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*.

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.

**Significance**

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). *ORANT1 (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-enolpyruvyl-shikimate 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 EOB II, 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

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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. 1A) 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. 1B and C and Fig. S1 A and B). In continuous dark, volatile peak strength diminished rapidly (Fig. 1D 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, EOB1, and EOBII (10–12). ODO1 exhibits an evening peak (Fig. 1K), whereas EOB1 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 benzyl-CoA: benzylic alcohol/phenylethanol benzyloxyltransferase (BPBT) gene, which is expressed in a circadian fashion even in continuous light (Fig. S1L). 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., argenate 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. EOB1 and EOBII did not show any rhythmic expression patterns under these conditions, suggesting that daily rhythmic expression of EOB1 and EOBII could largely be regulated by light/dark transitions. Also, this indicates that the daily rhythmic expression of EOB1 and EOBII does not control the daily ODO1 expression, even
leaves ceased in the first day in the dark (Fig. 2 A and B). Expression profiles of PhLHY and PhGI in continuous light 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 to 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. SB). As the ODOI (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 (SLHY) (Fig. S2A). 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. S2C). Similar to the Arabidopsis counterpart, PhGI expression peaks in the evening, demonstrating an antiphase 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 and B). 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 < 0.02; 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. hybrid* *PSEUDO RESPONSE REGULATOR 5* (PhPRR5; Fig. S2D)] and 15 genes involved in the FVBP pathway in flowers (Fig. 4A–E and Fig. S5 A–M). The expression levels of the genes [PhGI, PhPRR5, ODO1, EPSPS, chorisimate 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 coexpression 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. 4P and Q) and also resulted in transgenic plants that exhibited silencing 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. 4F–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. 4R–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. 5A and B). The glutathione S-transferase (GST)-fused PhLHY protein specifically bound to 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. 5A). In addition, PhLHY also bound to the EEs in EPSPS and IGS promoters (Fig. S8B). We also tested the functional interaction of PhLHY on the EEs and CBS in the ODO1 promoter in vivo by using transient assay (Fig. 5C). We found that coexpression 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. 5D), 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. hybrid* 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. 1A; *P* < 0.05, daily expression and scent emission patterns of transgenic lines differ significantly from WT *Petunia*; two-way ANOVA).
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 the expression of key transcriptional regulators for many steps in the FVBP pathway (35). 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 FVBP (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-overexpressed 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 EOB1, EOB2, 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 mirrors 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 PhLHY is maintained in Petunia floral tissues in continuous dark (Fig. 2). These data clearly suggest that PhLHY may interact with the promoters of other FVBP genes (in perhaps both suppression and activation).

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**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).
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 up-stream area of the pathway responsible for precursor availability. It is also possible that PhLHY is up-regulating certain morning-peak FVBP genes, such as IG3. 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 fluxes 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/m²/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 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′-CACCTTGAAGACCCCTTACCT-3′ and 5′-GTCTCCCTGTAGAATTCACA-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/dTOPO 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. 2A 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) (1) 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 OD₆₀₀ of 0.4–0.6. The leaf tissue excisions and Agrobacterium were co-cultivated for 1 d on plates containing co-inoculation 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]. T₁ and T₂ generations were selected by their expression patterns of PhLHY, and T₃ and T₄ 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 T₃ and T₄ 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 samples 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 cm³/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 N₂ 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, EOB1, EOB2, CMI, ADT, CFAT, BPBT, BSMT1, BSMT2, EGS, EPSPS, IGS, KAT1, ODO1, PAAS, PhGI, and

Fenske et al. www.pnas.org/cgi/content/short/1422875112
Phylogenetic Analysis. Amino acid sequences for LHY, CCA1, GI, and PRR5 homologs were aligned by using ClustalW (7), and the phylogenetic trees were generated using Mesquite (8). 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 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 accelerated maximum likelihood (RAxML) (13), applying the JTT model of amino acid likelihood. The bootstrap values were calculated over 1,000 iterations. The final analysis and presentation was 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, Br030496; BrLHYb, Br033291; SILHLY, Solyc10g005080; PtLHY1, Potri.014g106800; PtLHY2, Potri.014g106800; PtLHY1, BA938935; CsLHY, AY611129; PtLHY1, JQ429143; OsCCA1, NM_001067567; PpCCA1a, AB548831; PpCCA1b, AB548832; PtLHY1, AY29402; SccIca, TA31430 4588 TA26762 4588; AGI, ATIG22770; BrGI, NP_001288824; PiGI, XP_002307516; OsGI, BAF04134; AtpPrr5, AT5G24470; PpAPPR5, NP_001288827; and CsPrr5, AB534647.

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 OD600 of 0.5 and resuspended in MES buffer [50 mM MES, pH 5.7, 2 mM NaH2PO4, 0.5% (wt/vol) glucose, 100 mM acetoxyribose]. 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 halogen lamps (EKE 21V150W; Ushio) filtered with a heat cut filter. For the transient LUC reporter assay in P. hybrida flowers (Fig. S5), we generated ODO1 promoter-driven firefly LUC-reporter (pODO1:LUC) and PhLHY effector 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′-CAGTCTTCAATGTAACTCCGCAG-3′ and 5′-CCTACTGACTTACGATCTACCAC-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 effector, we used pK7WG2:PhLHY plasmid. For a negative control, we used pK7WG2 plasmid containing GFP cDNA. The GFP cDNA was amplified by using 5′-CACCAGTGAAAGCCGAGGAGG-3′ and 5′-CTACTGTTGTCATGCTCACCAC-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 pRT2-2-Rluc (17) with HindIII, and ligated into the binary vector pPZP221 (18). Two- to three-day-old flowers were coinfected with Agrobacterium containing a reporter (pODO1:LUC or pODO1:LUC mEE+mCBS), an effector [35S:LHY (pK7WG2 LHY)], 35SS:GFP (pK7WG2 GFP), or nothing, and 35S:Rluc. In addition, the Agrobacterium transformant that possesses tomato stunt bushy virus silencing-silencer p19 plasmid (35SP19) was added to all coinfections (19). To prepare for the transfection, after growing each Agrobacterium transformant overnight, the appropriate combinations of the transformant cultures (adjusted OD600 to 1.0) were mixed, spun down, and resuspended in MES buffer [50 mM MES, pH 5.7, 2 mM NaH2PO4, 0.5% (wt/vol) glucose, 100 mM acetoxyribose]. After 1 h of incubation in the MES buffer, 0.1 mL of the Agrobacterium solution was injected into the corollas of flowers via needless syringe at zeitgeber time 12 (ZT12). 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 Victor3 V plate reader (Perkin–Elmer).

For the time-course LUC activity analysis in Petunia flowers (Fig. S6), P. hybrida cv. Mitchell (W115) flowers were coinfected with Agrobacterium transformants harboring combinations of pODO1:LUC, 35S:PhLHY, and 35S:GFP. All flowers also received the Agrobacterium transformant containing 35SP19
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 α-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'-TATCAAGATTCCGACCTTACTCCTCGGAGGAAAC-3' and 5'-ATCATAGCGGCCGCTTAAGTAGAAG

was collected by centrifugation and resuspended in the following buffer: 20 mM Hepes-KOH, pH 7.2, 80 mM KC1, 10% (vol/vol) glycerol, 0.1 mM EDTA, 0.1 mM PMSF, 2.5 mM DTT, and Pierce Phosphatase 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 KC1, 0.1 mM EDTA, 5% (vol/vol) glycerol, 2.5 mM DTT, 0.2 μg/mL-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]TATAACCTAATAAAAAATCATTGATAAAAAATTA-3' and the competitor sequences are 5'-TATAACCTAATAAAAAATTCGATGATAAAAAATTA-3' (mutated nucleotides are shown in bold) for *pODO1* mutated EE1, 5'-TATAACCTTATAAAAAATCATTGATAAAAAATTA-3' for the *pODO1* EE2, 5'-AAGAAAGATGGTAGATTITTTATATATTTAGG-3' for the *pODO1* CBS, 5'-ACTTAATTGTAGATATTTTCCATGAAAATTA-3' for the *pEPSPS* (accession no. CS050416) EE, and 5'-AAGAGAGAGAGAGATATTTAACCCAAAAAAA-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 0.25x TBE. Fluorescent gel images were obtained by using a Typhoon FLA 9000 Biomolecular Imager (GE Healthcare Life Sciences).


Fig. S1. (Continued)
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.
Fig. S2. (Continued)
Fig. S2. (Continued)
Fig. S2. (Continued)
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), and *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). PnPRR5 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.
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.)
Fig. S5. (Continued)
Fig. S5. (Continued)
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, 88, and 8C) 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.)
Constitutive expression of PhLHY through transient transformation of flowers suppresses \( pO.DO1:LUC \) in vivo. \( O.DO1 \) promoter-driven LUC (\( pO.DO1: \) LUC) is used as a reporter (Fig. 5C). \( pO.DO1:LUC \) 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 (\( n=8 \)). (*\( P<0.05 \), \( pO.DO1:LUC/35S:PhLHY \) is the only expression profile differing significantly from the one in \( pO.DO1:LUC \); 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).
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