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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 HYPOCOTYL* (*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. *Cestrum 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 HYPOCOTYL (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 (Alabadí *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 Klasmann plug soil (Klasmann–Deilmann GmbH, Geesten, Germany) in the glasshouse. After 10 d, plants were transferred to 1 l 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 T_2 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 T_2 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, CACTCTTTTCAAGGAAGGTG; NaLHY-R, GTCGAAGGTG TTACAAGAGC; NaTOC1-F, ATCGTAGAACGGCAGCAC TT; NaTOC1-R, TCACAAACTGTCCCCTCACA; NaZTL-F, CCCTATTGACTCGCTTCTGC; NaZTL-R, GCCAAGGAC

TTCTTCAGCAC; NaFKF1-F, ACAAGCCTACATGGAGAG AA; NaFKF1-R, CCTCCAAGTCAATCGTGTAT; NaCAB2-F, GCCGGAAAGGCAGTGAAAC; NaCAB2-R, ACCGGGTCT GCAAGATGATC) 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, CCACACTTCCCACATTGCTGTCA; EF1a-R, CGC ATGTCCCTCACAGCAAAAC. 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 made the measurements.

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 mm^3. 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 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 calculations of 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 transcript 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*) (Figs 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

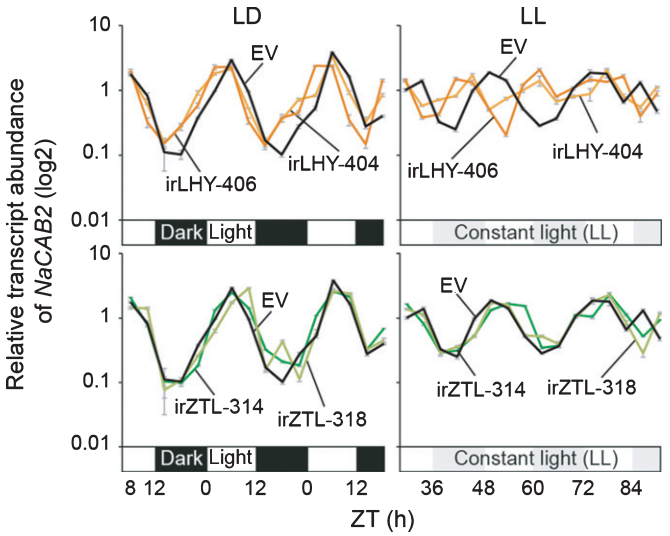

Fig. 1 Effect of silencing *NaLHY* and *NaZTL* on the internal rhythms in seedlings. Mean (± SE) transcript accumulation of *CAB2* in *Nicotiana attenuata* seedlings of empty vector, irLHY-406, irLHY-404, irZTL-314 and irZTL-318 grown under 12 h : 12 h, light : dark (LD) conditions, and seedlings in the same growth conditions but then exposed to constant light (LL) conditions. Seedlings were harvested every 4 h for 3 d. The relative transcript abundance of *NaCAB2* was divided by the transcript abundance of the ELONGATION FACTOR (EF) gene, normalized and linear detrended. Gray boxes indicate the subjective dark period of LL conditions. LHY, LATE ELONGATED HYPOCOTYL; ZTL, ZEITLUPE; EV, plant transformed with the empty vector used to generate transgenic lines; irLHY, *NaLHY*-silenced line; irZTL, *NaZTL*-silenced line; CAB2, CHLOROPHYLL A/B BINDING PROTEINS 2; ZT, zeitgeber time.

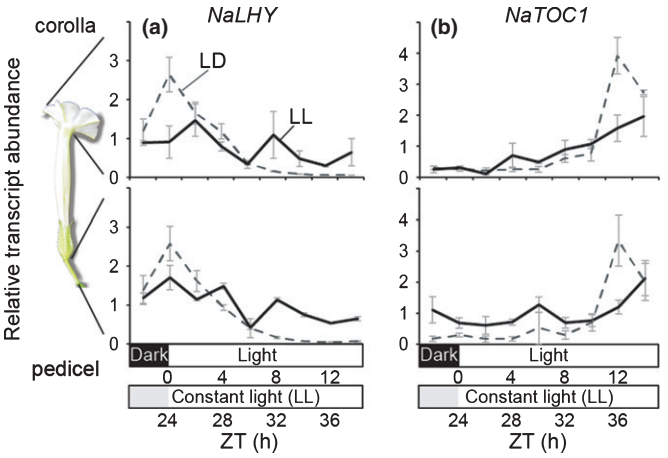

Fig. 2 *NaLHY* and *NaTOC1* transcript expression in corolla limbs and pedicels. Mean (± SE) levels of transcript accumulation of (a) *NaLHY* and (b) *NaTOC1* in corolla limbs and pedicels of *Nicotiana attenuata* under 16 h : 8 h, light : dark (LD) and constant light (LL) conditions. The relative transcript abundance of both genes was divided by the transcript abundance of the ELONGATION FACTOR (EF) gene. LHY, LATE ELONGATED HYPOCOTYL; TOC1, TIME OF CAB1 EXPRESSION; ZT, zeitgeber time.

comparison with EV plants (21.94 ± 0.14 h) under LL conditions (irZTL-314, 23.44 ± 0.05 h, $P = 0.01$; irZTL-318, 23.74 ± 0.26 h, $P = 0.009$). To further clarify the alteration of internal rhythms in irZTL plants, we measured the levels of *NaLHY* and *NaTOC1* transcripts in irZTL-314. As shown in Arabidopsis *ztl* mutants (Somers *et al.*, 2004), silencing *NaZTL* reduced the amount of *NaLHY* and *NaTOC1* transcripts under LD conditions (Fig. S1c).

In a previous study, we have shown that the ectopic overexpression of *NaLHY* and *NaZTL* in Arabidopsis seedlings results in elongated hypocotyls compared with the hypocotyls of WT seedlings (Yon *et al.*, 2012). To test whether silencing of *NaLHY* and *NaZTL* alters hypocotyl length in *N. attenuata*, we germinated the seeds under dim light conditions and, 10 d later, measured the hypocotyl lengths of these lines. Seedlings of irLHY and irZTL displayed significantly increased hypocotyl lengths compared with seedlings of EV plants (Fig. S1d).

Expression of *NaLHY* and *NaTOC1* in flower tissues

When *N. attenuata* plants were grown under LD conditions and exposed to LL conditions, the leaf maintained the circadian rhythm of *NaLHY* and *NaTOC1* transcript levels peaking at near subjective dawn and dusk, respectively, at least for 2 d (Yon *et al.*, 2012). We examined the transcript levels of *NaLHY* and *NaTOC1* in corolla limbs and pedicels; the aperture and BA emission 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 *NaLHY* and *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 *et al.*, 2012). However, we found that the rhythmic oscillation of *NaLHY* and *NaTOC1* transcripts in corolla limbs and pedicels was not perceptible under LL conditions (Fig. 2).

Silencing of *NaLHY* and *NaZTL* alters flower opening

To examine whether NaLHY and NaZTL regulate floral rhythms, first we examined the opening and closing of *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 *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 *N. attenuata* remained unchanged, as shown in several flowering plants (Bunning, 1956; Overland, 1960; Van Doorn & Van Meeteren, 2003; Yakir *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

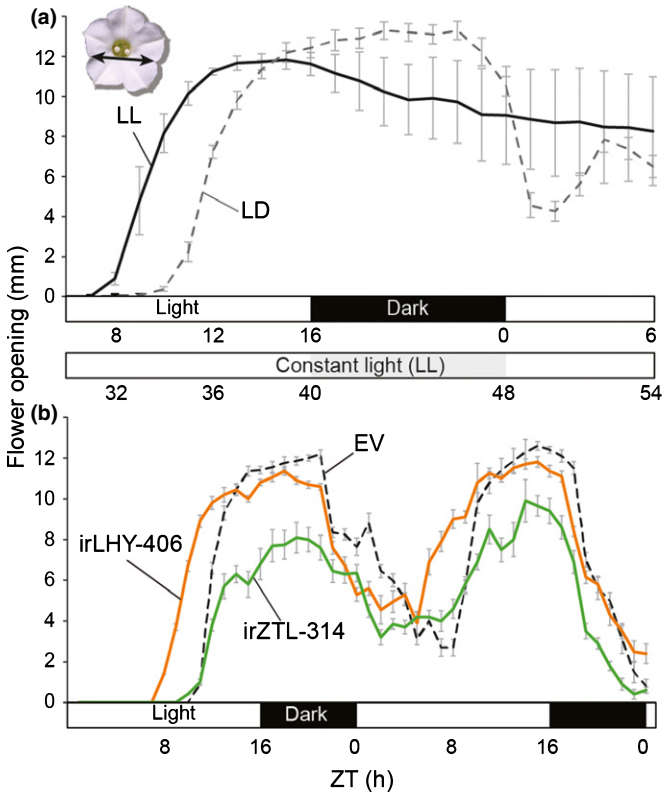

Fig. 3 Silencing of *NaLHY* and *NaZTL* alters flower opening. (a) Mean (± SE) distance between petal junctions on corolla limbs of wild-type *Nicotiana attenuata* plants under 16 h : 8 h, light : dark (LD) conditions and constant light (LL) conditions. We exposed LD-grown flowering plants to LL conditions for 24 h and measured the flower aperture. (b) Mean (± SE) distance between petal junctions on corolla limbs of empty vector (EV), irLHY-406 and irZTL-314 plants grown under LD conditions. LHY, LATE ELONGATED HYPOCOTYL; ZTL, ZEITLUPE; ZT, zeitgeber time.

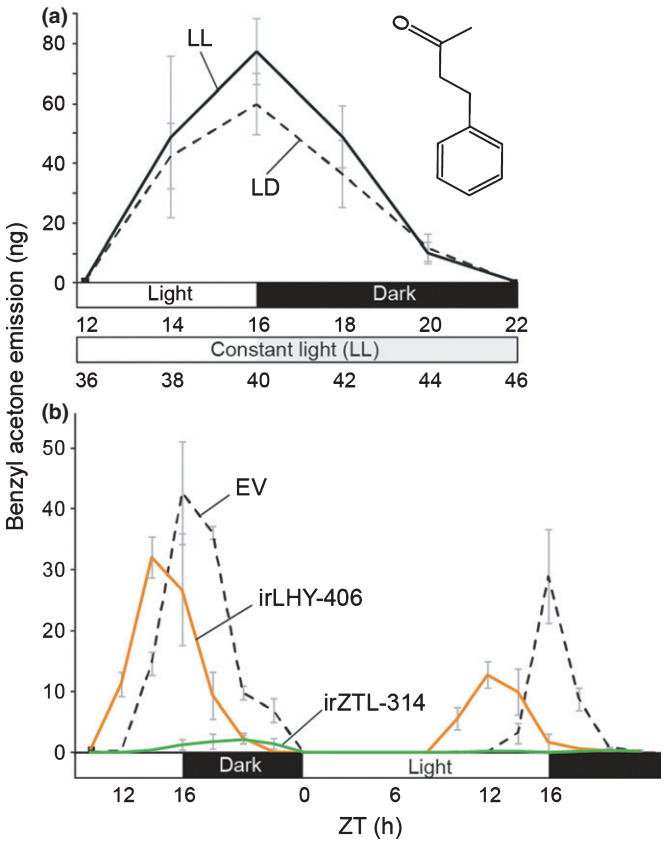

Fig. 4 Silencing of *NaLHY* and *NaZTL* alters the emission of attractive floral volatile, benzyl acetone (BA), from flowers. (a) Mean (± SE) levels of BA emission from *Nicotiana attenuata* wild-type plants under 16 h : 8 h, light : dark (LD) and constant light (LL) conditions. We exposed LD-grown flowering plants to LL conditions for 24 h and then measured BA emission using a z-Nose™ instrument for real-time measurements. (b) Mean (± SE) levels of BA emission from flowers in empty vector (EV), irLHY and irZTL plants grown under LD conditions. LHY, LATE ELONGATED HYPOCOTYL; ZTL, ZEITLUPE; ZT, zeitgeber time.

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 *c.* 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 using a z-Nose™ instrument for real-time measurements. The pattern of BA emission from flowers under LL conditions was similar to the pattern of BA emission from flowers under LD 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 (Fig. S5) 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).

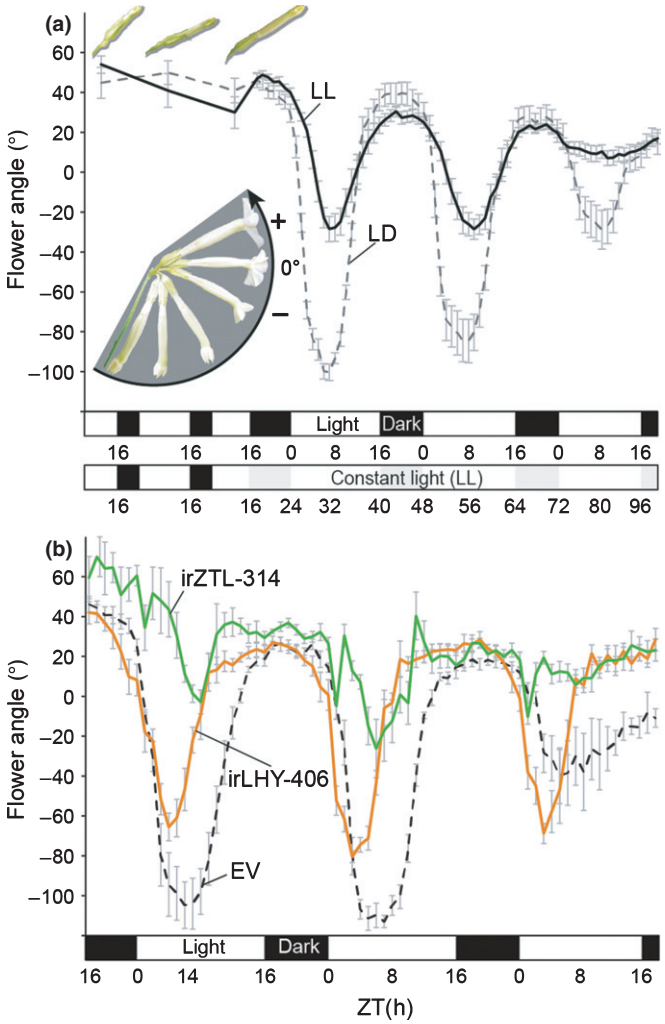

Fig. 5 Silencing of *NaLHY* and *NaZTL* alters vertical movement in flowers. (a) Mean (± SE) angles of flowers in *Nicotiana attenuata* wild-type plants under 16 h : 8 h, light : dark (LD) and constant light (LL) conditions. Flower movement is initiated in the morning of the first opening day and repeated 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.

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

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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