Silencing Nicotiana attenuata LHY and ZTL alters circadian rhythms in flowers

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Summary

• The rhythmic opening/closing and volatile emissions of flowers are known to attract pollinators at specific times. That these rhythms are maintained under constant light or dark conditions suggests a circadian clock involvement. Although a forward and reverse genetic approach has led to the identification of core circadian clock components in Arabidopsis thaliana, the involvement of these clock components in floral rhythms has remained untested, probably because of the weak diurnal rhythms in A. thaliana flowers.
• Here, we addressed the role of these core clock components in the flowers of the wild tobacco Nicotiana attenuata, whose flowers open at night, emit benzyl acetone (BA) scents and move vertically through a 140° arc.
• We first measured N. attenuata floral rhythms under constant light conditions. The results suggest that the circadian clock controls flower opening, BA emission and pedicel movement, but not flower closing.
• We generated transgenic N. attenuata lines silenced in the homologous genes of Arabidopsis LATE ELONGATED HYOCOTYL (LHY) and ZEITLUPE (ZTL), which are known to be core clock components. Silencing NaLHY and NaZTL strongly altered floral rhythms in different ways, indicating that conserved clock components in N. attenuata coordinate these floral rhythms.

Introduction

Linnaeus (1751) designed a garden, known as the ‘flower clock’, comprising different plant species with unique flower opening and closing times. The opening of dandelion (Taraxacum officinale) flowers in his garden indicated morning, whereas the opening of Mirabilis dichotoma flowers meant that it was c.16:00 h in the afternoon. Many flowering plants also emit floral scents at specific times during the day. Centrum nocturnum (night-blooming jasmine) (Overland, 1960), Nicotiana sylvestris and N. suaveolens (Loughrin et al., 1991; Kolosova et al., 2001) emit a bouquet of floral scents at night, and Antirrhinum majus (snapdragon) flowers emit methyl benzoate in the afternoon (Kolosova et al., 2001). These famous examples show that flowering plants have characteristic rhythms which synchronize with environmental factors, such as the active times of their pollinators (Somers, 1999; Fründ et al., 2011). In addition, classical experiments have demonstrated the retention of floral rhythms under constant light (LL) or dark (DD) conditions, suggesting that an internal biological clock, called a circadian clock, regulates flower opening as well as the emission of floral volatiles (Bunning, 1956; Overland, 1960; Sweeney, 1963; Loughrin et al., 1991; Kolosova et al., 2001; Van Doorn & Van Meeteren, 2003; Vandenbrink et al., 2014). Nevertheless, LL conditions are not the normal environmental context in which the circadian clock functions, as pointed out by Vanin et al. (2012) for the Drosophila system. More recently, de Montaigu et al. (2014) demonstrated the importance of the day–night cycle transitions for understanding the function of the clock in nature using the Arabidopsis system.

The plant circadian clock has been studied intensively in the genetic model species Arabidopsis thaliana (Nagel & Kay, 2012). Forward and reverse genetic approaches have revealed that this circadian clock consists of transcriptional and post-translational feedback loops. In Arabidopsis, two morning-expressed MYB transcription factors, LATE ELONGATED HYOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), bind to the promoter of the evening component, TIMING OF CAB EXPRESSION 1 (TOC1, also called PSEUDO-RESPONSE REGULATOR 1, PRR1), to repress TOC1 transcription during the day (Alabadi et al., 2001). Near dusk, the positive regulator, REVEILLE8, induces the expression of TOC1 transcripts (Hsu et al., 2013), and TOC1 protein suppresses the expression of LHY and CCA1 transcripts, establishing a transcriptional negative feedback loop (Gendron et al., 2012; Huang et al., 2012). Post-translational regulation also fine-tunes the
plant circadian clock. ZEITLUPE (ZTL) protein physically binds to TOC1 and PRR5 proteins under dark conditions, resulting in the degradation of TOC1 and PRR5 proteins (Más et al., 2003a, b; Kiba et al., 2007; Kim et al., 2007).

The alteration of the expression of these circadian clock genes has produced arrhythmic or dysrhythmic plants; these plants show defects in development (Nagel & Kay, 2012) and defense (Wang et al., 2011b; Goodspeed et al., 2012). For instance, several daily rhythmic traits, such as stomata aperture, leaf movement and the expression of photosynthetic machinery, are altered in clock-altered lines (Yakir et al., 2007). In addition, hypocotyl elongation, flowering time, meristem circumnutation and biotic/abiotic defense are also regulated by the circadian clock, and have been examined using clock-altered lines (Niinuma et al., 2005; Wang et al., 2011a; Nagel & Kay, 2012; Seo et al., 2012; Vandenbrink et al., 2014). However, little is known about whether diurnal rhythms in flowers are regulated by the circadian clock whose molecular details are now known. Are the floral rhythms regulated by the known circadian clock components? This question is frequently noted in the literature (Van Doorn & Van Meeteren, 2003; Yakir et al., 2007; Nitta et al., 2010).

To examine the influence of the core clock components on floral rhythms, we used the wild tobacco N. attenuata, which shows strong diurnal rhythms in flowers and whose plant–pollinator interactions have been well studied (Kessler et al., 2008, 2010). Nicotiana attenuata produces self-compatible flowers which are visited by nocturnal hawkmoths (e.g. Manduca sexta) and day-active pollinators, such as hummingbirds (Kessler et al., 2010). Approximately 95% of N. attenuata flowers open at night; at this time, they emit a bouquet of volatiles, mainly benzyl acetone (BA) (Euler & Baldwin, 1996), which attracts nocturnal hawkmoths (Kessler et al., 2010). These floral rhythms are repeated for 2 or 3 d, and, if pollination occurs, corollas senesce, capsules develop and seeds mature.

In a previous study, we identified the N. attenuata LHY (NaLHY), NaTOC1 and NaZTL, which are the homologous proteins of Arabidopsis LHY, TOC1 and ZTL, respectively (Yon et al., 2012). The oscillating patterns of these genes under light–dark (LD) cycles and LL conditions are similar to those of Arabidopsis clock components. To corroborate the functional conservation of the clock components, we provided data meeting the standards established in the Arabidopsis system. We generated the overexpression lines of NaLHY and NaZTL transcripts in Arabidopsis; these lines had elongated hypocotyls and flowered late compared with wild-type (WT) plants, phenotypes that copy those of Arabidopsis LHY- and ZTL-overexpressing lines (Schaffer et al., 1998; Somers et al., 2004). In addition, we showed that TOC1–ZTL protein interactions in Arabidopsis are also conserved in N. attenuata. NaZTL protein binds NaTOC1 and Arabidopsis TOC1 proteins as well, indicating that NaLHY, NaTOC1 and NaZTL are functionally homologous proteins of Arabidopsis. In this study, we show that silencing of NaLHY and NaZTL alters the internal rhythm of N. attenuata and three main rhythms in N. attenuata flowers: scent emission, corolla opening and flower movement.

### Materials and Methods

#### Plant growth conditions

We used Nicotiana attenuata Torr. Ex. Wats. (Solanaceae) plants (30th inbred generation), which originated from a population in Utah, USA. Seeds were sterilized and germinated on Petri dishes with Gamborg’s B5 medium, as described in Krügel et al. (2002). Petri dishes with 30 seeds were kept under LD (16 h : 8 h, light : dark) conditions in a growth chamber (Percival, Perry, IA, USA) for 10 d, and seedlings were transferred to small pots (TEKU JP 3050 104 pots; Pöppelmann GmbH & Co. KG, Lohne, Germany) with Klassmann plug soil (Klassmann–Deilmann GmbH, Geesten, Germany) in the glasshouse. After 10 d, plants were transferred to 11 pots. The glasshouse growth conditions are described in Krügel et al. (2002). For the LD and continuous light (LL) treatment, two growth chambers (Microclima 1000; Snijders Scientific, Tilburg, the Netherlands) were maintained at similar temperature conditions (26°C with ±1°C variation) with the glasshouse conditions. To measure hypocotyl length, seedlings were grown on vertically oriented agar plates under constant dim light conditions for 10 d (5.3 μmol m⁻² s⁻¹).

We measured hypocotyl length using ImageJ software (http://rsb.info.nih.gov/ij/index.html).

The silencing of NaLHY and NaZTL in N. attenuata

A specific fragment of NaLHY (NCBI accession number JQ424913) and NaZTL (JQ424912) (Supporting Information Table S1) was independently inserted into the pSOL8 (for NaZTL) and pRESC8 (for NaLHY) transformation vectors as an inverted repeat (ir) construct driven by the cauliflower mosaic virus (CaMV) 35S promoter (Gase et al., 2011). These vectors were transformed into N. attenuata WT plants using Agrobacterium tumefaciens-mediated transformation, and diploid transformed lines were selected as described in Gase et al. (2011). Homozygosity was confirmed in T₂ plants by hygromycin resistance, and selected lines were transferred to the glasshouse for further analysis. Transformed WT plants with an empty vector (EV) were used as controls for the characterization of the transgenic lines. Gene expression levels of each silenced line were determined by quantitative real-time polymerase chain reaction (qPCR) from rosette leaf tissues of selected T₂ plants collected at ZT0 (ZT, zeitgeber time) for irLHY and at ZT12 for irZTL. Total RNA was extracted using the TRIzol reagent (Invitrogen, Germany) and 1 μg of total RNA of each sample was used to synthesize a single-strand cDNA with reverse transcriptase (Fermentas, Germany). qPCR was conducted with a Stratagene MX3005p instrument and SYBR Green kit (Eurogentec, Cologne, Germany); data were processed with the instrument’s MxPro software v.4.1 (Stratagene, La Jolla, CA, USA). The sequences of the primers used for qPCR (NaLHY-F, CACCTCTTTTCAAGGAAAGGT; NaLHY-R, GTCGAAGGTGTTACAAGGAC; NaTOC1-F, ATCGTAGAACGGCAGCACTT; NaTOC1-R, TCACAAAATGTCCTCCTCACA; NaZTL-F, CCCTATGGACTCGCTTCTGC; NaZTL-R, GCCAAGGAC...
TTCTTCGACAC; NaFKF1-F, ACAAGCCTACATGGAGAGAA; NaFKF1-R, CCTCTAAGTCAATCCTGTAT; NaCAB2-F, GCCGAAAGGCACTTGAAAC; NaCAB2-R, ACCGGGTCTGCAAGTATGTC) were designed by GENEIOUS (v.5.7.7, http://www.geneious.com). We employed ELONGATION FACTOR 1a (NaEF) as reference gene, using the primers: EF1a-F, CCACCTTCCCCACATTGCTGATC; EF1a-R, CGC ATGTCCTCACAGGAAAC. Finally, we selected two independent lines of the clock-silenced lines: irLHY404, irLHY-406 and irZTL-314, irZTL-318.

**Measurements of floral rhythms**

Flower position was recorded at 1-h acquisition intervals using a time-lapse imaging set-up, composed of a digital camera IXUS 400 (Canon, Tokyo, Japan) and its remote control software ZOOMBROWSER v.5.6 (Canon). Selected flowers in photographs were analyzed using the software IMAGE TOOLS v.3.0 (UTHSCSA, San Antonio, TX, USA) and TRACKER v.4.72 (Cabrillo College, Aptos, CA, USA). Flower angles were measured with reference to the horizontal axis. Flower opening was measured using excised flowers with 6–12 biological replicates for each measurement. Photographs were taken every 1 h using a time-lapse imaging set-up. To quantify the opening, the inner distance between opposite lobes was measured in pixels and converted to millimeters. To measure each parameter in LL conditions, first we removed all open flowers from the plant grown under LD conditions, except those flowers that would be opening the next day. We exposed these plants to LL conditions for 24 h and then measured the developments.

BA emission from the first opening flowers was measured in real time using a portable gas chromatograph, z-Nose™ 4200 (Electronic Sensor Technology, Newbury Park, CA, USA). To trap the headspace volatiles released from individual flowers, 50-ml plastic tubes (Falcon Plastics, Oxnard, CA, USA) were cut in half, and the upper parts with a cap were used, with a headspace volume of c. 9000 mm³. A single hole was made in a cap to introduce a needle into the headspace of flowers.

To measure BA emission in LL conditions, we first removed all flowers from plants grown under LD conditions, except those flowers that would be opening the next day. We exposed these plants to LL conditions for 24 h and then measured the BA emissions. In this experiment, we used polydimethylsiloxane (PDMS) to trap BA in the flower headspace for a period of 2 h, starting at ZT16 until ZT4. Samples were analyzed with a TD-20 thermal desorption unit (Shimadzu, Duisburg, Germany) connected to a quadrupole GC-MS-QP2010Ultra (Shimadzu). The PDMS trapping procedure is described in Kallenbach et al. (2014). Peak areas were integrated and the concentration was calculated on the basis of BA standards.

**Analysis of rhythms and statistical test**

The rhythmic parameters of gene expression, period and phase were measured using the ARSER algorithm (Yang & Su, 2010). ARSER first removes any linear trends from the data, determines the period of the expression data and provides rhythmic parameters using harmonic regression analysis. Three biological replicates were used for this analysis. The period values obtained for each line were compared with control EV (under LD or LL conditions) using paired T-tests.

For the flower movement rhythmic parameters, we also used the ARSER algorithm (Yang & Su, 2010). To calculate the amplitude of WT flower movement in plants under LD and LL conditions, flower angle data were divided into three parts (first, second and third day) and time-series data in each part were concatenated before the ARSER analysis. To measure the rhythmic parameters of the clock-silenced lines, flower angle data from the first and second days were used. After calculating each flower by the ARSER analysis, mean (± SE) values of each clock-silenced line were calculated.

All statistical tests were performed using R 3.1.2 (http://www.r-project.org/) and R-Studio (v.0.98.976, http://www.rstudio.com/).

**Results**

**Effect of silencing NaLHY and NaZTL on the internal rhythm in N. attenuata seedlings**

We silenced the transcriptional levels of NaLHY and NaZTL in N. attenuata by transforming plants with gene-specific ir constructs, and identified several independent lines, which displayed >90% silencing efficiency at the peak expression times of the targeted gene, NaLHY at ZT0 and NaZTL at ZT12 (Fig. S1a). We also measured the levels of NaFKF1 transcripts in NaZTL-silenced lines (irZTL) to check unwanted co-silencing of the paralogous gene (Yon et al., 2012) in irZTL plants. There was no reduction in NaFKF1 expression in irZTL plants (Fig. S1b). EV-containing plants were used to control for possible transformation effects, which were not observed. To examine the internal rhythm of these lines, we measured the transcript abundance of N. attenuata CHLOROPHYLL A/B BINDING PROTEINS 2 (NaCAB2) (Figgs 1, S2), which have been used frequently to determine the internal rhythms of the Arabidopsis clock-altered lines (Somers et al., 1998; Nagel & Kay, 2012). Seedlings were grown under 12 h : 12 h LD conditions for 12 d and subsequently exposed to LL conditions. We collected samples of EV, irLHY (NaLHY-silenced line)-404, irLHY-406, irZTL (NaZTL-silenced line)-314 and irZTL-318 plants every 4 h for 3 d under LD and LL conditions. As shown in Arabidopsis (Somers et al., 2000; Mizoguchi et al., 2002), the period under LD conditions of NaCAB2 in EV plants (23.97 ± 0.03 h) was not different from the period of the NaLHY clock-silenced lines (irLHY-404, 24.24 ± 0.13 h, P = 0.176; irLHY-406, 24.34 ± 0.12 h, P = 0.096) or the NaZTL lines (irZTL-314, 24.10 ± 0.05 h, P = 0.114; irZTL-318, 24.00 ± 0.10 h, P = 0.778). Under LL conditions, the period of NaCAB2 oscillation was shortened when silencing NaLHY (irLHY-404, 17.10 ± 0.58 h, P = 0.015; irLHY-406, 19.93 ± 0.65 h, P = 0.0095) compared with the period in EV plants (21.94 ± 0.14 h). Silencing NaZTL slightly but significantly lengthened the period of NaCAB2 in
In a previous study, we have shown that the ectopic overexpression of \textit{NaLHY} and \textit{NaZTL} in \textit{Arabidopsis} seedlings results in elongated hypocotyls compared with the hypocotyls of WT seedlings (Yon \textit{et al}., 2012). To test whether silencing of \textit{NaLHY} and \textit{NaZTL} alters hypocotyl length in \textit{N. attenuata}, we germinated the seeds under dim light conditions and, 10 d later, measured the hypocotyl lengths of these lines. Seedlings of \textit{irLHY} and \textit{irZTL} displayed significantly increased hypocotyl lengths compared with seedlings of EV plants (Fig. S1c).

**Expression of \textit{NaLHY} and \textit{NaTOC1} in flower tissues**

When \textit{N. attenuata} plants were grown under LD conditions and exposed to LL conditions, the leaf maintained the circadian rhythm of \textit{NaLHY} and \textit{NaTOC1} transcript levels peaking at near subjective dawn and dusk, respectively, at least for 2 d (Yon \textit{et al}., 2012). We examined the transcript levels of \textit{NaLHY} and \textit{NaTOC1} in corolla limbs and pedicels; the aperture and BARhythm of flowers occurs in corolla limbs (Euler & Baldwin, 1996) and the vertical movement of flowers is mediated by the pedicel. We collected 12 flowers among 30 plants per line every 2 h from ZT22 to ZT14 under LD conditions and LL conditions (plants were exposed to LL for 24 h before sampling). The peak times of \textit{NaLHY} and \textit{NaTOC1} in corolla limbs and pedicels were ZT0 and ZT12, respectively, under LD conditions (Fig. 2); these patterns were similar to the transcript rhythms in leaves under the same conditions (Yon \textit{et al}., 2012). However, we found that the rhythmic oscillation of \textit{NaLHY} and \textit{NaTOC1} transcripts in corolla limbs and pedicels was not perceptible under LL conditions (Fig. 2).

**Silencing of \textit{NaLHY} and \textit{NaZTL} alters flower opening**

To examine whether \textit{NaLHY} and \textit{NaZTL} regulate floral rhythms, first we examined the opening and closing of \textit{N. attenuata} flowers under LD and LL conditions. For the LL experiments, we exposed LD-grown plants to LL conditions, 24 h before the flowers opened. The distance between the junctions on a corolla limb was measured to quantify the opening and closing of flowers (Fig. 3a, inset). The flower started to open around ZT10 in plants under LD (16 h : 8 h) conditions, and fully opened by ZT14 before dusk (Fig. 3a). Fully opened flowers displayed white flattened corolla limbs during the night (Kessler \textit{et al}., 2010). The flowers rapidly closed within 1 h of dawn the next day, but stopped in a half-opened position, which they retained during the following day (Fig. 3a). Under LL conditions, the speed of flower opening in \textit{N. attenuata} remained unchanged, as shown in several flowering plants (Bunning, 1956; Overland, 1960; Van Doorn & Van Meeteren, 2003; Yakir \textit{et al}., 2007), although the opening time shifted to start earlier. Flowers from plants exposed to LL did not close well (Fig. 3a), suggesting that an
internal clock in *N. attenuata* mainly regulates floral opening, but not its closing. There was no significant morphological difference between EV and clock-silenced plants (Fig. S3).

Next, we analyzed the timing of flower opening and closing in irLHY and irZTL grown under LD conditions (Fig. 3b). Flowers in irLHY lines began opening 2 h earlier than did EV flowers and reached full opening 2 h earlier at ZT12 (Fig. 3b). Interestingly, irZTL flowers began opening at the same time as EV flowers, but did not open completely; they were 60–80% open compared with EV flowers, which were fully open (Fig. 3b). By the next morning, irLHY and irZTL flowers closed rapidly within 1 h as did EV flowers, but the closing patterns of these flowers differed among the lines (Fig. 3b). Under LL conditions, flower opening in irLHY and irZTL began c. 4 h earlier and 4 h later, respectively, than in EV flowers (Fig. S4). Like EV flowers, irLHY-silenced flowers also did not close under LL conditions, and fully opened flowers were not observed in irZTL-silenced lines (Fig. S4).

Silencing of *NaLHY* and *NaZTL* alters floral scent emission

*Nicotiana attenuata* flowers emit several volatiles to attract pollinators at night (Kessler & Baldwin, 2007). The most abundant attractant, BA, is released from fully opened flowers: its release begins near dusk and lasts until the middle of the night (Fig. 4; Kessler et al., 2010). This emission is repeated for 2–3 d (Bhattacharya & Baldwin, 2012), synchronized with flower opening/closing times. We first monitored BA emission every 2 h in the headspace of WT flowers under LD and LL conditions (Fig. 4a), suggesting that an internal clock regulates BA emission in *N. attenuata*.

To determine whether NaLHY or NaZTL regulates the emission of floral volatiles, we monitored BA emission from the *NaLHY* and *NaZTL*-silenced lines. BA emission from irLHY flowers started earlier, but also declined earlier, than did BA emission from EV flowers under LD (Fig. 4b) and LL conditions. BA emission was correlated with early opening phenotypes, suggesting that rhythms in irLHY flowers shift to earlier times than do rhythms in EV flowers. Interestingly, BA was barely emitted from irZTL flowers (Fig. 4b).
Silencing of NaLHY and NaZTL alters vertical movement of flowers

Nicotiana attenuata flowers have an additional interesting rhythmic trait. Flowers in N. attenuata maintain an upright position c. 40° from the horizontal axis before opening (Fig. 5a). In the morning of the first opening day, flowers move to face down at > 90° below the horizontal axis (Fig. 5a). These flowers return to the upright position just before dusk (Fig. 5a, inset), when they fully open and emit BA. By the next morning, flowers face down again and have closed their corollas. This vertical movement of flowers is repeated for 2–3 d under LD conditions, with a diminished movement in the third day.

To examine whether this rhythmic movement is independent of LD cycles, we exposed LD-grown flowering plants to constant light (LL) conditions, 24 h before flowers opened, and measured the angle of flowers for 3 d (Fig. 5a). Flowers exposed to LL conditions started to move downward at the same time as LD-grown flowers, but the amplitude of movement in LL-exposed flowers (first day, 39.2°; second day, 28.1°; third day, 6.0°) was reduced in comparison with that of flowers grown under LD conditions (first day, 73.8°; second day, 65.5°; third day, 28.0°). The maximum upward angle in LL-exposed flowers was similar to the maximum angle in flowers grown under LD conditions (Fig. 5a). This result suggests that an endogenous clock regulates flower movement in N. attenuata, but that light signals are also needed to finely adjust the amplitude of the movement.

To clarify whether core clock components control this movement, we measured the angle of flowers in EV, irLHY and irZTL. Silencing of LHY and ZTL strongly altered flower movements in different ways (Figs 5b, S6). The timing of the downward movement in irLHY lines during the first day was similar to the timing of the same movement in EV flowers, but irLHY flowers moved upward c. 2 h earlier than did EV flowers; in addition, they showed a reduced amplitude of flower movement (Fig. 5b). This earlier vertical movement was associated with flower opening and initial scent emission occurring 2 h earlier in irLHY plants than in EV plants. The period of movement in irLHY flowers (22.4 ± 0.2 h) for the first 2 d was significantly shorter than that in EV flowers (23.5 ± 0.1 h, P < 0.05, one-way ANOVA followed by Bonferroni post-hoc tests). An alteration of the movement was also observed in irZTL lines; downward movement was almost abolished, but plants retained the weak diurnal pattern for the first 2 d (Fig. 5b). We also transferred LD-grown EV, irLHY and irZTL plants to LL conditions 24 h before we began to measure the flower angle. irZTL flowers did not show any vertical movement under LL conditions (Fig. S6a). Interestingly, silencing NaLHY abolished the vertical movement of flowers under LL conditions (Fig. S6a), but flower opening and BA emissions were maintained (Figs S4, S5).

Discussion

Following the first scientific report in 1729 that daily leaf movement in mimosa was retained under constant dark conditions, several daily rhythms in plants have been examined (McClung, 2006). Diurnal rhythms in flowers are one of the most popular examples known to both chronobiologists and nonscientists. Many reports, including time-lapse movies and nature documentaries, demonstrate that internal clocks regulate floral rhythms. However, these interesting floral rhythms have not been re-examined after the core circadian clock components were identified at a molecular level. Perhaps floral traits were not examined in clock-altered lines because of the lack of the strong floral rhythms in model plants. Here, we revisit a set of floral traits previously thought to be under circadian control with N. attenuata plants, which show three circadian rhythms in flowers. We provide fresh new evidence of the hypothesis that rhythmic traits in flowers are regulated by the circadian clock components at a molecular level. Whilst this paper was in

Fig. 5 Silencing of NaLHY and NaZTL alters vertical movement in flowers. (a) Mean (± SE) angles of flowers in Nicotiana attenuata wild-type plants under 16 h : 8 h, light : dark (LD) and constant light (LL) conditions. Flower movement is initiated in the morning of the first opening day and repeated over 2–3 d. Flower photographs were taken at six different times in a day and merged after removing background colors using Adobe Photoshop. (b) Mean (± SE) angle of flowers in empty vector (EV), irLHY and irZTL plants grown under LD conditions. LHY, LATE ELONGATED HYPOCOTYL; ZTL, ZEITLUPE; ZT, zeitgeber time.
review, a paper was published showing that the ectopic expression of petunia LHY (PhLHY) suppresses the emission of petunia floral volatiles (Fenske et al., 2015), a result that is similar to our observations with irZTL lines, which lacked BA emissions completely (Fig. 4). In addition, silencing of PhLHY results in the early emission of petunia floral volatiles (Fenske et al., 2015), a result again consistent with that shown here in N. attenuata irLHY lines, which also displayed early emissions (Fig. 4). These results suggest that the roles of the circadian clock in flowers are conserved, at least in the Solanaceae family.

Internal rhythms have been determined by the expression patterns of genes, such as CAB2, under LL conditions (Nagel & Kay, 2012). For instance, CAB2 periods in Arabidopsis lhy-12 and TOC1 RNAi plants were shorter than their periods in WT plants under LL conditions (Strayer et al., 2000; Mizoguchi et al., 2002; MÁS et al., 2003a), and the ztl-1 mutation lengthens the period of CAB2 expression under LL conditions (Somers et al., 2000). However, these patterns do not always correlate with important phenotypes, such as flowering time and hypocotyl length (Niwa et al., 2009), in different A. thaliana accessions and mutants grown under red light or LD conditions. Flowers in irLHY plants lost their rhythmic vertical movement under LL conditions (Fig. S6), whereas the period of NaCAB2 in irLHY seedlings was only shortened under LL conditions (Fig. 1). In addition, the floral phenotypes of irZTL plants were not well explained by the internal rhythms defined under LL conditions (Fig. 1). Internal rhythms in irZTL plants were slightly different from the internal rhythms of control plants. This difference might be a result of the incomplete silencing of NaZTL expression in transgenic lines which alters the internal rhythms in seedlings under LL conditions. An alternative explanation is that NaCAB2 expression does not fully reflect the internal rhythms of N. attenuata seedlings. However, the three circadian rhythms in N. attenuata flowers were almost completely abolished in irZTL flowers under LD and LL conditions, and transcript levels of NaLHY and NaTOC1 were strongly altered in irZTL flowers (Fig. S1), suggesting that silencing of NaZTL, even if incomplete, is sufficient to alter the circadian rhythms in flowers. Taken together, internal rhythms defined by the expression of a reporter gene in a single tissue are unlikely to fully explain the complex interactions between traits and the circadian clock (Niwa et al., 2009; Nagel & Kay, 2012).

In our previous study, the ectopic expression of NaLHY and NaZTL in Arabidopsis increased the hypocotyl length, which is similar to that in Arabidopsis LHY- and ztl-overexpressing lines (Yon et al., 2012). From these results, we expected that silencing of NaLHY and NaZTL would inhibit hypocotyl growth in N. attenuata. However, the hypocotyl length in irLHY and irZTL plants was longer than that in control plants (Fig. S1b). We hypothesize that NaLHY and NaZTL interact with the hypocotyl growth of N. attenuata in a different way than in Arabidopsis Columbia ecotype (Hall et al., 2003; Mizoguchi et al., 2005).

Peak times of Arabidopsis LHY and TOC1 transcript levels in leaves and roots (Schaffer et al., 1998; Strayer et al., 2000; James et al., 2008) are also well conserved in N. attenuata leaves and roots under LD conditions (Yon et al., 2012). In corolla limbs and pedicels, NaLHY and NaTOC1 transcripts also peaked at dawn (ZT0) and near dusk (ZT12), respectively, under LD conditions (Fig. 2). When we exposed LD-grown N. attenuata seedlings to LL conditions, the peak times of NaLHY and NaTOC1 transcript levels in the seedlings were maintained at least for 3 d under LL conditions, as reported in Arabidopsis seedlings (Millar et al., 1995; Schaffer et al., 1998; Yon et al., 2012). In the corolla limbs and pedicels, however, the oscillations of NaLHY and NaTOC1 transcript levels were quickly altered when LD-grown flowers were exposed to LL conditions. These results suggest that the circadian system in different parts of N. attenuata may have different sensitivities and responses to the non-natural LL conditions. Given the specific function of each tissue/organ, as well as its sensitivity to external stimuli, gating effects can be perceived asymmetrically in each tissue (Thain et al., 2002; Endo et al., 2014). Having different clocks or differential clock sensitivity in different plant parts can be advantageous in nature, given that each organ is located in different microenvironments, below- or aboveground, for example, requiring a differential fine-tuning to synchronize their functions to their particular environment. We hypothesize that flowers should be particularly sensitive in order to function and protect this organ from environmental insults, such as is seen in the rapid closing response of Gentiana algida flowers to sudden storm fronts, and their rapid reopening when conditions are again benign (Bynum & Smith, 2001).

Most insect-pollinated flowers have evolved special traits to attract pollinators (Raguso, 2004), including the ability to synchronize floral rhythms with times at which pollinators are active (Van Doorn & Van Meeteren, 2003). In nature, N. attenuata mainly produces night-opening flowers, which are synchronized with night-active pollinators, M. sexta moths (Kessler et al., 2010). The downward-facing movement of N. attenuata flowers probably prevents nectar from desiccating during the day in its native habitats, in particular the Great Basin Desert, Utah, USA, and the upward-facing movement might increase the accessibility of M. sexta moths during the night. In this study, we show that silencing of the conserved clock components altered the circadian rhythms in flowers, which sustain the pollination services mediated by insects for many wild plants as well as in domesticated crops (Potts et al., 2010). We conclude that the circadian clock in flowers is the ‘battery’ that makes the hands of Linnaeus’s multispecies ‘flower clock’ tick.

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Author contributions
I.T.B. and S-G.K. designed the research and conceived the project. F.Y., Y.J., S-G.K., L.C.L. and E.R. screened and characterized the transgenic lines and performed the experiments. F.Y., Y.J. and L.C.L. analyzed the data. F.Y., Y.J., L.C.L., I.T.B. and S-G.K. wrote the manuscript.

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### Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Silencing efficiency and hypocotyl length of irLHY and irZTL lines.

**Fig. S2** Protein alignment of CHLOROPHYLL A/B BINDING PROTEINS 2 (CAB2) orthologs in *Nicotiana attenuata* and Arabidopsis thaliana.

**Fig. S3** Flower morphology of the clock-silenced lines.

**Fig. S4** Flower opening in the clock-silenced lines under constant light (LL) and light–dark (LD) conditions.

**Fig. S5** Benzyl acetone emission in the clock-silenced flowers.

**Fig. S6** Vertical movement in the clock-silenced flowers under constant light (LL) and light–dark (LD) conditions.

**Table S1** Insertion fragments of irLHY and irZTL silenced lines

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