and

PhLHY) in WT *Petunia* against the pattern of expression in a paired transgenic line (line 37, 46, or 47).

Phylogenetic Analysis. Amino acid sequences for LHY, CCA1, GI, and PRR5 homologs were aligned by using ClustalW (7) on the Cyberinfrastructure or Phylogenetic Research (CIPRES) Science Gateway (www.phylo.org). A phylogenetic tree was generated through Bayesian analysis using MrBayes (8, 9), applying the Jones–Taylor–Thornton (JTT) model (10) of amino acid substitutions. The analysis was run over 5,000,000 Markov chain Monte Carlo generations, sampling every 1,000 with a "burn-in" proportion of 0.25. The final consensus tree was the product of 50% majority rule (11, 12). Maximum likelihood (ML) bootstrap values presented on the phylogenetic tree in Fig. S2*A* were calculated by generating a second phylogenetic tree with a topology constrained to be identical to the first via the ML method by using randomized axelerated maximum likelihood (RAxML) (13), applying the JTT model of amino acid substitution. The bootstrap values were calculated over 1,000 iterations. The final analysis and presentation were accomplished by using Mesquite (mesquiteproject.org) and FigTree (tree.bio.ed.ac.uk/software/figtree) software, respectively. The DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank accession numbers of genes (for deduced amino acid sequences) used in the phylogenetic tree are as follows: *PhLHY*, KP017483; *AtCCA1*, AY519511; *AtLHY*, AK316829; *BrCCA1*, HQ615939; *BrLHYa*, Bra030496; *BrLHYb*, Bra033291; *SlLHYI*, Solyc.10g005080; *PtLHY1*, Potri.002g180800; *PtLHY2*, Potri.014g106800; *PnLHY1*, BAH09384; *PnLHY2*, BAH09385; Cs*LHY*, AY611029; *NaLHY*, JQ424913; *OsCCA1*, NM_001067567; *PpCCA1*a, AB458831; *PpCCA1*b, AB458832; *PvLHY*, AJ420902; *SbCCA1*, TA31430_4558 TA26762_4558; *AtGI*, AT1G22770; *BrGI*, NP_001288824; *PtGI*, XP_002307516; *OsGI*, BAF04134; *AtPRR5*, AT5G24470, *PtAPRR5*, NP_001288827; and *CsPRR5*, ABV53464.

Fluorescent Imaging (Confocal Microscopy). To analyze the intracellular localization of PhLHY-GFP in the flower and the leaf, petals and young leaves *of P. hybrida* cv. Mitchell W115 were transiently transformed with *Agrobacterium* (GV3101) containing pK7WGF2 PhLHY (3) and RFP-H2B (14) Cultures of *Agrobacterium* containing these plasmids were grown to an OD_{600} of 0.5 and resuspended in MES buffer [50 mM MES, pH 5.7, 2 mM NaH_2PO_4, 0.5% (wt/vol) glucose, 100 mM acetosyringone]. After 1 h of incubation in the MES buffer, the *Agrobacterium* solution was injected into flower and leaf tissues via needleless syringe. Two days posttransfection, GFP and RFP images of the tissues were analyzed by using a confocal microscope (TCS SP5; Leica Microsystems).

Flowering Time and Hypocotyl Length Assays. To analyze the effects of *PhLHY* overexpression on flowering time regulation, the seeds of *Arabidopsis* WT (Col-0) and *35S*:*PhLHY* transgenic plants were sown on Sunshine Mix 4 (Sun Gro Horticulture) and stratified in darkness at 4 °C for 2 d to synchronize germination time. Plants were grown under long day (16 h light/8 h dark) conditions at 22 °C. Light was provided by full-spectrum white fluorescent bulbs with a fluence rate of 80 μmol/m^2/s. Flowering time was measured by counting the numbers of rosette and cauline leaves when plants bolted as previously described (2).

For hypocotyl length analysis in *Arabidopsis*, WT (Col-0) and *35S*:*PhLHY* transformants were sown on 1× LS media containing 3% (wt/vol) sucrose, then stratified at 4 °C for 2 d. Plants were grown under continuous light conditions at 22 °C. When the plants were 7 d old, they were scanned and hypocotyl length was measured by using ImageJ software (15). An independent-samples *t* test was performed by using R (www.r-project.org) to compare the lengths of WT and transgenic hypocotyls. For hypocotyl length analysis in *Petunia*, WT (W115) and our *35S*:*PhLHY* lines (nos. 37, 46, and 47) were sown on 1× LS media with 3% (wt/vol) sucrose and grown under continuous light conditions at 25 °C. When plants were 10 d old, they were scanned and analyzed as we did with *Arabidopsis*.

LUC Imaging. *Arabidopsis* WT seedlings (Col-0) and *PhLHY* overexpressors (*35S*:*PhLHY*) harboring a *CCA1*:*LUC* reporter gene (4) were grown on 1× LS media containing 3% (wt/vol) sucrose for 7 d under 12-h light/12-h dark conditions before beginning LUC imaging. At 24 h before imaging, the plants were sprayed with a 5-mM D-luciferin, 0.01% Triton-X solution. The plants were imaged for 5 d under continuous light or 12-h light/12-h dark conditions. Bioluminescence images were captured from plants every 2 h for 15-min exposures by using a high-sensitivity CCD camera (NightOWL; Berthold Technologies), and analyzed by using IndiGO software (Berthold Technologies). Luminescence data were gathered from at least 16 plants per line for *Arabidopsis* and 4 plants per line from *Petunia* flowers. Similar results were obtained from the repeated experiments. White light (~50 μmol/m^2/s) was obtained from halogen lamps (EKE 21V150W; USHIO) filtered with a heat cut filter.

For the transient LUC reporter assay in *P. hybrida* flowers (Fig. 5*D*), we generated *ODO1* promoter-driven firefly LUC reporters (*pODO1*:*LUC*) and PhLHY effecter plasmids. To generate the *pODO1*:*LUC* construct, the 1.2-kB fragment (−1207/−1) of the promoter was cloned from floral genomic DNA using 5′-CAGTTCTTTCAATGTAATTCCGCAG-3′ and 5′-CACTACTGACTCTCAGCTACCACC-3′ primers, and then inserted into the binary firefly LUC vector pFLASH (16). The *pODO1*:*LUC* mEE+mCBS was generated as by mutating the EE and CBS sites as described in *Materials and Methods* for the EMSA assay, before insertion into pFLASH. As the PhLHY effecter, we used pK7WG2-PhLHY plasmid. For a negative control, we used pK7WG2 plasmid containing *GFP* cDNA. The *GFP* cDNA was amplified by using 5′-CACCATGGTGAGCAAGGGCGAGGAG-3′ and 5′-CTACTTGTACAGCTCGTCCAT-3′ primers, and cloned into pENTR/D-TOPO plasmid (Invitrogen). To normalize for transformation efficiency, we used a binary vector containing *Renilla reniformis* LUC (Rluc) expression cassette. To generate the binary vector, we excised *35S* promoter controlled *Rluc* gene from pRTL2-Rluc (17) with HindIII, and ligated into the binary vector pPZP221 (18). Two- to three-day-old flowers were coinfiltrated with *Agrobacterium* transformants containing a reporter (*pODO1*:*LUC* or *pODO1*:*LUC* mEE+mCBS), an effector [*35S*:*LHY* (pK7WG2 LHY), *35S*:*GFP* (pK7WG2 GFP), or nothing], and *35S*:*Rluc*. In addition, the *Agrobacterium* transformant that possesses tomato stunt bushy virus silencing-suppressor p19 plasmid (*35S*:*p19*) was added to all coinfiltrations (19). To prepare for the transfection, after growing each *Agrobacterium* transformant overnight, the appropriate combinations of the transformant cultures (adjusted OD_{600} to 1.0) were mixed, spun down, and resuspended in MES buffer [50 mM MES, pH 5.7, 2 mM NaH_2PO_4, 0.5% (wt/vol) glucose, 100 mM acetosyringone]. After 1 h of incubation in the MES buffer, 0.1 mL of the *Agrobacterium* solution was injected into the corollas of flowers via needleless syringe at zeitgeber time 12 (ZT 12). All plants were entrained to 12-h light/12-h dark cycles and, after 24 h of incubation, flowers were harvested and flash-frozen at ZT 12. Samples were prepared and analyzed based on the protocol of the Dual-Luciferase Assay System (Promega). Soluble proteins were extracted with Passive Lysis Buffer (Promega) supplemented by Complete Protease Inhibitor Mixture tablets (Roche). The activities of firefly and *Renilla* LUCs in the plant extracts were analyzed by using a Victor^3 V plate reader (Perkin–Elmer).

For the time-course LUC activity analysis in *Petunia* flowers (Fig. S6), *P. hybrida* cv. Mitchell (W115) flowers were coinfiltrated with *Agrobacterium* transformants harboring combinations of *pODO1*:*LUC*, *35S*:*PhLHY*, and *35S*:*GFP*. All flowers also received the *Agrobacterium* transformant containing *35S*:*p19*

plasmid. After preparing the *Agrobacterium* solution as described earlier, the *Agrobacterium* solution was injected into the corollas of cut flowers (previously entrained to 12-h light/12-h dark) at ZT 12. Flowers were then immediately sprayed with a 5-mM D-luciferin, 0.01% Triton-X solution, and placed upright in a container filled with 5% (wt/vol) sucrose solution. Beginning at ZT 0 the next day, luminescence was recorded using the NightOWL imaging system (Berthold Industries) as described earlier. Plants were imaged for 48 h while within 12-h light/12-h dark conditions (with lights off for all image collection).

EMSA. For EMSA, we used GST-fused PhLHY protein. To produce the recombinant GST-PhLHY protein, the full length of *PhLHY* cDNA was amplified by using 5′-TATCAGAATTCGACCCTTACTCCTCTGGGGAGGAAC-3′ and 5′-ATCATAGCGGCCGCTTAAGTAGAAGCTTCTCCTTCCAAGC-3′ primers (the underlined sequences are restriction enzyme recognition sites of EcoRI and NotI, respectively).

The amplified PCR fragment was digested by EcoRI and NotI, and cloned into the EcoRI-NotI sites of the pGEX 4T-1 plasmid (GE Healthcare Life Sciences). The sequences of *PhLHY* cDNA in the pGEX-PhLHY plasmid were verified. The pGEX-PhLHY and pGEX 4T-1 plasmids were transformed into the BL21-CodonPlus (DE3; Stratagene) *Escherichia coli* strain to produce GST-PhLHY and GST proteins, respectively. Production of these proteins and preparation of cell extracts were performed as described previously (20). To induce the expression of GST-PhLHY and GST proteins, 0.1 mM of isopropyl β-D-1-thiogalactopyranoside was added into each bacterial culture (OD_{600}, 0.1). After an additional 4-h incubation at 37 °C, the cell culture was collected by centrifugation and resuspended in the following buffer: 20 mM Hepes·KOH, pH 7.2, 80 mM KCl, 10% (vol/vol) glycerol, 0.1 mM EDTA, 0.1 mM PMSF, 2.5 mM DTT, and Pierce Phospatase Inhibitor Mini Tablets (Thermo Scientific). After sonication and centrifugation, supernatants were collected and used for EMSA. EMSA was performed as previously described (21). A total of 1 μg of cell extracts containing GST-PhLHY or GST proteins were incubated with 100 nM of Cy5-labeled probe in a binding buffer [20 mM Hepes·KOH, pH 7.2, 80 mM KCl, 0.1 mM EDTA, 5% (vol/vol) glycerol, 2.5 mM DTT, 0.2 $\mu g \cdot \mu L^{-1}$ BSA, 500 ng poly dI-dC] and appropriate amounts of unlabeled competitor DNA (5-, 25- and 50-fold molar excess with respect to the labeled probe). The probe sequence of the EE (indicated by underline)-containing region of *ODO1* promoter (22) (*pODO1* EE1) is 5′-[Cy5]ATAAACCTAATAAAAAATATCTTGATAAAAATTAA-3′, and the competitor sequences are 5′-ATAAACCTAATAAAAAT**CGAG**TGATAAAAATTAA-3′ (mutated nucleotides are shown in bold) for the *pODO1* mutated EE1, 5′-ATAAACCTAATAAAAAATATCTCCATACATAATAC-3′ for the *pODO1* EE2, 5′-AAGAAAAGTTGGTAGATTTTTTATATATTTAGG-3′ for the *pODO1* CBS, 5′-ACTTAATTGTATTAGATATTTCTTGCACCTAAAA-3′ for the *pEPSPS* (accession no. CS050416) EE, and 5′-AAGAGAGAGAGAGAGATATTTTAACCCAAAAAAAA-3′ for the *pIGS* (GU983699) EE. After incubation for 30 min at room temperature, samples were separated by electrophoresis on 7% (wt/vol) acrylamide gels in 0.25× TBE. Fluorescent gel images were obtained by using a Typhoon FLA 9000 Biomolecular Imager (GE Healthcare Life Sciences).

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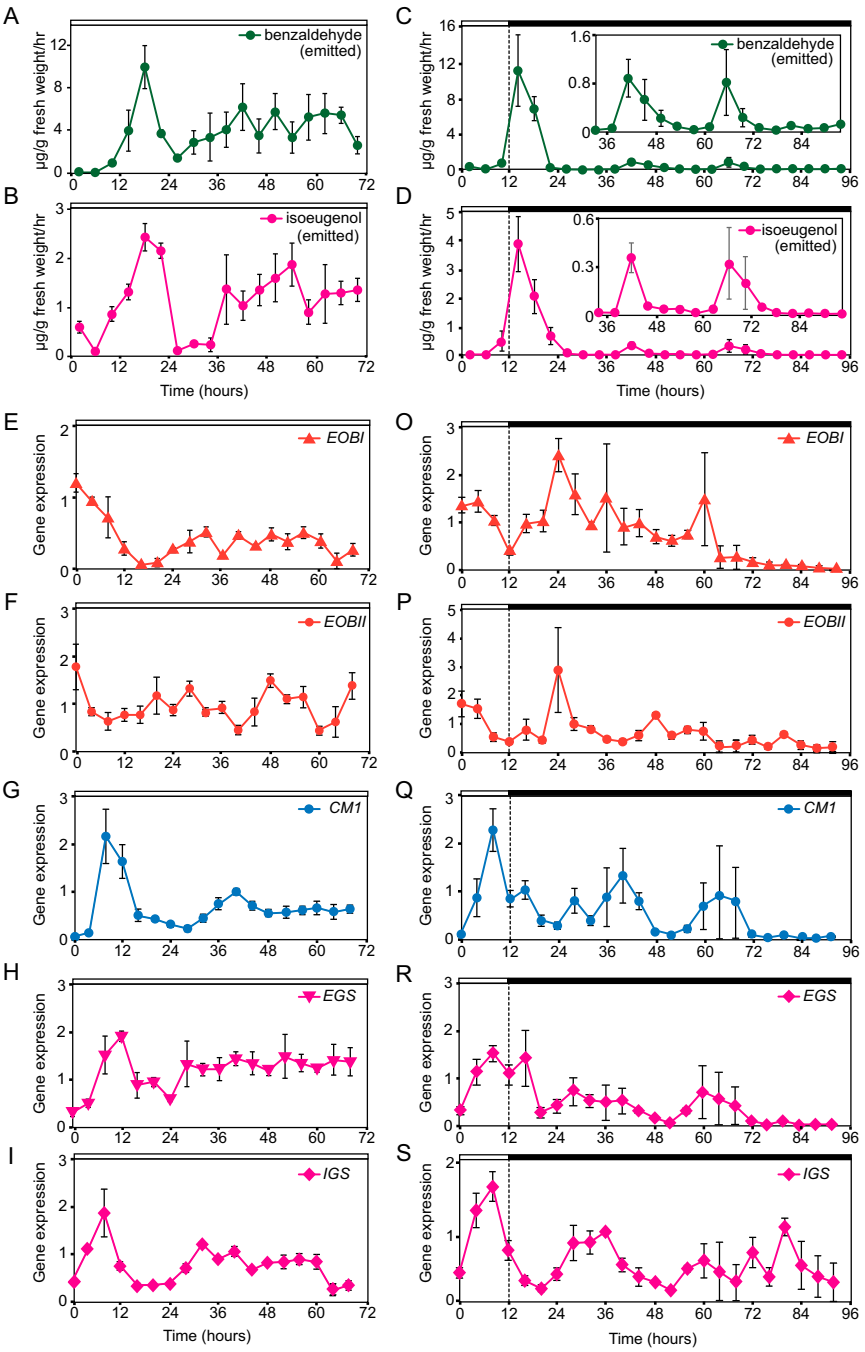

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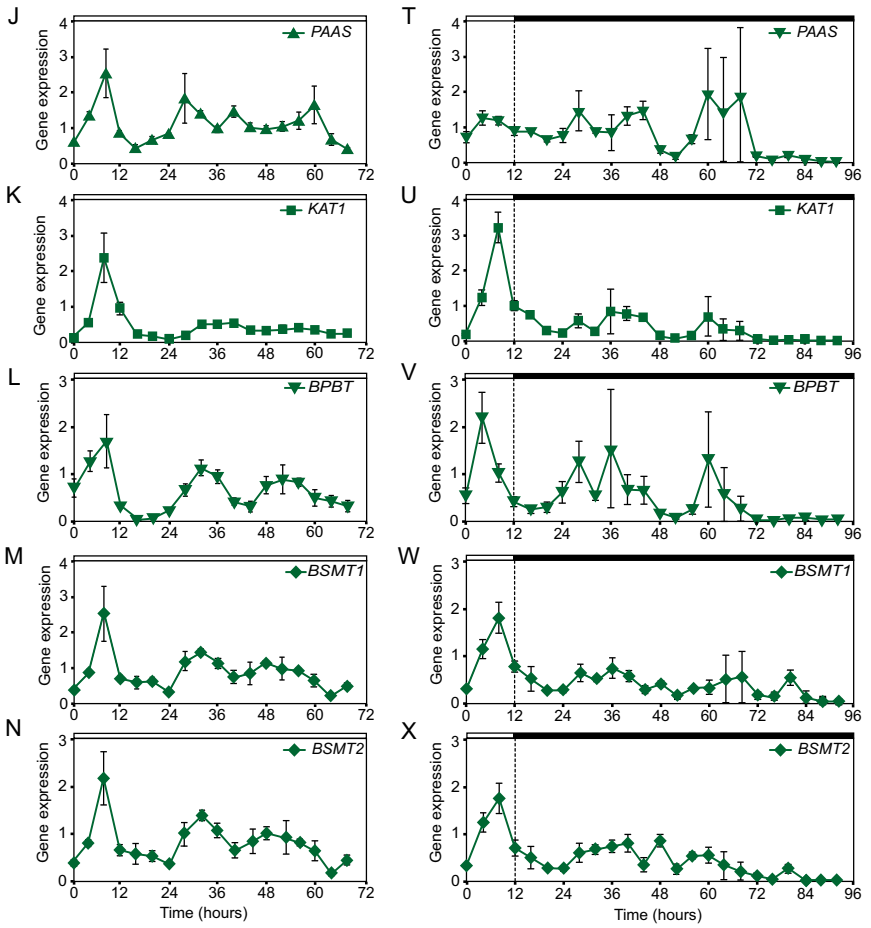
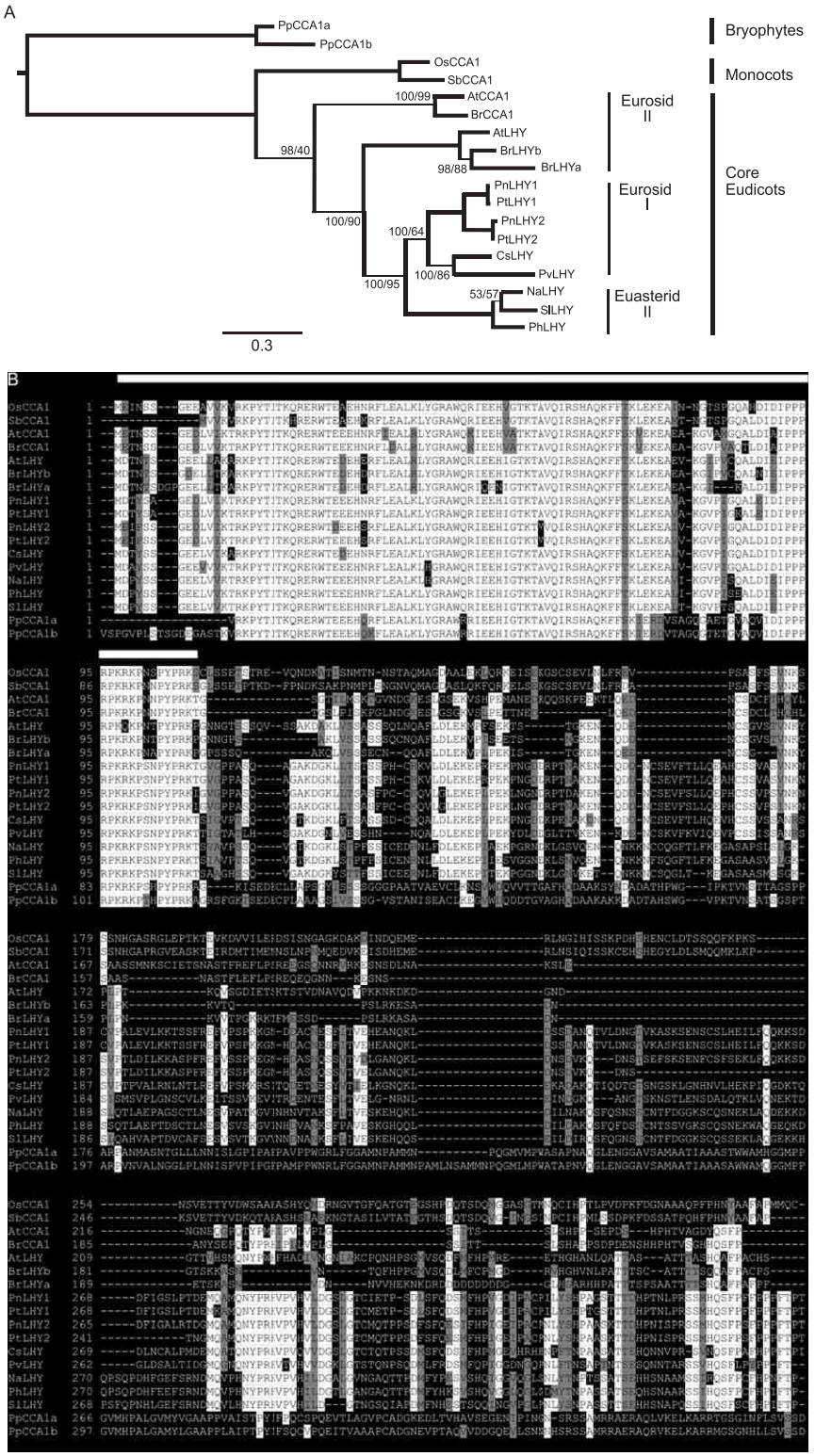


Fig. S1. The floral volatile emission and expression profiles of the genes in the FVBP pathway. (*A*–*D*) Scent expression patterns of methyl benzoate and benzyl benzoate under continuous light (*A* and *B*) and continuous dark (*C* and *D*) conditions. (*Insets*, *C* and *D*) Graphs with enlarged *y*-axes showing the same 32–96 time point results. (*E*–*X*) Expression patterns of the genes in the FVBP pathway under continuous light (*E*–*N*) and continuous dark (*O*–*X*) conditions. Values are relative to *UBQ*, and normalized by the average expression values of hours 0–12. Results represent mean ± SEM from three biological replicates. The line and marker color of the graphs corresponds to its placement within the greater FVBP pathway. White and black bars at the top indicate periods of light and dark, respectively.



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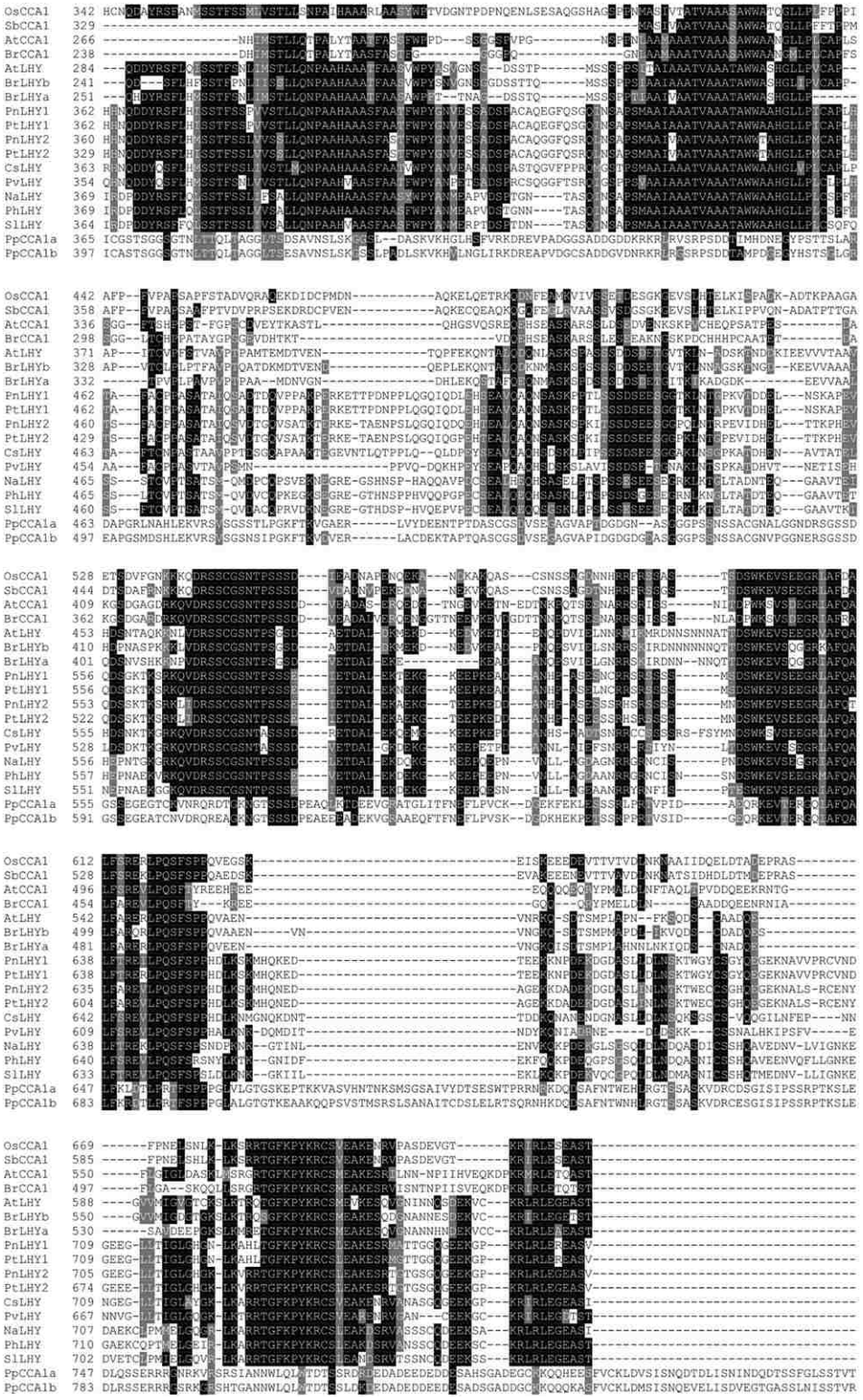

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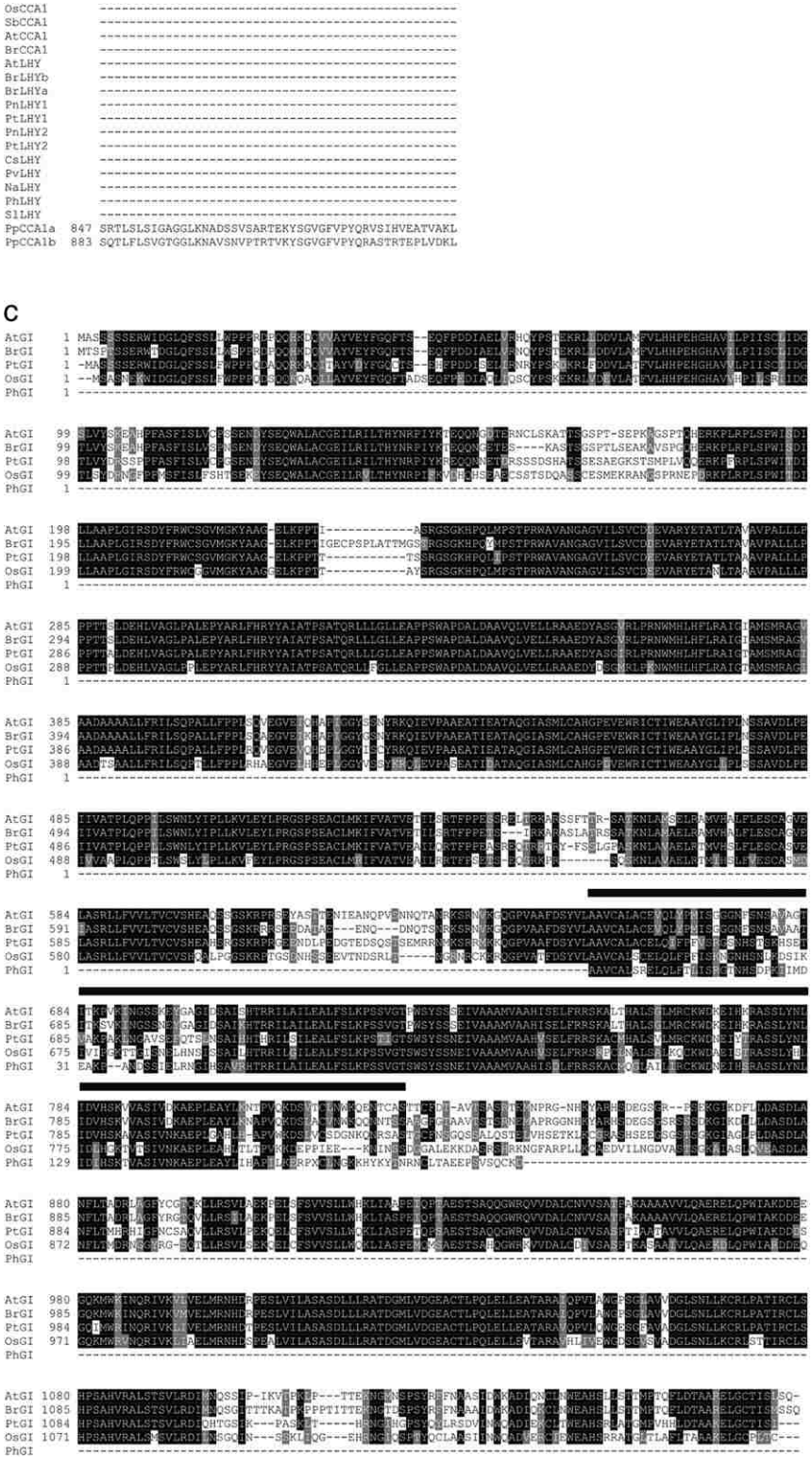

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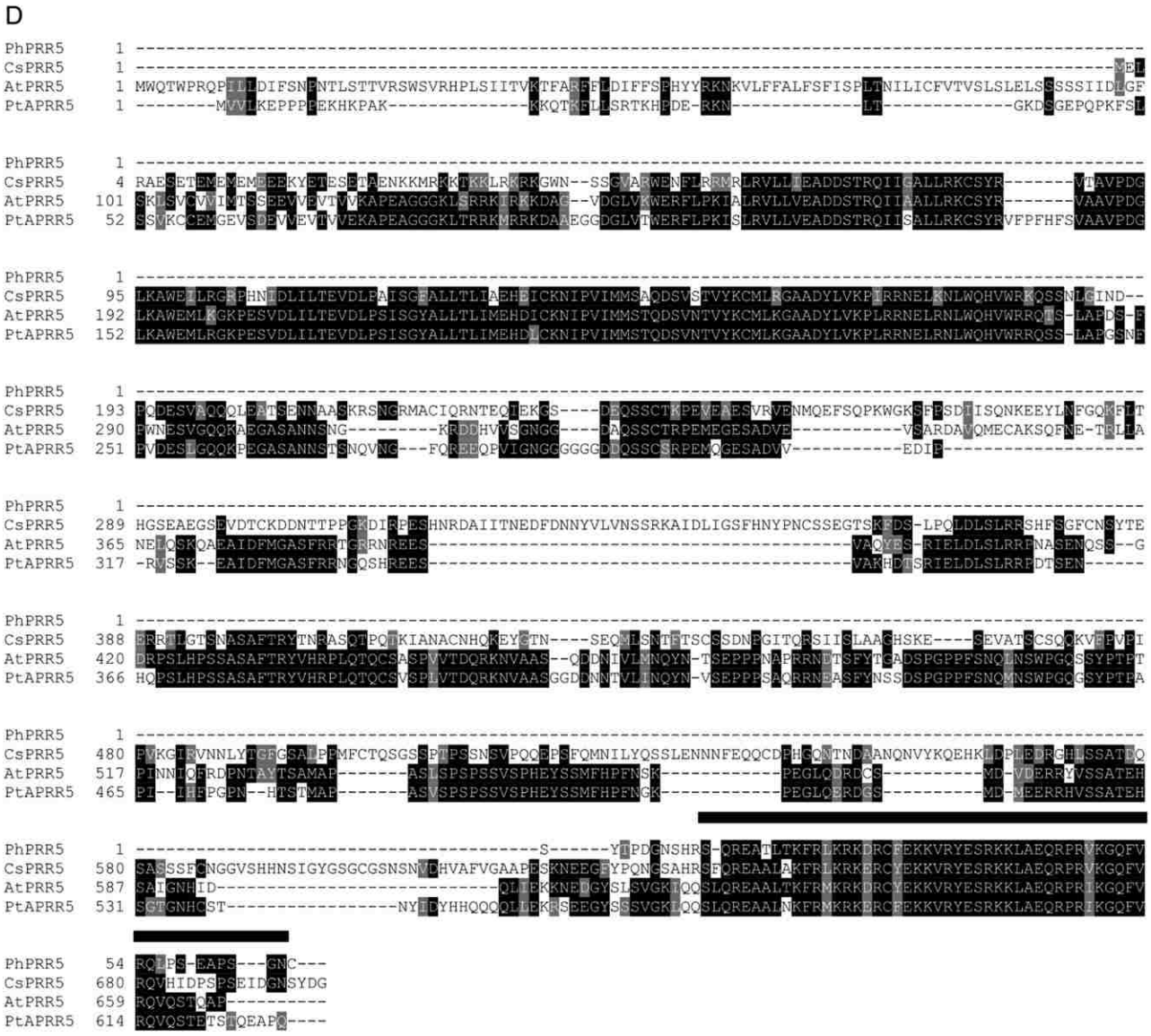


Fig. S2. (*A*) *PhLHY* is a homolog of the circadian clock gene LHY. Composite phylogenetic tree displaying the relationship of *PhLHY* with *LHY* and *CCA1* homologs. *LHY* and *CCA1* homologs used are from *Phaseolus vulgaris* (PvLHY), *Castanea sativa* (CsLHY), *Populus trichocarpa* (PtLHY1, PtLHY2), *Populus nigra* (PnLHY1, PnLHY2), *Nicotiana attenuata* (NaLHY), *Solanum lycopersicum* (SlLHY), *Arabidopsis thaliana* (AtLHY, AtCCA1), *Brassica rapa* (BrCCA1, BrLHYa, BrLHYb), *Sorghum bicolor* (SbCCA1), and *Oryza sativa* (OsCCA1), with designated outgroup *Physcomitrella patens* (PpCCA1a, PpCCA1b). Support values preceding branching are from Bayesian posterior probability analysis and maximum-likelihood analysis in format: Bayesian value/maximum likelihood value. Support values below 100/100 are shown, corresponding to adjacent thinned branches. (*B*) Amino acid alignment of LHY and CCA1 protein orthologs found in flowering and nonflowering plants. Proteins aligned are as follows: *P. vulgaris* (PvLHY), *C. sativa* (CsLHY), *P. trichocarpa (*PtLHY1, PtLHY2), *P. nigra* (PnLHY1, PnLHY2), *N. attenuata* (NaLHY), *S. lycopersicum* (SlLHY), *A. thaliana* (AtLHY, AtCCA1), *B. rapa* (BrCCA1, BrLHYa, BrLHYb), *S. bicolor* (SbCCA1), *O. sativa* (OsCCA1), and *P. patens* (PpCCA1a, PpCCA1b). Areas shaded in black represent portions of the proteins that display a high degree (>0.5) of agreement throughout all analyzed orthologs. (*C*) Amino acid alignment of GI homologs found in plants and *P. hybrida*. To identify putative *GI* homolog in *P. hybrida*, we screened *P. hybrida* EST database and found that the EST clone FN036363 contained a DNA fragment that showed a strong homology to *GI* cDNA. Proteins aligned as follows: *A. thaliana* GI (AtGI), *B. rapa GI* (BrGI), *P. trichocarpa* (PtGI), *O. sativa* (OsGI), and *P. hybrida* GI, (PhGI). PhGI amino acid sequences are deduced from the DNA sequences found in the EST clone, identified by a black bar running over the sequence. The same DNA sequences were used for designing qPCR primers. (*D*) Amino acid alignment of PRR5 homologs found in plants and the *P. hybrida*. To identify putative *PRR5* homolog in *P. hybrida*, we screened *P. hybrida* EST database and found that the EST clone FN035819 contained a DNA fragment that showed a strong homology to *PRR5* cDNA. Proteins aligned as follows: *C. sativa* PRR5 (CsPRR5), *A. thaliana* PRR5 (AtPRR5), *P. trichocarpa* APRR5, (PtAPRR5), and *P. hybrida* (PRR5). PhPRR5 amino acid sequences are deduced from the DNA sequences found in the EST clone, identified by a black bar running over the sequence. The same DNA sequences were used for designing qPCR primers.

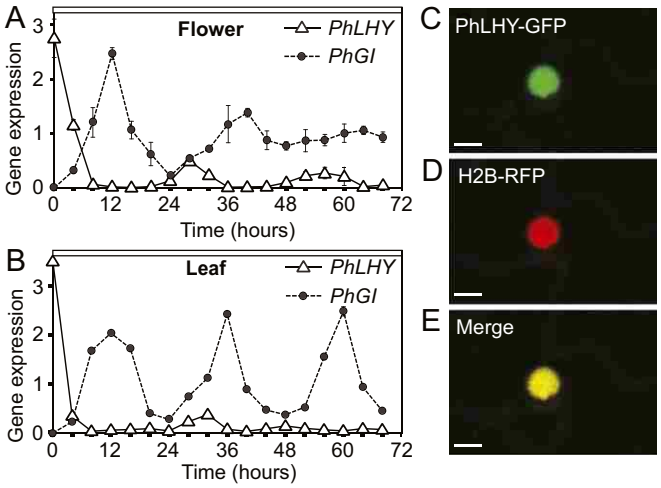


Fig. S3. Putative *Petunia* clock gene homologs show rhythmic gene expression patterns in *Petunia* leaf and flower tissue under continuous light conditions, and PhLHY is localized in the nucleus in leaf cells. (*A* and *B*) Under continuous light conditions, *PhLHY* and *PhGI* oscillations both dampen in flower (*A*), but only *PhLHY* dampens in leaf (*B*). Results represent mean ± SEM from three biological replicates. White bar at the top indicates period of light. (*C*–*E*) Confocal microscope images of the *Petunia* leaf epidermal cell. GFP fluorescence of PhLHY-GFP protein (*C*), RFP fluorescence of H2B-RFP protein used as a reference for nuclear localization (*D*), and a merged image of these (*E*) are shown. (Scale bar: 10 mm.)

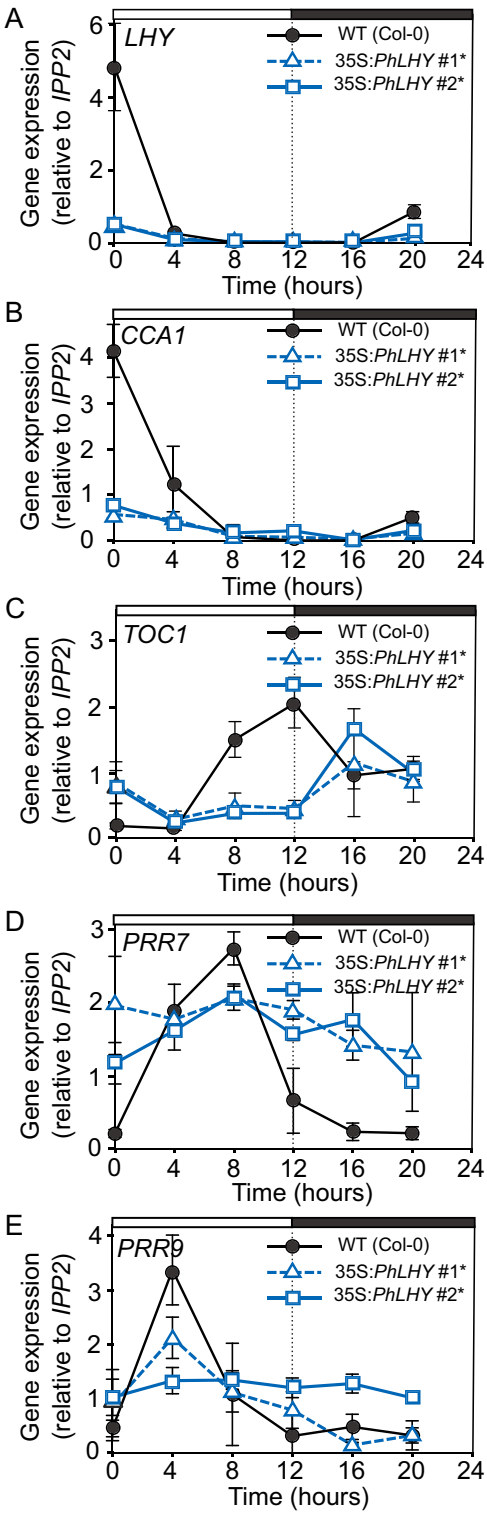
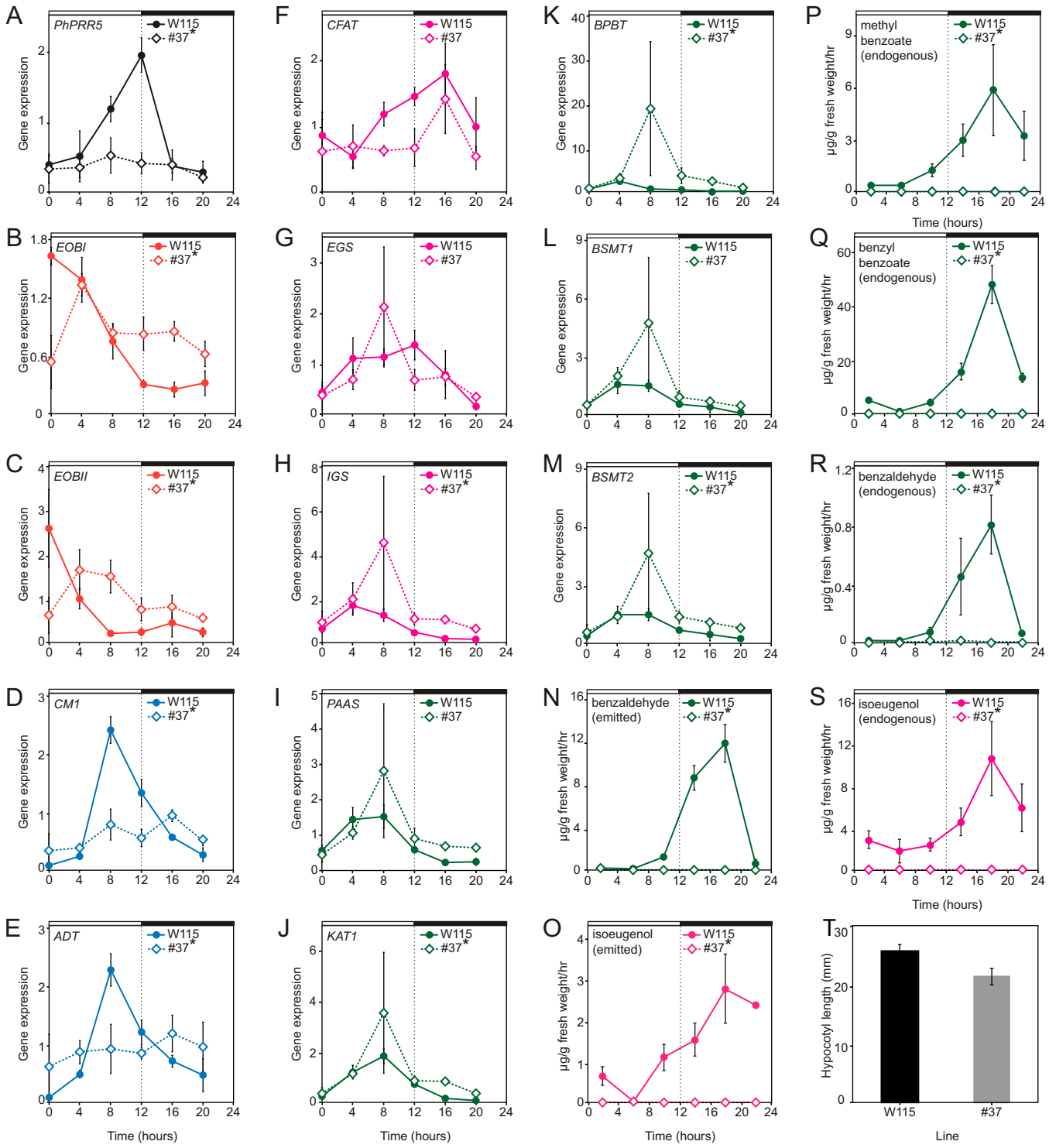


Fig. S4. Comparison of gene expression profiles of five core clock genes in *35S:PhLHY Arabidopsis* transgenic lines and WT Col-0 under 12-h light/12-h dark conditions over 24 h. The genes analyzed were *LHY* (*A*), *CCA1* (*B*), *TOC1* (*C*), *PRR7* (*D*), *and PRR9* (*E*). Results shown represent means ± SEM from three biological replicates. Black and white bars at the top indicate periods of light and dark, respectively. (*$P < 0.05$, expression pattern differs from the one in WT plants; two-way ANOVA.)



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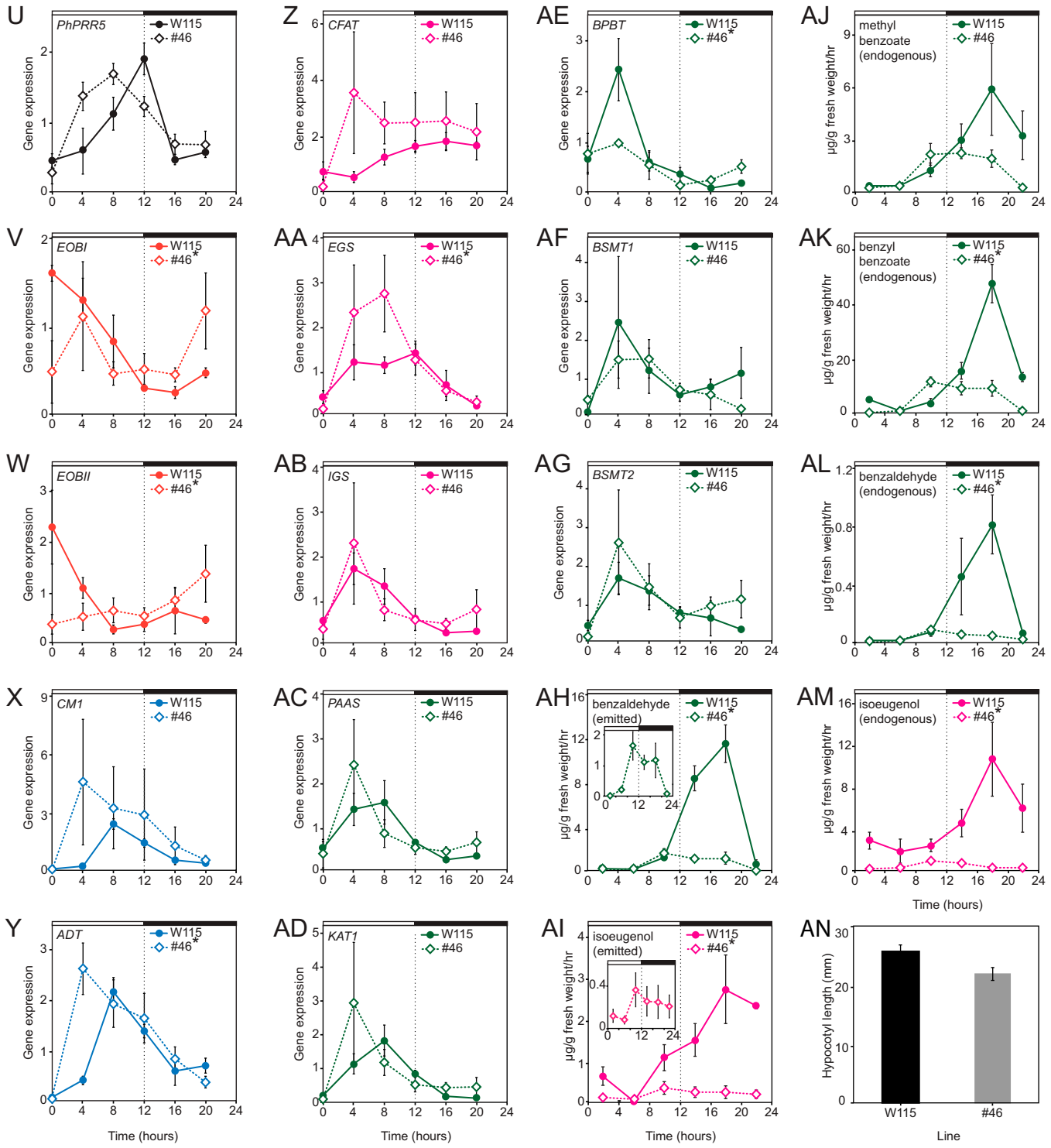

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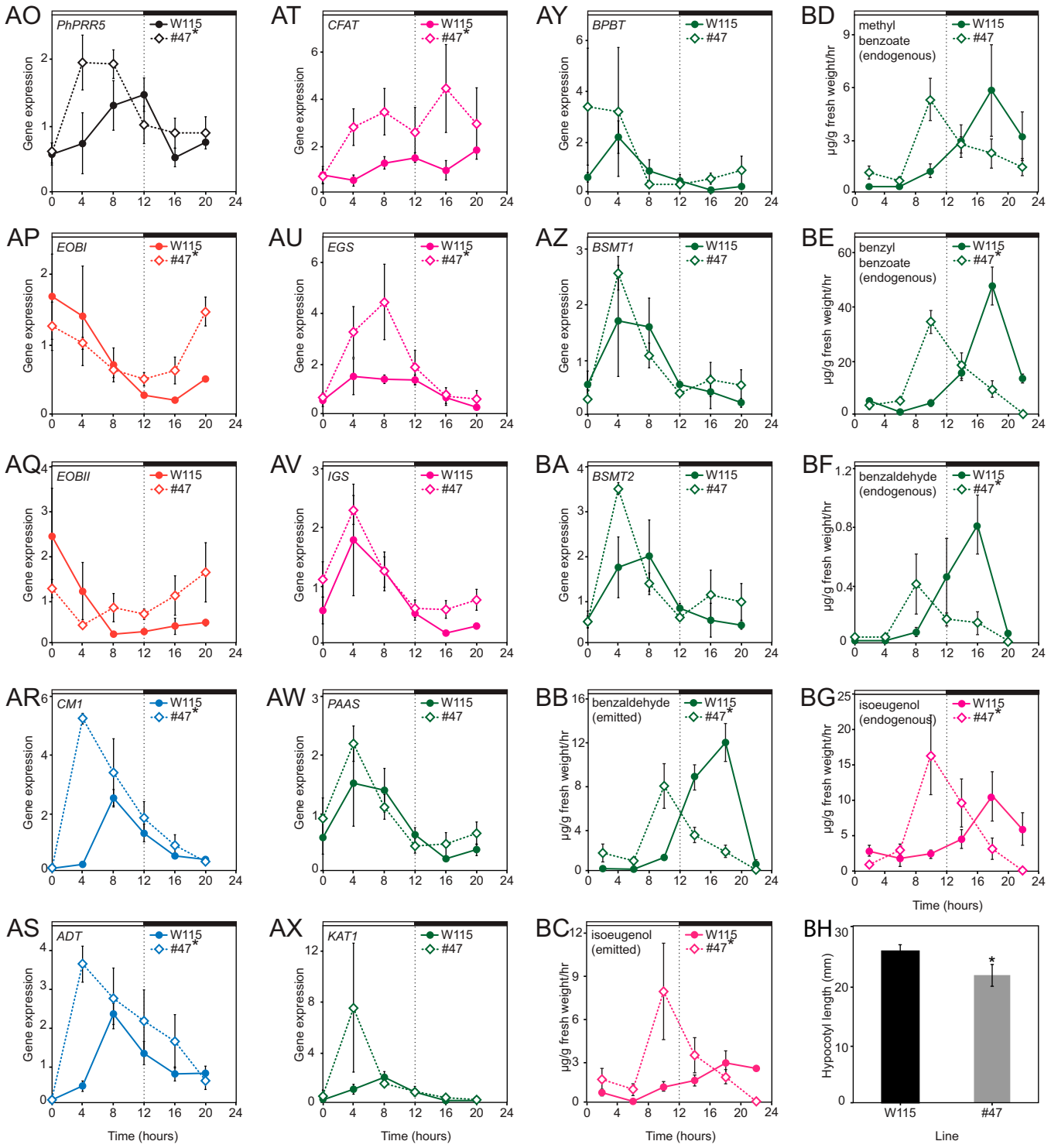

Fig. S5. *PhLHY* influences gene expression patterns and emission of floral volatiles in the FVBP pathway. (*A–M*, *U–AG*, and *AO–BA*) Daily expression patterns of transcription factor genes and enzyme genes related to the FVBP pathway in a transgenic line with constitutive (line 37) and reduced (lines 46 and 47) *PhLHY* expression (line 37) under 12-h light/12-h dark conditions. Values are relative to *UBQ*, and normalized by the average expression values of hours 0–12. (*N, O*, *AH–AI*, *BB*, and *BC*) Daily scent expression patterns of methyl benzoate and benzyl benzoate in lines 37, 46, and 47 and (*Insets*) graphs with enlarged *y*-axes showing the same 0–24 time point results. (*P–S*, *AJ–AM*, and *BD–BG*) Daily endogenous volatile compounds in lines 37, 46, and 47. Results in Fig. S5 represent mean ± SEM from three biological replicates. The line and marker color of the graphs corresponds to its placement within the greater FVBP pathway. White and black bars at the top indicate periods of light and dark, respectively (*$P < 0.05$, expression profiles, scent emission patterns, and daily endogenous volatile compounds of transgenic lines differ significantly from WT *Petunia*; two-way ANOVA). (*T*, *AN*, and *BH*) Developing hypocotyl length in millimeters in a comparison between transgenic lines 37, 46, and 47 and WT *Petunia* W115. (*$P < 0.05$, developing hypocotyl lengths that differ significantly from W115 *Petunia*; Student *t* test.)

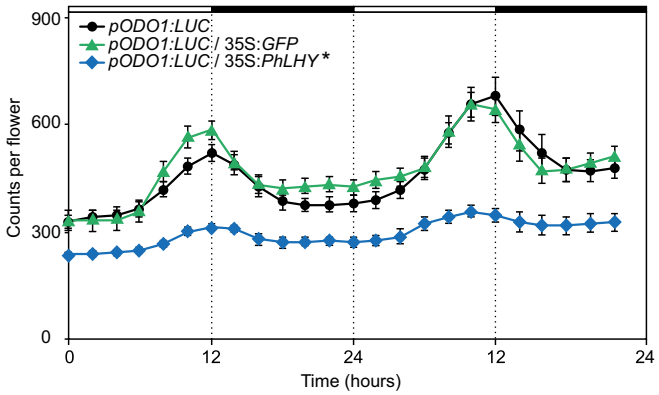

Fig. S6. Constitutive expression of PhLHY through transient transformation of flowers suppresses *pODO1:LUC* in vivo. *ODO1* promoter-driven LUC (*pODO1:LUC*) is used as a reporter (Fig. 5*C*). *pODO1:LUC* was coinfiltrated with *35S:PhLHY* or *35S:GFP*. White and black bars at the top indicate periods of light and dark, respectively. Results represent means ± SEM ($n = 8$). (*$P < 0.05$, *pODO1:LUC/35S:PhLHY* is the only expression profile differing significantly from the one in *pODO1:LUC*; two-way ANOVA.)

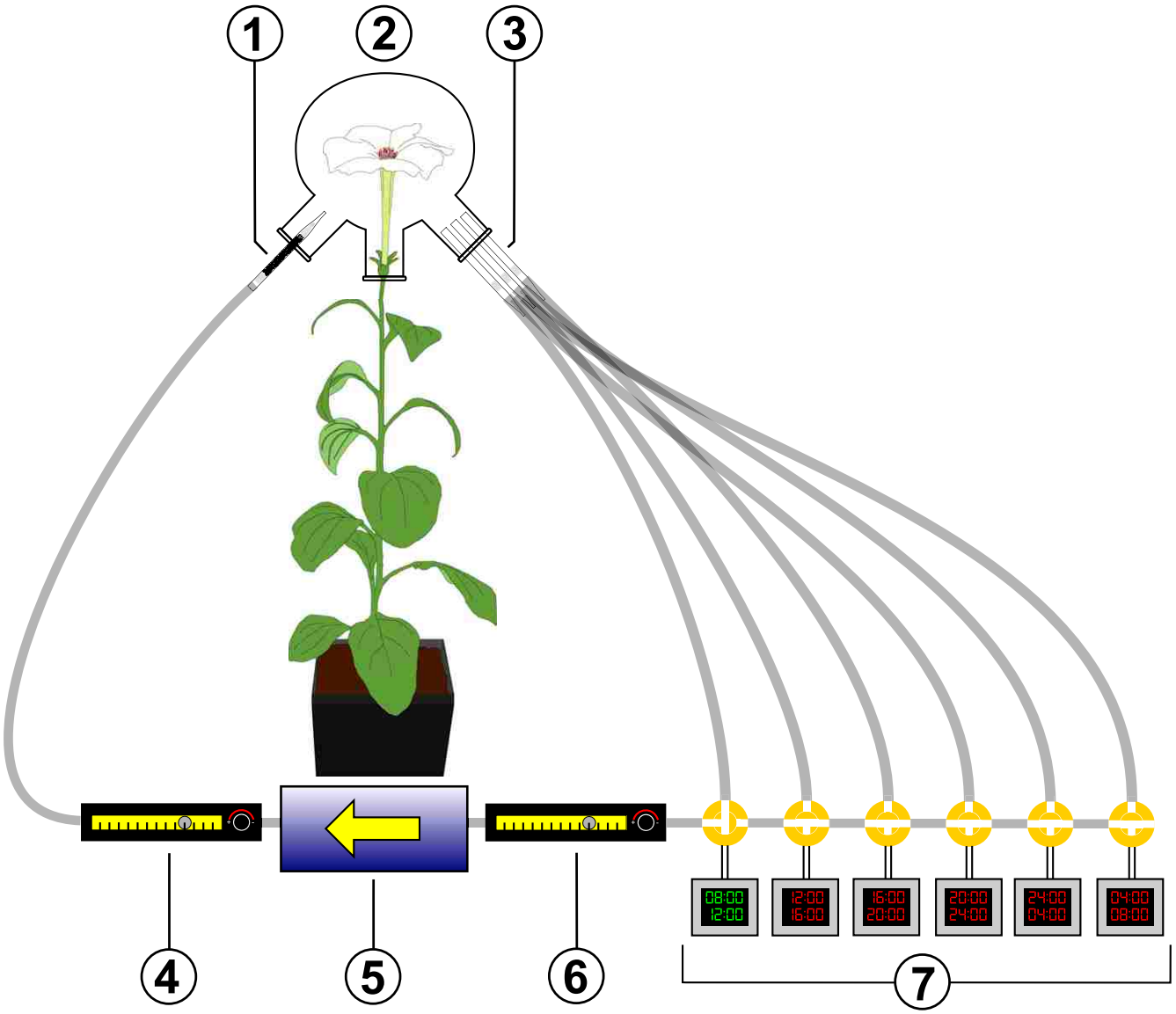

Fig. S7. Diagram of volatile collection apparatus. 1, Charcoal filter for introduced air; 2, Floral chamber (three-necked flask); 3, Volatile collection traps (Poropak); 4, Flow control for introduction of filtered air; 5, Unidirectional air pump (electric motor); 6, Flow control for suction to volatile collection traps; and 7, Timer-regulated solenoid switches (one trap open per time point).

Table S1. Quantitative PCR primer sequences used in this study

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession no.	Species
ADT	GCGTGAAGCAGTAGACGACA	GACGTTGCTTGAATCGTCCT	FJ790412	*P. hybrida*
BPBT	TGGTGGACCAGCTAAAGGAG	CAATTGAGCATCACCCTTCA	AY611496	*P. hybrida*
BSMT1	GTGGTCGAAAAACCCGAATA	CACGACCACCTATGAAGTGC	AY233465	*P. hybrida*
BSMT2	TCATAGGTGGTCGAGGTGCT	GACATGGGATAATTCCCTGTGG	AY233466	*P. hybrida*
CFAT	CCAATGCCTAGCCCTAACAA	GGACGCTTCTTCACATCACA	DQ767969	*P. hybrida*
CM1	CCCTGCTGTTGAAGAGGCTA	GCATGATCCAGTCCCCATAC	CO805161	*P. hybrida*
EGS	TCTGACCCTGCTAAGGGAAA	TTTGATCAGCCAATTGCATC	EF467241	*P. hybrida*
EOBI	CCTTAGCTCGATCTGCTGGT	CACCTGTTTCCCCACTTAGC	KC182627	*P. hybrida*
EOBII	CAAGCAGCTTCTTCAGAGCAAA	AATTAGGGCCTGCTTGGAAAGT	EU360893	*P. hybrida*
EPSPS	TGGCTCAAGGGATACAAACC	GCTGTAGCCACTGATGCTGA	M21084	*P. hybrida*
IGS	CCACGTCAAAAGAGTGAGCA	CCAGTGGTTTTCTCCCAAGA	DQ372813	*P. hybrida*
KAT1	GCTACAGGTGCACGTTGTGT	AAAGATCGTCCACAGCATCC	FJ657663	*P. hybrida*
ODO1	CATGCACCACTGATGAATCC	ATGGCGAATCGATAAGAGGA	AY705977	*P. hybrida*
PAAS	TGTCGATGAAACCCAAGTGA	ACCACATTCCAGGCCATATC	DQ243784	*P. hybrida*
PAL	GGGTCCTTCAAGGCATGATA	GTTGCCAAAGATTCCAGCAT	AY705976	*P. hybrida*
PhCAB	CTTGCCAAGTCGTGTTGATG	TTCACCTTGAGCTCAGCAAA	K00972	*P. hybrida*
PhGI	TCTGCCGTCCGTCATACTCG	ATGCAAGCCTTGGAGCGTCT	FN036363	*P. hybrida*
PhPRR5	TTCGTTTGAAGCGGAAAGAT	TACCCGATGGAGCCTCACTA	FN035819	*P. hybrida*
PhLHY	ACCGACAATGGAACTTGGAG	TTCTCCTTCCAAGCGAAGTC	KP017483	*P. hybrida*
UBQ	TGGAGGATGGAAGGACTTTGG	CAGGACGACAACAAGCAACAG	SGN-U207515	*P. hybrida*
CCA1	CCAGATAAGAAGTCACGCTCAGAA	GTCTAGCGCTTGACCCATAGCT	AT2G46830	*A. thaliana*
LHY	GACTCAAACACTGCCCAGAAGA	CGTCACTCCCTGAAGGTGTATTT	AT1G01060	*A. thaliana*
IPP2	GTATGAGTTGCTTCTCCAGCAAAG	GAGGATGGCTGCAACAAGTGT	AT3G02780	*A. thaliana*
PRR7	CTGCACTCGTTATATCGTTACTG	GGCATGATCACCTCTGTTAG	AT5G02810	*A. thaliana*
PRR9	CCAATGAGGAAAAACGAG	GCACCACTTCCTTGATCTG	AT2G46790	*A. thaliana*
TOC1	CTCTCCTTTCAGAGTGTTCTTATC	CACAGGGATTCTGCGAAG	AT5G61380	*A. thaliana*

Circadian clocks of both plants and pollinators influence flower seeking behavior of the pollinator hawkmoth *Manduca sexta*

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Abstract

Most plant-pollinator relationships occur during a certain time of day. Partially facilitating this daily meeting, many flowers are known to show their attractive qualities, such as scent, in a rhythmic fashion. Less is known about the regulation of these daily interactions from the pollinator's perspective. We examine the role of the circadian clock in modulating the interaction between *Petunia* and one of its pollinators, the hawkmoth *Manduca sexta*. We find that desynchronization of the *Petunia* clock affects moth preference for *Petunia* flowers. Moth locomotor activity is clock-regulated but light-repressed. Male moths show a time-dependent burst increase in flight activity during subjective night. In addition, moth antennal sensitivity to the floral scent benzaldehyde exhibits a 24h rhythm in both continuous light and dark. This study highlights the importance of the clock as a crucial factor in maintaining specialized plant-pollinator relationships.

Introduction

Plants and their pollinators provide one of the most studied examples of mutualistic interactions. To facilitate these interactions, many flowers produce and display phenotypes that operate as "advertisements" to the pollinators including shape, color[1], scent[2], etc. Evolution of these advertisements allows for some plant species to tune their relationship to a specific pollinator or set of pollinators, adopt a generalist approach to attracting pollinators, or a combination of both[3]. Experimental manipulation of floral traits has allowed researchers to examine the ease with which changes in floral advertisements can affect pollinator visitation. For instance, changes in floral color have been shown to affect the preference of bees and hummingbirds for *Mimulus* flowers[4,5], and also the preference of bees and hawkmoths for *Petunia* flowers[6].

A critical aspect for selectively attracting certain pollinators is temporal control of the flower advertisements and resources. Facilitating this, many components of floral attraction have been shown to oscillate on a daily schedule, including floral opening, scent emission [7], nectar production [8], orientation [9], etc. Many pollinators are generally known to have temporal restrictions on their activity[10], though detailed time courses of pollinator behaviors are few[11,12]. These observations indicate the ecological and evolutionary significance of their internal timekeeper mechanisms, the circadian clock. However, it has remained largely unknown whether and how the circadian clock of plants and insects is involved in maintaining plant-pollinator interactions.

The hawkmoth *Manduca sexta* has a wide distribution across the Americas, where its larval form the tobacco hornworm is a well-known pest of Solanaceae crops[13]. The adult moth is generally known as a nocturnal and/or crepuscular nectivore of various plant species (often Solanaceae), and is a model organism in the fields of animal behavior, neuroscience, and insect

flight[14,15]. *Manduca sexta* maintains a nocturnal relationship with the flowers of *Petunia axillaris* in the wilds of Uruguay [16]. Typical of nocturnal hawkmoth-pollinated species, *P. axillaris* has highly reflective white flowers with long narrow corolla tubes, and emits a robust bouquet of scent at night[17]. This nocturnal scent release involves compounds produced primarily from the Floral Volatile Benzenoid/Phenylpropanoid (FVBP) pathway, which is active during the evening[16]. The FVBP pathway has been studied extensively in the research model *Petunia hybrida* cv. Mitchell [18], which has a nearly identical floral morphology and scent profile to its parent species *P. axillaris*. Evening-expressed transcriptional regulators, such as *ODORANT1* (*ODO1*), *EMISSION OF BENZENOIDS II* (*EOBII*), and *EMISSION OF BENZENOIDS I* (*EOBI*), upregulate the transcription of enzymes involved in the processing of precursor molecules through the FVBP pathway [19-21]. The timing of daily scent emission is regulated by the circadian clock (ref-your review). To restrict the expression of FVBP genes to the evening and *Petunia*'s characteristic scent emission to the night, the morning-expressed clock component LATE ELONGATED HYPOCOTYL (LHY) directly represses the expression of *ODO1* and other key genes during the daytime [22].

The influence of the *M. sexta* clock on this plant-pollinator relationship is less characterized. Circadian clock-dependent behavioral and physiological responses to pheromones and food-related odors were described in other insect species, especially *Drosophila melanogaster* [23-27]. In other insects, circadian modulation of both locomotor activity and antennal sensitivity are likely mechanisms of rhythmic behavioral responses, though little evidence exists for plant-pollinator interactions. Antenna from *M. sexta* exhibit a stronger response to scent collected from *P. axillaris* flowers during the night vs the day[16], but no study to date in *M. sexta* has examined whether changes in antennal sensitivity to a standard quantity of scent changes

over the course of 24 hours. In a laboratory setting, tobacco plants with genetically-altered clocks showed a change in fitness compared to WT when exposed to *M. sexta* as a pollinator [28].

Here we provide evidence that the synchrony of the plant and insect circadian rhythms is important, and additional evidence that the *M. sexta* clock modulates its half of this interaction by gating locomotor activity and floral odor sensitivity to the night.

Results

Synchronization of circadian timing is important for floral visitation by *M. sexta*.

Previously, we examined the role of the circadian clock in regulating the emission of floral scent from *Petunia hybrida* cv. Mitchell, a commercial species derived from the *M. sexta*-pollinated *P. axillaris*, with which it shares similar phenotypes (white flowers, heavy emission of benzenoid volatiles at night). We found that the morning-expressed clock gene LHY directly represses the synthesis of scent during the daytime, restricting scent synthesis and emission to the night. Nightly emission of scent could confer a fitness advantage to plants in part by restricting pollination to a subset of efficient pollinators. A plant's temporal control of floral emission only describes one half of this nightly meeting however, the pollinator must also play its part. To examine the importance of clock synchronization to successful floral visitation, moths with a circadian time (the time of the internal clock) of 12 (CT12, evening) were subjected to make a choice between two plants of wild-type *P. axillaris*, one which was also experiencing CT12, and another plant experiencing an alternate phase of the circadian clock than that of the moth clock (Fig. 1). Moths showed a lower preference for plants as the plant's circadian time became earlier than CT12, with moths choosing CT12 plants over CT0 plants in ~90% of trials (PI= -0.82;

binomial exact test, p-value= $1.4x10^{-6}$). Significantly greater preference was shown for plants experiencing CT16 over CT12, likely because CT16 is closer to the peak emission time of floral scent in *P. axillaris*[16] (PI= 0.34; binomial exact test, p-value= 0.03).

Further evidence of the clock's importance to floral visitation was gathered by examining pollinator choice among plants with genetically altered clocks (Fig. 2). In our previous work, a line of plants with arrhythmic clock functionality was produced by constitutive expression of the clock gene *LHY*. In this experiment, wild-type plants and the transgenic plants were grown under the exact same conditions, but the timing of their internal clocks differed. Plants with an arrhythmic clock (*35S:PhLHY* #37) showed an almost complete absence of FVBP floral scent emissions. Moths chose *P. hybrida* with arrhythmic clocks (*35S:PhLHY*) over wildtype *P. hybrida* cv. Mitchell plants in only 10% of trials (PI= -0.79; binomial exact test, p-value= $3.35x10^{-7}$) (Fig. 2B-C). In addition, transgenic lines with shorter clock periods (*35S:PhLHY* #46 and #47) also received significantly lower preference from hawkmoths (Fig. 2C).

M. sexta locomotor activity is clock-regulated but also light-dependent.

Though *M. sexta* is generally known as a nocturnal and/or crepuscular species (refs), a thorough documentation of its circadian rhythmic activity has not been performed. To examine the circadian clock's effect on *M. sexta*'s general locomotor activity, naïve male moths were entrained to a 12L:12D cycle before exposure to a variety of light conditions. In 12L:12D conditions, moths begin to fly shortly after lights go out at Zeitgeber time 12 (ZT12), increasing in activity until a peak around the middle of the night, after which activity begins to decline until ZT24 (Fig. 3A). Around ZT0, a brief but significant spurt of activity occurs immediately after the overhead lights illuminate. In continuous dark (DD) conditions, moths exhibit oscillatory

flight behavior during subjective night, confirming the role of an endogenous circadian mechanism for locomotor activity (Fig. 3B). In continuous light (LL), flight activity is severely dampened throughout the experiment, implying that light represses general locomotor activity in *M. sexta* (Fig. 3C). To highlight the interplay of the clock and light conditions in determining locomotor activity in *M. sexta*, a 12-hour t-cycle was performed (Fig. 3D). A t-cycle is useful for extracting the separate roles of clock and lights inputs to rhythmic behaviors. If a behavior is only light regulated, then one can predict blanket activity only during light or dark periods (depending on the behavior). If a behavior is solely clock-regulated, one can expect rhythmic behavior to ignore light-dark transitions for the 24 hours of the t-cycle experiment. If a behavior is light-dependent and clock-regulated, activity is predicted to occur when the right light conditions align with the appropriate phase of time. During the hours corresponding with subjective day (CT0-12) in our t-cycle experiment, flight activity was low across the different light conditions. A low period of activity is also maintained throughout the first half of subjective night (CT12-18), when the lights are on. In the second half of subjective night (CT16-20), a peak of activity occurs immediately after lights out, which gradually declines until dawn, closely mirroring the activity shown in 12L:12D and DD conditions for these timepoints. Thus, *M. sexta* flight activity was observed when light conditions were aligned with a specific time. Taken together, these actograms provide evidence of strong clock regulation of the *M. sexta* locomotor, with additional suppressive regulation from high light input.

The circadian clock gates the sensitivity and behavioral response of *M. sexta* to floral odor signals.

While moths exhibit clock-regulated nocturnal activity in their locomotion (Fig. 3), can their behavior be altered by the presence of floral odors? To test this, we recorded moth activity in DD conditions, and administered a "pulse" of floral scent by placing cut and visually-hidden *P. axillaris* flowers in the intake of the wind tunnel during the night for 1 hour (Fig. 4C). A significant increase in moth flight activity over unprovoked moths (Fig. 4B) occurred during this hour, indicating that moths will increase locomotor activity in search of unseen but smelled flowers. Is this response to floral presence also time dependent? "Gating" is a term used to describe the ability of the clock to restrict sensitivity for a specific stimulus to a window or "gate" of time. To determine if the circadian clock gates the response of *M. sexta* to floral odors, the effect of day and night-time scent pulses on moth flight activity was analyzed more fully by also administering a pulse in the morning. When naïve moths were exposed to a CT4 scent pulse in DD, a much smaller increase in moth activity than that seen for the CT16 floral pulse appeared (Fig. 4D). Floral scent pulses at CT4 and CT16 contributed no measurable increase in activity in LL conditions (Fig. 4H-I), providing further evidence that hawkmoth activity is light repressed. To determine if scent from the *P. axillaris* vegetation could also affect male moth behavior, we also administered a "vegetative pulse" by placing 8 two-month-old, non-flowering plants in they intake of the windtunnel at the same day and night timepoints. When the vegetative pulse was introduced at CT4 and CT16, no deviation from unscented activity was observed in males (Fig. E-F; J-K), indicating that, at least in male *M. sexta* adults, vegetative scent is not an instigator of foraging behavior.

Given that moths exhibited a time-dependent behavioral response to odors, we were curious if this behavior could be partially explained by changing sensitivity in chemoreception to floral odors. Electroantennograms have traditionally been used to examine the strength of the antennal (olfactory) sensitivity in insects[29]. To assess whether antennal sensitivity to floral scent changed throughout the day, electroantennogram (EAG) responses to *P. axillaris* odorants were recorded in 24-hour time courses. In 12L:12D conditions, a clear rhythm in response to the floral odorant benzaldehyde occurs, with a minimum of response in the daytime separating periods of maximum response during the morning and night (Fig. 4E). Experiments in free running conditions (DD and LL) showed nearly identical rhythms (though both are slightly reduced in overall amplitude from LD), indicating that the observed rhythm results from an endogenous mechanism (Fig. 4F-G).

Given that a 24h rhythm of odorant sensitivity was found in *M. sexta* antennae, we wanted to examine what pieces of the odorant reception mechanism could be involved. Recent work has sought to uncover the molecular mechanisms regulating odorant reception in insect antennae[30]. In brief, primarily hydrophobic odorants enter pore tubules of the sensillum and bind to odorant-binding proteins (OBPs) in order to traverse the aqueous sensillar lymph to odorant receptors (ORs). There seems to be a large diversity of OBPs and ORs across each insect species, with few highly conserved homologs. In particular, a protein originally described as a traditional OR has been shown to act as a coreceptor for other ORs, and is highly conserved among insects and designated odorant receptor coreceptor (Orco). An examination of gene expression in moth antennae during continuous dark conditions (where EAG daily rhythms continued) showed clock activity as measured by *per* mRNA expression (Fig. 5G), but no daily cycles for *Orco* expression (Fig. 5H), indicating that a general suppression of odorant reception does not occur through

clock modulation of *Orco*. We also examined two general odorant binding proteins which show some conservation in insects, General Odorant Binding Protein 1 (GOBP1) and General Odorant Binding Protein 2 (GOBP2), both of which did not oscillate in their expression (Fig 5I-J).

Discussion

Clock synchrony is important to plant-pollinator interactions.

Over the past several decades, knowledge of circadian clocks and their importance to biology has advanced significantly. Clocks are now known to exist in all species, with the few exceptions seen in environments without 24h cycles (deep sea, subterranean, etc.). Additionally, clock regulation of physiology and behavior appears to be robust: a famous example is found in *Arabidopsis thaliana*, where one third of mRNA transcripts are known to be under clock control [31]. Clocks are thought to be so conserved and physiologically integrated in order to allow organisms a way to prepare for situations which can be readily predicted to occur in the future of the organism, giving them a "leg up" on the competition.

Given that so many pollinators are generally known to have temporal rhythms in their activity, and associate with many plants which also exhibit rhythmic attractive qualities, we were interested in whether a shift in the synchronization of the plant-pollinator clocks would result in decreased success for the interaction. We found that changing the phase of the clock, either through entrainment or transgenic manipulation, did generally result in decreased preference for those plants by moths. One exception is plants at ZT16 were preferred versus plants at ZT12, though this makes sense as ZT16 flowers are closer to the emission maximum than ZT12 flowers. A recent study also showed that plants with altered clocks had changes to their fitness, as measured by seed production as a result of outcrossing [28].

Coevolutionary relationships between plants and pollinators can likely be modified or swapped entirely based on phenotypic changes in any of the involved species. For plants, these phenotypic changes could include flower shapes, colors, scent composition, nectar volume; while in pollinators these could include chemosensory and visual capabilities, metabolic rates, mouth part morphology, etc. Many of the above examples are modulated in circadian rhythms, and so it is important to consider modification to clock regulation -or the clock itself- is also an important factor in the establishment and disestablishment of plant-pollinator interactions.

Many plants have adapted to specialized relationships with pollinators, and have evolved a series of traits to maximize the efficacy of those interactions. In the same sense that changes in flower shape, color, or scent composition can be considered a mechanism by which plants coevolve with pollinators, changes in the timing of those traits by the clock can also be considered a

"Pollination syndromes" are convenient terms to describe suites of traits which allow for specialization with certain types of pollinators. While controversial, it's undeniable that many traits provide obvious advantages for distinct specializations (eg. white flowers reflect more light than any other color at night, and so provide optimal visibility to nocturnal pollinators).

Moth behavior is clock-regulated but light-dependent.

Moth locomotor activity is clearly driven by circadian rhythms, as is evident in continuous dark (Fig. 3C and Fig. 4A). However, in all continuous light experiments, flight behavior was severely reduced throughout the timecourse, indicating that moths avoid flight in high light conditions regardless of the clock's phase. This arrhythmicity in behavior due to continuous light

has also been observed in other insects, including *Drosophila melanogaster* [32,33] and *Calliphora vicina* [34]**.** In *C. vicina*, the clock itself maintains a rhythm, indicating that the constant dampening of locomotor activity occurs through means other than the clock [34]. The small amount of activity that was seen in *M. sexta* males occurred in the last few hours of the first day (Fig. 3C, 4B), which is delayed from the peak activity time in LD or DD conditions, indicating that clock pace could also be slowed in LL conditions, a phenomenon seen in other insects during continuous light of relatively low intensity [32-34].

In all experiments where moths exit a period of darkness and receive a sudden onset of light, moths exhibit a brief but significant increase in flight activity (Fig. 3A, 3C-D, Fig. 4F-J). This effect seems to be much greater at ZT0 than at ZT12 (Fig. 3C). This dark-to-light transition behavior can also be observed in other nocturnal insects, such as bed bugs [35]. This behavior potentially exemplifies an escape response caused by a sudden increase in light, a situation which would occur when insect hiding places/harborages are exposed.

Behavioral response to floral scent modulated by the circadian clock.

Moths showed a greater response to floral odor stimuli at night (Fig. 4A-C), indicating that the clock gates the ability of the moth to respond to the odor. Temporal modulation of behavioral responses to odors has been documented before in insects, though in non-floral settings, usually in response to pheromone or food-related odors. An early example of temporal modulation to pheromones can be found in *Epiphyas postvittana*, where male moths were shown to exhibit strong response to female sex pheromones during the evening, with greatly reduced sensitivity during the other portions of the day [36]. Food-related examples can be found in disease vectors

including the tsetse fly, *Triatoma infestans*, and *Rhodnius prolixus*, which all show rhythmic responses to host odors [26,27,37].

While no apparent response to vegetative tissues was shown in any of the scent pulse experiments (Fig. 4F-J), it is important to note that all moths in this study were male. Female moths of *M. sexta* are well known to oviposit on solanaceous host plants, like *Solanum*, *Nicotiana*, and also *Petunia* [38]. It is thus possible and even likely that female moths would show an increase in flight activity in response to emissions from vegetative tissue. Whether this response would also be clock-regulated is another question. Female moths of *H. virescens* do show differential oviposition responses to herbivore-induced volatiles from vegetative tissues collected from day and night timepoints [39].

To further investigate a potential mechanism of the time-dependent response to floral odors, we examined *M. sexta* olfactory sensitivity in a series of time courses. Indeed, moths showed a clear 24h oscillation of sensitivity in 12L:12D, LL, and DD conditions (Fig. 5 A-F). While a novel finding in plant-pollinator interactions, circadian modulation of antennal sensitivity has been documented in other species. In *Drosophila melanogaster*, flies showed increased sensitivity to ethyl acetate, an attractive compound, as well as to benzaldehyde (which, in contrast to *M. sexta*, *D. melanogaster* avoids) [23].

A key finding in understanding circadian rhythmicity in antennal responses was shown in *D. melanogaster*, where rhythmic EAG responses were observed in flies lacking the nerve cells containing the central oscillators [25]. A targeted reduction of CLK and CYC expression in antennal tissues abolished the EAG rhythms, indicating that peripheral clocks in the antenna are responsible for regulating rhythmicity in olfactory sensitivity[25]. At the same time, a study in cockroaches found that severing the optic nerve also abolished rhythmicity in EAG recordings,

indicating that the central input can effect olfactory sensitivity [40]. While we show that the antennal clock in *M. sexta* is cycling (Fig. 5G), the conserved components of the odorant reception machinery that we tested (Orco, GOBP1, GOBP2; Fig. 5H-J) did not cycle. Further examination of the other Odorant Binding Proteins, Odorant Receptors, and other pieces will be necessary to elucidate the mechanism behind rhythmic EAG responses in *M. sexta*.

Beyond circadian sensitivity however, it is possible that other factors also contribute to the time-dependent behavioral response seen in Fig. 4, including general locomotor activity. Oscillation of EAG response continues in LL conditions (Fig. 5F), indicating the lack of response to ZT16 floral scent pulse in LL conditions (Fig. 4G) is likely due to impairment of locomotor activity. To fully understand the insect's temporal response to odors, additional studies must further explore the mechanisms by which the central and antennal clocks interact with olfaction machinery in the antennae. While work in this field will be much easier in *D. melanogaster*, applying knowledge gained from fruit flies to important pollinator species will allow for insight into how plant-pollinator interactions are tuned by their clocks.

Methods

Plant cultivation and insect rearing

Petunia plants (*Petunia axillaris* and *Petunia hybrida*) were grown in an enriched potting soil (Sunshine 4 Mix, Sun Gro Horticulture) in a custom growth room at 25˚C and under a 12L:12D lightcycle. Light from full spectrum fluorescent lamps (Octran F032/950/48; Osram-Sylvania) was set to an approximate fluence rate of 100 µmol/m^2/s. Generation of all transgenic lines was described previously [22].

To avoid mating and oviposition behaviors, all experimental *M. sexta* moths were male, and obtained from a rearing facility in the Department of Biology at the University of Washington, Seattle, USA. Larval diet is described previously [41]. Pupa were entrained and allowed to eclose in a 12L:12D light cycle, 25˚C, 65% humidity. Once eclosed, adult moths used for experiments were kept flower-naïve to eliminate learned behaviors and food-deprived in order to eliminate any unforeseen (and untested) food-related impacts on the clock.

Actograms and behavioral assays

All behavior experiments were conducted in a plexiglass windtunnel (Fig. S1), dimensions of 2.5x1x1 m^3 (LxWxH). Airflow was set to 0.1 m/s. Overhead fluorescent lights were used to simulate daylight, at 100 µmol/m^2/s. Night light (dark period) was simulated by turning off the overhead lights and turning on an indirect light source from 50 ft away. Photon density during dark periods was too low to measure using our photometer (Licor LI-250A), and even though it was not possible for examiners to see chamber activity without the aid of infrared lighting and cameras, moths seemed to be able to navigate quite readily.

Prior to experiments, 15 adult male *M. sexta* were introduced to the chamber 1 day early and entrained to 12L:12D light cycle. During recording, the number of moths flying in every 10-minute period was counted. During dark periods, infrared lighting was used in tandem with a camera (Basler Pilot GigE, Basler Vision Technologies) to visualize movement. Infrared lighting was kept on for all light periods. Each experiment was repeated 3 times.

For behavioral choice assays, naïve male moths obtained from the rearing facility were entrained to a 12L:12D light cycle at 25˚C, 50% humidity in a Percival environmental chamber for 1 day

after eclosion. Standard error of the mean was calculated as [42]:

$$\text{s.e.m.} = \left(\frac{p(1-p)}{n} \right)^{\frac{1}{2}},$$

where p is the observed proportion and n is the number of observations. Statistical analyses were performed in R (www.r-project.org) using the binomial exact test.

Prior to scent pulse assays, whole flowers (2-3 days in age) were cut from plants in the growth room, placed in a glass vial containing 5% sucrose solution and carried to the wind tunnel room in a sealed container. At the appropriate timepoint, flowers (still in vials) were arranged in a regular pattern (5 down the height axis, 3 across the width axis). Squares of brown construction paper (also used to line the floor of the wind tunnel's test chamber) were taped in front of the flowers, to block visual availability of the flowers to moths in the wind tunnel, and assist in dispersing the floral odors. After the timepoint, flowers were immediately placed into the sealed container and removed from the room.

Electroantennogram

Moths were reared and trained as mentioned above and placed in the wind tunnel for 1 additional day of entrainment prior to the experimental day. About 10 minutes prior to the time point, antennae were excised from moths and the basal end hydrated in electrode gel (Spectra 360, Parker Laboratories). For dark time points, antennal harvest took place under dim red light, and was immediately transferred in dark containers to the EAG room, where EAG recordings took place in the dark. The antenna from a single male moth was connected to two glass-electrodes filled with conductive gel. The EAG signal was recorded by Ag-AgCl wires connected to the

headstage of an extracellular amplifier (1800, A-M Systems, Sequim, WA), to achieve 100× amplification, and collected using WinEDR acquisition software (WinEDR v3.5; University of Strathclyde, Glasgow). The signal was filtered and digitized at 400 Hz sampling rate. Olfactory stimuli were delivered to the antenna by pulses of air from a constant air stream diverted through a glass syringe containing a piece of filter paper bearing the odor stimuli. The odor pulses were injected into a charcoal-filtered air stream flowing to the side of the antenna at a rate of 100 ml/min. The stimulus was pulsed by means of a solenoid-activated valve controlled by the WinDaq acquisition software. Odor syringes containing 10uL of a 1:100 dilution of synthetic odorants diluted in mineral oil were prepared. Scent pulses were delivered every <insert> seconds for 5 total pulses. EAG amplitudes (mV) were measured for each odor pulse, and the average of the 5 pulses was recorded as a datapoint. Each timepoint represents an accumulation of data from 12 individual antennae from as many moths.

Gene expression analysis

Male moths (*M. sexta*) entrained post eclosure in 12L:12D cycle were introduced to the windtunnel chamber 1 day prior to the experiment. At each timepoint, all moth tissues were immediately flash frozen in liquid N_2. While still frozen, individual tissues were dissected and separated into 2mL tubes with 2 steel "BBs". Tissue was ground in tubes while frozen (Retsch Technology, Mixer Mill MM 400). Total RNA was extracted by TRIzol-based method, and received DNAse treatment. cDNA synthesis and qPCR analyses were performed as described previously [43]. Primers for qPCR as follows (per primers from Schuckel et al.[44]): RPS13-F 5'-GTCTTGCCCCTGACCTACCT-3' ; RPS13-R 5'-TGGCAGCACACTCTTTGTCT-3'; PER-F 5'-CCGCATCCGCCGCTACC-3'; PER-R 5'-TGCAATCATGGCGGTGAAC-3'; ORCO-F 5'-

ACAGCCACCCACCCATTGTTCACG-3'; ORCO-R 5'-GGTCTCGTTCGTCTCCTTGTT-3'; GOBP1-F 5'- GCCACTTCAACCTGCTCACC -3'; GOBP1-R 5'-GGTCCTCTTCTGCGTCGTGT -3'; GOBP2-F 5'-ACACGCATCCATCACGTCAA-3'; GOBP2-R 5'-CGTCGTATTGCTTCTCGCAGT-3'.

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Author contributions statement

M. P. F., T. I., and J. A. R. designed experiments. M. P. F., L. A. N., and E. H. performed experiments. M. P. F., L. A. N., T. I. and J. A. R. wrote the manuscript.

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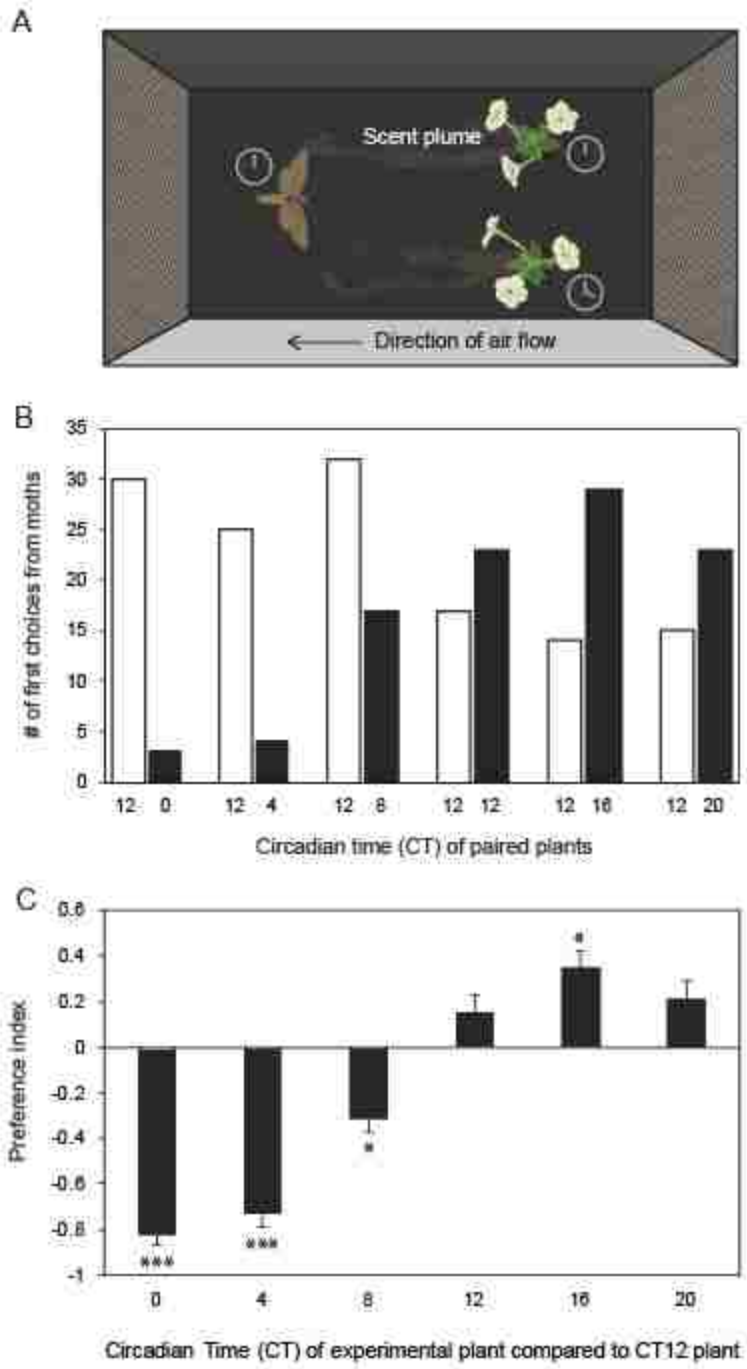

Fig. 1. Synchronization of plant and pollinator clocks important for floral visitation. (*A*) Diagram of experimental setup: an adult male *M. sexta* moth at zeitgeber time 12 (ZT12) is given 5 minutes to make a first choice between two stimuli: the control plant (also at ZT12) and an experimental plant entrained to another internal time (with a comparative control for CT12).

Plants were randomly assorted. (B) Raw data from the choice experiment. Each pair indicates the circadian time of the two plants in the choice assay, a control at CT12 (shown in white), and an experimental plant at another time (shown in black). (*C*) Preference index of the choice experiment data. Each bar represents the preference for 30+ individuals (tested separately) at one timepoint comparison of the 24-hour experiment. Error bars represent the standard error of the binary distribution. Asterisks denote choices which are significantly different from random in a binomial exact test (* = $p < 0.5$, *** = $p < 0.001$). See Fig. S1 for full schematic of wind tunnel apparatus.

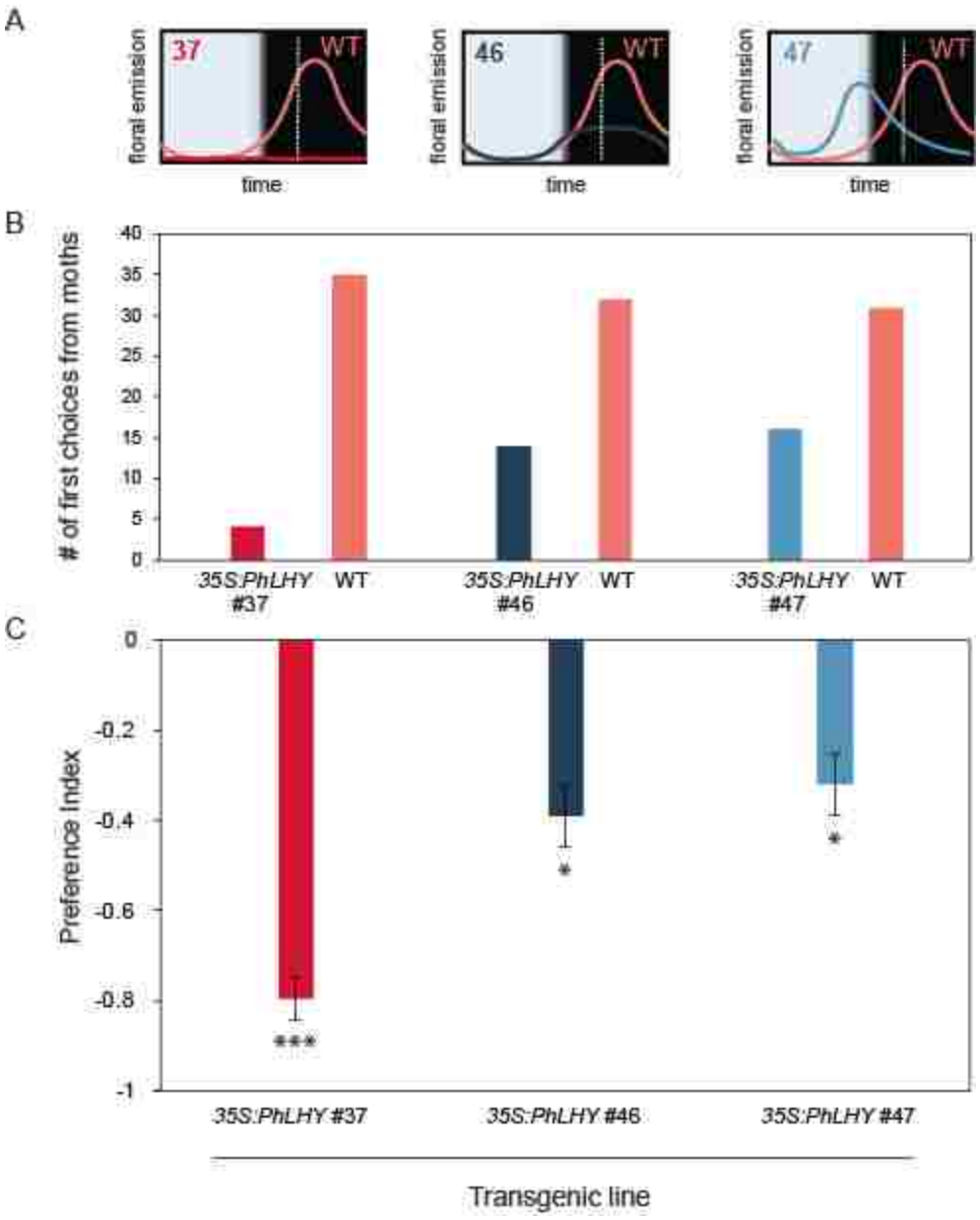

Fig. 2. Transgenic *Petunia hybrida* with disrupted clocks receive reduced preference from *M. sexta* vs wildtype *P. hybrida*. Moths entrained to 12L:12D cycle were given a choice between 2 plants, a wildtype *P. hybrida* plant and a transgenic plant with altered clock rhythm: (37: arrhythmic, 46: early phase-shift, 47: early phase shift). (*A*) Emission profiles of the plant lines for each experiment is shown in the above the corresponding bar (emission profiles synthesized from previously published data [22]. All organisms were entrained to a 12L:12D cycle, and the

choice experiment carried out at ZT16 (denoted as the white dotted line in the emission profile). (*B*) The raw data of each choice experiment between clock-altered line and WT *P. hybrida*. Error bars represent the standard error of the binary distribution. Asterisks denote choices which are significantly different from random in a binomial exact test (* = $p < 0.5$, ** = $p < 0.01$, *** = $p < 0.001$). (*C*) Preference index of the choice experiment data. Each bar represents the preference for 39+ individuals (tested separately) for each choice experiment. Error bars represent the standard error of the binary distribution. Asterisks denote choices which are significantly different from random in a binomial exact test (* = $p < 0.5$, *** = $p < 0.001$). See Fig. S1 for full schematic of wind tunnel apparatus.

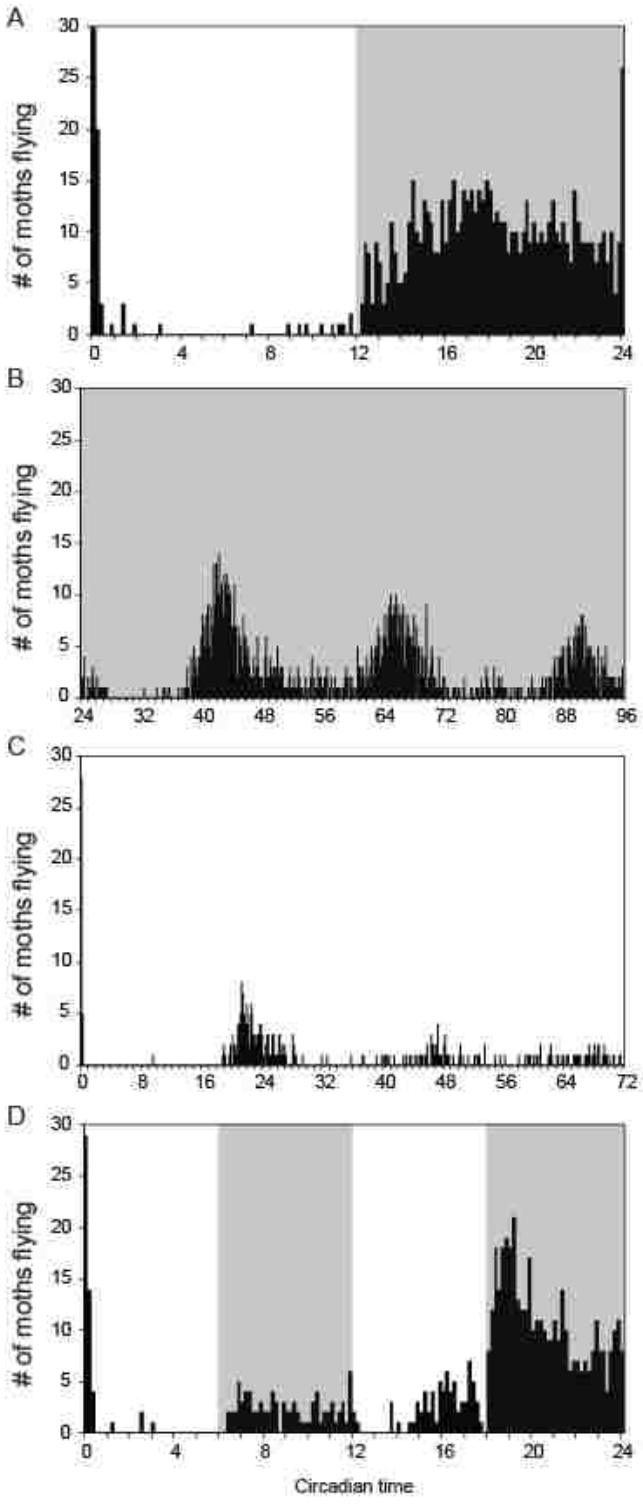

Fig. 3. *M. sexta* activity is clock regulated but light-repressed. Actograms of the number of male *M. sexta* moths flying in each 10-minute window of 24 hours or 72 hours. Experiments were repeated independently with 15 male moths three times. The results show the accumulated

counts of flying moths in each time window. (*A*) 12L:12D. (*B*) Continuous dark for 3 days (3D DD). (*C*) Continuous light for 3 days (3D LL). (*D*) 12 hour t-cycle.

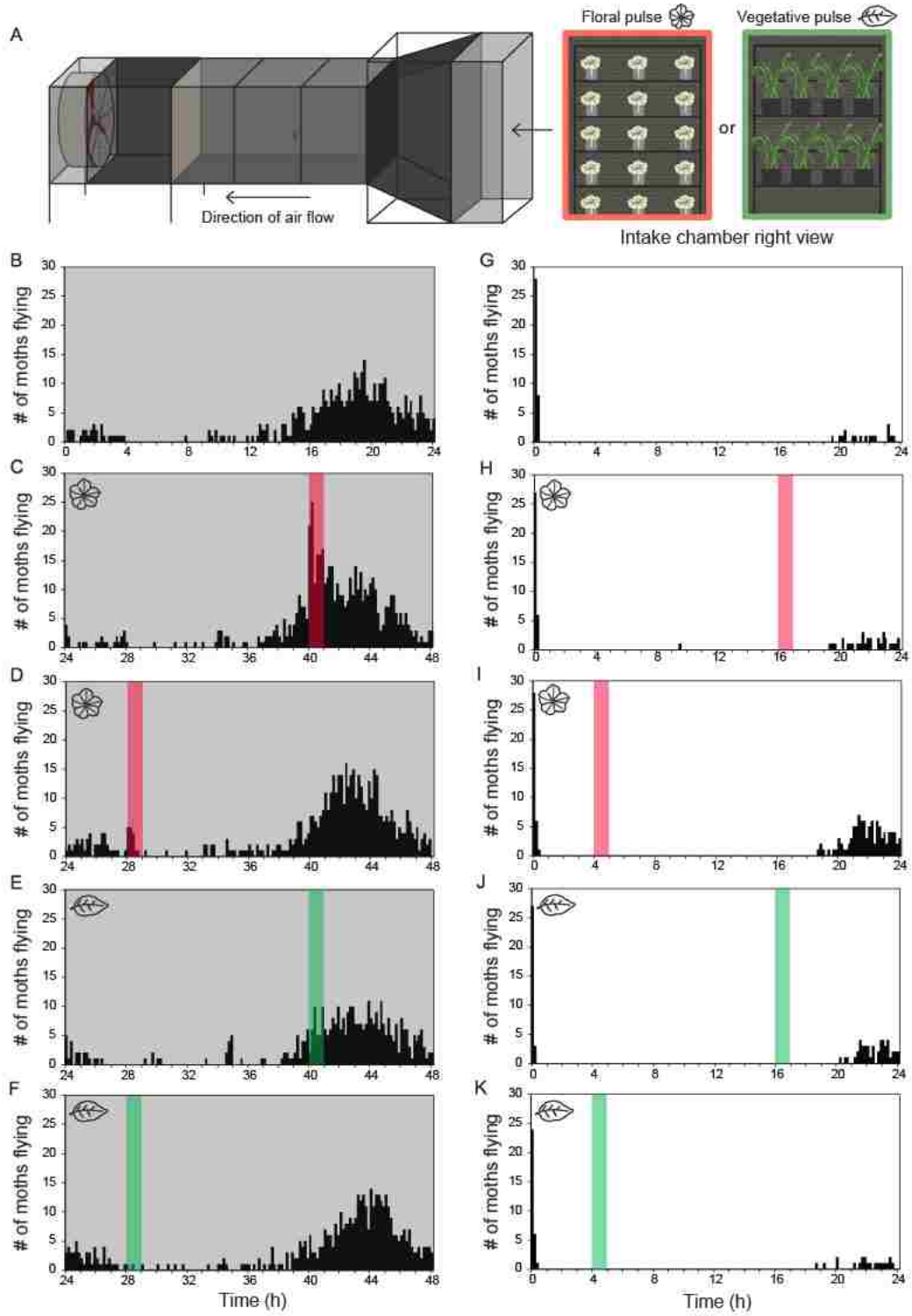

Fig. 4. Circadian clock and light modulate behavioral response to floral odor. (*A*) Wind tunnel used for scent pulse experiments with diagrams of the floral and vegetative pulse setups administered to the intake of the windtunnel. All floral scent pulses administered by adding 15 cut flowers entrained to a night timepoint, ZT16. All vegetative plant pulses were consisted of 8 two-month-old plants with no flowers. (*B-H*) Actograms of flight activity with subjective day/night stimulation pulses of floral scent or vegetative plant emissions. Each experiment consisted of 15 male moths entrained to 12L:12D and was repeated 3 times (*B*) DD actogram (*C*) DD actogram with ZT40-41 "floral night pulse". (*D*) DD actogram with ZT28-29 "floral day pulse". (*E*) DD actogram with ZT40-41 "vegetative night pulse". (*F*) DD actogram with ZT28-29 "vegetative day pulse". (*G*) LL actogram (*H)* LL actogram with ZT40-41 "floral night pulse". (*I*) LL actogram with ZT28-29 "floral day pulse". (*J*) LL actogram with ZT40-41 "vegetative night pulse". (*K*) LL actogram with ZT28-29 "vegetative day pulse".

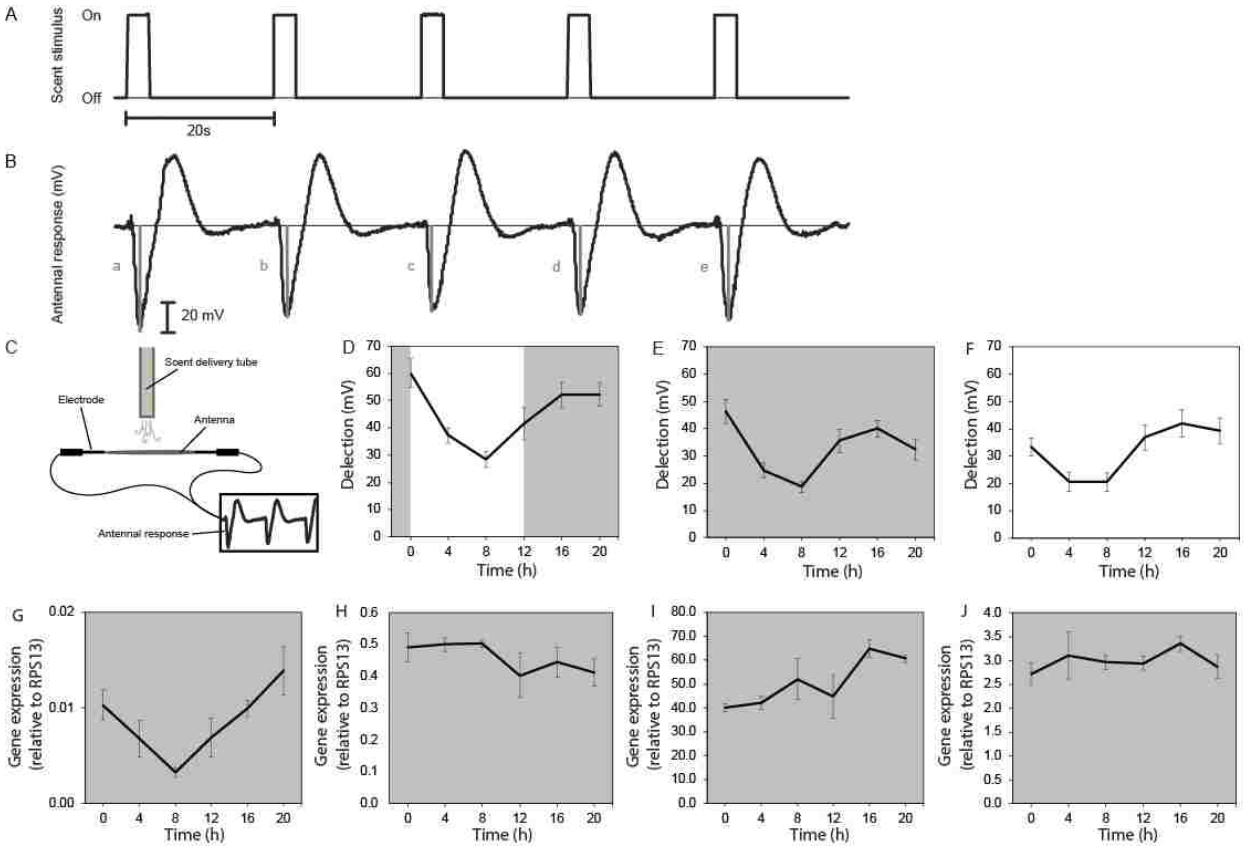

Fig. 5. *Manduca sexta* exhibit a circadian rhythm in antennal sensitivity to the floral odor benzaldehyde, regardless of light conditions. (*A*) For each electroantennogram, an antenna is subjected to a series of 5 brief scent pulses every 20 seconds. (*B*) The maximal deflection amplitude is recorded for each of the 5 scent pulses. Each data point is the mean of the 5 recorded deflection values (n = 12). (*C*) Illustration of electroantennogram setup (*D*) Benzaldehyde EAG in 12L:12D. (*E*) Benzaldehyde EAG in DD. (*F*) Benzaldehyde EAG in LL. (*G*) per expression, DD qPCR (*H*) Orco expression, DD qPCR (*I*) GOBP1 expression, DD qPCR (*J*) GOBP2 expression, DD qPCR.

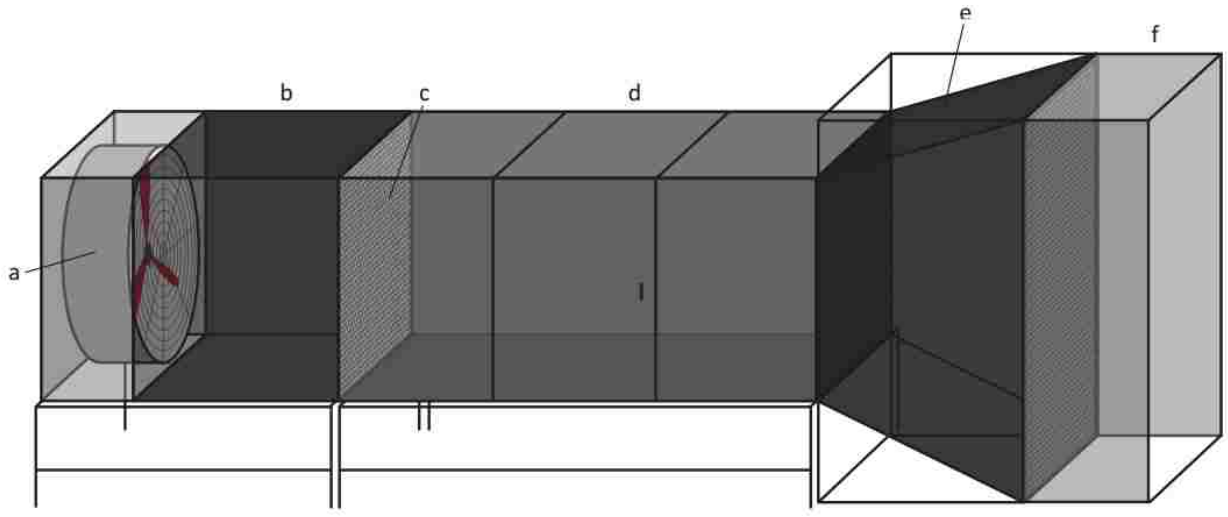

Fig. S1. Diagram of the wind tunnel apparatus used for all behavioral experiments. (*A*) Fan. (*B*) Diffuser. (*C*) Flow straightener. (*D*) Experimental chamber. (*E*) Contraction section. (*F*) Intake and flow straightener.

Summary and Conclusions

Time represents an important variable for many aspects of biological interactions. The expansive reach of the clock in regulating the behaviors in nearly all lifeforms allows for the synchronization of many factors at once, including communication between species. The work presented here investigates the role of the clock in regulating the attraction of pollinators by floral scent. In summary:

Circadian rhythms in floral scent release

Daily rhythms in plant behavior have been observed for hundreds of years, one of which is the emission of floral scent. Early empirical evidence can be found in Lillian Overland's work at the Missouri Botanical Gardens in 1960, where she reported a nocturnal scent release in *Cestrum nocturnum* (Night-blooming jasmine)[1]. Most interestingly, this nocturnal, 24 hour rhythm continued to occur every 24 hours even in continuous light. A limitation of Overland's study was the lack of available technology which could accurately quantify scent particles emitting from the flowers (Lillian and coworkers used their sense of smell to assign a reading of "odor" or "no odor".

The utilization of gas-chromatography mass-spectrometry allowed researchers to accurately identify scent compounds and quantify their rhythmic release, from organisms such as orchids[2,3], tobacco[4], etc.

Further technological advancement allowed for a mechanistic analyses of floral scent release, beginning with an understanding of genes involved in the metabolism of scent production. This research necessitated the use of model organisms, and species such as snapdragon, tobacco, and petunia were enlisted by scientists in the pursuit of mapping the metabolic pathways regulating scent emission. In the nocturnal-emitting *Petunia hybrida*, there has been a focus on characterizing the floral volatile benzenoid/phenylpropanoid (FVBP) pathway which produces most of the volatile mass emitted from the flowers[5-7]. A limited number of regulatory components had been identified prior to this dissertation's genesis, such as *ODORANT1* (*ODO1*)[8], *EMISSION OF BENZENOIDS I* (*EOBI*)[9] and *EMISSION OF BENZENOIDS II* (*EOBII*)[10].

Circadian clock regulation of floral scent metabolism

This dissertation began as an investigation into the circadian regulation of floral scent production in *Petunia hybrida*. An examination of the FVBP pathway in *P. hybrida* reveals that the regulators of that pathway express in 24 hour rhythms, such as *ODO1*. 3 clues to *ODO1*'s rhythmic expression can be found in its promoter region, which includes 3 predicted binding sites for the clock transcription factor LHY. LHY often acts as a negative repressor, and during its peak expression in the morning, LHY binds to promoters, restricting their expression to the evening. I hypothesized that LHY protein in Petunia was binding to the *ODO1* promoter during the morning, restricting the expression of ODO1 and genes it regulates to the evening, and thus causing the emission of scent to occur only during the night in *Petunia*.

In Chapter 2 of this dissertation, I identified and cloned a possible candidate for *LHY* in expressed sequence tags (ESTs). The candidate gene, labeled *Petunia hybrida LHY* (*PhLHY*) localized to the nucleus and expressed in a circadian rhythm with a peak in the morning[11]. Recent publications confirm that *CCA1*/*LHY* has only one orthologue in *Petunia*[12]. Constant expression of *PhLHY* through a *35S:PhLHY* construct led to clock arrhythmia, and a complete loss of scent emission[11]. An investigation of FVBP enzymatic and regulatory genes revealed that expression of many genes, including *EPSPS*, *PAL*, and *ODO1* were strongly reduced[11]. In transgenic plants with early expression peak for *PhLHY*, *Petunia* flowers emitted scent on an early schedule (afternoon), and *ODO1* and other FVBP genes had earlier peaks of expression[11]. PhLHY protein was also verified to physically bind to evening elements (EEs) on the promoter of *ODO1*. This study conclusively identifies the clock gene *LHY* as crucial component in setting the timing of scent metabolism and emission in *Petunia*. A paper published right after this chapter also shows evidence of *LHY* (and *ZTL*) regulation of floral scent release, as well as floral opening and positioning in the closely related species *Nicotiana attenuata*[13]. The genomes for *Petunia axillaris* and *Petunia integrifolia* were published soon after Chapter 2's publication, allowing for a holistic examination of *LHY* and other transcription factors' interactions with the FVBP pathway. Like many other studies in floral scent release, this project focused on the clock's genetic manipulation of FVBP

metabolism, rather than the physical aspects of emission. A recent publication identified an active transporter, *PhABCG1*, which when repressed by RNAi reduced volatile emissions by over 50%[14]. The identification of other transporters, and an analysis of their regulation could reveal if the clock also affects rhythmic emission of scent through active transport.

Circadian clock regulation of floral visitation

Many plant-pollinator interactions are known to occur during a certain window of time. *Petunia* and *Manduca* compose a famous nocturnal interaction in South America, which we hoped to understand the coordination of both from the plant perspective, as well as the pollinator's perspective. After verifying the role of LHY as a key regulator of timing for the plant's floral emissions, we set out to investigate the role of the pollinator's clock in its ability to interact with *Petunia*.

In Chapter 3, we found that male moths of *Manduca sexta* could distinguish between plants with clocks which were in sync with themselves and plants which were "jet-lagged" through different growth conditions or had altered clock pace by genetic manipulation. We found that the hawkmoth locomotor was regulated by the circadian clock but also strongly repressed by light. By emitting scent pulses in continuous dark during either subjective day and night, male moths were shown to exhibit a stronger response during subjective night. This led us to ask whether time-dependent moth response to floral scent was simply locomotor based or could be regulated by antennal sensitivity. We found that antennal sensitivity did oscillate in 24-hour rhythms regardless of light conditions (after entrainment to 12L:12D). Chapter 3 highlights the importance of both plant and insect clocks as a sometimes critical component of the plant-pollinator interaction, one by which mutation and evolution could potentially create or destroy individual relationships.

Further questions

Many questions remain unanswered and open to further study.

There are still interactions between the clock and the metabolic pathways regulating scent production in Petunia that remain undiscovered. Does LHY's interactions with Petunia's FVBP pathway resemble clock regulation of scent metabolism in other plants, especially in species outside of Solanaceae? Given that we now know that active transport[14], and not simply diffusion are responsible for scent release, could it be that the clock also regulates the physical emission of scent through these transporters?

In Chapter 3, we find that males moths of *M. sexta* show an increase in flight activity when exposed to floral scent, but not for vegetative scent. Would females behave differently, considering that they oviposit on *Petunia* tissues?

What is behind the circadian rhythm found in *M. sexta* electroantennogram responses to floral scent? In chapter 3, we find that the important odorant coreceptor Orco, perhaps the only known component in odorant reception shown to be widely conserved in plants, does not exhibit rhythmic mRNA expression in continuous dark. This is perhaps not surprising, as an oscillation in Orco's expression would cause a general repression of odorant reception, according to the current understanding of odorant reception in moths[15]. It is possible that changing sensitivity to floral scent is achieved the clock regulation of other components, such as odorant receptors (ORs) or odorant binding proteins (OBPs). At least we find that he general odorant binding proteins GOBP1 and GOBP2 do not oscillate in their mRNA expression patterns in continuous dark. Even if we find odorant reception components in *Manduca sexta* or *Drosophila melanogaster* tied to the clock, how relevant would this mechanism be for other insect species? As far as the neurological processing of scent information, does the brain attenuate signals from the antennae to regulate a behavioral response?

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