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Herbivory elicits changes in green leaf volatile production via jasmonate signaling and the circadian clock

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Abstract

The timing of plant volatile emissions is important for a robust indirect defense response. Green leaf volatiles (GLVs) are emitted by plants upon damage but can be suppressed by herbivore-associated elicitors, and the abundance and composition of GLVs vary depending on the timing of herbivore attack. We show that the GLV biosynthetic enzyme *HYDROPEROXIDE LYASE* (*HPL*) is transcriptionally regulated by the circadian clock in *Nicotiana attenuata*. In accordance with transcript abundance of *NaHPL*, GLV aldehyde pools in intact leaves peaked at night and at subjective night under diurnal and continuous light conditions, respectively. Moreover, although the basal abundance of *NaHPL* transcripts is upregulated by jasmonate (JA) signaling, JA does not regulate the reduction of *NaHPL* transcript abundance in damaged leaves by simulated herbivore treatment. Unexpectedly, the plant circadian clock was strongly altered when *Manduca sexta* larvae fed on *N. attenuata*, and this was also independent of JA signaling. Lastly, the temporal dynamics of *NaHPL* transcripts and total GLV emissions were strongly altered by *M. sexta* larval feeding. Our data suggest that the temporal dynamics of emitted GLV blends result from a combination of damage, JA signaling, herbivore-associated elicitors, and the plant circadian clock.

KEYWORDS

green leaf volatiles (GLVs), herbivore-associated elicitors, *HYDROPEROXIDE LYASE* (*HPL*), jasmonate signaling, *Manduca sexta*, *Nicotiana attenuata*, plant circadian clock, plant–herbivore interaction

1 | INTRODUCTION

Plants emit complex blends of volatile compounds that can help them adapt to biotic and abiotic stresses and provide information to mutualists like pollinators and the natural enemies of herbivores (Dicke & Baldwin, 2010; Hare, 2011). Plant volatile blends mainly consist of terpenoids, aromatics, and fatty acid or branched chain amino acid derivatives (Baldwin, 2010; Dudareva et al., 2013). Among the plant volatiles, green leaf volatiles (GLVs) are a group of fatty acid-derived compounds released immediately upon leaf damage from most plants, and in large amounts (μg/g FM) (Ameye et al., 2017). GLVs can attract predators or parasitoids of herbivores (Shiojiri, Ozawa, & Takabayashi, 2006; Halitschke, Stenberg, Kessler, Kessler, & Baldwin, 2008), have antimicrobial activity (Croft, Juttner, & Slusarenko, 1993), and prime plant defense (Engelberth, Alborn, Schmelz, & Tumlinson, 2004). GLVs are formed from lipids through sequential enzymatic steps in the oxylipin pathway by lipase, lipoxygenase (LOX), and hydroperoxide lyase (*HPL*; Matsui, 2006). One of the most abundant GLVs, (*Z*)-3-hexenal, originates from the cleavage of *α*-linolenic acid through the activity of *HPL* and is converted in part to (*E*)-2-hexenal. GLV aldehydes can be further metabolized by alcohol dehydrogenase (Fauconnier et al., 1999) or nicotinamide adenine dinucleotide phosphate-dependent reductase (Matsui, Sugimoto, Mano, Ozawa, & Takabayashi, 2012; Tanaka et al., 2018) and alcohol acyltransferase (D'Auria, Pichersky, Schaub, Hansel, & Gershenzon, 2007) to the alcohol and ester forms of GLVs.

The *HPL* gene has been reported to be stress-inducible (Xiao et al., 2012); mechanical wounding strongly increases transcript abundance of *HPL* in many plant species (Bate et al., 1998; Gomi, Yamasaki, Yamamoto, & Akimitsu, 2003; Howe, Lee, Itoh, Li, & DeRocher, 2000) and methyl jasmonate treatment also increases the abundance of *HPL* transcripts in *Arabidopsis thaliana* (Brassicales: Brassicaceae) (Matsui, Wilkinson, Hiatt, Knauf, & Kajiwara, 1999). In contrast, herbivory suppresses wound-induced GLV emissions and transcript abundance of *HPL* in *Nicotiana attenuata* (Solanales: Solanaceae; Gaquerel, Weinhold, & Baldwin, 2009; Halitschke, Ziegler, Keinänen, & Baldwin, 2004). In *A. thaliana*, *HPL* transcripts are suppressed by the attack from *Pieris rapae* but not by *Spodoptera exigua* (Savchenko, Pearse, Ignatia, Karban, & Dehesh, 2013). This suggests that herbivory-associated suppression of *HPL* transcript accumulation can be herbivore-specific. Moreover, the effect of herbivory on *HPL* transcript abundance is time-dependent: herbivore-associated elicitor treatments increased the transcript abundance of *HPL* after 15 min and then reduced their abundance until 2 hr posttreatment in *N. attenuata* (Halitschke et al., 2004). A recent study showed that stress-induced accumulation of *HPL* transcripts is mediated by methylerythritol cyclodiphosphate (MEcPP) in *A. thaliana*, an isoprenoid precursor from the methylerythritol 4-phosphate (MEP) pathway (Xiao et al., 2012).

Emission of plant volatiles varies throughout the day and different compounds may be emitted with different rhythms (Turlings & Erb, 2018). The timely emission of specific herbivore-induced plant volatiles determines the effectiveness of the volatile-mediated indirect defense response (Joo et al., 2018), oviposition behavior of conspecific insects (de Moraes, Mescher, & Tumlinson, 2001), and larval feeding activity (Shiojiri, Kishimoto, et al., 2006). Many factors can affect the temporal dynamics of plant volatile emissions. For instance, light differentially regulates two biosynthetic pathways of terpenoid volatiles in plants: light activates the MEP pathway and suppresses the mevalonic acid pathway (Paré & Tumlinson, 1997; Rodríguez-Concepción 2006; Pokhilko et al. 2015). The plant circadian clock also regulates the timing of plant volatile emissions (Dudareva, Klempien, Muhlemann, Kaplan, Muhlemann, & Kaplan, 2013 et al. 2005; Fenske et al. 2015; Yon et al., 2016).

The circadian clock is thought to help plants anticipate environmental changes (Greenham & McClung, 2015). Plant metabolism is synchronized with environmental rhythms (Wijnen & Young, 2006) and around 30% of expressed genes in *A. thaliana* have circadian patterns of transcript accumulation (Covington, Maloof, Straume, Kay, & Harmer, 2008; Harmer et al., 2000; Pan et al., 2009). The circadian clock can increase plant Darwinian fitness by coordinating plant metabolism with environmental factors, for example, light/dark cycles (Dodd et al., 2005), herbivory (Goodspeed, Chehab, Min-Venditti, Braam, & Covington, 2012), and pollination (Atamian et al., 2016; Yon, Kessler, Joo, Kim, & Baldwin, 2017). Emission of GLVs is strongly affected by the timing of herbivore attack (Joo et al., 2018). The GLV biosynthetic genes *LOX* and *HPL* also have diurnal rhythms of transcript abundance in several plant species (Allmann, Halitschke, Schuurink, & Baldwin, 2010; Christensen et al., 2015; Pan et al., 2009). Moreover, the transcript abundance of the *HPL* gene shows a strong circadian rhythm in *A. thaliana* (Mizuno & Yamashino, 2008; Pan et al., 2009).

Although the costs and benefits of volatile emission for plants depend on the specific volatile blends and the ecological context (Allmann & Baldwin, 2010; Kessler, Diezel, Clark, Colquhoun, & Baldwin, 2013; Webster, Gezan, Bruce, Hardie, & Pickett, 2010), the timing of GLV production can be an important factor for plant indirect defense. However, it is not known how temporal regulation interacts with herbivore induction to affect GLV production, nor has the role of the circadian clock in this process been investigated. Here, we used the wild tobacco *N. attenuata* and its specialist herbivore *Manduca sexta* to understand how plants shape the temporal dynamics of GLV biosynthesis and release in response to herbivore attack. First, we asked whether the circadian clock regulates GLV biosynthesis and emission. We then analyzed the influence of herbivory on GLV biosynthesis. Lastly, we investigated interactive effects of the circadian clock and herbivory on GLV biosynthesis and emission.

2 | MATERIALS AND METHODS

2.1 | Plant materials

The wild-type (WT) inbred line of *N. attenuata* originated from a collection at the DI ranch in southwestern Utah, United States of America, and was inbred for 31 generations. Homozygotes of the third transformed generation of lines harboring a single RNAi construct (inverted-repeat [ir] or antisense [as]), irLHY (LATE ELONGATED HYPOCOTYL, line A-11-406-3), irAOC (ALLENE OXIDE CYCLASE, line A-07-457-1), asHPL (line A-04-428), or irLOX2 (line A-04-52-2), and the empty vector (EV) control (pSOL3, line A-04-266-3) were used for experiments. All transgenic lines were previously described and screened in comparison with multiple lines harboring the same construct (Allmann et al., 2010; Halitschke et al., 2004; Kallenbach, Bonaventure, Gilardoni, Wissgott, & Baldwin, 2012; Yon et al., 2016); the EV line has been used and shown to have a WT phenotype in volatile emission and other traits over several years of field studies, for example (Kessler, Gase, & Baldwin, 2008; Schuman, Barthel, & Baldwin, 2012). Petri dishes with 30 seeds were kept under light/dark cycle (LD; 16 hr light and 8 hr dark) conditions in a growth chamber (Percival, Perry, Iowa, USA) for 10 days, 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) for further experiments.

2.2 | Herbivory treatments

To test for effects of herbivore-associated elicitors, plants were treated with wounding and distilled water (W) or diluted regurgitant (R) of *M. sexta* larvae as a standardized means to mimic feeding damage by *Manduca* spp. (Halitschke, Schittko, Pohnert, Boland, & Baldwin, 2001). Regurgitant was collected from 3rd–4th instar *M. sexta* larvae feeding on WT or EV *N. attenuata* plants in the glasshouse. Pure collected R was stored at −20°C under Ar and diluted 1:5 with distilled water just prior to experiments. A similar, mature, nondamaged, and nonsenescent rosette leaf was randomly chosen from each plant. The chosen leaves were wounded using a pattern wheel six times over

the adaxial side of the leaf parallel to the midrib. Water or diluted R (20 µL) was rubbed into the leaf puncture wounds gently with gloved fingers. Untreated plants were used as controls. To test the effects of real herbivore attack, three neonates of *M. sexta* were placed on three randomly chosen rosette leaves (1 neonate per leaf) on a plant in the early elongation stage. After placing the neonate, dead neonates were replaced by new neonates within 8 hr.

2.3 | Transcript abundance

To determine the rhythmicity of transcript abundance of *NaHPL* in EV and irLHY plants under different light conditions, seedlings were directly collected into a plastic 1.5 mL microcentrifuge tube and immediately frozen in liquid nitrogen. To determine the rhythmicity of transcript abundance of *NaHPL* in response to treatments, leaves from WT, EV, and irAOC plants were cut at the petiole and wrapped with aluminum foil, and immediately frozen in liquid nitrogen. Before analysis, all samples were ground to a fine powder with a mortar and pestle and transferred to plastic 2 mL or 15 mL tubes for storage. Total RNA was extracted from *N. attenuata* using the Plant RNeasy Extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop (Thermo Scientific, Wilmington, USA) and cDNA was synthesized from 500 ng of total RNA using RevertAid H Minus reverse transcriptase (Fermentas) and oligo (dT) primer (Fermentas). qPCR was performed in a Mx3005P PCR cycler (Stratagene) using the SYBR GREEN1 kit (Eurogentec). The elongation factor gene EF1a was used as a standard for normalization. The sequences of primers used for qPCR are provided in Table S1.

2.4 | Measurement of GLV internal pools

To measure internal GLV pools, we grew EV, asHPL, rLOX2, and irRCA plants in a glasshouse, and EV and irLHY plants in climate chambers in order to permit exposure to different light conditions (diurnal and continuous light). For glasshouse experiments, after 10 days on Petri dishes under the conditions described under plant materials, seedlings were transferred to small pots (TEKU JJP 3050 104 pots, Poeppelmann GmbH & Co. KG, Lohne, Germany) in the glasshouse and then to 1 L pots 10 days later with soil, fertilization, and watering regimes as previously described. Plants were grown under the temperature in the glasshouse ranged from ca. 35°C (highest daytime temperature) to 19°C (lowest nighttime temperature), 16 hr light/8 hr dark (supplemental lighting by Philips Sun-T Agro 400 W and 600 W sodium lights), and 55% humidity (Krügel, Lim, Gase, Halitschke, & Baldwin, 2002; Schuman, Palmer-Young, Schmidt, Gershenzon, & Baldwin, 2014). For different light treatment experiments, after potting, plants were placed in one of two climate chambers (Johnson Controls Unitary Products, Norman, OK, USA; dimensions: 4.00 ml × 2.22 mw × 2.35 mh) either under a diurnal cycle (12 hr light/12 hr dark or 16 hr light/8 hr dark) or continuous light at 65% relative humidity and 26°C (Herden et al., 2016).

Silicone tubing (ST) preparation, volatile sampling, and sampling analysis methods have been described in detail (Kallenbach et al., 2014; Kallenbach, Veit, Eilers, & Schuman, 2015). Briefly, we prepared 5-mm long ST pieces (1 mm i.d. × 1.8 mm o.d.). Whole single rosette leaves (+1) from EV, asHPL, irLOX2, and irLHY were harvested, immediately frozen, and ground, and approximately 50 mg of the leaf material (precise mass recorded) were aliquoted in glass vials. We directly added 1 mL of saturated $CaCl_2$ solution to inhibit enzyme activity, containing an internal standard (394 ng/mL of (*Z*)-4-hexenol). We then added an ST piece and incubated overnight (>8 hr) on a desktop shaking incubator at 600 rpm at room termperature. STs were rinsed with distilled water and dried under gentle nitrogen flow. STs were placed into 85 mm thermal desorption (TD) sampler tubes (Supelco) and samples were analyzed using a TD-20 thermal desorption unit (Shimadzu) connected to a quadrupole gas chromatography–mass spectrometry (GC–MS)–QP2010Ultra (QP-5050, Shimadzu) equipped with a wax column (ZB-WAX, 30 m long, 0.25 mm i.d., 0.25 µm film thickness, Zebron) as previously described (Kallenbach et al., 2014) but with the following modifications. The time for thermal desorption was increased to 15 min and nitrogen flow was increased to 100 mL to eliminate residual water and any associated contamination. The column was held at 40°C for 5 min and then heated at 5°C/min to 115°C, then at 30°C/min to 230°C for cleaning. The MS program ended at 18 min following the elution of all GLVs to avoid contaminants during the cleaning phase, and a few peaks identified as originating from STs and not coeluting with any GLVs were also removed from the MS method. Peak areas were integrated and normalized by fresh mass and internal standard ((*Z*)-4-hexenol). For quantification, an external standard curve was generated by spiking a dilution series of a standard mixture into the same saturated $CaCl_2$ solution containing the (*Z*)-4-hexenol internal standard, incubated with an ST as described. Quantification was based on response curves of each compound to the internal standard.

2.5 | Relative quantification of herbivore-induced volatiles

To collect herbivore-induced volatiles from plants grown in the climate chamber, we used a headspace sampling method with Poropak Q filters containing 20 mg of Poropak Q by pushing air filtered via activated charcoal into the headspace around the plant, and pulling it over the plant and through the filter as previously described (Kallenbach et al., 2014; Schuman et al., 2014). Three neonates of *M. sexta* were placed on three rosette leaves of each plant (Kim et al., 2011; the source/sink transition leaf and the first two fully expanded leaves: positions 0, +1, and + 2) and whole plant shoots were enclosed immediately using a clean, transparent oven bag of equal volume (500 mL) secured around the top edge of the pot below, and a filter and PTFE air infuser above, installed so that the air infuser was below the filter. The Poropak-Q filter was exchanged with a new filter every 4 hr over 2 days, starting immediately after treatment. Plant volatiles from the filters were eluted using 250 µL dichloromethane (Sigma-Aldrich). Eluents from all filters (1 µL) were analyzed using a liquid autosampler (AOC-20i, Shimadzu) connected to a quadrupole GC–MS-QP2010Ultra (QP-5050, Shimadzu) bearing a nonpolar ZB-5 column (30 m long, 0.25 mm i.d., 0.25 µm film thickness, Zebron; Kallenbach

et al., 2014). Peak areas were integrated and the concentration calculated by comparison with an external standard curve.

2.6 | Statistical analyses

Statistical significance of differences in transcript abundance, internal GLV pools, and GLV emissions were analyzed by one-way or two-way analyses of variance (ANOVAs). Comparison between gene transcript abundance between treatments and genotypes were analyzed by *t* tests. All statistical analyses were performed using the statistical package R version 3.3.1 and R Studio version 0.99.903 (R studio, 2015). The significance level was set at $\alpha = 0.05$.

3 | RESULTS

3.1 | The plant circadian clock regulates GLV biosynthesis

To understand the role of the plant circadian clock in the biosynthesis of GLVs, we first analyzed transcript abundance of the GLV biosynthetic genes *NaLOX2* and *NaHPL* every 4 hr over 3 days under light/dark cycles (black line, LD) and constant light conditions (grey line, LL) in EV seedlings (Figure 1). Under LD, transcript abundance of both genes peaked around dusk. Under LL, the phase of *NaLOX2* transcripts was not consistent from day to day, with peaking times on the first, second, and third days at Zeitgeber time (ZT) 20, ZT 36, and ZT 60, respectively (Figure 1a). Moreover, the transcript levels of *NaLOX2* gradually increased under LL, so that the maximum transcript level on the 3rd day was more than four-folds higher than the maximum on the 1st day (Figure 1a). However, *NaHPL* transcript abundance exhibited a robust circadian rhythm which peaked consistently at subjective dusk under LD and LL (Figure 1b).

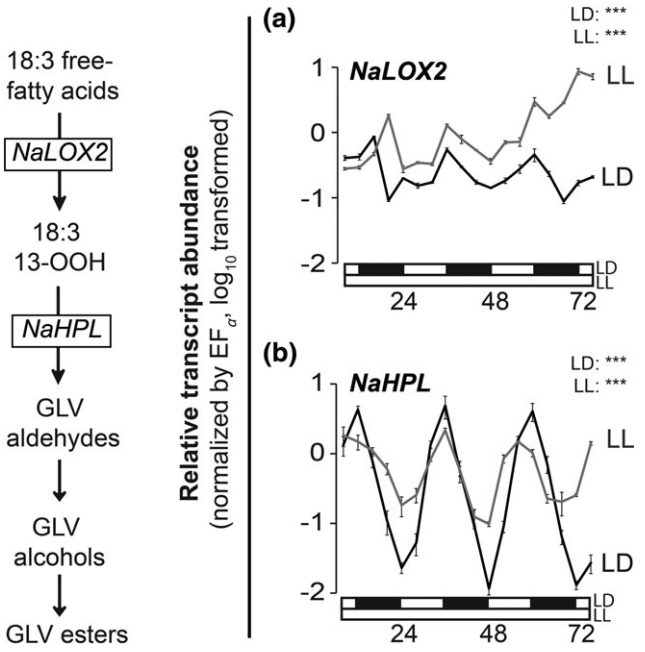

FIGURE 1 Transcript accumulation of the GLV biosynthetic gene *NaHPL* has a circadian rhythm. Mean (±SE, $n = 3$) relative transcript abundance of *NaLOX2* and *NaHPL* were measured in *Nicotiana attenuata* EV seedlings grown under 12 hr light/12 hr dark conditions (LD) or under constant light conditions (LL). Seedlings were harvested every 4 hr for 3 days. (a, b) Transcripts of GLV-biosynthetic genes oscillated diurnally in abundance, but only *NaHPL* oscillation was maintained under constant light conditions. Values are shown on a $\log_{10}$ scale. ***$p < 0.001$; *p*-values from one-way analysis of variance analyses with time as the factor. White bars, light period in LD; black bars, dark period in LD; GLV, green leaf volatile; *LOX2*, *lipooxygenase 2*; *HPL*, *hydroperoxide lyase*; EV, empty vector-containing plant

Although GLV emissions did not show circadian rhythms (Figure S1), herbivore-induced GLV emissions differed at different times of day (Joo et al., 2018). Volatile emission is a complex process, so there can be discrepancies between production and emission (Widhalm, Jaini, Morgan, & Dudareva, 2015). Particularly, GLVs are rapidly released, likely from storage and rapidly activated by herbivory (Matsui et al., 2012; Paré & Tumlinson, 1997). Therefore, we further hypothesized that plants have internal pools of GLVs in intact leaves that are regulated by known GLV biosynthetic genes, *NaLOX2* and *NaHPL* (Allmann et al., 2010; Halitschke et al., 2004). To detect the internal GLV pools in EV-containing plants, *NaHPL*-silenced plants (asHPL), and *NaLOX2*-silenced plants (irLOX2), we used a sorbent extraction method employing ST in mature leaf tissue from rosette-stage plants grown under LD. The internal GLV pools in intact leaves comprise fewer compounds than the set of GLVs emitted from damaged leaves; GLV aldehydes are the most abundant GLVs in intact leaf tissue, whereas GLV aldehydes and alcohols are similarly abundant in emissions from damaged leaves (Allmann & Baldwin, 2010). The total amount of internal GLV pools was mainly synthesized by *NaLOX2*, but time-dependent accumulation was dependent on *NaHPL* (Figure 2). Moreover, reducing the photosynthetic rate by silencing *RuBisCo activase* (*NaRCA*) affected the total production of GLVs, but only at the peaking time, ZT 16 (Mitra & Baldwin, 2008; Figure S2).

Because the internal GLV pools had time-dependent accumulations, we further tested the role of the plant circadian clock in the biosynthesis of GLVs in *N. attenuata*. We analyzed transcript abundance of *NaHPL* and internal GLV pools under LD and LL in EV and *NaLHY*-silenced plants (irLHY), which have early-phased rhythms in *NaCAB2* transcripts and floral behaviors in *N. attenuata* (Joo, Fragoso, Yon, Baldwin, & Kim, 2017; Yon et al., 2016). Under LD, the transcript levels of *NaHPL* in irLHY plants still had a ca. 24 hr period, but the peaking time was shifted approximately 4 hr earlier than it was in EV plants. Under LL, silencing *LHY* shortened the period of circadian rhythms in *NaHPL* expression and reduced the amplitudes of *NaHPL* transcript abundance (Figure 3a). In EV plants, the initial product of HPL activity, (*Z*)-3-hexenal, and its isomer (*E*)-2-hexenal, had a strong time-dependent accumulation under LD and maintained their time-dependent accumulation under LL (Figure 3b–c). Pools of (*Z*)-3-hexenal and (*E*)-2-hexenal in irLHY plants exhibited an earlier peaking time than in EV plants under LD: between ZT 4 and ZT 8 (Figure 3b–c). Furthermore, GLV pools in irLHY plants exhibited two peaking times per day under LL: in the middle of day, and in the middle of the night, following the transcript abundance of *NaHPL* in irLHY plants (Figure 3).

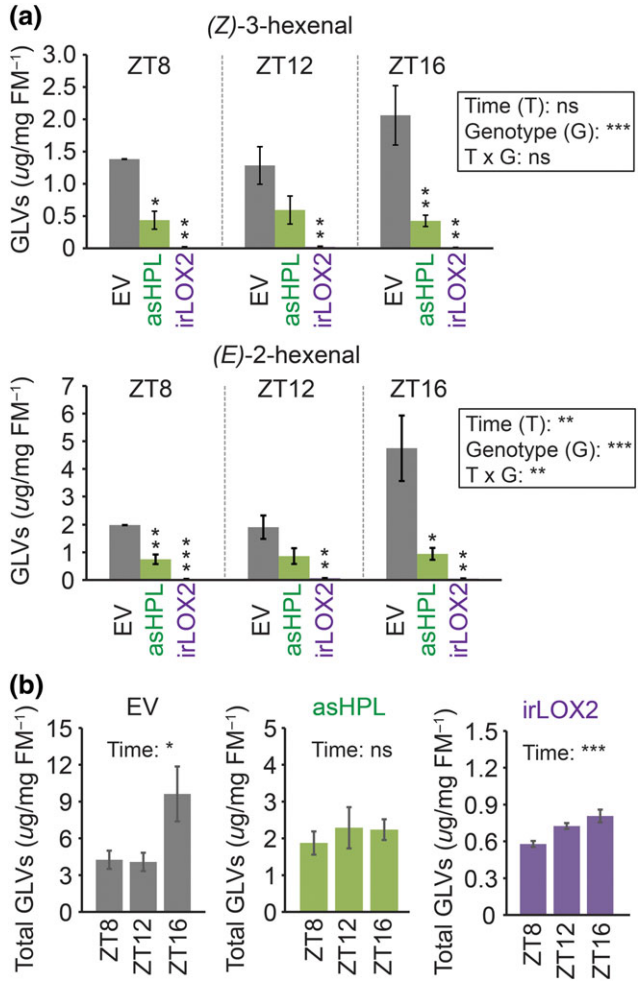

FIGURE 2 *NaHPL* and *NaLOX2* are involved in the time-dependent accumulation of green leaf volatiles (GLVs). (a) Mean (± SE, $n = 6$) accumulation of (*Z*)-3-hexenal and (*E*)-2-hexenal were measured in EV, asHPL, and irLOX2 leaf tissue at different times; ZT 8, ZT 12, and ZT 16. (b) Mean (±SE, n = 6) accumulation of total internal GLV pools were measured in EV, asHPL, and irLOX2 plants at different times; ZT 8, ZT 12, and ZT 16; note differences in scale. Asterisks indicate significant differences within genotype over time (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). EV, empty vector control; asHPL, antisense *NaHPL*; irLHY, inverted repeat *NaLHY*

3.2 | JA and herbivore-associated elicitors differentially affect the transcript abundance of *NaHPL*

To examine whether the diurnal rhythm of *NaHPL* transcript expression is related with jasmonates (JA), we measured the transcript abundance of *NaHPL* every 4 hr over 1 day in EV plants, and plants rendered deficient in *NaAOC* by RNAi (irAOC), which are severely impaired in JA biosynthesis (Kallenbach et al., 2012). Transcript abundance of *NaHPL* were reduced in irAOC plants, but they retained their time-dependent accumulation (Figure 4a). These data suggest that JA is required for the basal accumulation of *HPL* transcripts but did not play a significant role in the rhythmicity of *HPL* expression.

M. sexta R elicitation increases the JA levels in attacked and systemic leaves, and JA induces *HPL* transcripts. Therefore, we expected that herbivory elicitation would increase the levels of *HPL* transcripts in leaves. To examine this hypothesis, we treated leaves on EV and irAOC plants with wounding and water (W + W) or wounding and *M. sexta* R (W + R) at ZT 8, ZT 14, and ZT 0, and collected leaves at ZT 15 and ZT 1, which are the peak and the trough times of *NaHPL* transcript accumulation (Figure 4b). Although the basal abundance of *NaHPL* transcripts was JA-dependent, *NaHPL* transcript levels were significantly reduced by W + R treatments both in EV and irAOC treated leaves at the peaking time (Figures 4b and S3). These data suggest that the suppression of *NaHPL* transcript abundance is herbivore elicitor-specific. Lastly, we analyzed the effects of W + R treatments on transcript abundance of *NaHPL* in local and systemic leaves every 4 hr over 1 day after treatments. *NaHPL* transcript levels were induced by the W + R treatment in systemic leaves, but effects of simulated herbivory were highly time-dependent in local leaves; transcripts were suppressed during the first 5 hr after treatment, but induced at later time periods (Figure 4c).

3.3 | The circadian clock is altered by herbivory

We next tested whether the circadian clock and herbivory independently affect GLV biosynthesis. We collected leaf samples from WT plants every 4 hr over 2 days during *M. sexta* feeding (Figure 5a) and measured the transcript abundance of two circadian clock components: *NaLHY* and *TIMING OF CAB EXPRESSION 1* (*NaTOC1*) in damaged leaves from these plants versus equivalent samples from undamaged control plants. Interestingly, the rhythmicity of both *NaLHY* and *NaTOC1* were significantly altered by the herbivory treatment (Figure 5b), but the correlation coefficients between *NaLHY* and *NaTOC1* were not significantly decreased (Figure S4). The rhythmicity of a marker gene (*CHLOROPHYLL A/B BINDING PROTEIN 2*, CAB2) for endogenous rhythmicity was also significantly changed by *M. sexta* feeding treatments (Figure S4).

To investigate the specific role of herbivore-associated elicitors, we measured the transcript abundance of the circadian clock genes in samples from the experiment shown in Figure 4b. Basal transcript abundance of *NaTOC1* did not differ between EV and irAOC plants, and W + R treatments significantly suppressed *NaTOC1* in both EV and irAOC plants (Figure 6a–b). In contrast, *NaLHY* transcript abundance was not affected by either W + W or W + R treatments (Figure S5).

3.4 | Interactive effects between the circadian clock and herbivory on GLV biosynthesis and emissions

To investigate the time-dependent effects of herbivory on *NaHPL* transcripts, we analyzed the transcript abundance of *NaHPL* in samples from the experiment shown in Figure 5a. The abundance of *NaHPL* transcripts had a strong diurnal rhythm and peaked between ZT12 and ZT16 in control plants (Figure 7a, $F_{5,\ 35} = 12.13$, $P < 0.001$, one-way ANOVA). In response to herbivore attack, the transcript abundance of *NaHPL* increased after 8 hr of herbivore damage and remained elevated in the second day compared with *NaHPL* transcripts in control plants (Figure 7a, $F_{1,\ 71} = 75.70748$, $P < 0.001$, two-way ANOVA), but *NaHPL* transcripts in treated samples also maintained their temporal dynamics (Figure 7a, $F_{5,\ 35} = 1.51$,

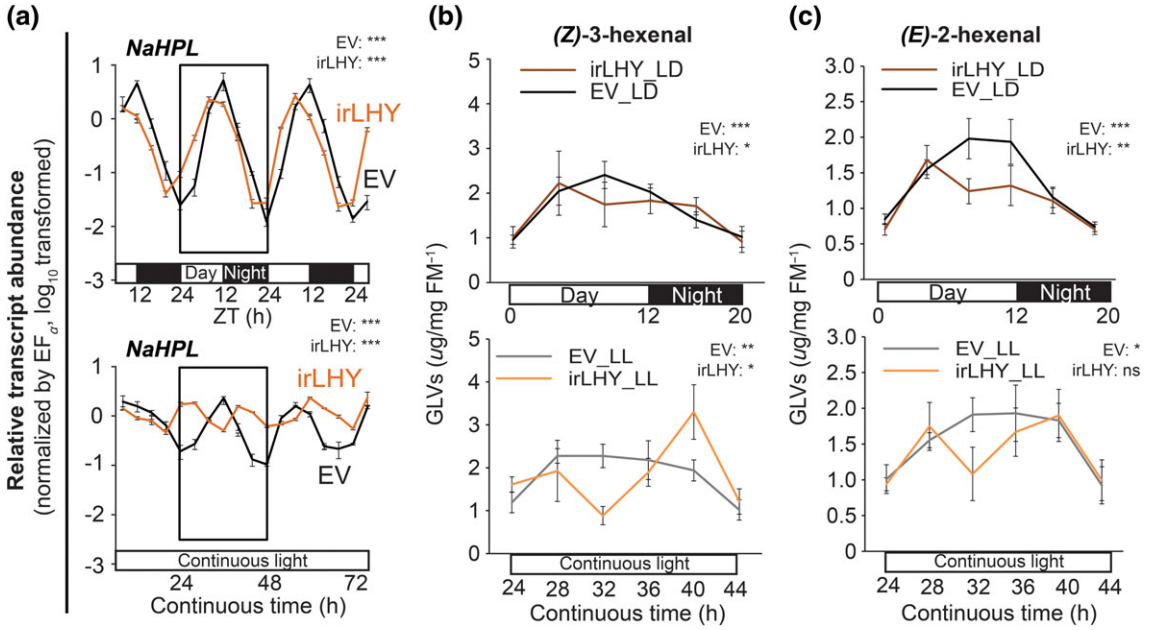

FIGURE 3 Internal green leaf volatile (GLV) pools fluctuate with a circadian rhythm. (a) Transcript abundance of *NaHPL* in irLHY plants showed an early shifted phase in LD and a shortened period and amplitude in LL compared with empty vector plants (mean ± SE, n = 3). (b, c) Mean (± SE, *n* = 5) internal pools of (*Z*)-3-hexenal and (*E*)-2-hexenal were measured from two groups of plants entrained under a diurnal environment (LD), one of which was transferred to continuous light (LL) 1 day before sample collection. White and black boxes indicate light and dark period, respectively. **p* < 0.05, ***P* < 0.01, ****p* < 0.001; *p*-values from one-way analyses of variance with time as the factor; ns, no significant difference. Black lines indicate LD and gray indicate LL. irLHY, inverted repeat *NaLHY*

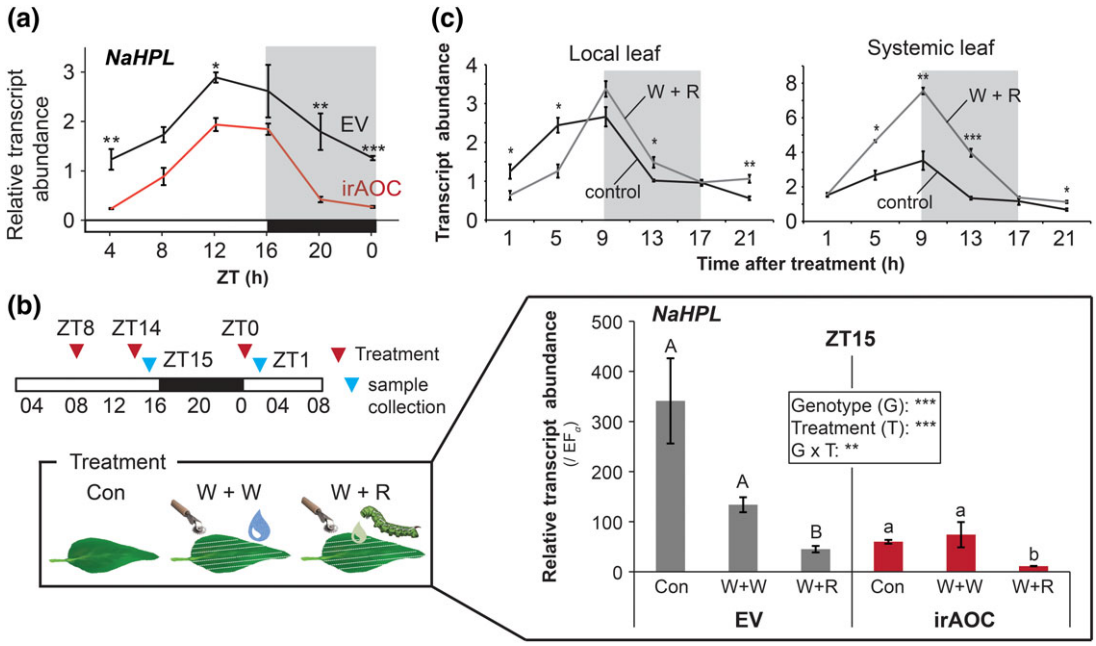

FIGURE 4 Basal transcript abundance of *NaHPL* is JA-dependent, but the elicitation-induced reduction at peaking time is not mainly regulated by JA. (a) Basal *NaHPL* transcript levels (mean ± SE, n = 3) in irAOC plants and EV controls over 24 h. (b) Leaves (n = 3, 1/plant) were wounded with a pattern wheel and diluted R from *M. sexta* larvae (W + R) or water (mock, W + W) was added; control leaves remained undamaged (Con). Mean transcript abundance (± SE) of *NaHPL* was measured at evening (ZT 15) in EV and irAOC plants. (c) The kinetics of *NaHPL* transcript accumulation in local (treated) versus systemic leaves. Black- and grey-colored lines refer to control (undamaged) and OS-induced leaves, respectively. The y axis shows the relative transcript abundance measured by microarray. Data are shown as mean ± SEM. The data were extracted from the microarray dataset in Kim *et al*. (2012). Asterisks indicate significant differences between treatments within time points (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001) as determined by one-way analysis of variance (ANOVA). Different letters indicate significant differences between leaves determined by ANOVAs with *post-hoc* tests with Tukey correction. *AOC*, *allene oxide cyclase*

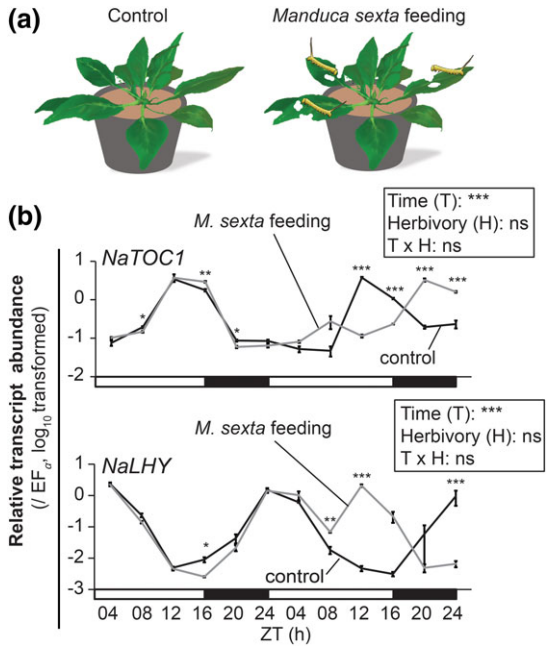

FIGURE 5 Transcriptional rhythms of two core clock genes are altered by *Manduca sexta* feeding. (a) Three neonates of *M. sexta* were placed per plant (1/leaf, 3 leaves/plant) and leaf samples were collected every 4 hr for 2 days. (b) Mean (± SE, n = 3) transcript abundance of two circadian clock components, *NaTOC1* and *NaLHY*, were measured in leaves attacked by *M. sexta*. Asterisks indicate significant differences between treatments within time points (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *P*-values are shown for ANOVAs comparing transcript abundance of genes between time (T) and treatment (herbivory, H). LHY, late elongated hypocotyl; TOC1, timing of CAB expression 1

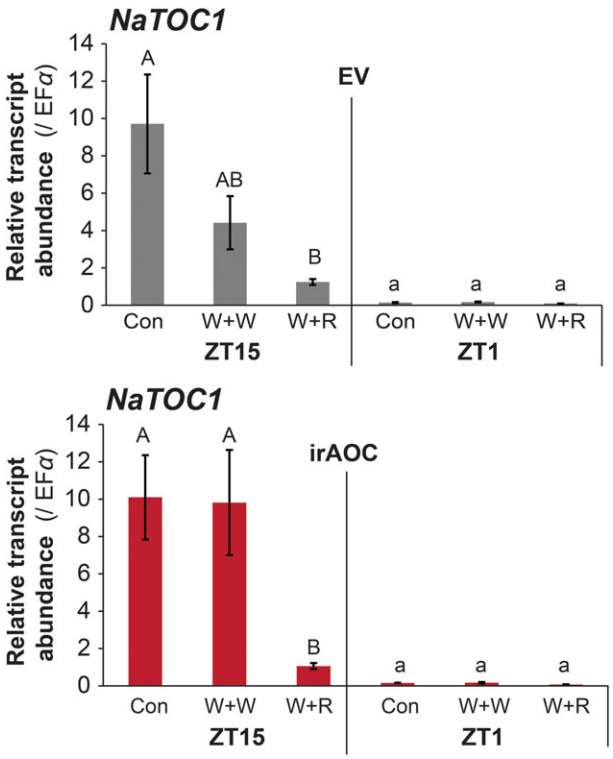

FIGURE 6 Feeding-induced changes in clock gene transcript abundance depend on elicitors in herbivore R, but not JA signaling

P = 0.02, one-way ANOVA). Moreover, the time-dependent accumulation of *NaHPL* transcripts was also significantly affected by the herbivory treatment (Figure 7a, $F_{11, 71} = 14.04$, $P < 0.001$, interaction term in a two-way ANOVA).

We further measured the emission of GLVs in leaves subjected to feeding by *M. sexta* larvae. Although plants produced a significant amount of GLVs immediately after the start of herbivore feeding, plants continued to emit GLVs as the herbivores fed (Herden et al., 2016). Consistent with the transcript abundance of *NaHPL*, the rhythmicity of GLV emissions also exhibited two peaks in a day (Figure 7b). These data suggest that herbivore-induced alterations in transcript abundance of *NaHPL* could also affect the temporal dynamics of GLV emission.

4 | DISCUSSION

4.1 | Circadian regulation of GLV biosynthesis

Although GLVs increase plant indirect defense in many different plant species (Ameye et al., 2017), *Geocoris* spp., which are natural enemies of *M. sexta*, are day-active carnivores and they can distinguish day-specific from night-specific GLV blends of *N. attenuata* (Joo et al., 2018). Although total amounts of GLVs peaked at dusk, circadian regulation of GLV biosynthesis may also be important for qualitative diurnal changes mediating attractiveness of GLV blends to *Geocoris* spp. But *Geocoris* are not the only biotic player that responds to GLV emissions; GLVs also can be induced by fungal infection and GLVs suppress fungal growth (Kishimoto, Matsui, Ozawa, & Takabayashi, 2008). GLV aldehydes in particular have strong activity against many different fungal pathogens (de Lucca, Carter-Wientjes, Boué, & Bhatnagar, 2011; Hamilton-Kemp, McCracken, Loughrin, Andersen, & Hildebrand, 1992). Moreover, virulence of the fungal plant pathogen *Botytis cinerea* is higher at dusk than at dawn, and this is regulated by the fungal circadian clock (Hevia, Canessa, Müller-Esparza, & Larrondo, 2015). Airborne fungal spores are furthermore most abundant at dusk (Abdel Hameed, Khoder, Yuosra, Osman, & Ghanem, 2009; Abdel-Fattah, Moubasher, & Swelim, 1981). Therefore, the peaking of GLV aldehydes near dusk may function to suppress fungal colonization at dusk, but this hypothesis should be tested in future studies.

4.2 | Effects of herbivory on GLV biosynthesis

Herbivory treatments suppress photosynthetic activity, and herbivore-associated elicitors can exacerbate this suppression (Meza-Canales, Meldau, Zavala, & Baldwin, 2017; Nabity, Zavala, & DeLucia, 2009). Thus, herbivore-associated elicitor-specific signals may decrease *NaHPL* transcript levels through the level of MEcPP (Xiao et al., 2012; Figures 4b and S2). Different responses of *NaHPL* transcript abundance in local and systemic leaves could be caused by opposing regulation by JA, which is systemically induced in response to

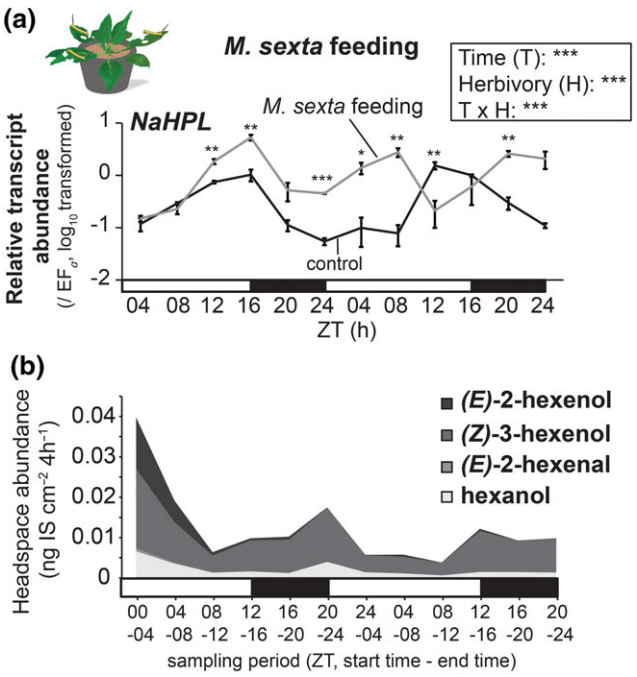

FIGURE 7 Most green leaf volatiles (GLVs) are emitted immediately in response to *Manduca sexta* feeding, but only (*Z*)-3-hexenol and (*E*)-2-hexenol show enhanced rhythmic emissions during the second day of attack. (a) Three neonates of *M. sexta* were placed per plant (1/leaf, 3 leaves/plant) and damaged leaf samples were collected every 4 hr for 2 days. Mean (± SE, n = 3) transcript abundance of *NaHPL* was measured. (b) Plant volatiles were collected every 4 hr for 2 days. Plant volatiles were sampled by Poropak-Q filters and analyzed by GC–MS (mean ± SE, n = 5). Circadian-regulated total GLV emissions are altered by *M. sexta* feeding, which is following the transcript abundance of *NaHPL*. Asterisks indicate significant differences between treatments within time points (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *P*-values are shown for analyses of variance comparing transcript abundance of genes between time (T) and treatment (herbivory, H) or genotype (G) and treatment (T)

herbivore feeding and herbivore-associated elicitors, which remain at the locally damaged leaf (Figure 4c). Because GLVs can act as feeding stimulants (Halitschke et al., 2004), these differential responses may be adaptive for the plant, by limiting feeding activity of herbivores on attacked leaves whereas, for example, attracting natural enemies of herbivores by increasing biosynthesis of GLVs in systemic unattacked leaves.

Crosstalk between JA and GLV signaling has been suggested because HPL and allene oxide synthase (AOS) share the same LOX-derived substrates, and the two cascades exhibit some competition for common substrate (Halitschke et al., 2004). However, silencing of JAZh, an inhibitor of JA-regulated gene transcription, increases emission of GLVs in response to simulated herbivore attack (Oh, Baldwin, & Galis, 2012). Also, JA levels did not change in irLOX2 plants (Allmann et al., 2010). These data suggest that metabolic competition between JA and GLVs may be buffered by limits on 13-hydroperoxide accumulation (Bonaventure, Schuck, & Baldwin, 2011). Moreover, differences in subcellular localization could avoid direct substrate competition between HPL and AOS (Froehlich, Itoh, & Howe, 2001). Here, we also have shown that the basal transcript abundance of *NaHPL* is JA-dependent. Therefore, the crosstalk between JA and GLVs could be regulated by both the metabolic competition and direct gene regulation.

4.3 | Herbivore-associated alterations in the circadian clock

Previous studies have shown that the synchronization between plant inducible secondary metabolites (glucosinolates) and herbivore activity increase plant resistance (Goodspeed et al., 2012, 2013). As inducible resistant metabolites, glucosinolates require myrosinase to be activated (Howe & Jander, 2008). However, it is still unknown whether plant-inducible defense metabolites can synchronize with herbivore activity under continuous herbivore attack. The robustness of the plant circadian clock is often decreased by environmental factors, for example, cold temperatures (Bieniawska *et al*., 2008) and fungal infection (Wang *et al*., 2011). Exogenous treatment with phytohormones also affects the robustness of the plant circadian rhythm in different ways (Hanano et al., 2006). Because herbivory profoundly affects levels of phytohormones, and the transcriptome and metabolome in plants (Halitschke et al., 2001, 2003; Gaquerel et al., 2009), herbivory could affect the plant circadian clock as well. Here, we showed that the rhythm of circadian clock gene transcripts was altered during herbivore feeding, and that circadian-regulated metabolites became arrhythmic (Figure 7). Therefore, current assumptions (Goodspeed et al., 2012, 2013) about the role of the plant circadian clock in defense may need to be reconsidered. Taken together, these results suggest the role of the circadian clock in herbivore resistance can be separated into three phases: before herbivore attack, early phase after herbivore attack, and late phase after herbivore attack. Control by the circadian clock may be greater during the first phase, and its importance may decrease with time during a plant-herbivore interaction because herbivore attack strongly disrupts the clock system and activates induced responses. In other words, the clock seems to be hijacked by herbivore-associated elicitors during the activation of induced defenses. It remains an open question how predictable these dynamics are during herbivore feeding; for example, does this overlay of induced responses on circadian basal processes result in predictable dynamics, or does the interaction of two signaling systems generate stochasticity, resulting in something closer to the "moving target" model of plant defense (Adler & Karban, 1994)?

In summary, here, we show that GLV biosynthesis has a circadian rhythm. Although the biosynthesis of GLVs is JA-dependent, herbivore-associated elicitors suppress GLV biosynthesis. Moreover, the rhythm of circadian gene transcripts was strongly altered by herbivory treatment in a manner that was also both elicitor-specific and time-dependent. We found that both the circadian clock and herbivory interactively regulated GLV biosynthesis. This study provides evidence of how a plant shapes the temporal dynamics of GLVs by integrating both circadian control, and induced responses to herbivory.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

Abdel Hameed, A. A., Khoder, M. I., Yuosra, S., Osman, A. M., & Ghanem, S. (2009). Diurnal distribution of airborne bacteria and fungi in the atmosphere of Helwan area. *Egypt. Science of the Total Environment*, *407*, 6217–6222. https://doi.org/10.1016/j.scitotenv.2009.08.028

Abdel-Fattah, H. M., Moubasher, A. H., & Swelim, M. A. (1981). Studies on air-borne fungi at Qena. *Zeitschrift für Allgemeine Mikrobiologie*, *21*, 177–179. https://doi.org/10.1002/jobm.3630210302

Adler, F. R., & Karban, R. (1994). Defended fortresses or moving targets ? Another model of inducible defenses inspired by military metaphors. *American Naturalist*, *144*, 813–832. https://doi.org/10.1086/285708

Allmann, S., & Baldwin, I. T. (2010). Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. *Science*, *329*, 1075–1078. https://doi.org/10.1126/science.1191634

Allmann, S., Halitschke, R., Schuurink, R. C., & Baldwin, I. T. (2010). Oxylipin channelling in *Nicotiana attenuata*: *Lipoxygenase 2* supplies substrates for green leaf volatile production. *Plant, Cell and Environment*, *33*, 2028–2040. https://doi.org/10.1111/j.1365-3040.2010.02203.x

Ameye, M., Allmann, S., Verwaeren, J., Smagghe, G., Haesaert, G., Schuurink, R. C., & Audenaert, K. (2017). Green leaf volatile production by plants: A meta-analysis. *New Phytologist*..

Atamian, H. S., Creux, N. M., Brown, E. A., Garner, A. G., Blackman, B. K., & Harmer, S. L. (2016). Circadian regulation of sunflower heliotropism, floral orientation. *And Pollinator Visits. Science*, *353*(6299), 587–590.

Baldwin, I. T. (2010). Plant volatiles. *Current Biology*, *20*, R392–R397. https://doi.org/10.1016/j.cub.2010.02.052

Bate, N. J., Sivasankar, S., Moxon, C., Riley, J. M., Thompson, J. E., & Rothstein, S. J. (1998). Molecular characterization of an *Arabidopsis* gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible. *Plant Physiology*, *117*, 1393–1400. https://doi.org/10.1104/pp.117.4.1393

Bonaventure, G., Schuck, S., & Baldwin, I. T. (2011). Revealing complexity and specificity in the activation of lipase-mediated oxylipin biosynthesis: A specific role of the *Nicotiana attenuata* GLA1 lipase in the activation of jasmonic acid biosynthesis in leaves and roots. *Plant, Cell and Environment*, *34*, 1507–1520. https://doi.org/10.1111/j.1365-3040.2011.02348.x

Christensen, S. A., Huffaker, A., Kaplan, F., Sims, J., Ziemann, S., Doehlemann, G., … Schmelz, E. A. (2015). Maize death acids, 9-lipoxygenase–derived cyclopente(a) nones, display activity as cytotoxic phytoalexins and transcriptional mediators. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 11407–11412. https://doi.org/10.1073/pnas.1511131112

Covington, M. F., Maloof, J. N., Straume, M., Kay, S. A., & Harmer, S. L. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biology*, *9*, R130. https://doi.org/10.1186/gb-2008-9-8-r130

Croft, K., Juttner, F., & Slusarenko, A. J. (1993). Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv phaseolicola. *Plant Physiology*, *101*, 13–24. https://doi.org/10.1104/pp.101.1.13

D'Auria, J. C., Pichersky, E., Schaub, A., Hansel, A., & Gershenzon, J. (2007). Characterization of a *BAHD acyltransferase* responsible for producing the green leaf volatile (*Z*)-3-hexen-1-yl acetate in *Arabidopsis thaliana*. *Plant Journal*, *49*, 194–207. https://doi.org/10.1111/j.1365-313X.2006.02946.x

Dicke, M., & Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: Beyond the "cry for help". *Trends in Plant Science*, *15*, 167–175. https://doi.org/10.1016/j.tplants.2009.12.002

Dodd, A. N., Salathia, N., Hall, A., Kévei, E., Tóth, R., Nagy, F., … Webb, A. A. R. (2005). Plant circadian clocks increase photosynthesis, growth, survival. *And Competitive Advantage. Science*, *309*(5734), 630–633.

Dudareva, N., Klempien, A., Muhlemann, K., Kaplan, I., Muhlemann, J. K. J. K., & Kaplan, I. (2013). Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytologist*, *198*, 16–32. https://doi.org/10.1111/nph.12145

Engelberth, J., Alborn, H. T., Schmelz, E. A., & Tumlinson, J. H. (2004). Airborne signals prime plants against insect herbivore attack. *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 1781–1785. https://doi.org/10.1073/pnas.0308037100

Fauconnier, M. L., Mpambara, A., Delcarte, J., Jacques, P., Thonart, P., & Marlier, M. (1999). Conversion of green note aldehydes into alcohols by yeast alcohol dehydrogenase. *Biotechnology Letters*, *21*, 629–633. https://doi.org/10.1023/A:1005593821577

Froehlich, J. E., Itoh, A., & Howe, G. A. (2001). Tomato *Allene Oxide Synthase* and fatty acid *Hydroperoxide Lyase*, two cytochrome P450s involved in oxylipin metabolism, are targeted to different membranes of chloroplast envelope. *Plant Physiology*, *125*, 306–317. https://doi.org/10.1104/pp.125.1.306

Gaquerel, E., Weinhold, A., & Baldwin, I. T. (2009). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphigidae) and its natural host *Nicotiana attenuata*. VIII. An unbiased GCxGC-ToFMS analysis of the plant's elicited volatile emissions. *Plant Physiology*, *149*, 1408–1423. https://doi.org/10.1104/pp.108.130799

Gomi, K., Yamasaki, Y., Yamamoto, H., & Akimitsu, K. (2003). Characterization of a hydroperoxide lyase gene and effect of C6-volatiles on expression of genes of the oxylipin metabolism in *Citrus*. *Journal of Plant Physiology*, *160*, 1219–1231. https://doi.org/10.1078/0176-1617-01177

Goodspeed, D., Chehab, E. W., Min-Venditti, A., Braam, J., & Covington, M. F. (2012). *Arabidopsis* synchronizes jasmonate-mediated defense with insect circadian behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 4674–4677. https://doi.org/10.1073/pnas.1116368109

Goodspeed, D., Liu, J. D., Chehab, E. W., Sheng, Z., Francisco, M., Kliebenstein, D. J., & Braam, J. (2013). Postharvest circadian entrainment enhances crop pest resistance and phytochemical cycling. *Current Biology*, *23*, 1235–1241. https://doi.org/10.1016/j.cub.2013.05.034

Greenham, K., & McClung, C. R. (2015). Integrating circadian dynamics with physiological processes in plants. *Nature Reviews Genetics*, *16*, 598–610. https://doi.org/10.1038/nrg3976

Halitschke, R., Schittko, U., Pohnert, G., Boland, W., & Baldwin, I. T. (2001). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specifi. *Plant Physiology*, *125*, 711–717. https://doi.org/10.1104/pp.125.2.711

Halitschke, R., Stenberg, J. A., Kessler, D., Kessler, A., & Baldwin, I. T. (2008). Shared signals—"Alarm calls" from plants increase apparency to herbivores and their enemies in nature. *Ecology Letters*, *11*, 24–34.

Halitschke, R., Ziegler, J., Keinänen, M., & Baldwin, I. T. (2004). Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and

defense signaling crosstalk in *Nicotiana attenuata*. *Plant Journal*, *40*, 35–46. https://doi.org/10.1111/j.1365-313X.2004.02185.x

Hamilton-Kemp, T., McCracken, J. R. C., Loughrin, J., Andersen, R., & Hildebrand, D. (1992). Effects of some natural volatile compounds on the pathogenic fungi *Alternaria alternata* and *Botrytis cinerea*. *Journal of Chemical Ecology*, *18*, 1083–1091. https://doi.org/10.1007/BF00980064

Hare, J. D. (2011). Ecological role of volatiles produced by plants in response to damage by herbivorous insects. *Annual Review of Entomology*, *56*, 161–180. https://doi.org/10.1146/annurev-ento-120709-144753

Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H.-S., Han, B., Zhu, T., … Kay, S. A. (2000). Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science*, *290*, 2110–2113. https://doi.org/10.1126/science.290.5499.2110

Herden, J., Meldau, S., Kim, S.-G., Kunert, G., Joo, Y., Baldwin, I. T., & Schuman, M. C. (2016). Shifting *Nicotiana attenuata*'s diurnal rhythm does not alter its resistance to the specialist herbivore *Manduca sexta*. *Journal of Integrative Plant Biology*, *58*, 656–668. https://doi.org/10.1111/jipb.12458

Hevia, M. A., Canessa, P., Müller-Esparza, H., & Larrondo, L. F. (2015). A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 8744–8749. https://doi.org/10.1073/pnas.1508432112

Howe, G. A., & Jander, G. (2008). Plant immunity to insect herbivores. *Annual Review of Plant Biology*, *59*, 41–66. https://doi.org/10.1146/annurev.arplant.59.032607.092825

Howe, G. A., Lee, G. I., Itoh, A., Li, L., & DeRocher, A. E. (2000). Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of *Allene Oxide Synthase* and fatty acid *Hydroperoxide Lyase*. *Plant Physiology*, *123*, 711–724. https://doi.org/10.1104/pp.123.2.711

Joo, Y., Fragoso, V., Yon, F., Baldwin, I. T., & Kim, S. G. (2017). Circadian clock component, LHY, tells a plant when to respond photosynthetically to light in nature. *Journal of Integrative Plant Biology*, *59*, 572–587. https://doi.org/10.1111/jipb.12547

Joo, Y., Schuman, M. C., Goldberg, J. K., Kim, S. G., Yon, F., Brütting, C., & Baldwin, I. T. (2018). Herbivore-induced volatile blends with both "fast" and "slow" components provide robust indirect defence in nature. *Functional Ecology*, *32*, 136–149. https://doi.org/10.1111/1365-2435.12947

Kallenbach, M., Bonaventure, G., Gilardoni, P. A., Wissgott, A., & Baldwin, I. T. (2012). *Empoasca* leafhoppers attack wild tobacco plants in a jasmonate-dependent manner and identify jasmonate mutants in natural populations. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, E1548–E1557. https://doi.org/10.1073/pnas.1200363109

Kallenbach, M., Oh, Y., Eilers, E. J., Veit, D., Baldwin, I. T., & Schuman, M. C. (2014). A robust, simple, high-throughput technique for time-resolved plant volatile analysis in field experiments. *Plant Journal*, *78*, 1060–1072. https://doi.org/10.1111/tpj.12523

Kallenbach, M., Veit, D., Eilers, E. J., & Schuman, M. C. (2015). Application of silicone tubing for robust, simple, high-throughput, and time-resolved analysis of plant volatiles in field experiments. *Bio-Protocol*, *5*, 1–8.

Kessler, D., Diezel, C., Clark, D. G., Colquhoun, T. A., & Baldwin, I. T. (2013). *Petunia* flowers solve the defence/apparency dilemma of pollinator attraction by deploying complex floral blends. *Ecology Letters*, *16*, 299–306. https://doi.org/10.1111/ele.12038

Kessler, D., Gase, K., & Baldwin, I. T. (2008). Field experiments with transformed plants reveal the sense of floral scents. *Science*, *321*, 1200–1202. https://doi.org/10.1126/science.1160072

Kim, S.-G., Yon, F., Gaquerel, E., Gulati, J., & Baldwin, I. T. (2011). Tissue specific diurnal rhythms of metabolites and their regulation during herbivore attack in a native tobacco. *PLoS ONE*, *6*(10), e26214. https://doi.org/10.1371/journal.pone.0026214

Kishimoto, K., Matsui, K., Ozawa, R., & Takabayashi, J. (2008). Direct fungicidal activities of C6-aldehydes are important constituents for defense responses in *Arabidopsis* against *Botrytis cinerea*. *Phytochemistry*, *69*, 2127–2132. https://doi.org/10.1016/j.phytochem.2008.04.023

Krügel, T., Lim, M., Gase, K., Halitschke, R., & Baldwin, I. T. (2002). Agrobacterium-mediated transformation of Nicotiana attenuata. *A Model Ecological Expression System. Chemoecology*, *12*, 177–183.

de Lucca, A. J., Carter-Wientjes, C. H., Boué, S., & Bhatnagar, D. (2011). Volatile trans-2-hexenal, a soybean aldehyde, inhibits *Aspergillus flavus* growth and aflatoxin production in corn. *Journal of Food Science*, *76*, M381–M386. https://doi.org/10.1111/j.1750-3841.2011.02250.x

Matsui, K. (2006). Green leaf volatiles: Hydroperoxide lyase pathway of oxylipin metabolism. *Current Opinion in Plant Biology*, *9*, 274–280. https://doi.org/10.1016/j.pbi.2006.03.002

Matsui, K., Sugimoto, K., Mano, J., Ozawa, R., & Takabayashi, J. (2012). Differential metabolisms of green leaf volatiles in injured and intact parts of a wounded leaf meet distinct ecophysiological requirements. *PLoS One*, *7*, e36433. https://doi.org/10.1371/journal.pone.0036433

Matsui, K., Wilkinson, J., Hiatt, B., Knauf, V., & Kajiwara, T. (1999). Molecular cloning and expression of *Arabidopsis* fatty acid hydroperoxide lyase. *Plant & Cell Physiology*, *40*, 477–481. https://doi.org/10.1093/oxfordjournals.pcp.a029567

Meza-Canales, I. D., Meldau, S., Zavala, J. A., & Baldwin, I. T. (2017). Herbivore perception decreases photosynthetic carbon assimilation and reduces stomatal conductance by engaging 12-oxo-phytodienoic acid, mitogen-activated protein kinase 4 and cytokinin perception. *Plant Cell and Environment*, *40*, 1039–1056. https://doi.org/10.1111/pce.12874

Mitra, S., & Baldwin, I. T. (2008). Independently silencing two photosynthetic proteins in *Nicotiana attenuata* has different effects on herbivore resistance. *Plant Physiology*, *148*, 1128–1138. https://doi.org/10.1104/pp.108.124354

Mizuno, T., & Yamashino, T. (2008). Comparative transcriptome of diurnally oscillating genes and hormone-responsive genes in *Arabidopsis thaliana*: Insight into circadian clock-controlled daily responses to common ambient stresses in plants. *Plant and Cell Physiology*, *49*, 481–487. https://doi.org/10.1093/pcp/pcn008

de Moraes, C. M., Mescher, M. C., & Tumlinson, J. H. (2001). Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature*, *410*, 577–580. https://doi.org/10.1038/35069058

Nabity, P. D., Zavala, J. A., & DeLucia, E. H. (2009). Indirect suppression of photosynthesis on individual leaves by arthropod herbivory. *Annals of Botany*, *103*, 655–663. https://doi.org/10.1093/aob/mcn127

Oh, Y., Baldwin, I. T., & Galis, I. (2012). *NaJAZh* regulates a subset of defense responses against herbivores and spontaneous leaf necrosis in *Nicotiana attenuata* plants. *Plant Physiology*, *159*, 769–788. https://doi.org/10.1104/pp.112.193771

Pan, Y., Michael, T. P., Hudson, M. E., Kay, S. A., Chory, J., & Schuler, M. A. (2009). Cytochrome P450 monooxygenases as reporters for circadian-regulated pathways. *Plant Physiology*, *150*, 858–878. https://doi.org/10.1104/pp.108.130757

Paré, P. W., & Tumlinson, J. H. (1997). *de novo* biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiology*, *114*, 1161–1167. https://doi.org/10.1104/pp.114.4.1161

R studio (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing Vienna, https://www.R-project.org/.

Savchenko, T., Pearse, I. S., Ignatia, L., Karban, R., & Dehesh, K. (2013). Insect herbivores selectively suppress the HPL branch of the oxylipin pathway in host plants. *Plant Journal*, *73*, 653–662. https://doi.org/10.1111/tpj.12064

Schuman, M. C., Barthel, K., & Baldwin, I. T. (2012). Herbivory-induced volatiles function as defenses increasing fitness of the native plant in nature. *eLife*, *1*, e00007. https://doi.org/10.7554/eLife.00007

Schuman, M. C., Palmer-Young, E. C., Schmidt, A., Gershenzon, J., & Baldwin, I. T. (2014). Ectopic terpene synthase expression enhances

sesquiterpene emission in *Nicotiana attenuata* without altering defense or development of transgenic plants or neighbors. *Plant Physiology*, *166*, 779–797. https://doi.org/10.1104/pp.114.247130

Shiojiri, K., Kishimoto, K., Ozawa, R., Kugimiya, S., Urashimo, S., Arimura, G., … Takabayashi, J. (2006). Changing green leaf volatile biosynthesis in plants: An approach for improving plant resistance against both herbivores and pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(45), 16672–16676.

Shiojiri, K., Ozawa, R., & Takabayashi, J. (2006). Plant volatiles, rather than light, determine the nocturnal behavior of a caterpillar. *PLoS Biology*, *4*, 1044–1047.

Tanaka, T., Ikeda, A., Shiojiri, K., Ozawa, R., Shikia, K., Nagai-Kunihiro, N., … Matsui, K. (2018) (in press)). Identification of a hexenal reductase that modulates the composition of Green leaf volatiles. *Plant Physiology*. https://doi.org/10.1104/pp.18.00632

Turlings, T. C. J., & Erb, M. (2018). Tritrophic interactions mediated by herbivore-induced plant volatiles: Mechanisms, ecological relevance, and application potential. *Annual Review of Entomology*, *63*, 1.

Webster, B., Gezan, S., Bruce, T. J. A., Hardie, J., & Pickett, J. A. (2010). Between plant and diurnal variation in quantities and ratios of volatile compounds emitted by *Vicia faba* plants. *Phytochemistry*, *71*, 81–89. https://doi.org/10.1016/j.phytochem.2009.09.029

Widhalm, J. R., Jaini, R., Morgan, J. A., & Dudareva, N. (2015). Rethinking how volatiles are released from plant cells. *Trends in Plant Science*, *20*, 545–550. https://doi.org/10.1016/j.tplants.2015.06.009

Wijnen, H., & Young, M. W. (2006). Interplay of circadian clocks and metabolic rhythms. *Annual Review of Genetics*, *40*, 409–448. https://doi.org/10.1146/annurev.genet.40.110405.090603

Xiao, Y., Savchenko, T., Baidoo, E. E. K., Chehab, W. E., Hayden, D. M., Tolstikov, V., … Dehesh, K. (2012). Retrograde signaling by the plastidial metabolite MEcPP regulates expression of nuclear stress-response genes. *Cell*, *149*, 1525–1535. https://doi.org/10.1016/j.cell.2012.04.038

Yon, F., Joo, Y., Cortes Llorca, L., Rothe, E., Baldwin, I. T., & Kim, S. G. (2016). Silencing *Nicotiana attenuata LHY* and *ZTL* alters circadian rhythms in flowers. *New Phytologist*, *209*, 1058–1066. https://doi.org/10.1111/nph.13681

Yon, F., Kessler, D., Joo, Y., Kim, S. G., & Baldwin, I. T. (2017). Fitness consequences of a clock pollinator filter in *Nicotiana attenuata* flowers in nature. *Journal of Integrative Plant Biology*, *59*, 805–809. https://doi.org/10.1111/jipb.12579

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Treatment with wounding and herbivore regurgitant elicited transient GLV emission, but the emissions of GLVs do not have a circadian rhythm (A, B) WT plants were grown in a climate chamber to identify circadian-regulated GLVs. WT plants were entrained in LD and transferred to LL. Plant volatiles were sampled by Poropak-Q filters and analyzed by TD-GC–MS (mean ± SE, n = 5 for LD and 6 for LL samples). The release of wounding plus regurgitant elicited GLVs did not have circadian rhythm. Indicated p-values are from one-way ANOVA analyses. Black arrow represents time of W + R elicitation. IS, internal standard; LD, light/dark cycle (12 h day/12 h night); LL, free-running condition (24 h day)

Figure S2. *NaHPL* is involved in time-dependent accumulation of total GLVs. (A) Mean (± SE, n = 6) accumulation of the total internal GLV pools were measured in EV and irRCA plants at different times; ZT8, ZT12, and ZT16.Asterisks indicate significant differences between treatments within time points (*, P < 0.05; **, P < 0.01; ***, P < 0.001). *RCA*, *RUBISCO ACTIVASE*

Figure S3. Basal transcript abundance of *NaHPL* is JA-dependent but the wound-induced reductions are time-dependent. Leaves (n = 3, 1/plant) were wounded with a pattern wheel and diluted regurgitant from *M. sexta* larvae (W + R) or water (mock, W + W) was immediately applied to the resulting puncture wounds; control leaves remained undamaged (Con). Mean transcript abundances (± SE) of *NaHPL* were measured in the morning (ZT1) in EV and irAOC plants.

Figure S4. Transcriptional rhythm of CAB2 and correlations between two circadian clock components are altered by *M. sexta* feeding. (A) Three *M. sexta* neonates were placed per plant (1/leaf) and leaf samples were collected every 4 h for 2 days. Mean (± SE, n = 3) transcript abundances of NaCAB2 were measured in leaves attacked by *M. sexta* larvae. (B) A regression analysis conducted between *NaLHY* and *NaTOC1* with and without herbivory. Asterisks indicate significant differences between treatments within time points (*, P < 0.05; **, P < 0.01; ***, P < 0.001). P-values were produced by an ANCOVA test and t tests.

Figure S5. Elicitations did not change the transcript abundances of *NaLHY*. Mean transcript abundance (± SE) of *NaLHY* was measured at ZT15 and ZT1 in plants subjected to W + W and W + R elicitation treatments. Different letters indicate significant differences among leaves determined by two-way ANOVAs with *post-hoc* tests with Tukey correction.

Table S1. Primers used in this study

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