Coping with variable herbivore community: ecological consequences of temporal dynamics and tissue-specific defense in *Nicotiana attenuata*

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1. Introduction

1.1. Beyond model organism and laboratory-based phenotyping and why it needs to combine with the natural history observation

Moving forward by looking backwards (Baldwin, 2011)

After Watson and Crick found the "common genetic material" of the all living organisms (Watson & Crick, 1953), Francis Crick (1996) made a claim that "The ultimate aim of the modern movement in biology is to explain all biology in terms of physics and chemistry." Molecular biologists have dramatically increased the mechanistic knowledge about the organisms by the well framed inductive method and it has advanced much faster than the other biological fields (strong inference, Platt, 1964). Great advances in molecular biology have been achieved by the experiments conducted in the laboratory with many representative model organisms, which are highly reproducible and falsifiable. Theoretical background of classical model organism systems is that all organisms share the same genetic material, DNA, and RNA, and these common traits make the model organisms representing of the "rest of life" for many biological questions (Alfred & Baldwin, 2015). Laboratory-based phenotyping also has been regarded as an important process, because a scientist tightly controls the experimental conditions for producing reproducible data. Since genes can function at any level of the biological phenomena from cell to community levels (Baldwin, 2012), those reductionist approaches in biology have been accumulated the knowledge successful in the early days of biology.

However, if these inductive assumptions of the molecular biology are not fully supported by the deductive reasoning of the certain research field, the knowledge from the "those inferences" could be groundless knowledge in biology. Every organism is the results of evolutions by natural selection in their natural habitats. If laboratory conditions do not fully mimic the environments of the native habitat, it is hard to test their adaptation. Therefore, there are many pieces of evidence that functional analysis in model organisms with laboratory-based phenotypes was not reproducible in nature. For instance, *(E)-β*-farnecene (TBF) is a well-known alarm pheromone of aphids which is produced when they are attacked by the predator and some plants produce this insect pheromone to decrease aphid abundances (Gibson & Pickett, 1983). This insect pheromone has been regarded as an

alternative target of plant engineering to increase plant resistance to aphid (Gatehouse, 2008) and the functional consequence of TBF has been proved under the laboratory environment (Beale *et al.*, 2006). However, unexpectedly, the genetically engineered wheat, which consistently produces TBF did not significantly increase the plant resistance to aphid in the field than did the wild type wheat (Bruce *et al.*, 2015). Continuous emitting of TBF might not work in the field as a semiochemical to aphids (Kunert *et al.*, 2010). In addition, reducing the lignin contents in plants can improve the quality of biofuel crops. Although the genetic manipulation of the lignin biosynthetic gene (silencing-*Cinnamyl Alcohol Dehydrogenase*, CAD) decreases lignin contents of the stem in glasshouse-grown popular plants, the lignin contents in the field-grown plants were not consistent in different planting sites (Lapierre *et al.*, 1999; Pilate *et al.*, 2002). In *Nicotiana attenuata*, lignin contents in silencing-CAD plants are fully recovered in the native habitat; the strong wind and UV in nature can stimulate the alternative biosynthetic pathway of lignin (Kaur *et al.*, 2012). Therefore, the function of a gene should be tested in nature with enough natural history observations of the study organisms; the gene functions in organismic levels are often different from its biochemical function (Baldwin, 2012).

1.2. Gene functions in nature

Many molecular biologists have tried to answer functional questions to understand the adaptive value of the phenotypes with the knowledge obtained from the model systems. In parallel, ecologists have been asking the ecological processes in a fundamentally molecular level (Purugganan & Gibson, 2003). Adaptation is the process that an organism or species becomes better suited to its environment. Adaptation is a relative concept so that it can be a benefit in a certain environment, and it also can be detrimental in the other environment. Therefore, adaptive values of phenotypic plasticity should be tested under ecologically relevant environments (Schmitt *et al.*, 2003). For example, the model plant, *Arabidopsis thaliana* ecotypes are classified regarding a vernalization requirement for flowering: summer-annual (rapid-cycling genotype, e.g. *col*) and winter-annual (e.g. *Ler*) (Simpson & Dean, 2002). Flowering times of plants are strongly associated (or correlated) with the latitude of plant's native habitat (Stinchcombe *et al.*, 2004). However, this well-known hypothesis was recently tested. Wilczek and coworkers (2009) planted two different accessions with several flowering time mutants of *A. thaliana* in 6 different habitats (which

have different latitudes). Interestingly, they concluded that two accessions could be both summer-annual and winter-annual ecotypes and only their sensitivities to environmental signals varied. Phenotypes of the model organisms are frequently used to answer both mechanistic and functional questions. However, if natural history observation is not properly combined, the functional knowledge which is solely concluded from the laboratory, e.g. life-cycle of *A. thaliana*, can be groundless. Further, the comparison of two well-known insect behaviors ("nictation" and "siesta") will illustrate why field-based phenotypes are necessary for the functional analysis (**Figure 1**).

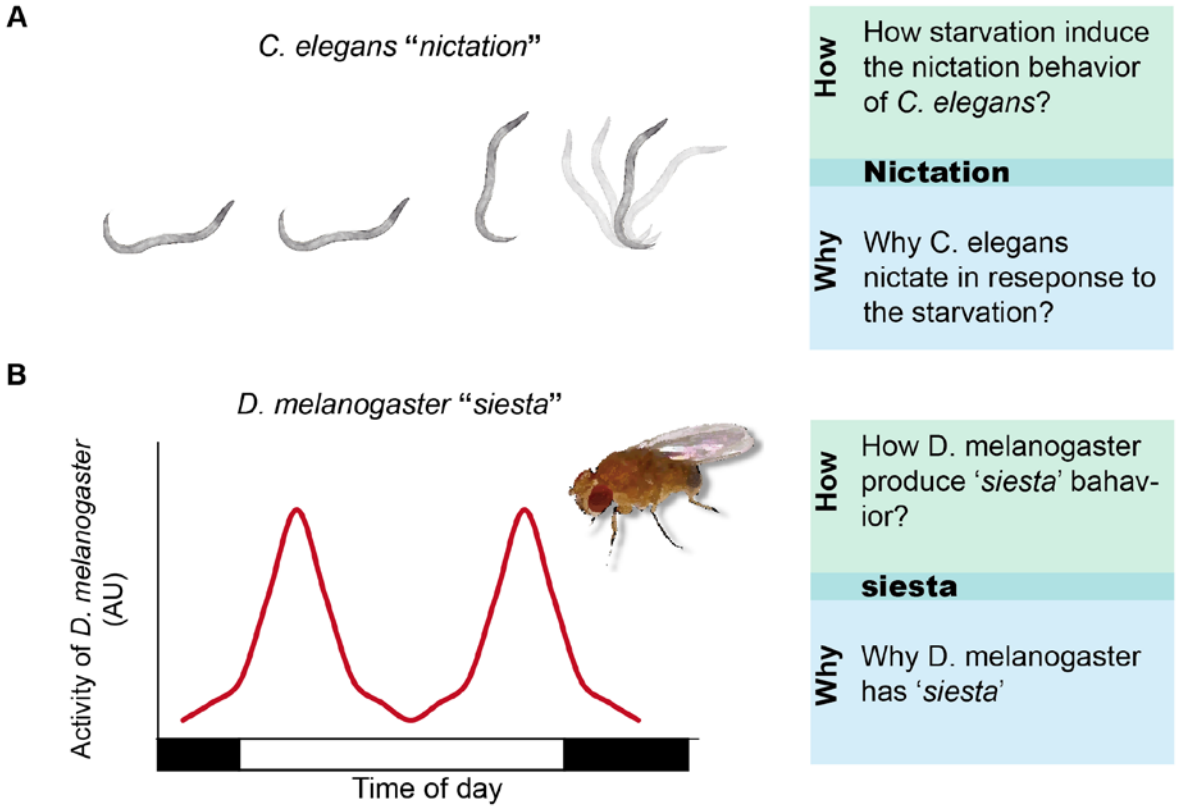

Figure 1. Graphical summary of *Caenorhabditis elegans* 'nictation behavior' (A) and *Drosophila melanogaster* 'siesta behavior' (B).

Each phenotype is used to understand the molecular background of the important biological phenomenon: 'nictation behavior' for the starvation responses (Campbell & Gaugler, 1993) and '*siesta*' for the circadian rhythm in animal (Blau & Rothenfluh, 1999).

When the *Caenorhabditis elegans* larvae starve, they turn into the dauer stage to avoid unfavorable conditions (Fielenbach & Antebi, 2008). Interestingly, the dauer larvae are still able to move and erect themselves with a waving behavior, which is called the nictation **(Figure 1A)** (Lee *et al.*, 2011). Many developmental genetic studies have shown that how the starvation induces the nictation of *C. elegans* (Félix & Braendle, 2010). Furthermore, Lee *et al.*, (2011) revealed that nictation behavior of *C. elegans* increases the chance to move out from the current unfavorable place by attaching to legs of flies in the laboratory. Interestingly, this observation in the lab is also supported by the behavior traits of *C. elegans* in their native habitat (Campbell & Gaugler, 1993). In this case, mechanistic information was beneficial to understand the function reason of the insect behaviors.

In *Drosophila*, sleep and waking patterns are the phenotypic screening parameters to examine the animal endogenous clock system (Shaw *et al.*, 2000). The fruit fly is known to be inactive during the midday, which is called '*siesta*'(Blau & Rothenfluh, 1999; Majercak *et al.*, 1999). Many studies suggest several functional reasons of *'siesta'* behavior, e.g. increasing mating efficiency, avoiding strong daylight **(Figure 1B)** (Fujii *et al.*, 2007; Rieger *et al.*, 2007; Ferguson *et al.*, 2015). Unexpectedly, Vanin and colleagues (2011) have shown that fruit fly has additional activity during the day in fluctuated environment and the fruit fly does not take a rest during the mid-day. It suggests that '*siesta*' behavior is a more likely "artifact" in the laboratory. The chronobiologists have identified successfully the genes that act as the mechanical gears of the clock using the behaviors of *D. melanogaster* under the artificial laboratory environment. However, if scientists want to make arguments about evolutionarily appropriate behaviors in flies, it is evident that scientist is better not to use flies that rest and wake at odd times like '*siesta*'.

These case studies point out why the level of analysis should be considered and why the natural observation is necessary for the functional analysis even in model organisms. If we know the natural history of the species, we can find the ecologically relevant functional question. Each of questions in 'how' and 'why' is not competing (Sherman, 1987). I rather want to emphasize how combining natural history with molecular biology techniques, which increases our knowledge about nature (Millar, 2016). These are the academic motivations of this dissertation why I have studied the molecular mechanisms of ecological interactions and conducted the field experiments as well for research questions.

1.3. Keeping robust biological phenomenon under variable environments

Although many ecologists underestimate the usages of the model organisms by saying like “no two cells are alike” (Platt, 1964), biology is the science of heterogeneous system. In nature, biotic and abiotic factors are not homogeneous, so it is challenging fully mimic in the laboratory. The environmental heterogeneity is ubiquitous in nature, and it generates different ecological niches (Tilman, 1982). In community ecology, the environmental heterogeneity is often regarded as one of the most important positive factors for species richness because it increases a chance for species coexistence, persistence, and diversification (Jeremy & Lundholm, 2009; Stein *et al.*, 2014). Moreover, a single organism also faces biotic and abiotic heterogeneity over time and space scales, and these heterogeneities often increase the phenotypic plasticity of organisms (Karban, 2011). Therefore, to survive under heterogeneous environment, plants require evolving plastic responses over time and space.

Timing is everything in ecological interactions because many of ecological interactions are not homogenous throughout a different time (**Figure 2**). In particular, the ecological interactions between the herbivores and the hostplant depend largely on their temporal and spatial characteristics, e.g. the sequence of arrival and localization relative to the attacked tissues on the same hostplant, as well as on their feeding guilds (Erb *et al.*, 2011; Kant *et al.*, 2015; Lortzing & Steppuhn, 2016). Perhaps as an emergent property of the coordination between individual organisms and abiotic cycles, circadian clocks also allow organisms to coordinate with each other’s diurnal activity patterns (Wang *et al.*, 2011; Goodspeed *et al.*, 2012; Lai *et al.*, 2012; Jander, 2012). Ontogenetic events furthermore determine timing and prioritization of phenotypes due to e.g. developmental necessity, adaptation to environmental changes, or the transition from vegetative growth to reproduction. However, it is largely unknown whether the timing is crucial in plant-insect interactions and it is less known how plants keep robust interactions with environments under temporally heterogeneous environments.

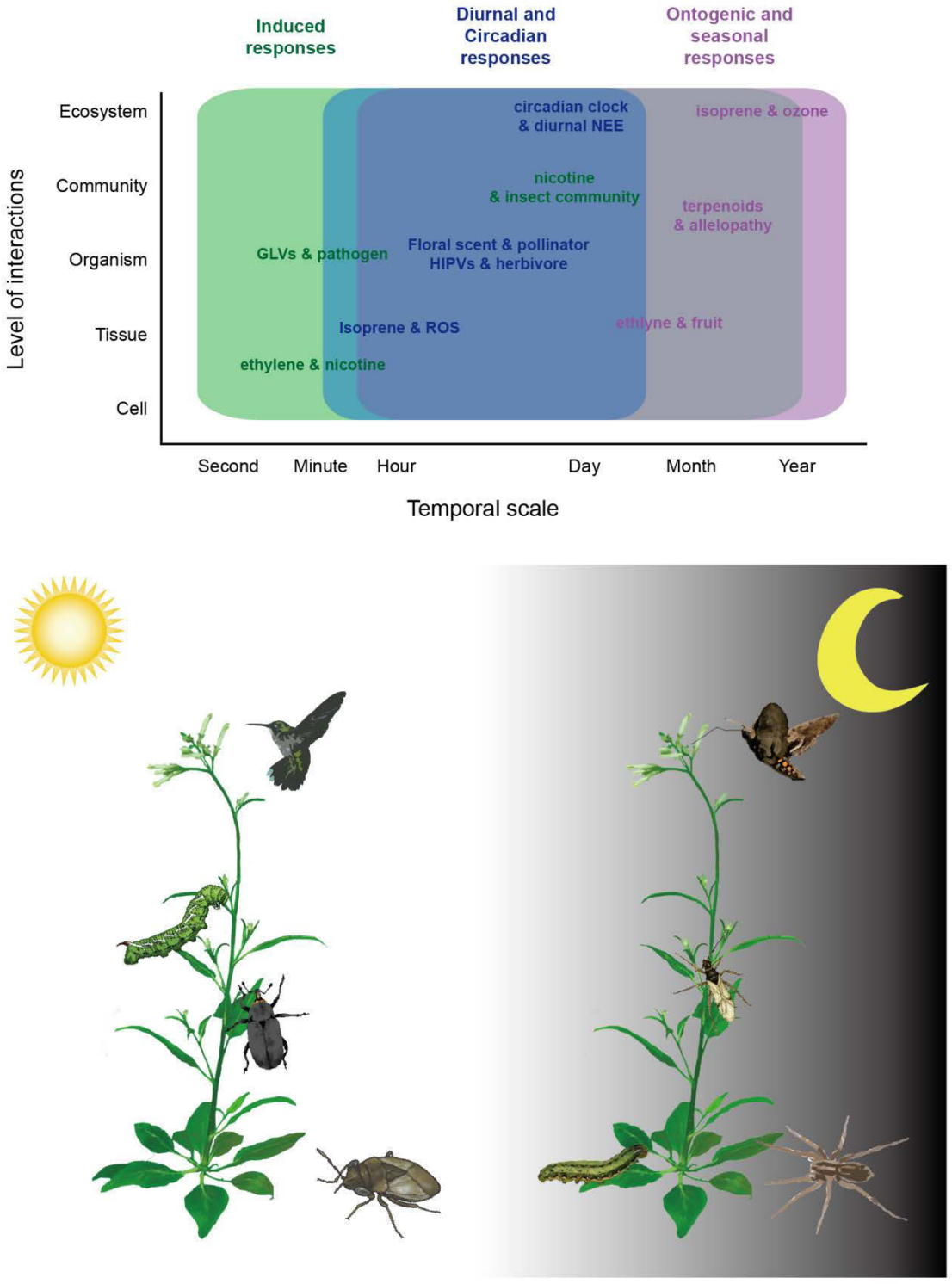

Figure 2. Temporal heterogeneity of ecological interactions (A) A conceptual scheme of the functions of plant volatiles over ecological and temporal scales. Ecological interactions highly vary in different levels of interactions and temporal scale. (B) Diurnal variations of ecological interactions in *Nicotiana attenuata.* Plants face diverse insect community at different time of a day.

Plants have diurnal rhythms in response to biotic and abiotic signals, e.g. light, temperature, and pollinator activity. Many organisms including plants have their own endogenous oscillator, which is called by the circadian clock, to keep internal rhythms without *zeitgeber*. The plant circadian clock has been intensively dissected in a molecular level when a non-destructive technique was developed to examine a plant endogenous rhythm. Kay and colleagues have developed the luciferase system for the model plant, *Arabidopsis thaliana*, which is successfully worked in *Drosophila* clock studies (Millar *et al.*, 1992). Over 30 clock components have found in *Arabidopsis* using this system and these components shape multiple interlocked feedback loops (Sanchez & Kay, 2016). It may be important to maintain the function under heterogeneous environments by having temporal plastic responses (Troein *et al.*, 2009). However, it is widely unknown the role of the plant circadian clock in nature.

Insect communities also heterogeneous even in a single plant, so plants have various types of feeding guilds of herbivores which colonize in the different parts of the plant, e.g. leaf chewer, stem borer, and root feeder (**Figure 3**). Interguild- and intraguild interactions of herbivorous insects are important for shaping the plant and insect community in nature. Co-occurring herbivorous insect in a common host plant can be in competition with one another (Hardin, 1960) if the ecological niche of the single host plant does not differentiate temporally and spatially (Schoener, 1974; Connell, 1983). Although the competition theory is widely accepted in herbivore interactions in a same host plant and plant systemic signals increase the chance of competitive interactions, the importance of competition among the herbivorous insect remains controversial because plant-mediated effects are not always symmetric (Kaplan & Denno, 2007). Especially, plant-mediated herbivorous insect interactions also can be synergistic and neutral from other herbivores because plant systemic signals are often asymmetric (Kaplan *et al.*, 2008; van Dam & Heil, 2011; Erb *et al.*, 2015). Plant secondary metabolites are highly heterogeneous in different plant tissues (Li *et al.*, 2016). Therefore, it is likely that plants have the tissue-specific resistance strategies to defense tissue-specific herbivores. However, we have comparatively less known about how plants cope with spatially-separated herbivores in a same hostplant.

The main aim of the dissertation is investigating how plants survive in various herbivore community in spatiotemporal scales. Ecological interactions vary on many spatial and temporal scales because niche can be differentiated in different time and different space.

Simplified interactions or laboratory-based phenotypes are not inadequate to test those questions because ecological interactions are often context-dependent (Chamberlain *et al.*, 2014). Therefore, I have investigated spatiotemporal heterogeneity of biotic and abiotic factors and their ecological consequences in nature and then further studied how plants cope with the environmental heterogeneities.

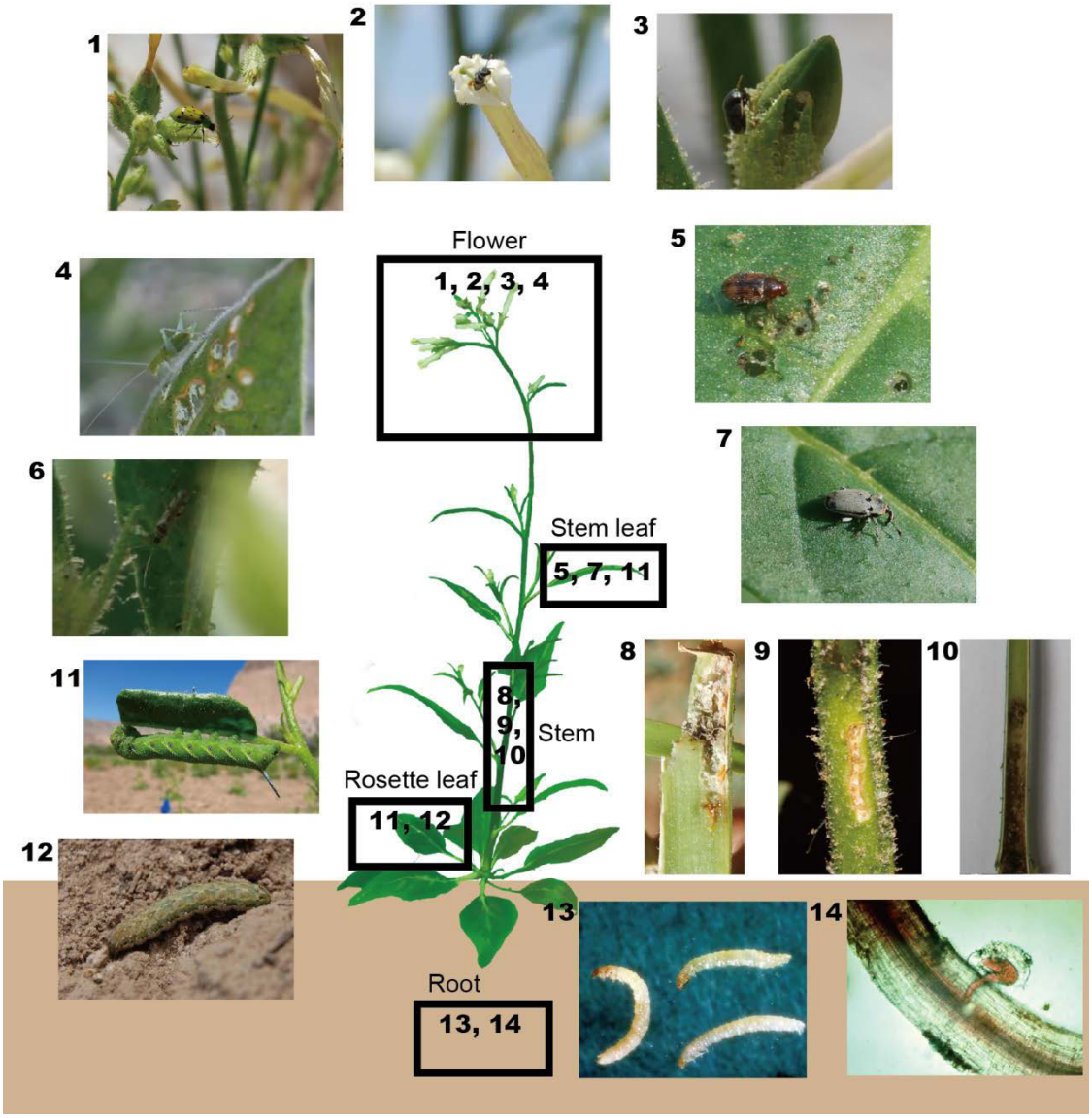

Figure 3. Spatial heterogeneity of ecological interactions and the ecological model plant *Nicotiana attenuata* and its native herbivores in the Great Basin Desert, Utah, USA. *N. attenuata* interact with various insects in different tissues. 1. Spotted cucumber beetle (*Diabrotica undecimpunctata*), 2. Bee, 3. Negro bug (*Corimelaena extensa*), 4. Tree cricket (*Oecanthus fultoni*), 5. Flea beetle (*Epitrix* sp.), 6. Mirids (*Tupiocoris notatus*), 7. Tobacco

weevil (*Trichobaris mucorea*), 8. Gall midge (unknown species), 9. Oviposition damage by tree cricket (*Oecanthus fultoni*), 10. Tobacco weevil larvae (*Trichobaris mucorea*), 11. Tobacco hornworm (*Manduca sexta*), 12. Armyworm (*Spodoptera sp.*), 13. Flea beetle larvae (*Epitrix* sp.). All pictures were taken by Youngsung Joo, except 11 (Anne Weinhold), 13, and 14.

1.4. Timing of photosynthesis

Plants face highly fluctuated light under their native habitat. The complex mechanism in plant circadian clock has been regarded as an adaptation for surviving under environmental noises (Troein *et al.*, 2009; Sanchez & Kay, 2016). Plants have evolved to track the highly predictable solar radiation that results from the rotation of the earth on its tilted axis, so photosynthesis is one of the best examples of the circadian clock-regulated biological processes (Müller *et al.*, 2014). While photosynthesis is a very complex process consisting of several interrelated physiological and molecular processes, each part of the photosynthetic process exhibits strong circadian rhythms (Müller *et al.*, 2014). Net carbon assimilation rates were reduced in *CCA1*-overexpressed plants, and plant fitness is maximized when the period of internal rhythms defined under constant light conditions matched the period of external light/dark cycles (Dodd *et al.*, 2005, 2014).

Especially, Dodd *et al.* (2014) claimed that the circadian clock is important for the anticipation of dawn because of the clock mutants, which have a different circadian period than that of wild-type (WT) plants, are unable to anticipate dawn and dusk. Many genes encoding different parts of the photosynthetic apparatus are transcriptionally induced before dawn, and carbon fixation is reduced in clock-altered *A. thaliana* plants. It suggests that the circadian clock may 'warm up' the photosynthetic machinery in anticipation of the sun rising to maximize carbon fixation (Harmer *et al.*, 2000; Covington *et al.*, 2008). This important inference, which is commonly assumed by the circadian research community, has not been rigorously examined in plants growing under real-world conditions. Therefore, I have investigated the clock function for photosynthesis in nature in the **manuscript I**.

1.5. Timing of perfuming for semiochemicals

(Summarized from Shuman, Valim, and Joo, 2016)

Volatile compounds are small molecules (generally < 300 Da): sufficiently lightweight and low-polarity to have high vapor pressures under normal environmental conditions (Dudareva *et al.*, 2006). These molecules may come from any of several biosynthetic pathways which are closely linked to pathways or products of general metabolism, i.e., fats and other lipids, amino acids and proteins (Dudareva *et al.*, 2006; Baldwin, 2010).

Plant volatiles have significant roles within plant tissues in physiology, signaling, and defense. When emitted through the cuticle, stomata or injured tissue, or from specialized structures (Widhalm *et al.*, 2015), they may be perceived by a host of other organisms as well as by remote parts of the plant (Heil & Ton, 2008; Baldwin, 2010). The composition of volatile blends can convey detailed information about the physiological and ecological status of plants which may be used by microbes, animals, and other plants, both detrimental and beneficial (Dicke & Baldwin, 2010). The timing of both production and emission of floral and vegetative volatiles is thus essential to their functions in within-plant signaling, as well as in orchestrating interactions with other organisms, and may determine their potential for abuse by enemies.

Behaviors of insects are highly time-dependent, so timely regulation of plant metabolism is required to keep robust plant-insect interactions. Although rhythmic emissions of plant volatiles are well known, the functionality of the rhythm itself has not been reported. Plant indirect defenses are perhaps more susceptible to disturbance by temporal shifts in the insect communities around plants than direct defenses are. Plant indirect defenses rely on attracting predators or parasitoids after herbivore attacks. Herbivore-induced plant volatiles (HIPVs) are not the direct reward for predators, but it makes herbivorous preys more apparent to the predator. Therefore, to keep robust plant indirect defense, timely coincidences among tri-trophic levels are required: plant volatiles, herbivore existence, and carnivore activity. In **manuscript II**, I investigated how plants cope with temporally heterogeneous insect communities. Moreover, it is also poorly known the function of the clock in diurnal rhythms in HIPVs, so I have investigated the clock function in HIPVs and their ecological consequences in **Manuscript III**.

As well as the plant indirect defense, plant pollination also requires temporal coincidence between floral advertisements and pollinator activities. I have investigated the role of the circadian clock in floral volatiles and their ecological consequence in **Manuscript**

IV and V.

1.6. Tissue-specific defense for spatially heterogeneous insect damages in a plant

Individual plants provide space for various herbivore communities, and multiple herbivores can often colonize different parts of the same plant. Plants can, therefore, play an important role in shaping community composition in ecosystems by mediating interactions among herbivores (Ohgushi, 2016). Plant-mediated interactions among different folivores or between above- and below-ground herbivores are relatively well understood (Soler *et al.*, 2013). However, although the stem is essential for structural support and nutritional transport, it is largely unknown how the stem responds to stem-feeding herbivores, or whether significant interactions between leaf herbivores and stem herbivores exist. Many plants systemically induce the resistance to herbivores in response to multiple herbivore attacks via jasmonic acid (JA) signaling (Howe & Jander, 2008). Systemic induction, e.g. via JA signaling, thus has the potential to homogenize the different niches of a plant, although other plant systemic signals could also shape synergistic and neutral interactions among herbivores in a plant. In **manuscript VI**, I have investigated how plants defend against the stem herbivore attack (tissue-specific resistance) and its ecological consequence (the interaction between the leaf herbivore and the stem herbivore).

1.7. *Nicotiana attenuata* as a study plant

Nicotiana attenuata Torr. ex. S. Watson (Solanaceae) is a wild tobacco species found as a summer annual native plant to Southwestern North America (**Figure 3**). As a post-fire pioneer species, seeds of *N. attenuata* preferentially germinate in the nutrition-rich, e.g. nitrogen, soils after being exposed to smoke-related cues (Baldwin & Morse, 1994). *N. attenuata* less competes with other plant species for their resource because they are the post-fire pioneer species. However, *N. attenuata* is exposed to extremely variable and unpredictable herbivore communities (**Figure 3**), e.g. the piercing-sucking herbivores *Tupiocoris notatus* (mirid) and *Empoasca* spp. (leaf hopper), the chewing herbivores *Trichobaris mucorea*. *Epitrix* spp. (flea beetles), *Spodoptera* spp. (armyworm) and larvae of the specialists *Manduca sexta* (tobacco hornworm) and *Manduca quinquemaculata* (tomato hornworm). Moreover, *N. attenuata* is native to the Great Basin Desert of the southwestern

USA, so plants also experience strongly variable abiotic factors (e.g. temperature, light intensity, and UV). Therefore, in response to these extreme environmental selective pressures, *N. attenuata* has evolved highly plastic adaptive responses to cope with highly various environmental factors (Baldwin, 1998). As well as ecological characteristics of *N. attenuata,* this species also have great advantage for the genetic manipulations and the resources. *N. attenuata* is self-compatible, easily cultivated and regenerated after *Agrobacterium*-mediated transformation (Krügel *et al.*, 2002). These properties make *N. attenuata* a perfect and attractive model plant for my dissertation.

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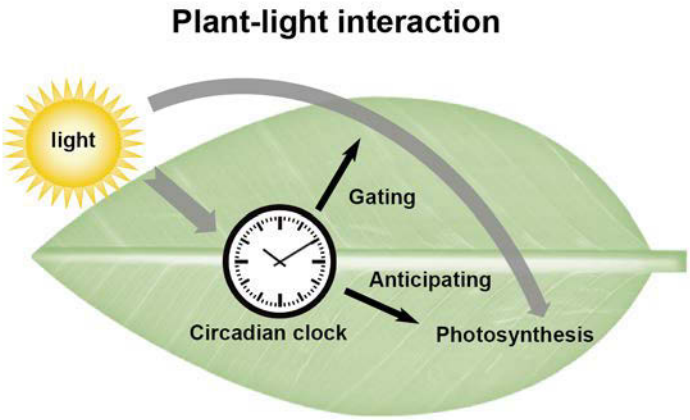
2. Manuscript Overview

Manuscript I

The circadian clock component, LHY, tells a plant when to respond photosynthetically to light in nature

Youngsung Joo, Variluska Fragoso, Felipe Yon, Ian T. Baldwin*, Sang-Gyu Kim*

* Co-corresponding authors (Accepted in *Journal of Integrative Plant Biology*)



In this manuscript, I tested whether the circadian clock primes the photosynthetic machinery to anticipate light at dawn under real world conditions with a native plant. I showed that it does not; I found that the clock is more important for dusk anticipation, and it also does something more interesting: the clock tells a plant when to ignore the light, particularly, not to respond to light in the middle of the night. Furthermore, I revealed that plants modulate the responsiveness to red light and it mediateby phytochrome A. In the ongoing work with clock mutants in the field, this is emerging as a central theme: the clock tells plants when to pay attention to environmental signals and when not to.

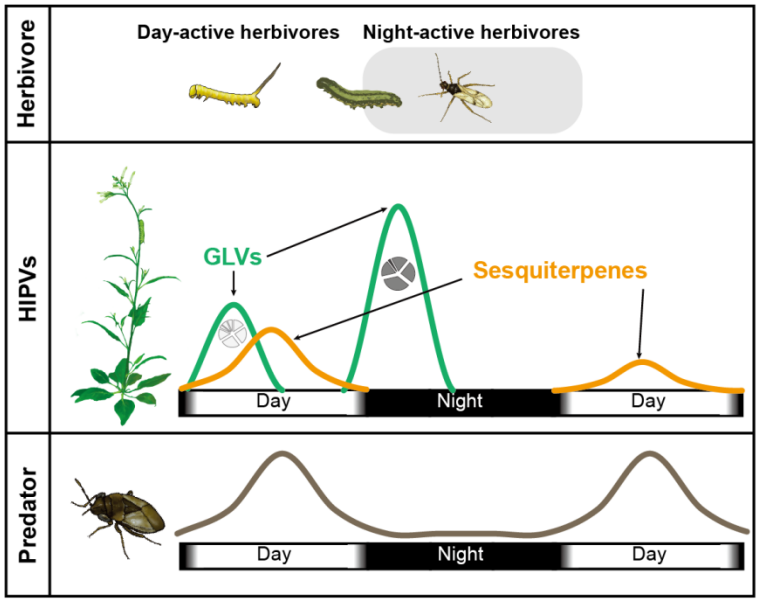
I.T.B. and S.K. conceived the project. Y.J., I.T.B. and S.K. designed the research. Y.J., S.K., F.Y. and V.F. performed experiments. Y.J. and V.F. analyzed the data. Y.J., I.T.B. and S.K. wrote the manuscript.

Manuscript II

Herbivore-induced volatile blends with both "fast" and "slow" components provide robust indirect defense in nature

Youngsung Joo, Meredith C. Schuman*, Jay K. Goldberg, Sang-Gyu Kim, Felipe Yon, Christoph Brütting, and Ian T. Baldwin* (under review in *Functional Ecology*)

* Co-corresponding authors



In this manuscript, I investigated how temporal dynamics of HIPV blends from plants in nature are affected by both time of day, and elicitation time by herbivores, and whether these dynamics influence the responses of native predators. By the supplementation test, I found that the timing of herbivore elicitation strongly affected HIPV blend composition. Green leaf volatiles (GLVs) and sesquiterpenes attracted predators at different times of day and during different time periods after an attack by the genetic manipulation. Combinations in temporal dynamics of wild-type HIPV blends resulted in the reliable predator attraction, despite the variable timing of herbivore attack.

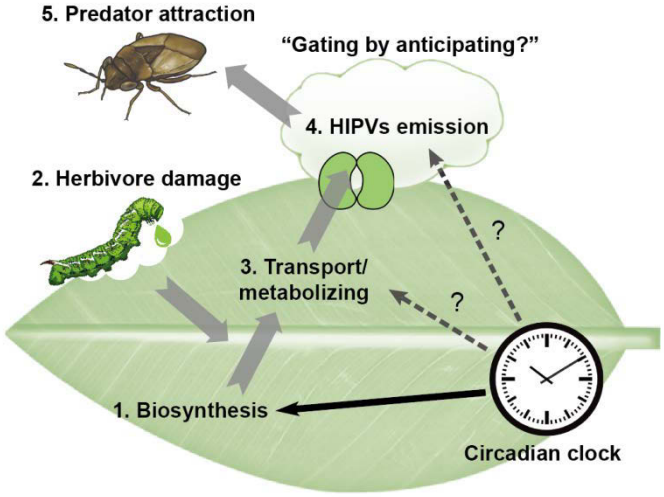
Conceptualization and Supervision: S.K., I.T.B., and M.C.S. Project Administration: I.T.B. Funding Acquisition: I.T.B. Methodology: Y.J., S.K., I.T.B., and M.C.S. Investigation: Y.J., J.K.G., S.K., F.Y., and M.C.S. Formal Analysis, Data Curation and Validation: Y.J., J.K.G., and M.C.S. Visualization: Y.J. Writing – Original Draft: Y.J. and M.C.S. Writing – Review & Editing: I.T.B.

Manuscript III

The circadian clock in *Nicotiana attenuata* times accumulation, but not emission, of herbivore-induced plant volatiles that function as indirect defenses

Youngsung Joo, Meredith C. Schuman, Jay K. Goldberg, Felipe Yon, Antje Wissgott, Sang-Gyu Kim*, and Ian T. Baldwin* (*In preparation)*

* Co-corresponding authors



In this manuscript, I investigated the role of the plant circadian clock in plant inducible defense in response to the herbivore attack. Here I showed that the circadian clock in *Nicotiana attenuata* regulates biosynthesis of constitutively present green leaf volatiles (GLVs) which are, however, modified and released in typical ways upon herbivory. Although the circadian clock did not regulate emissions of herbivore-induced plant volatiles directly, the clock also modulates the time-dependent responsiveness of other herbivore-induced plant volatiles.

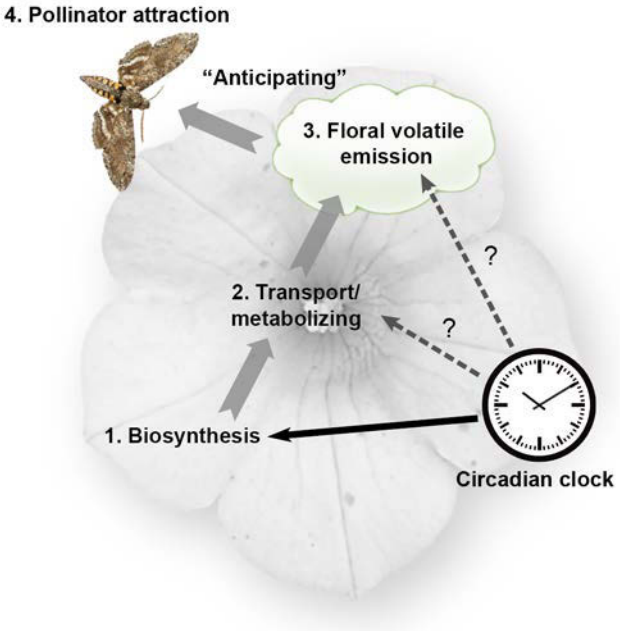
Conceptualization and Supervision: S.K., I.T.B., and M.C.S. Project Administration: I.T.B. Funding Acquisition: I.T.B. Methodology: Y.J., S.K., I.T.B., and M.C.S. Investigation: Y.J., J.K.G., S.K., F.Y., and M.C.S. Formal Analysis, Data Curation and Validation: Y.J., J.K.G., and M.C.S. Visualization: Y.J. Writing – Original Draft: Y.J. and M.C.S. Writing – Review & Editing: I.T.B.

Manuscript IV

Silencing *Nicotiana attenuata* LHY and ZTL alters circadian rhythms in flowers

Felipe Yon, **Youngsung Joo**, Lucas Cortes Llorca, Eva Rothe, Ian T. Baldwin, Sang-Gyu Kim

Published in the *New Phytologist* 2016, 209(3):1058-1066, doi: 10.1111/nph.13681



In this manuscript, I examined the expression of clock-regulated genes, e.g. *NaCAB2,* in different circadian clock transgenic lines to know endogenous rhythms. I also measured temporal dynamics of floral volatiles under a diurnal and free-running condition in WT and clock transgenic plants to test the function of the plant circadian clock in the pollination. This work allows me to think about the role of the circadian clock can be different in various ecological interactions.

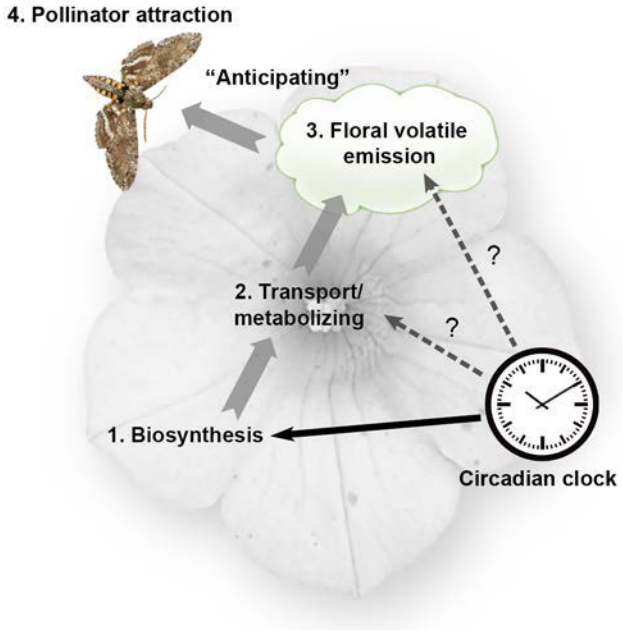
Conceptualization and Supervision: S.K. and I.T.B. Project Administration: I.T.B. Funding Acquisition: I.T.B. Methodology: F.Y., Y.J., L.C.L, and S.K. Investigation: F.Y., Y.J., L.C.L, E.R., and S.K. Formal Analysis, Data Curation and Validation: F.Y., Y.J., L.C.L, and S.K. Writing – Original Draft: F.Y., Y.J. and S.K. Writing – Review & Editing: I.T.B.

Manuscript V

Fitness consequences of altering floral circadian oscillations for *Nicotiana attenuata*

Felipe Yon, Danny Kessler, **Youngsung Joo**, Lucas Cortes Llorca, Sang-Gyu Kim, Ian T. Baldwin

Published in the *Journal of Integrative Plant Biology* 2017, 59(3):180-189, doi: 10.1111/jipb.12511



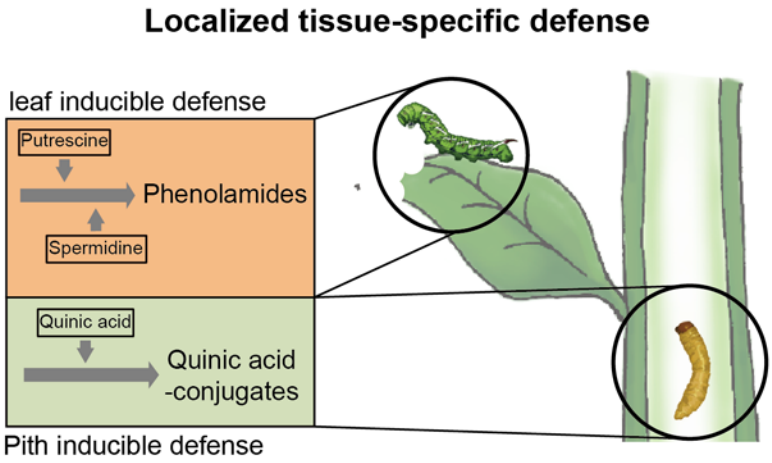
In this manuscript, I examined the floral volatile emissions in different circadian clock transgenic lines to know whether the circadian regulated floral advertisement is essential for plant Darwinian fitness. Unexpectedly, the fitness consequence of the circadian clock is not adaptive in the glasshouse. Here we only tested the fitness consequence with one pollinator, *M. sexta*. It strongly suggests that the importance of circadian clock may be context-dependent and the necessities of the test in nature.

Conceptualization and Supervision: S.K. and I.T.B. Project Administration: I.T.B. Funding Acquisition: I.T.B. Methodology: F.Y., Y.J., K.D and S.K. Investigation: F.Y., Y.J., K.D, and S.K. Formal Analysis, Data Curation and Validation: F.Y., Y.J., and K.D. Writing – Original Draft: F.Y. Review: Y.J., K.D., and S.K. Writing – Review & Editing: I.T.B.

Manuscript VI

What happens in the pith stays in the pith; tissue-localized defense responses facilitate niche differentiation between two spatially separated herbivores

(Gisuk Lee*, **Youngsung Joo***), Sang-Gyu Kim, Ian T. Baldwin (under review in *The Plant Journal*) * **Co-first authors**



We first asked whether a significant interaction between leaf and stem herbivores exists when they simultaneously colonize plants by conducting reciprocal feeding assays in both the field and the glasshouse. To investigate defense responses in the pith, we conducted phytohormone and secondary metabolites analysis using transgenic plants impaired in JA signaling and in the specific secondary metabolites elicited in the pith. Lastly, we investigated how plants shape the interaction between these herbivores by measuring systemic changes in leaf and stem tissues, respectively.

Conceptualization and Supervision: S.K. and I.T.B. Project Administration: I.T.B. Funding Acquisition: I.T.B. Methodology: I.T.B.,G.L. and Y.J. Investigation: G.L. and Y.J. Formal Analysis, Data Curation and Validation: G.L. and Y.J. Visualization: G.L. and Y.J. Writing – Original Draft: G.L., Y.J. and S.K: Rewriting – Review & Editing: I.T.B.

3. Manuscript

Manuscript I

JIPB Journal of Integrative Plant Biology

Circadian clock component, LHY, tells a plant when to respond photosynthetically to light in nature

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doi: 10.1111/jipb.12547

Research Article

Abstract The circadian clock is known to increase plant growth and fitness, and is thought to prepare plants for photosynthesis at dawn and dusk; whether this happens in nature was unknown. We transformed the native tobacco, *Nicotiana attenuata* to silence two core clock components, *NaLHY* (irLHY) and *NaTOC1* (irTOC1). We characterized growth and light- and dark-adapted photosynthetic rates (A_c) throughout a 24 h d in empty vector-transformed (EV), irLHY, and irTOC1 plants in the field, and in NaPhyA- and NaPhyB1-silenced plants in the glasshouse. The growth rates of irLHY plants were lower than those of EV plants in the field. While irLHY plants reduced A_c earlier at dusk, no differences between irLHY and EV plants were observed at dawn in the field. irLHY, but not EV plants, responded to light in the night by rapidly increasing A_c. Under controlled conditions, EV plants rapidly increased A_c in the day compared to dark-adapted plants at night; irLHY plants lost these time-dependent responses. The role of NaLHY in gating photosynthesis is independent of the light-dependent reactions and red light perceived by NaPhyA, but not NaPhyB1. In summary, the circadian clock allows plants not to respond photosynthetically to light at night by anticipating and gating red light-mediated in native tobacco.

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INTRODUCTION

The circadian clock (from the Latin *circa*; approximately, and *dies*; day) increases the Darwinian fitness of many organisms grown under light/dark cycles (Yerushalmi and Green 2009). Daily rhythms in organisms persist in non-rhythmic environments due to the action of an internal circadian clock which is entrained by external signals (Greenham and McClung 2015). Many genetic components of the plant circadian clock have been identified from high-throughput screens with the genetic model plant, *Arabidopsis thaliana* (Millar et al. 1992). Among them, LATE ELONGATED HYPOCOTYL (LHY)/ CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and TIMING OF CAB EXPRESSION 1 (TOC1), which are highly conserved in plants, play a central role in synchronizing internal rhythms with external signals (Nagel and Kay 2012).

The regulation of photosynthesis by the circadian clock has been well studied as plants have evolved to track the highly predictable variation in solar radiation that results from the rotation of the earth on its tilted axis (Müller et al. 2014). While photosynthesis is a complex process consisting of several interrelated physiological and molecular processes, each part exhibits strong circadian rhythms, for example, net carbon assimilation rate, stomatal opening, chlorophyll fluorescence, and chlorophyll contents (Gorton et al. 1989; Hennessey and Field 1991; Rascher et al. 2001; Müller et al. 2014; Pan et al. 2015). Net carbon assimilation rates were reduced in *CCA1*-overexpressing plants, and plant fitness is maximized when the period

of internal rhythms defined under constant light conditions matched the period of external light/dark cycles (Dodd et al. 2005). In addition, the anticipation of dawn and dusk by the circadian clock may be important because the circadian clock mutants are unable to anticipate the proper phase of dawn and dusk (Dodd et al. 2014). Transcript levels of photosynthesis-related genes also support the idea that the circadian clock may "warm up" the photosynthetic machinery in anticipation of light at dawn to maximize carbon fixation and "cool down" the photosynthetic machinery at dusk to maximize water efficiency (Harmer et al. 2000; Dodd et al. 2005, 2014; Covington et al. 2008). This important inference about the potential role of the clock in photosynthesis has not been rigorously examined, and not in plants growing in nature.

In addition, the circadian clock may also play roles in photosynthesis by modulating responsiveness (="*gating*") to changes in light (Hotta et al. 2007), shade avoidance (Salter et al. 2003), cold (Dong et al. 2011) and drought (Legnaioli et al. 2009), although the molecular mechanism of this circadian gating remains relatively unknown (Hubbard and Webb 2015). Stomatal responsiveness to light signals shows time-dependent patterns under a free-running condition, which are gated by the circadian clock (Gorton et al. 1993) and stomatal regulation by ABA signaling is mediated by TOC1 (Legnaioli et al. 2009; Lee et al. 2016). Since stomatal opening is essential for the assimilation of carbon, circadian gating of stomata behavior can dramatically affect photosynthetic performance in nature.

The circadian regulation of photosynthesis can be mediated by photoreceptors. Light entrains the circadian clock and acts as both energy source and signal for photosynthesis and stomatal opening (Gorton et al. 1993; Somers et al. 1998b; Bae and Choi 2008). Light regulates stomatal aperture through its influence on guard cells (Shimazaki et al. 2007). Blue light elicits stomatal opening by activating a membrane H^+-ATPase in guard cells (Shimazaki et al. 2007). In addition, the blue light receptor, phototropin, maintains the robust rhythm of light-dependent reactions of photosynthesis under blue light (Litthauer et al. 2015).

The regulation of photosynthesis and related processes by the circadian clock has been established by many laboratory experiments, but this function of the clock for plants growing in nature remains unknown. Some clock functions established by laboratory experiments have been found to be artifacts when tested under the natural light conditions of the real world conditions (see Vanin et al. 2012). To test the role of the circadian clock in the plant's ability to photosynthetically anticipate certain phases, for example, dawn and dusk, and modulate a plant's responsiveness to light, we transformed *Nicotiana attenuata*, a native tobacco to the Great Basin Desert of the USA, which has been used for three decades as a model ecological study system (Baldwin 2001), to silence two core clock components, *NaLHY* and *NaTOC1* (Yon et al. 2012, 2016), and examined their photosynthetic performance when grown in the plant's native habitat (Figure 1A).

RESULTS

LHY, but not TOC1, represses photosynthesis at night, and anticipates dusk rather than dawn in nature

Previously, we had shown that the phase of rhythmic transcript abundances of *N. attenuata CHLOROPHYLL A/B BINDING PROTEINS 2* (*NaCAB2*), a marker of endogenous circadian rhythms, in *LHY*-silenced *N. attenuata* (irLHY) plants was advanced (shifted to earlier times) compared with that of control plants (Yon et al. 2016), as reported in *Arabidopsis* (Alabadí et al. 2002; Mizoguchi et al. 2002). We observed a similar pattern of *NaCAB2* and *NaTOC1* transcript levels in field-grown irLHY plants (Figure 1B). In the field, the growth of irLHY plants was delayed compared to EV plants (Figure 1C), while there was no significant difference in the final rosette sizes between EV plants and irLHY plants. This result is consistent with a previous study with rice (Izawa et al. 2011).

Empty vector-transformed and the circadian clock gene-silenced plants (irLHY and irTOC1) did not differ in their photosynthetic performances at midday in the field (Figure S1). Since A_c changes predictably throughout the day, A_c was measured at different times of the day (Figure 1D) to evaluate if A_c and stomatal conductance (g_s) followed the early phase predicted by the patterns of *NaTOC1* and *NaCAB2* transcript accumulations in irLHY plants. Interestingly, A_c and g_s of irLHY were similar to those of EV plants at dawn and midday (Figures 1E, 1F, S2A), but decreased earlier at dusk than did EV plants (Figures 1G, S2B). Moreover, when plants were exposed to

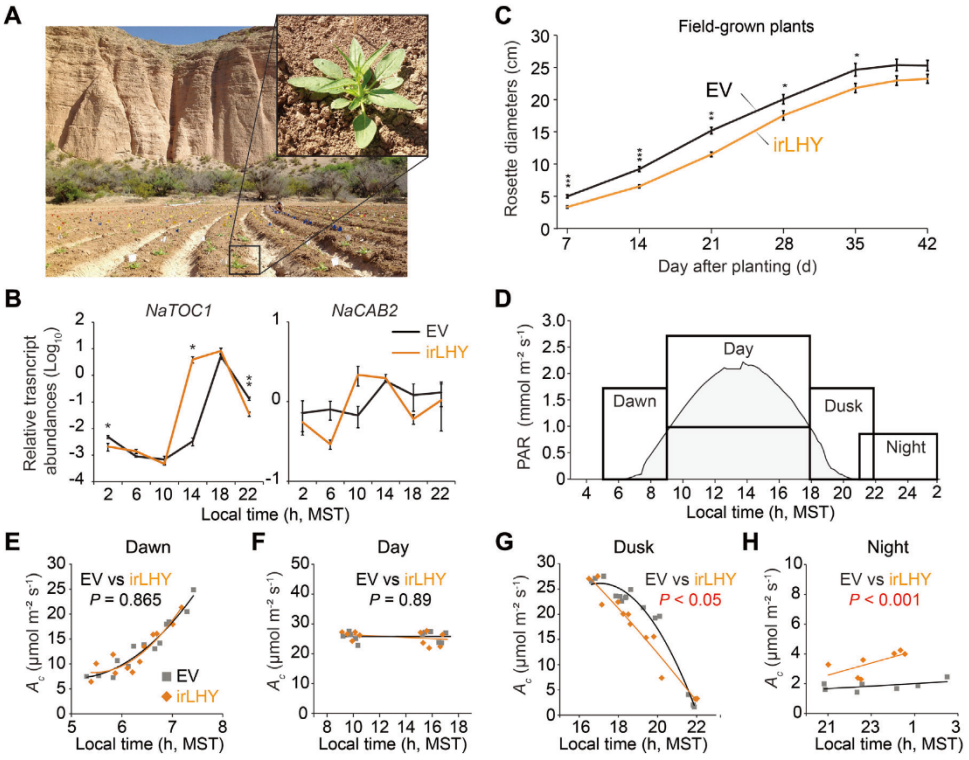

Figure 1. The growth and photosynthetic measurement of *NaLHY*-silenced plants in nature

(**A**) Plant growth and photosynthesis were measured in the Great Basin Desert, Utah. (**B**) Mean (±*SE*, $n = 3$) transcript abundances of *NaCAB2* and *NaTOC1* in irLHY plants increase earlier than those of EV plants. (**C**) irLHY plants grew more slowly than do EV plants when planted into a field plot in Utah (mean ± *SE*, $n = 20$). (**D**) Photosynthetically active radiation (PAR) in the field throughout the day during which photosynthesis measurements were conducted in panels E–H. See Figure S9 for PAR values throughout the growing season. (**E–H**) A_c, quantified 2 min after 1,000 µmol/m^2 per s light exposure during the dawn, day, dusk, and night, reveal that while the A_c of irLHY plants did not differ during the dawn and the day but differed during the dusk. In addition, irLHY plants responded more rapidly to light during the night with increased rates. Local time is Mountain Standard Time (MST). LHY, LATE ELONGATED HYPOCOTYL; EV, plant transformed with the empty-vector used to generate transgenic plants; irLHY, *NaLHY*-silenced plants; A_c, net photosynthetic rate (=carbon assimilation rate). *P*-values reflect the result of ANCOVA tests of the relationship between A_c and time in EV and irLHY plants and two-tailed Student's *t*-tests. CAB2, CHLOROPHYLL A/B–BINDING PROTEIN 2; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

light during the night, the A_c of irLHY were immediately greater than those of EV plants (Figure 1H) and increased more rapidly during this "unexpected" night-time light exposure (Figure 2). Although irTOC1 plants displayed a short-period under free-running conditions, as was previously reported in *Arabidopsis* TOC1 mutants (Figure S3, Somers et al. 1998b), irTOC1 plants displayed photosynthetic responses that were indistinguishable from those of EV plants (Figure S4).

Gating effects in photosynthetic responses by the circadian clock

Higher rates of assimilation during the night in field-grown irLHY plants may have resulted from responses to the particular environmental conditions of the field, so we re-measured the responses in glasshouse-grown plants. We measured A_c, g_s, and transpiration rate (E) after dark adaptation followed by light exposures during the day and the night (Figure 3A). EV and the circadian clock gene-silenced plants showed the same

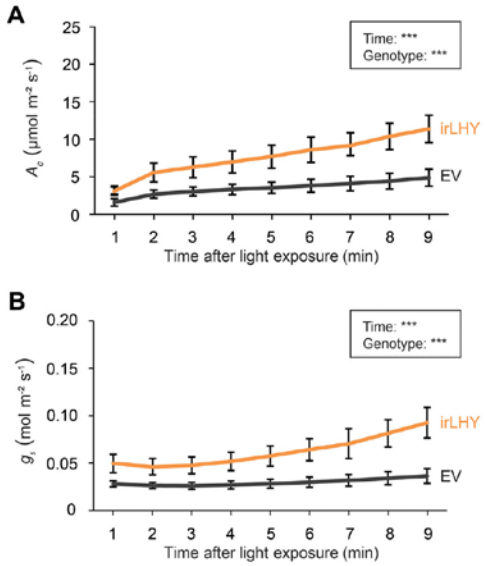

Figure 2. *NaLHY*-silenced plants in nature have higher photosynthetic responses when exposed to light in the night

(**A**, **B**) Light responses were measured during the midnight (PAR: 1,000 μmol/m^2 per s, $CO_{2\ ref}$: 400 μmol/m^2 per s). Mean (±*SE*) levels of A_c and g_s in EV and *NaLHY*-silenced (irLHY) plants ($n = 4$) at the time point shown after the light exposure. irLHY showed consistently higher A_c and g_s in response to "unexpected" light exposures than did EV plants. g_s, stomatal conductance; $^{***}P < 0.001$. Asterisks indicate significant differences between genotypes and time points. The results of two-way ANOVA analyses are summarized in the box of each graph.

photosynthetic performances at midday in the glasshouse (Figure S5). Respiration rate did not differ between day and night after dark adaptation (Figure 3B), but g_s and *E* of dark-adapted EV were still higher during the day than during the night (Figure 3C, D). While A_c, g_s, and *E* of dark-adapted EV plants increased during light treatments, all values increased more rapidly during the day than during the night (Figure 3E–G). Silencing *NaLHY* abolished the differences observed in EV plants in A_c, g_s, and *E* between the treatments during the day and during the night (Figure 3H–J).

The relationships between g_s and A_c in dark-adapted EV plants differed slightly between the day and the night, but the relationship between g_s and A_c did not, and was strongly positively correlated during both the day and the night ($R^2 = 0.94$, $P < 0.001$); these results suggest that the gating of A_c results mainly from the gating of g_s (Figure 4A, B). From these results, we conclude that *NaLHY*, but not *NaTOC1* (Figure S6), is required for the gating of photosynthetic responses by a light signal that depends on the time of day in *N. attenuata*.

Gating of photosynthetic responses is independent of the light dependent reactions

CAB proteins play a major role in light-dependent reactions of photosynthesis (Jansson 1994; Pietrzykowska et al. 2014). As transcript levels of several *CAB* genes in *N. attenuata* also oscillated under both light/dark conditions and constant light conditions (Figure S7), we hypothesized that the time-specific responses of photosynthesis would be mediated by the light-dependent reactions in *N. attenuata*. To test this inference, we measured the maximum photosystem II (*PSII*) efficiency (F_v/F_m) during dark acclimation and the efficiency of *PSII* ($\phi PSII$) and non-photochemical quenching (*qN*) after light exposure (Figure 5), following the same experimental procedure (except for the measuring intervals) depicted in Figure 3A. During dark adaptation, F_v/F_m values are excellent predictors of the maximum quantum yield of *PSII*, and $\phi PSII$ and *qN* reflect changes in *PSII* efficiency with light exposure (Schonknecht et al. 1995; Baker 2008). There were no differences in *Fv/Fm* between dark-adapted EV during the day and the night (Figure 5A). In addition, *PSII* efficiency increased with the light treatment, but there were no differences in any of the efficiency parameters of the light-dependent reactions between dark-adapted EV during the day and the night (Figure 5B, C).

Red light perception by phytochrome A is required for the gating of photosynthesis

We further investigated whether a specific light signal is involved in the gating of photosynthesis. The results showed that A_c and g_s under red light were higher in dark-adapted EV than in dark-adapted irLHY during the day, but not under blue light (Figure 6A, B). These data led us to examine the role of a specific class of red light receptors, the phytochromes, for their role in the gating in photosynthesis under light/dark cycles. To this end, we examined the gating effects in different phytochrome-silenced plants (Fragoso et al. 2017). Carbon assimilation rates at different light intensity were similar

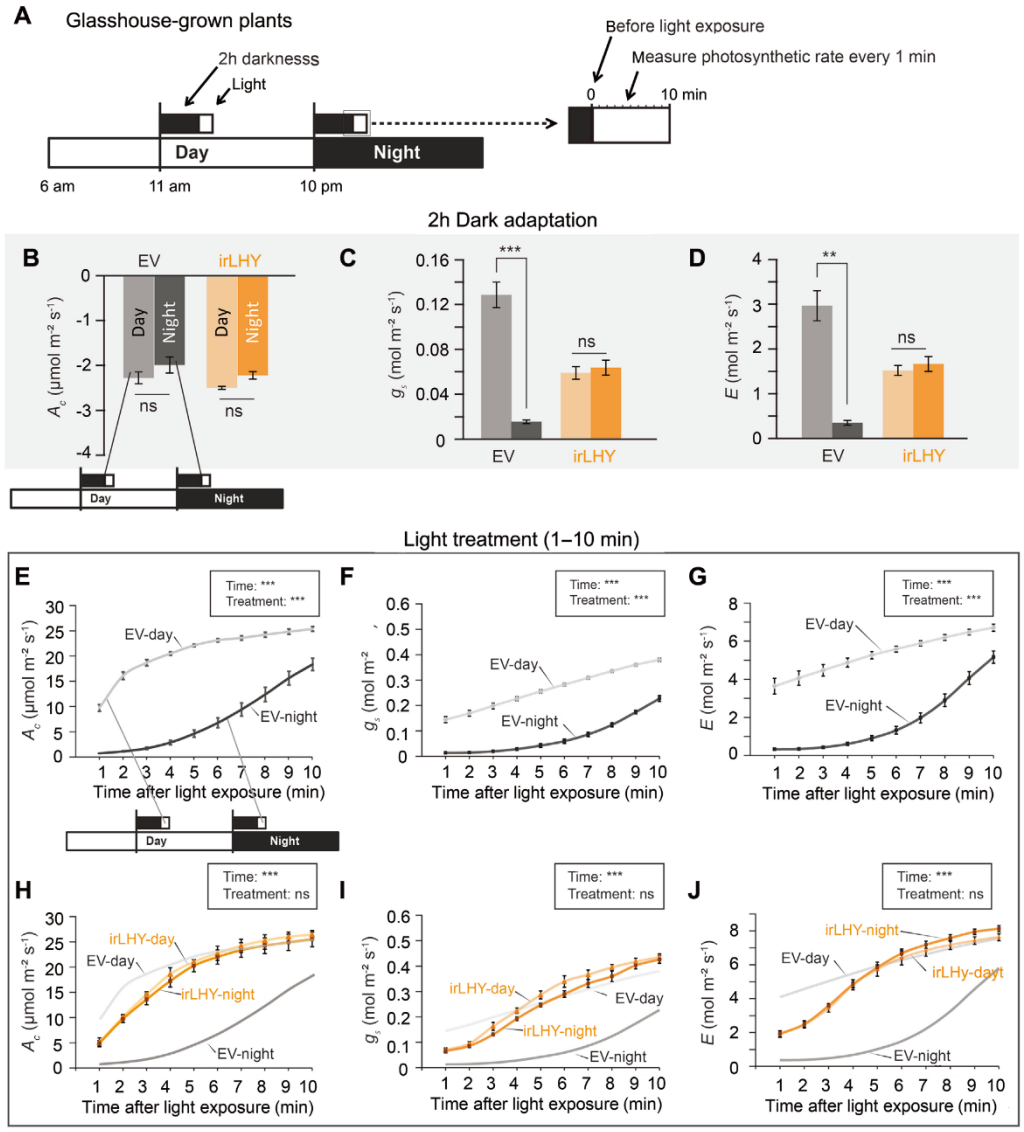

Figure 3. A functional NaLHY informs plants when to gate photosynthetic increases in response to light
(**A**) The photosynthetic responses of dark-adapted plants to a standardized light exposure revealed the role of the morning element of the circadian clock, LHY, in the gating of photosynthesis. Respiration, g_s, and E were measured when plants were dark-adapted for 2 h without light (PAR: 0 μmol/m² per s, $CO_{2\ ref}$: 400 μmol/m² per s). Light responses were measured in dark-adapted plants (PAR: 1,000 μmol/m² per s, $CO_{2\ ref}$: 400 μmol/m² per s). (**B–D**) Plants increased A_c over time after dark adaptation. A_c increased faster in dark-adapted plants during the day than during the night. (**E–J**) A_c, g_s, and E were compared in plants during the day and the night after dark adaptation and after light exposure for 10 min every 1 min and all parameters differed significantly between the day and the night in EV plants, but not irLHY plants. Glasshouse-grown plants (mean ± SE; $n = 3$ per each line) were transferred at the early-elongated stage to a climate chamber to allow for whole-plant dark adaptation before the measurement. E, transpiration rate; $^{***}P < 0.001$; ns, not significant. The results of two-way ANOVA analyses are summarized in the box of each graph; data presented in bar graphs were analyzed by two-tailed Student's t-tests.

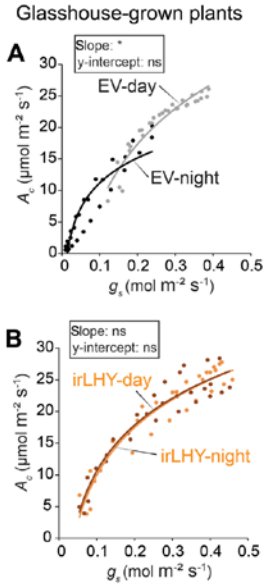

Figure 4. Stomatal conductance (g_s) versus net photosynthetic rates (A_c) in EV and irLHY plants in response to light exposure after dark adaptation

(**A**, **B**) Regression analyses in different plants revealed a significant difference in photosynthetic responses of dark-adapted EV plants between the day and night treatments. However, there was no significant difference in the responses of dark-adapted irLHY plants. Differences in the slopes of the regression lines and in the y-intercepts of the regression lines between during the day and the night are indicated in the inset boxes. $^{*}P < 0.05$; ns not significant. *P*-values were produced by an ANCOVA tests. We used A_c and g_s values in Figure 3E–J for this covariance analysis.

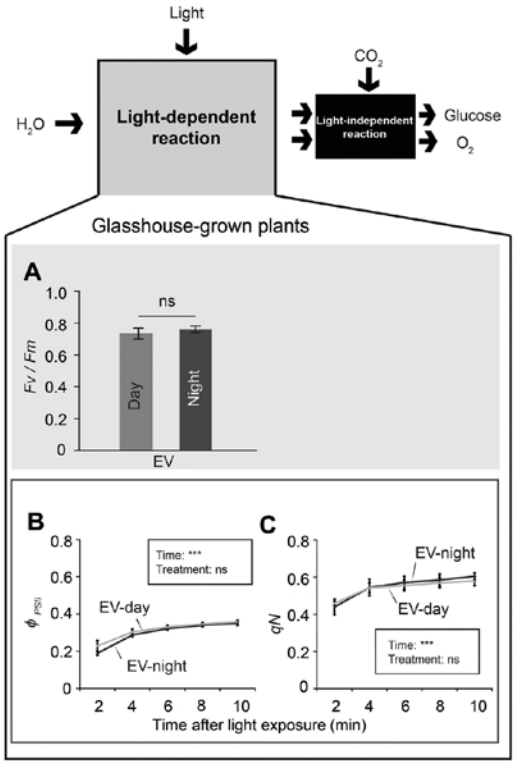

Figure 5. The gating of the circadian clock of photosynthesis is independent of the light-dependent reactions of photosynthesis

(**A–C**) The efficiency of the light-dependent reactions did not differ to a light exposure during the day and the night after dark adaptation. $\phi PSII$ and *qN* were compared in plants during the day and the night after dark adaptation and after light exposure for 10 min every 2 min; otherwise experimental conditions were identical to those described in Figure 3A. Glasshouse-grown plants (mean ± SE; $n = 3$) were transferred at the early-elongated stage to a climate chamber for whole-plant dark adaptation before the measurements. All parameters did not differ significantly between the day and the night in EV plants. F_v/F_m, ratio of variable to maximum fluorescence; $\phi PSII$, quantum yield of photosystem II photochemistry; *qN*, non-photochemical quenching; $^{***}P < 0.001$; ns, not significant. The results of two-way ANOVA analyses are summarized in the black box of each graph; data presented in bar graphs were analyzed by two-tailed Student's *t*-tests.

between irPhyA and EV, but significantly lower in irPhyB1 than in EV plants (Figure S8). Although gene silencing efficiencies were not as strong as in the irPhyA transgenic line (54% vs. 80%, respectively; Fragoso et al. 2017), these data suggest that NaPhyB1 plays a dominant role in the control of photosynthesis in *N. attenuata* (Figure S8), as has also been shown for *Arabidopsis* (Somers et al. 1998a; Bae and Choi 2008). We next measured photosynthetic responses in irPhyA and irPhyB1 plants following the same experimental procedure depicted in Figure 3A. All plants showed time-dependent photosynthetic responses after 2 h of dark treatment (Figure 6C–E). irPhyB1 plants showed lower g_s than EV plants which is correlated with the reduction in *Ac*. Interestingly, light-induced photosynthetic responses in irPhyB1 plants retained their strong time-dependent responses between treatments during

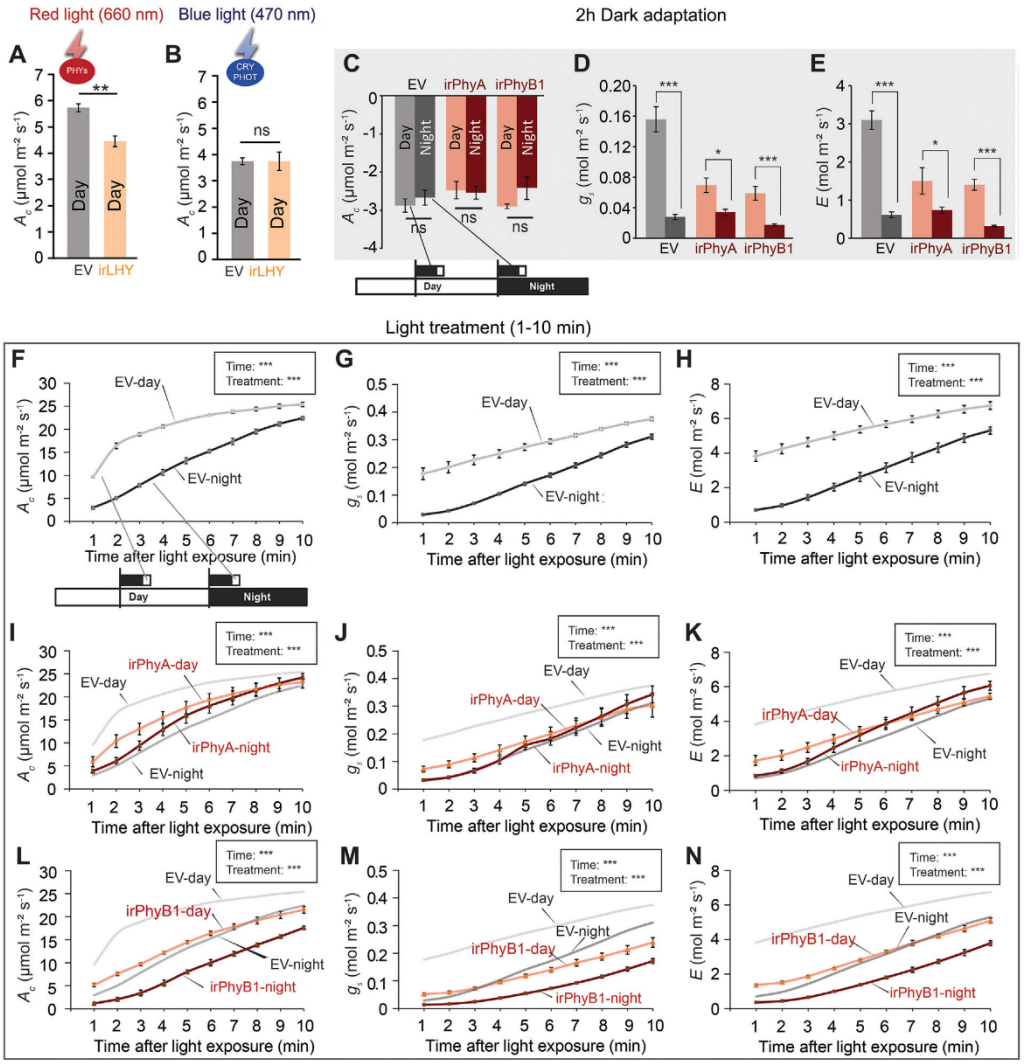

Figure 6. The gating of the circadian clock of photosynthesis mediated by LHY is in turn mediated by NaPhyA-perceived red light

(**A**, **B**) To evaluate which light signal mediates differences in photosynthetic responses, we exposed dark-adapted plants during the day to red (665 nm, 100 μmol/m^2 per s, $CO_{2\ ref}$: 400 μmol/m^2 per s) or blue light (470 nm, 100 μmol/m^2 per s, $CO_{2\ ref}$: 400 μmol/m^2 per s). Only red light exposure revealed differences in the photosynthetic responses between EV and irLHY plants ($P < 0.001$). (**C**–**N**) The same experimental procedure used with the circadian clock-silenced plants was used with phytochrome-silenced plants, irPhyA and irPhyB1. (**C**–**E**) Both irPhyA and irPhyB1 displayed diurnal responses in photosynthesis. (**F**–**N**) We repeated same experiment with EV plants to compare with phytochrome-deficient plants. A_c, g_s, and E in EV plants differed significantly between the day and the night like Figure 3. (**I**–**N**) irPhyB1 have different photosynthetic responses at different times of the day, but the gating effects of light in irPhyA were attenuated. Glasshouse-grown plants (mean ± SE; $n = 3$ per each line) were transferred at the early-elongated stage to a climate chamber for whole-plant dark adaptation before the measurements. Phy, phytochrome; irPhyA, *NaPhyA*-silenced line; irPhyB1, *NaPhyB1*-silenced line; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The results of two-way ANOVA analyses are summarized in the black box of each graph; data presented in bar graphs were analyzed by two-tailed Student's *t*-tests.

the day and the night (Figure 6L–N), but the time-dependent differences were attenuated in irPhyA plants (Figure 6I–K).

DISCUSSION

The circadian clock anticipates rhythmic changes in the environment to synchronize metabolic processes with environmental rhythms and also modulate responsiveness to external stimuli (Harmer et al. 2000; Hotta et al. 2007). This study revealed that the circadian clock in nature anticipates dusk, rather than dawn as was generally thought (McClung 2006) and gates photosynthesis with regard to expectations about the occurrence of light. Nighttime stomatal closure is reportedly important for plant growth by increasing water-use efficiency (Coupel-Ledru et al. 2016), hence gating the "unexpected light" response could be an adaptive trait for *N. attenuata* that grows in desert habitats. Anticipation of dusk for photosynthesis is consistent with previous ideas from glasshouse studies (Harmer et al. 2000; Dodd et al. 2005, 2014) and it might increase water use efficiency and decrease ROS stress by "cooling down" the photosynthetic machinery in advance of night (Lai et al. 2012; Resco de Dios et al. 2013). The circadian clock is entrained by the light period to anticipate a specific phase because the light period is highly predictable in nature (Michael et al. 2003; Nagano et al. 2012). Light intensity, unlike the light period is highly heterogeneous, often unpredictable, in nature (Figure S9, Izawa 2012). Hence it's not surprising that the circadian clock plays roles in both of these two different characteristics of light in nature: anticipation and gating. The conclusions of this work are also consistent with an important inference made by an *in silico* analysis: that the plant circadian clock has evolved both to anticipate phases and maintain the robustness of the phases in response to environmental noise (Troein et al. 2009).

We have shown that the silencing of *NaLHY* resulted in the failure to maintain CO_2 assimilation during dusk and the loss of repression of photosynthetic reactions in response to unexpected light at night; silencing *NaTOC1* did not result in these changes. However, how NaLHY specifically mediates these responses remains an open question. The effects of NaLHY in the late afternoon may also not be a direct consequence of lowered NaLHY protein and transcript levels, as these are already very low at the end of the day (Kim et al. 2003; Yon et al. 2012). Rhythmic alterations are much more accentuated in irLHY than in irTOC plants and these differences could result from changes in the period in the circadian clock. Silencing NaLHY in *N. attenuata* advances the endogenous rhythm to those found in the lhy/cca1 double mutant of *A. thaliana* (Alabadí et al. 2002; Yon et al. 2016) and this may result from the lack of a CCA1 homologue in *N. attenuata* as is the case with many other plant species (Takata et al. 2008; Yon et al. 2012). Additional research is needed to understand the mechanisms by which NaLHY modulates photosynthesis.

Circadian regulation of the light-dependent reaction of photosynthesis is also well known from studies measuring different chlorophyll fluorescence parameters (Rascher et al. 2001; Gould et al. 2009; Litthauer et al. 2015). Our data showed the gating in photosynthesis is independent of light-dependent reactions in the photosynthesis (Figure 5). Since light-adapted plants rapidly attain maximum photosynthetic rates, we used dark-adapted plants to test the plant's responsiveness to light signals at different times of the day. $\phi PSII$ values in *Arabidopsis* leaves have been reported to show circadian rhythms which peak at the subjective night under constant blue light conditions (Litthauer et al. 2015). We used dark-adapted plants to test time-dependent photosynthetic responses to blue and red light together, and hence the results of this work are not readily compared to those of the published literature. In addition, $\phi PSII$ in dark-adapted leaf did not show strong diurnal rhythms, perhaps because $\phi PSII$ values are known to be very heterogeneous at particular times (Rascher et al. 2001). Hence the inference that the light-dependent reactions of photosynthesis are not involved in the gating of photosynthesis in dark-adapted plants is consistent with literature results.

Differences between day and night in the plant's responses to light in dark-adapted plants were strongly correlated with stomatal conductance. The results of the ABA treatment experiment were also consistent with this result ($R^2 = 0.76$, $P < 0.001$, Figure S10). Although stomatal conductance may play a major role in the gating of photosynthesis in response to light, the regressions of A_c and g_s between the day and the night were not identical. This suggests other factors in the light-independent reactions of photosynthesis may also be involved in the gating of photosynthesis. Non-stomatal processes

are also important in the circadian regulation of photosynthesis (Hennessey and Field 2001). In addition, sugar also affects photosynthesis (Blasing et al. 2007; Haydon et al. 2014). Therefore, the gating of photosynthesis might result from a complex interplay of different processes.

Both red/blue light are important for the gating of stomatal opening (Gorton et al. 1993; Shimazaki et al. 2007). Although the *NaLHY*-mediated gating of photosynthesis is mainly contributed by stomatal conductance, we found that only red light is involved (Figure 6A). These results contrast with those of earlier studies about interactions between blue light signaling and photosynthesis through stomata (Noordally et al. 2013; Litthauer et al. 2015). Roles of blue light receptors in stomatal conductance are well studied, but those of the phytochromes are not (Shimazaki et al. 2007). However, the blue light responses of stomata are also strongly affected by red light, for example, under high light intensity (Shimazaki et al. 2007). Two major phytochromes, PhyA and PhyB, have been intensively studied and are known to have distinctive functions and different light stabilities (Bae and Choi 2008). *PhyA*-deficient mutants of *Arabidopsis* do not exhibit strong growth or developmental defects when grown under controlled environmental conditions (Reed et al. 1994). However, PhyA plays important roles under specific light conditions: seed germination, seedling de-etiolation, shade avoidance responses (Yanovsky et al. 1995; Krzymuski et al. 2014), phototropic stimulation under low light intensities (Sullivan et al. 2016), and in the induction of early response genes during dark-to-light transitions (Tepperman et al. 2001). Moreover, Dalchau et al. (2010) proposed that both clock-regulated blue and red light signaling are required for the maintenance of the appropriate Ca^{2+} ions rhythms in the cytosol ($[Ca^{2+}]_{cyt}$), which are important for stomatal opening. In particular, changes in $[Ca^{2+}]_{cyt}$ resulting from red light exposure is mediated by PhyA in *Arabidopsis* (Dalchau et al. 2010). irPhyA plants had photosynthetic activities indistinguishable from those of EV plants in the glasshouse (Figure S8). However, irPhyA plants lacked a gated response to light in the night, which suggests that NaPhyA enables plants to gate photosynthetic responses to red-light signals in a time-specific manner.

The functions of the plant clock in photosynthesis cannot solely be interpreted as being either "anticipation" or "*gating*" for plants grown in nature. "Anticipation" and "*gating*" frequently coexist in the regulation of a single output, for example, light/ CAB (Millar et al. 1992; Millar and Kay 1996), cold/ *COLD BINDING FACTORs* (CBFs) (Fowler et al. 2005; Dong et al. 2011), ABA/ MYB96 (Lee et al. 2016) and pathogen/ *R*-gene mediated plant immunity (Wang et al. 2011). Therefore, the continued examination of the function of the circadian clock in native habitats will provide additional interesting insights into the adaptive significance of the circadian clock.

MATERIALS AND METHODS

Field and glasshouse plant growth conditions

The wild type *Nicotiana attenuata* plants originated from a collection at the DI ranch in southwestern Utah USA, which was inbred for 31 generations, and used to produce the transformants. For seed germination, seeds were sterilized for 5 min and incubated for 1 h in 50 µL of 1 mmol/L GA_3 and 5 mL of 1:50 diluted liquid smoke to break seed dormancy. We used Gamborg's B5 medium (Duchefa) with 86 mg/L hygromycin to select transgenic plants harboring specific transformation constructs. For the diurnal and continuous light treatments, Petri dishes with seedlings were transferred to two growth chambers (Microclima 1000, Snijders Scientific, Netherlands), which were maintained under the same environmental conditions. All details of the seed germination, glasshouse-growth conditions, and plant transformation procedures have been described in Krügel et al. (2002).

A specific fragment of *NaLHY* and *NaTOC1* was independently inserted into the pRESC9 and pRESC8 binary vector, respectively; these vectors were transformed into *N. attenuata* WT plants to silence *NaLHY* and *NaTOC1* transcript levels. The silencing efficiency was more than 90% at the time of maximum transcript accumulation for each gene (Yon et al. 2012, 2016). To silence the phytochromes in *N. attenuata*, we inserted a specific fragment of *NaPhyA* and *NaPhyB1* into pRESC8 and pSOL8 binary vector, respectively (Fragoso et al. 2017). As a control, we used an empty vector (EV) line A-04-266-3 transformed with pSOL3NC, which is known to be completely comparable to wild-type plants

(Bubner et al. 2006). irLHY and irTOC1 were previously characterized (Figures S11, S3, Yon et al. 2012, 2016). Phytochrome-silenced plants were fully characterized; homozygous, inverted-repeat (ir) RNAi transformed lines with single transgene insertions of the second transformed generation (T_2) were used for experiments (Fragoso et al. 2017). The *N. attenuata* genome does not harbor a *CCA1* homologue (Yon et al. 2016).

To grow *N. attenuata* plants in the field, seedlings were transferred into previously hydrated 50 mm peat pellets (Jiffy 703, Always Grows, Sandusky, OH, USA) 14 d after germination. The seedlings were gradually exposed to the native environments to adapt them to the high sunlight and low relative humidity of the Great Basin Desert habitat. Adapted size-matched seedlings were transplanted into a field plot at the Lytle Ranch Preserve, which is located at latitude 37.146, longitude 114.20 (Santa Clara, UT, USA) (Figure 1A). The three different transformed genotypes were randomly planted in rows with 1.5 m among plants. Seeds of the transformed *N. attenuata* lines (irLHY and irTOC1) were imported and released under US Department of Agriculture Animal and Plant Health Inspection Service (APHIS) notification numbers 11-350-101r and 12-333-101r.

To measure photosynthesis and chlorophyll fluorescence in the climate chambers, plants were transferred from the glasshouse 1 d before the measurement. The growth chamber conditions were maintained at 26°C, 16 h light/8 h dark (lighting by Philips Sun-T Agro 400 W and 600 W sodium lights consistent with the conditions of the glasshouse), and 60% humidity. For dark treatments, an identical climate chamber maintained under the same temperature and humidity conditions, but without light, was used. For dark adaptations, plants were kept in the dark for 2 h during the day (11 am to 1 pm) or during the night (10 pm to 12 am) and subsequently exposed to light.

Gas exchange and leaf chlorophyll fluorescence measurements

Gas exchange and chlorophyll fluorescence measurements were conducted with plants in the early elongating stage of growth with the first fully-expanded stem leaves in both the glasshouse and the field experiments: approximately 5–6-week-old plants in the glasshouse and approximately 4 weeks after planting into the field (e.g., the early elongated vegetative stage). The measurements were conducted with a LI-6400XT portable photosynthesis analysis system (Li-Cor Bioscience, Lincoln, NE, USA), with an integrated fluorometer in the leaf chamber. A cloudless day was chosen to conduct the detailed photosynthetic measurements because light environments in the field often fluctuate and confound quantitative assessments of photosynthetic performance.

The rate of net carbon assimilation rate (A_c), stomatal conductance (g_s), and transpiration rate (E) were automatically calculated by the LI-6400XT with the general gas exchange formula of von Caemmerer and Farquhar (1981). All measurements were taken under constant airflow (500 μmol/s) and block temperature (25°C). We controlled the temperature also in the field measurement because we were more interested in the natural variation in light intensity. Gas exchange was measured during dawn, day, dusk, and night in the field. Each time period was based on natural light intensity: dawn (from 5:00 to 8:00, Mountain Standard Time), day (from 9:00 to 17:00), dusk (from 17:00 to 21:30), and night (from 21:00 to 3:00). To examine the photosynthetic activity of the circadian clock-altered lines in the field, we randomly choose EV plants in the field at each time period: dawn (EV, $n = 13$; irLHY, $n = 12$; irTOC1, $n = 10$), day (EV, $n = 11$; irLHY, $n = 10$ irTOC1, $n = 12$), dusk (EV, $n = 15$; irLHY, $n = 15$; irTOC1, $n = 14$), night (EV, $n = 7$; irLHY, $n = 7$; irTOC1, $n = 7$). A_c and stomatal conductance quantified 2 min after 1,000 μmol/m² per s light exposure ($CO_{2\ ref}$: 400 $\mu mol_{CO2}/m^2$ per s). Light-treated plants were not used again in the same day to exclude effects of the pre-light treatment.

To examine the maximum photosynthetic activity of the circadian clock-altered line in the field and glasshouse, measurements were conducted at constant CO_2 (400 μmol/m² per s) and constant temperature (25°C). We used 1,000 μmol/m² per s and 2,000 μmol/m² per s of the light intensity in the glasshouse and the field, respectively to match the maximum light intensity of each growth condition. A/Ci curves were performed using the same light intensity and temperature with the maximum photosynthetic activity measurement at different reference CO_2 levels (0, 200, 400, 600, 800, and 1,000 μmol/m² per s). Light response curves for phytochrome-silenced plants were performed from dark to light (0, 100, 400, 600, 1,000, 1,500, and 2,000 μmol/m² per s) at constant CO_2

(400 μmol/m² per s) and constant temperature (25°C).

To measure the photosynthetic activity after dark adaptation, we transferred glasshouse-grown plants 1 d before and treated the dark for 2 h (light intensity: 0 μmol/m² per s, temperature: 25–26°C, and humidity: 60%). After the dark adaptation, A_c, g_s, and E were quantified every minute for 10 min after 1,000 μmol/m² per s light exposures ($CO_{2\ ref}$: 400 $\mu mol_{CO2}/m^2$ per s) during the day (ZT 7) and the night (ZT 18). To evaluate the light effects in the time-dependent responsiveness of photosynthesis, we followed the same experimental procedure described above measurements, except for the light intensity. Since the maximum blue light intensity of Li-6400XT is 100 μmol/m² per s, we exposed plants to 100 μmol/m² per s for both red and blue light treatments. To test the effects of stomatal opening in light response of dark-adapted plants, a petiole-feeding assay was performed. The first stem leaves of glasshouse-grown plants were carefully excised at the base of their petioles. The leaves were transferred to vials with water and kept for 2 h in the growth chamber (light intensity: 200 μmol/m² per s, temperature: 25–26°C, and humidity: 60%). After the acclimation in the growth chamber, we changed vials with water or two different ABA-containing solutions (0.1 or 1 mmol/L). Then, we turned off the light in the growth chamber for 2 h to measure the photosynthetic activity after dark adaptation. After the dark adaptation, A_c, g_s, and E were quantified every minute for 10 min after 200 μmol/m² per s light exposures ($CO_{2\ ref}$: 400 $\mu mol_{CO2}/m^2$ per s) during the day (ZT 7). We used low light (200 μmol/m² per s) because the leaves were acclimated with the low light in the growth chamber. Under dark conditions, plant cannot photosynthesize and release respiratory CO_2; here we used negative values of CO_2 flux to indicate respiratory release. We used weak green light only while changing plants in the dark chamber to minimize the effects of light.

To measure chlorophyll fluorescence, the minimal fluorescence (F_o) was detected with weak irradiation and a saturating flash (>6,000 μmol/m²), which was applied to determine the maximum fluorescence (F_m) after 2 h dark adaptation. Subsequently, actinic light (1,000 μmol/m² per s) irradiated for 10 min and another saturation flash (>6,000 μmol/m²) were applied every 2 min to measure $F_{m'}$. Further chlorophyll fluorescence parameters were also automatically calculated by the LI-6400XT.

RT-PCR and quantitative PCR

We harvested three similar, mature and non-senescent stem leaves from field-grown plants. Leaves were cut at the petiole and wrapped in aluminum foil, and immediately frozen on dry ice. Samples were stored at −20°C until transport to the Max Planck Institute for Chemical Ecology (Jena, Germany) on dry ice and kept at −80°C. Total RNA was extracted from *N. attenuata* using Plant RNeasy Extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA was quantified using NanoDrop (Thermo Scientific, Wilmington, USA). The cDNA was synthesized from 500 ng of total RNA using RevertAid H Minus reverse transcriptase (Fermentas, Germany) and oligo (dT) primer (Fermentas, Germany). Real-time quantitative PCR was performed in Mx3005P PCR cycler (Stratagene, Germany) using SYBR GREEN1 kit (Eurogentec, Cologne, Germany). The elongation factor (EF) gene was used as a control. The sequences of primers used for qPCR (*NaLHY*-F, CACTCTTTTCAAGGAAGGTG; *NaLHY*-R, GTCGAAGGTGTTACAAGAGC; *NaTOC1*-F, ATCGTAGAACGGCAGCACTT; *NaTOC1*-R, TCACAAACTGTCCCCTCACA; *NaCAB2(b)*-F, GCCGGAAAGGCAGTGAAAC; *NaCAB2(b)*-R, ACCGGGTCTGCAAGATGATC; EF1a-F, CCACACTTCCCACATTGCTGTCA; EF1a-R CGCATGTCCCTCACAGCAAAAC; *NaCABc*-F, AACTTCGGCATTTCAAACTA; *NaCABc*-R, GGGAGAGTTTCACTGCCT; *NaCABd*-F, GCTGCATTTTCAAGAGTATTTC; *NaCABd*-R, CACAGTCTTCCTCATGGTG; *NaCABe*-F, TATGCATTAGAGTTATCATTCTAC; *NaCABe*-R, CAGTCTTCCTCATGGTAACT, *NaPhyA*-F, TGTTGTCTCGTGGCTTTCTG; *NaPhyA*-R, TTCCTGCCATCATCCTTTTC, *NaPhyB1*-F, TCGAGACTCGCCTACGGC; *NaPhyB1*-R, AGCGCCATCGCACTTCAC) were designed by Geneious (Version 5.7.7, http://www.geneious.com).

Measurements of light intensity

Photosynthetically active radiation (PAR) was measured by an automatic weather station (microclimate monitoring system, Decagon Devices, Washington, USA) and data were stored in a data logger (Em50 data logger, Decagon Devices) every 30 min during the entire field season. PAR was measured by a PAR photon flux sensor (QSO-S, Decagon Devices) located 1 m above the soil surface. The weather station was located in the center of the field plot.

Measurements of plant growth rates in the field and glasshouse

Developmentally-synchronized EV, irLHY, and irTOC were planted into the field plot. We grew size-matched plants (at planting) in the field under non-competitive conditions and measured rosette sizes at different times before the plants started elongating. Rosette diameter was measured as the maximum diameter with a ruler and leaf tips were flattened against the ruler for all measurements. Rosette diameters were measured five times every week, except during the last week of the field season, when plants were measured twice.

Statistics

To compare differences in photosynthetic responses in the field and relationships among photosynthetic parameters, the data were analyzed by analyses of covariance (ANCOVA). We performed polynomial regression tests to determine the best fit model. For non-linear regressions ($r^2 < 0.9$), values were log-transformed for the ANCOVA analyses. Photosynthetic responses after light exposures in the different treatments or transgenic lines were compared using two-way ANOVAs. Student's t-test was used for simple comparisons. All statistical analyses were performed using the statistical package R. Significance level was set at $\alpha = 0.05$.

Data accessibility

All DNA sequences are available in GenBank database: NaLHY, JQ424913; NaTOC1, JQ424914; NaPHYA, GEDD01000001; NaPHYB1, GEDD01000002.

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AUTHOR CONTRIBUTIONS

I.T.B. and S.K. conceived the project. Y.J., I.T.B., and S.K. designed the research. Y.J., S.K., F.Y., and V.F. performed experiments. Y.J. and V.F. analyzed the data. Y.J., I.T.B., and S.K. wrote the manuscript. All authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

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Figure S1. Maximum photosynthetic responses are similar between EV and clock-silenced plants grown in the field

(**A–C**) Mean (±SE, $n = 5$) levels of net photosynthetic rates (A_c), transpiration rate (E), and stomatal conductance (g_s) in EV and clock-silenced lines between 14:00 and 15:00 (MST) with the following parameters: photosynthetically active radiation = 2,000 μmol/m² per s, $CO_{2\ ref} = 400$ μmol/m² per s. ns, not significant (two-tailed Student's t-test).

Figure S2. Trends in stomatal conductance track those in carbon assimilation

Stomatal conductance and net photosynthetic rate were measured at the same time in the field. While the stomatal conductance of irLHY plants did not differ during the dawn and the day (**A**), the values for irLHY plants decreased more rapidly during the dusk and night (**B**). ns, not significant; $^*P < 0.05$; The results of ANCOVA analyses are summarized in the box of each graph.

Figure S3. Effect of silencing NaTOC1 on the internal rhythms in seedlings

Mean (±SE) transcript accumulation of CAB2 in *N. attenuata* seedlings of empty vector, irTOC1-205, and irTOC1-212 lines grown under 12 h:12 h, light:dark (LD) conditions, and seedlings in the same growth conditions but subsequently 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 (EFa) gene, normalized, and linear detrended.

Figure S4. Photosynthetic responses of irTOC1 plants are similar to those of EV plants when exposed to light in the night

(**A**) Transcript abundances of *NaCAB2*, a molecular marker gene reflects the internal rhythm in a plant. Leaf samples were collected every 4 h for 1 d from field-grown EV and irTOC1 plants. (**B**) irTOC1 plants grew similar with EV plants when planted into a field plot in Utah (mean ± SE, $n = 20$). (**C–F**) Net photosynthetic rates (A_c) were measured during a day with the following parameters: photosynthetically active radiation = 1,000 μmol/m^2 per s, $CO_{2\ ref}$ = 400 μmol/m^2 per s. All photosynthetic values were recorded 2 min after light exposure. Gray and blue lines represent EV and irTOC1 plants, respectively. *P*-values reflect the result from an ANCOVA test of relationship between net photosynthetic rate and the time in EV and irTOC1 plants. TOC1, TIMING OF CAB EXPRESSION 1; *NaCAB2*, *N. attenuata* CHLOROPHYLL A/B BINDING PROTEINS 2; EV, empty vector transformed plant; MST, Mountain Standard Time.

Figure S5. Maximum photosynthetic responses are similar between EV and clock-silenced plants grown in the glasshouse

Mean (±SE, $n = 3$) levels of (**A**) net photosynthetic rates (A_c), (**B**) transpiration rates (E), and (**C**) stomatal conductance (g_s) in EV and clock-silenced lines ($n = 5$) between 10:00 and 11:00 (EST) with the following parameters: photosynthetically active radiation = 1,000 μmol/m^2 per s, $CO_{2\ ref}$ = 400 μmol/m^2 per s. (**D**) A_c versus *Ci* curve between EV and clock-silenced plants (mean ± SE; $n = 3$). *Ci*, intercellular CO_2 concentration; ns, not significant (two-tailed Student's *t*-test).

Figure S6. A functional NaTOC1 does not inform plants when to pay attention to light

Mean (±SE) levels of A_c, g_s, E, and *Ci* were measured at the end of dark adaptation (2 h). A_c, g_s, and E (mean ± SE; $n = 3$) were compared in plants during the day and the night after dark adaptation and after light exposure for 10 min every 1 min. (**A–D**) All parameters differed significantly between the day and the night in irTOC1 plants as they did in EV plants. A_c, net photosynthetic rates; g_s, stomatal conductance; E, transpiration rate; *Ci*, intercellular CO_2 concentration. ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$ (two-tailed Student's *t*-test).

Figure S7. Phylogenetic trees of CHLOROPHYLL A/B BINDING PROTEIN (CAB) in several plant species

(**A**) Phylogenetic trees of CAB proteins in several plant species. Full-length amino acid sequences were aligned using the Geneious software. Unweighted Pair Group Method with the Arithmetic mean (UPGMA) method was used from the numbers of amino acid substitutions by applying the Jukes-Cantor model. The numbers represent the number of amino acid substitutions per site. (**B**) We selected eight *NaCAB* genes, which belong to the same clade (Clade I) as *AtCAB2* and measured the transcript levels under light/dark cycles and continuous low light conditions. Among eight *NaCAB* genes in Clade I, 4 *NaCAB* genes showed strong circadian rhythms in transcript levels. Black boxes indicate the dark period and white boxes indicate the light period. At, *Arabidopsis thaliana*; Na, *Nicotiana attenuata*; Os, *Oryza sativa*; Sl, *Solanum lycopersicum*; So, *Spinacia pleracea*; Zm, *Zea mays*; ZT, zeitgeber time.

Figure S8. Silencing *NaPhyB1* reduces the net photosynthetic rate in *N. attenuata*

Mean (±SE) levels of net photosynthetic rate (A_c) versus intensity of photosynthetically active radiation (PAR) in EV, irPhyA, and irPhyB1 plants ($n = 5$), which were grown under white light and then exposed to the indicated PAR levels during measurements. A_c levels in irPhyB1 were significantly lower than A_c levels in EV or irPhyA at every light intensity. There was no significant difference in A_c levels between EV and irPhyA. Two independently transformed lines of irPhyA (1; A-14-200, 2; A-14-213) and irPhyB1 (1; A-14-178, 2; A-14-246) were used for this experiment. ns, not significant; $^{***}P < 0.001$ (two-tailed Student's *t*-test).

Figure S9. Photosynthetically available radiation throughout the growing season at the field plot at the Great Basin Desert during the 2013 field season

Photosynthetically active radiation (PAR) was measured by a PAR photon flux sensor (QSO-S, Decagon Devices, Washington, USA) at 1 m above ground level and data were

stored in a data logger (Em50 data logger, Decagon Devices) every 30 min during the entire field season. Light intensity in the field gradually changed over the day, but the patterns of light intensity were not always homogeneous, as shown in the highlighted days, which revealed the heterogeneity in PAR levels throughout the day in the field.

Figure S10. Effect of ABA treatment on the gating response in dark-adapted plants

We compared the relationships between *Ac* and g_s in response to different levels of ABA treatments. Plants were grown under glasshouse conditions (16 h d and 8 h night, 26°C). Detached leaves were petiole-fed with 0, 1 mmol/L, and 0.1 mmol/L ABA solutions and dark-adapted in a dark chamber at (ZT 5) for 2 h. *Ac* and g_s were measured with the following parameters: photosynthetically active radiation = 200 μmol/m^2 per s, $CO_{2\ ref}$ = 400 μmol/m^2 per s.

Figure S11. Transcript abundances of NaLHY in 11 independently transformed irLHY plants

Mean levels of relative transcript abundance of NaLHY at zeitgeber time (ZT) 0 in wild-type and in 11 independently silencing-LHY transgenic lines (n = 3 per line).

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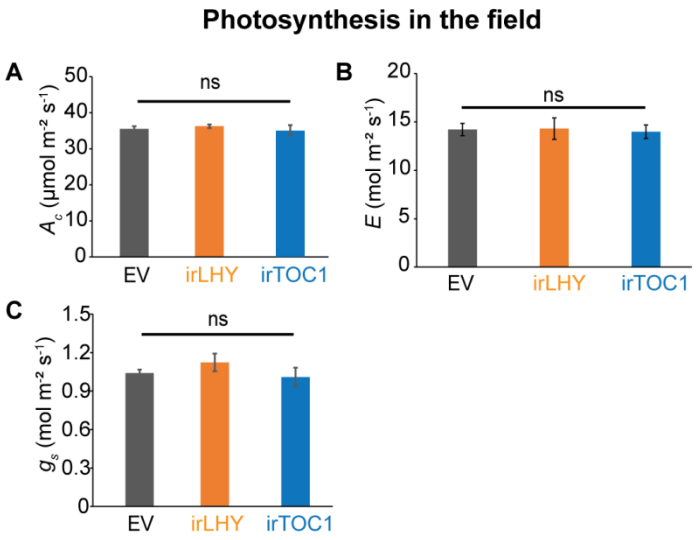
Supporting Information



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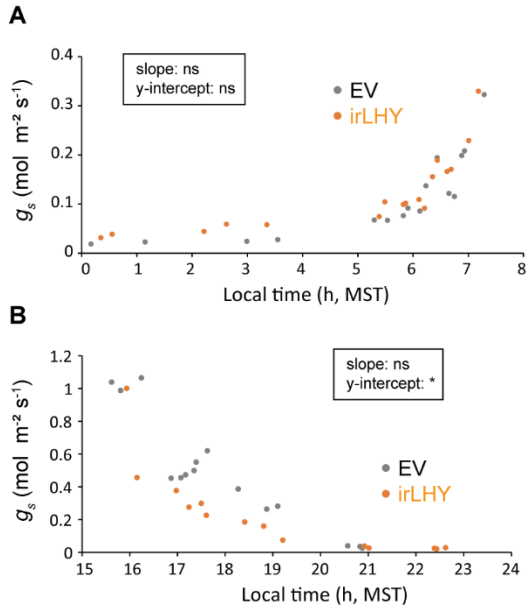

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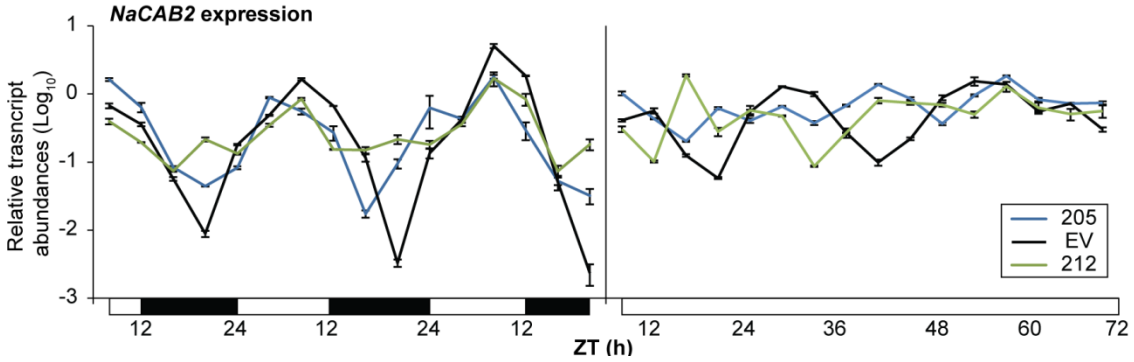

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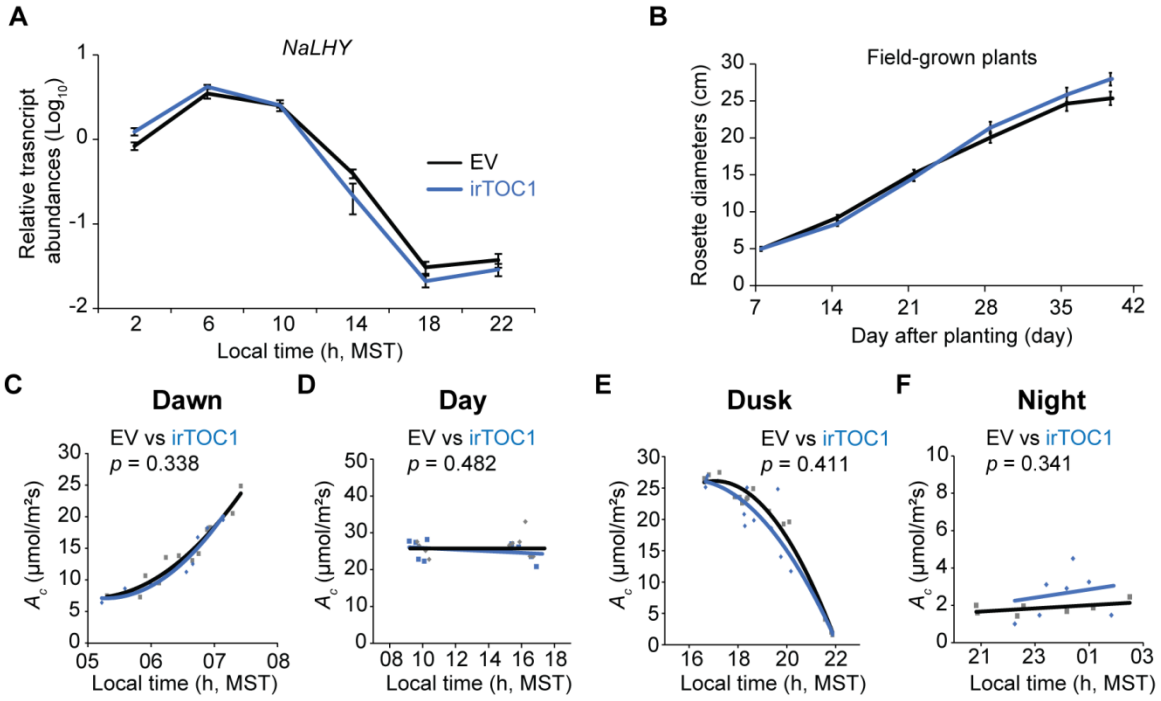

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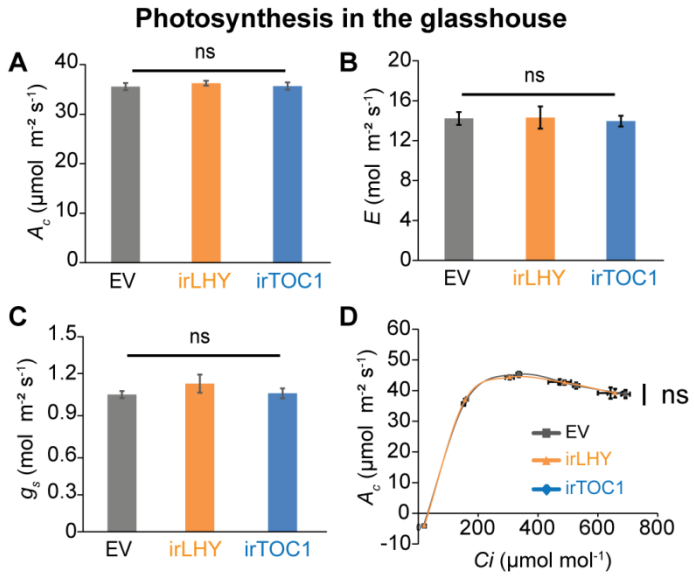

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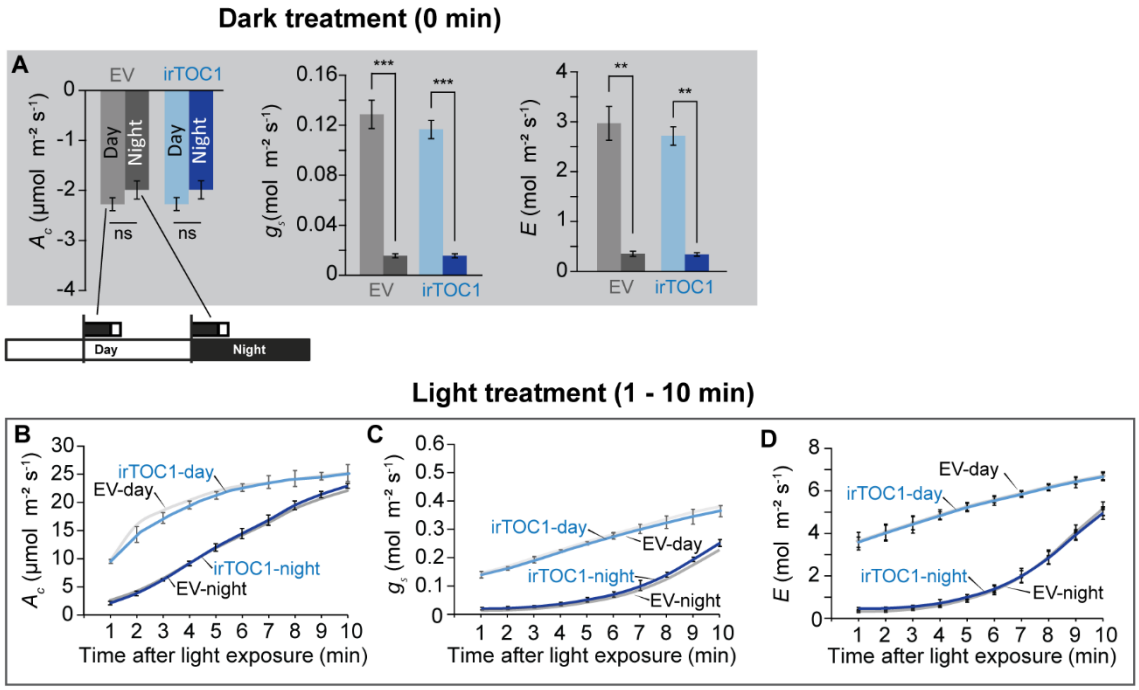

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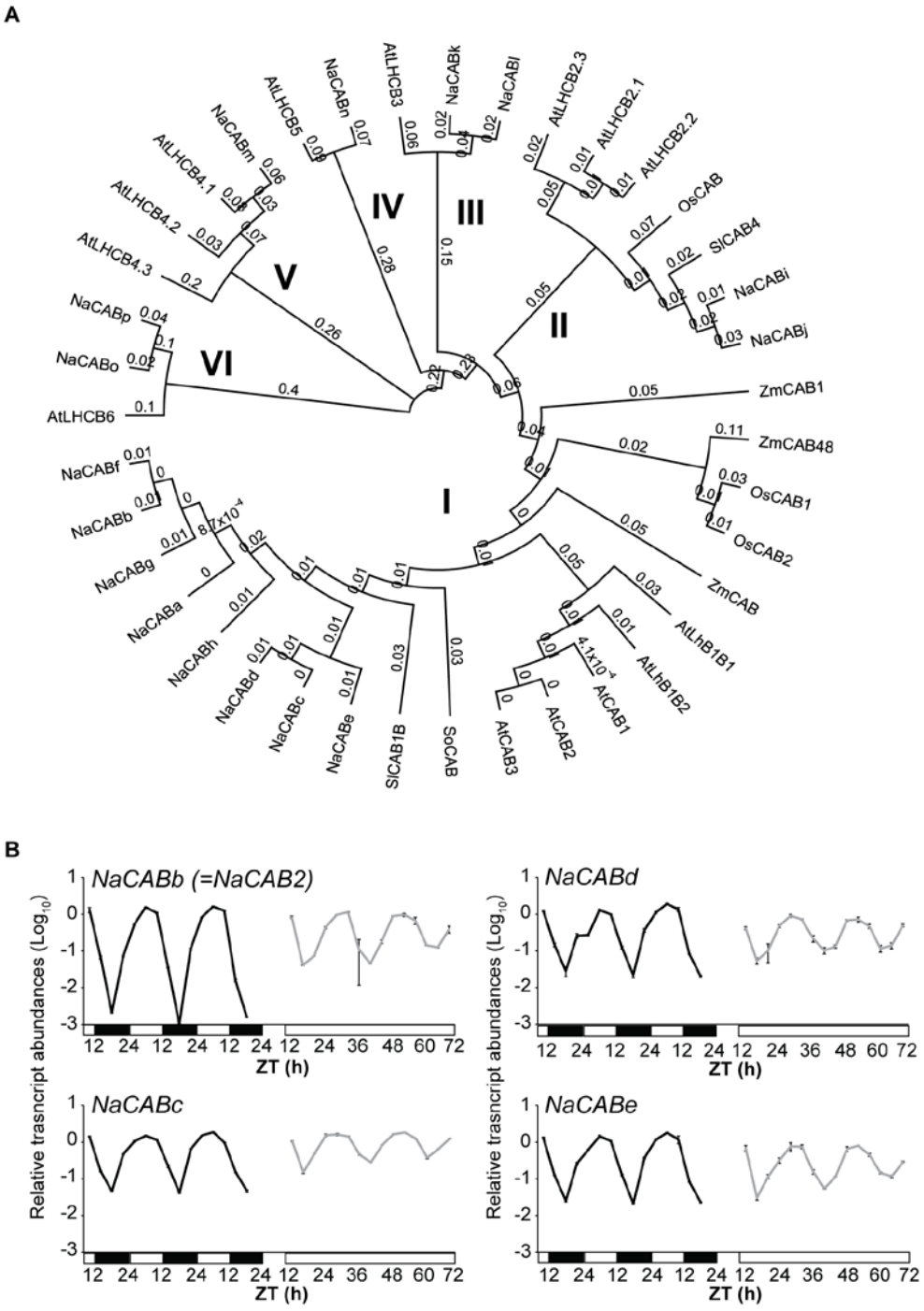

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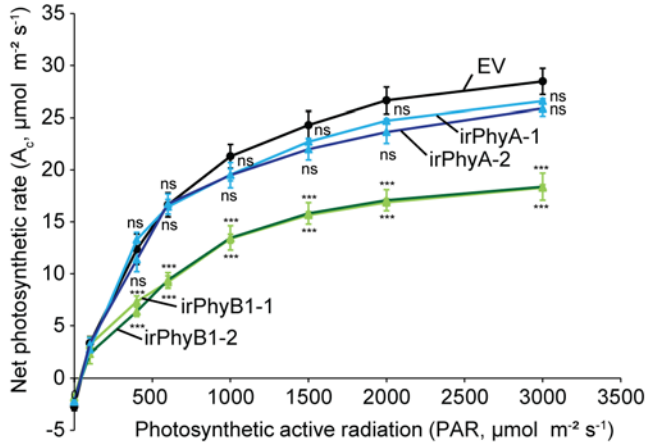

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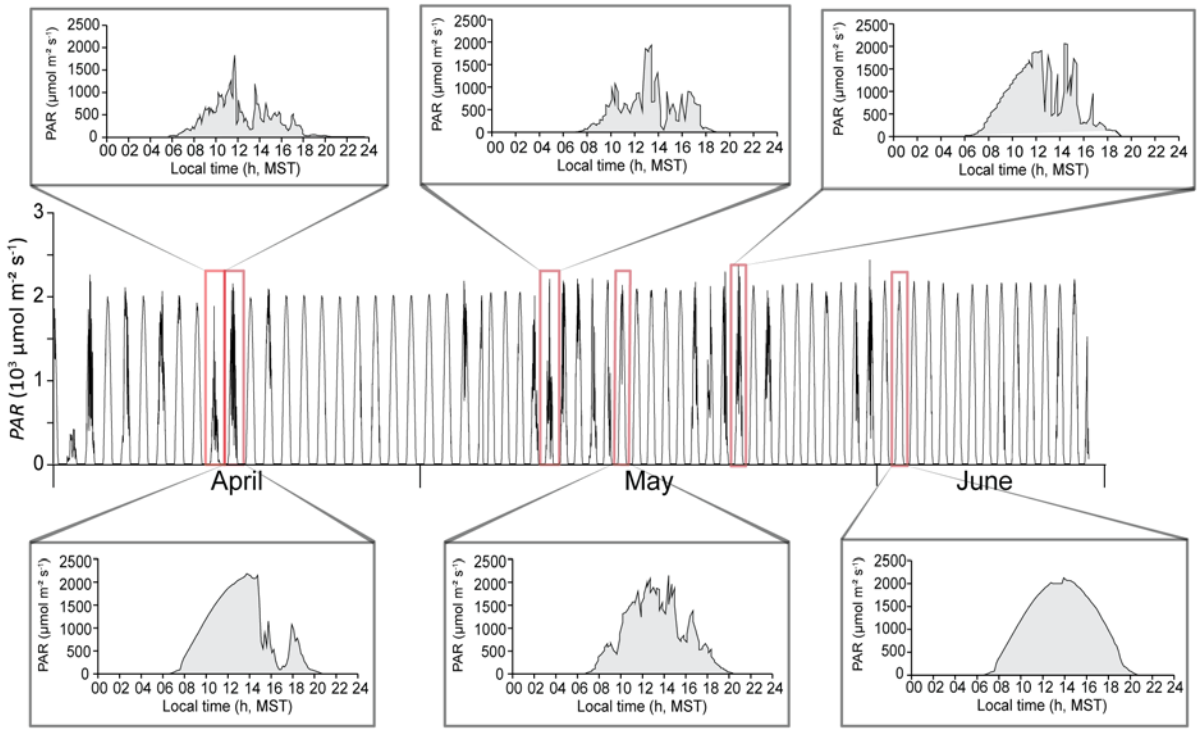

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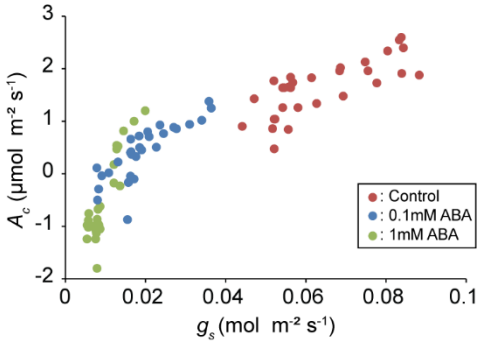

Figure S10. Effect of ABA treatment on the gating response in dark-adapted plants. We compared the relationships between *Ac* and g_s in response to different levels of ABA treatments. Plants were grown under glasshouse conditions (16h day and 8h night, 26 °C).

Detached leaves were petiole-fed with 0, 1 mM, and 0.1 mM ABA solutions and dark-adapted in a dark chamber at (ZT 5) for 2h. *Ac* and g_s were measured with the following parameters: photosynthetically active radiation = 200 µmol $m^{-2}s^{-1}$, $CO_{2\ ref}$ = 400 µmol $m^{-2}s^{-1}$.

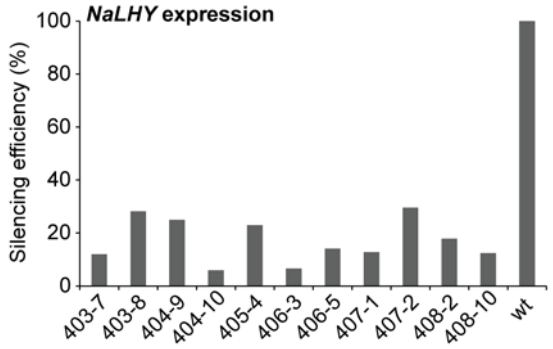

Figure S11. Transcript abundances of NaLHY in 11 independently transformed irLHY plants. Mean levels of relative transcript abundance of NaLHY at zeitgeber time (ZT) 0 in wild-type and in 11 independently silencing-LHY transgenic lines (n = 3 per line).

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RESEARCH ARTICLE

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Herbivore-induced volatile blends with both "fast" and "slow" components provide robust indirect defence in nature

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Abstract

1. Plants emit volatile blends specific to particular herbivore interactions, which predators and parasitoids learn to associate with prey, increasing herbivore mortality and thereby plant fitness in a phenomenon termed indirect defence.
2. Herbivore-induced plant volatile blends commonly include both rapid, transient green leaf volatiles (GLVs) and delayed, enduring sesquiterpenes. A few laboratory studies indicate that insects can use plant volatiles to time behaviour, but it is not known whether and how the temporal dynamics of plant volatile blends influence their function in indirect defence.
3. We characterized the activity of the native herbivores *Manduca sexta* and *Tupiocoris notatus* and their predators, *Geocoris* spp., on their host plant *Nicotiana attenuata* in their natural habitat. Diurnal predator activity only partially overlapped with variable herbivore activity, and herbivore attack at the beginning or end of the photophase elicited plant volatile blends with distinct GLV and sesquiterpene profiles.
4. In field trials, day-active *Geocoris* spp. predators preferred morning- over evening-typical GLV blends. Using plants genetically transformed so as to be unable to produce specific volatiles, we found that GLVs increased predation after dawn elicitations, whereas sesquiterpenes increased predation after dusk elicitations in field trials.
5. We conclude that predators respond to temporal differences in plant volatile blends, and that the different dynamics of specific volatiles permit effective indirect defence despite variable herbivore activity in nature.

KEYWORDS
Geocoris sp., green leaf volatiles, herbivore-induced plant volatiles, *Manduca sexta*, *Nicotiana attenuata*, sesquiterpenes, temporal dynamics, *Tupiocoris notatus*

1 | INTRODUCTION

Flowering plants (angiosperms) and the insect herbivores that attack them are among the most diverse groups of multicellular life on Earth. Perhaps it is not surprising that plants can induce defences with great specificity in response to attack by particular insect herbivores (reviewed in Schuman & Baldwin, 2016), as exemplified by the specificity of herbivore-induced plant volatiles (HIPVs, reviewed e.g. in Howe & Jander, 2008). Much work on plant–insect interactions is done under controlled conditions required to dissect intricate mechanisms of specificity in elicitation and response. However, to be useful for wild plants, responses must keep functioning in the face of environmental disturbances: they must be robust (Kitano, 2004). Most plant and insect communities in nature are unevenly distributed over space and

time (Agrawal, Lau, & Hambäck, 2006) and changes in spatiotemporal co-occurrence may alter the course of plant–insect interactions (Brown, 2003; Kolasa & Rollo, 1991; López-Carretero, Díaz-Castelazo, Boege, & Rico-Gray, 2014). Thus it is important to ask how robust are plant defences when faced with the variable herbivore communities that characterize natural interactions.

Plant indirect defences are perhaps more susceptible to disturbance by shifts in the insect communities around plants than are direct defences. That is because indirect defences rely on attracting predators or parasitoids to attack herbivores, rather than direct effects on herbivores (Kessler & Heil, 2011). To be effective, indirect defences must reward carnivores for patrolling or inhabiting the plant, as is the case for extrafloral nectar or domatia; or else they must make herbivores more apparent, by acting as reliable indicators of herbivory, which is how HIPVs are thought to work (Dicke & Baldwin, 2010; Hare, 2011; Kessler & Heil, 2011).

Herbivore-induced plant volatiles comprise a variety of structures from different biosynthetic pathways including terpenoids, fatty acid derivatives such as the ubiquitous green leaf volatiles (GLVs), amino acid derivatives and methanol (Baldwin, 2010; Dudareva, Negre, Nagegowda, & Orlova, 2006; Schuman, Allmann, & Baldwin, 2015). Herbivore-induced plant volatiles are perceived by diverse organisms and affect many aspects of plants' ecological interactions (Dicke & Baldwin, 2010; Hare, 2011). Specific HIPV blends can attract natural enemies of herbivores, e.g. predators and parasitoids (Allmann & Baldwin, 2010; de Moraes, Lewis, Pare, Alborn, & Tumlinson, 1998; Gols, Bullock, Dicke, Bukovinszky, & Harvey, 2011; Kessler & Baldwin, 2001), and thereby indirectly increase plant Darwinian fitness in nature (Schuman, Barthel, & Baldwin, 2012). The ratio of particular HIPV blend components, as well as their presence or absence, are critical for determining insects' responses (Bruce & Pickett, 2011; McCormick, Gershenzon, & Unsicker, 2014). Different biosynthetic classes of HIPVs have different emission patterns in terms of rapidity, rhythm and abundance after herbivore attack, and these patterns additionally vary depending on the timing of attack (Arimura et al., 2008; de Moraes, Mescher, & Tumlinson, 2001; Loughrin, Manukian, Heath, Turlings, & Tumlinson, 1994). HIPV blend composition therefore changes both with elicitation time and over time post-elicitation (Hare & Sun, 2011a). Loughrin et al. (1994) hypothesized that the diurnal patterns of HIPV emission may be synchronized with activity of natural enemies, just as floral volatile emissions are known to be synchronized with pollinator behaviour. However it is not known whether, or how, these temporal dynamics affect tri-trophic interactions in nature.

Interestingly, Zhang and colleagues demonstrated in laboratory bioassays that feeding by the pea leafminer *Liriomyza huidobrensis* initiated rhythmic HIPV emission in *Phaseolus lunatus* (lima bean) under diurnal conditions, and that a greater concentration of volatiles increased parasitoid (*Opius dissitus*) locomotion and oviposition under light, resulting in coordinated rhythmic parasitoid behaviour synchronized by light–dark cycles; furthermore, Y-tube assays indicated that *O. dissitus* parasitoids could distinguish HIPVs from different times of day (Zhang, Wei, Guo, Liu, & Kang, 2010). However, laboratory observations often have not predicted the outcomes of field trials due to both the higher complexity of the field environment and differences between natural and laboratory conditions (Bruce et al., 2015; Vanin et al., 2012). Similarly, although temporal dynamics of HIPVs are well described in the literature, fine-resolution volatile trappings to measure diurnal rhythms of HIPVs have been conducted only in glasshouses, climate chambers or laboratories (de Moraes et al., 2001; Gouinguené & Turlings, 2002; Loughrin et al., 1994; Turlings, Lengwiler, Bernasconi, & Wechsler, 1998) due to the challenges of field volatile sampling, although HIPV emissions are thought to be affected by many abiotic factors (Dudareva, Klempien, Muhlemann, & Kaplan, 2013; Gouinguené & Turlings, 2002) which likely confound extrapolations of laboratory results into field settings. Finally, many of these studies have been performed with cultivated plants, and though these data are relevant for agricultural biocontrol, it remains unclear how relevant they are to understanding indirect defence in native plants.

The interaction of the wild tobacco *Nicotiana attenuata* with its specialist herbivores *Manduca sexta* and *Tupiocoris notatus* and their natural predators, *Geocoris* spp. has been well studied in their native environment in southwestern Utah, providing an ecologically relevant system in which to test hypotheses about HIPV-mediated indirect defence in nature (Halitschke, Stenberg, Kessler, Kessler, & Baldwin, 2008; Kessler & Baldwin, 2001; Schuman et al., 2012). *Nicotiana attenuata* produces an HIPV blend comprising terpenoids, GLVs and other fatty acid derivatives and aromatics, regulated by wounding and elicitors in herbivore regurgitant (R) or oral secretions (OS) (Gaquerel, Weinhold, & Baldwin, 2009; Halitschke, Schittko, Pohnert, Boland, & Baldwin, 2001; Schuman, Heinzel, Gaquerel, Svatos, & Baldwin, 2009). The jasmonate-regulated sesquiterpene (*E*)-α-bergamotene and GLV components have been shown to reduce herbivore loads by attracting predators (Allmann & Baldwin, 2010; Halitschke et al., 2008; Kessler & Baldwin, 2001; Schuman et al., 2009, 2015) and predator attraction by the GLV component can increase plant fitness (Schuman et al., 2012). Two separate 13-lipoxygenase (LOX) isoforms determine the emission of green leaf volatiles (GLVs) and sesquiterpene HIPVs in *N. attenuata*: *NaLOX2* provides substrates for GLV biosynthesis, whereas *NaLOX3* independently provides substrate for jasmonate biosynthesis (Allmann, Halitschke, Schuurink, & Baldwin, 2010) and jasmonate signalling is required to elicit sesquiterpene HIPVs (Halitschke et al., 2008; Schuman et al., 2015).

Terpenoids and GLVs contribute to HIPV blends in nearly all plant species studied, and to some extent, their observed emission patterns may be due to broadly conserved biosynthetic constraints (Schuman & Baldwin, 2016; Turlings et al., 1998). We built on our knowledge of the *N. attenuata* system to test hypotheses about the importance of temporal differences in GLV and sesquiterpene HIPV emission dynamics. We recorded the behavioural dynamics of *M. sexta* and *T. notatus* herbivores and *Geocoris* spp. predators in nature on a timescale relevant for HIPV-mediated indirect defence. In *N. attenuata*'s native habitat, we characterized the time-resolved emission of GLVs and sesquiterpene HIPVs following elicitation at different times of day, and we obtained similar results in a controlled glasshouse environment. Lastly, by manipulating *NaLOX2* and *NaLOX3* expression and

using synthetic blends representative of daytime-typical emissions, we tested hypotheses about the relevance of different temporal dynamics of GLVs and jasmonate-induced sesquiterpenes for mediating the indirect defence of *N. attenuata* plants, in a field plantation and in wild populations, as measured by predation rates of *M. sexta* eggs from *N. attenuata* by naturally occurring *Geocoris* spp. predators.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

The wild-type (WT) inbred line of *N. attenuata* originated from a collection at the DI ranch in southwestern Utah USA and was inbred for 31 generations. Homozygotes of the third transformed generation (T_3) of inverted repeat (ir)LOX2 (line A-04-52-2) or irLOX2/3 (line A-07-707-2) and the pSOL3 empty vector (EV) control (line A-04-266-3) with single transgene insertions were used for experiments. The irLOX2 and irLOX2/3 lines were previously described and screened in comparison to multiple lines using the same construct (Allmann et al., 2010; Schuman et al., 2015) and the EV line has been shown to have a WT phenotype in volatile emission and other traits over several years of field studies (e.g. Kessler, Gase, & Baldwin, 2008; Schuman et al., 2012). Vector construction and the pSOL3 plasmid have been described previously (Bubner, Gase, Berger, Link, & Baldwin, 2006). Seeds of the transformed *N. attenuata* lines (EV, irLOX2 and irLOX2/3) were imported and released under US Department of Agriculture Animal and Plant Health Inspection Service (APHIS) notification numbers 07-341-101 m (import), 12-333-101r (release of EV in 2013) and 13-350-101r (release of irLOX2, irLOX2/3 and EV in 2014). Seeds were germinated and seedlings were adapted to field conditions as previously described (Kessler et al., 2015; Krügel, Lim, Gase, Halitschke, & Baldwin, 2002). Adapted size-matched seedlings were transplanted into a field plot at the Lytle Ranch Preserve Snow Ranch property, located at latitude 37.141 N, longitude 114.03 W (Santa Clara, UT, USA). Plants were watered using a trench irrigation system until roots had established, and then as needed.

For glasshouse experiments, seedlings were transferred to small pots (TEKU JJP 3050 104 pots, Poeppelmann GmbH & Co. KG, Lohne, Germany) in the glasshouse and then transferred to 1 L pots 10 days later with soil, fertilization and watering regimes as previously described and grown under 24–26°C, 16 hr light (supplemental lighting by Philips Sun-T Agro 400 W and 600 W sodium lights) and 55% humidity (Herden et al., 2016; Krügel et al., 2002).

2.2 | Quantification of *Geocoris* spp. abundance and predation activity

Eggs from laboratory-reared *M. sexta* were used for predation assays. Eggs were frozen at −20°C in Jena, Germany within 3 days of oviposition, transferred on cool packs to the Lytle field station and then kept at −20°C prior to experiments. *M. sexta* larvae were freshly hatched from eggs kindly provided by C. Miles at the State University of New York in Binghamton. Predation rates (between 18 May and 11 June in 2014 and between 26 May and 3 June in 2015) were recorded for three larvae per plant, or for five eggs per plant fixed with alpha-cellulose glue, on a lower stem leaf (1/plant) in a standardized position and eggs were glued to the underside of the leaf as previously described (Kessler & Baldwin, 2001; Schuman et al., 2012).

To quantify *Geocoris* spp. abundance and predation activity over the day, we counted the number of *Geocoris pallens* adults and the number of *M. sexta* eggs predated from WT plants (n = 36 plants) in our field plot from 9 to 11 June 2013. Plants in transects were at least 1 m apart, which is sufficient to allow *Geocoris* spp. to distinguish volatiles from individual plants (Schuman et al., 2012). During these observations most *Geocoris* individuals in this population were adults and we only observed *G. pallens* on focal plants; in general, we observed *G. pallens* and rarely *Geocoris punctipes* on *N. attenuata* (Schuman, Kessler, & Baldwin, 2013) and these are the most abundant predators of *M. sexta* eggs and young larvae on *N. attenuata* (Kessler & Baldwin, 2001; Schuman et al., 2013). These plants had been used for a headspace supplementation assay but we found that *Geocoris* spp. did not differentiate among the blends used in this assay and thus we pooled the data for *Geocoris* spp. activity. Quantifications were done every 4 hr during the day by two or three people in parallel who checked all eggs (9 and 10 June) or counted all *Geocoris* spp. adults per plant (11 June), scanning from the top to the base of the plant; in total each count took <30 min for all plants. The interval of 4 hr was chosen to correspond to feeding damage observations and volatile analysis intervals; the timescale of 24–48 hr corresponds to the time over which HIPVs have been shown to increase predation rates here and in previous studies from the same area (Allmann & Baldwin, 2010; Halitschke et al., 2008; Kessler & Baldwin, 2001; Schuman et al., 2012, 2015). The same approach was used to assay *Geocoris* spp. abundance on the EV, irLOX2, and irLOX2/3 plants used for the predation assays on these lines for the training period of 2 days during which all plants were baited with equal numbers of *M. sexta* larvae (see below).

2.3 | Quantification of herbivore feeding activity

To quantify the feeding activity of chewing herbivores, we modified the method in Herden et al. (2016) for field analysis. Feeding activity was assayed in food-grade plastic boxes (20 cm × 10 cm × 10 cm) with mesh-ventilated covers between 12 and 19 May 2013. We placed three 1st-instar *M. sexta* larvae or 3rd-instar *Spodoptera exigua* larvae per plastic box (n = 6 boxes). Undamaged leaves were harvested and petioles wrapped with Parafilm to prevent dehydration. We took pictures before exposing leaves to larvae to quantify the leaf area damaged by larval feeding. Damaged leaf area was calculated using the IMAGEJ program (imagej.nih.gov/ij) and calibrated with a 100 mm^2 paper. The boxes were placed near the field plot but we avoided direct sunlight to prevent overheating. Durations of observations coincided with the period of the second larval instar during which larvae have established on plants but are still vulnerable to *Geocoris* spp. predation, and the feeding intervals of 4 hr corresponded to observation intervals of *Geocoris* spp. activity and of HIPV sampling.

Ambient temperature was measured by a temperature sensor every 10 min during the field season (ECT Air Temperature Sensor, Decagon Devices, Pullman, WA, USA) as part of an automatic weather station (microclimate monitoring system, Decagon Devices) and data were stored in a data logger (Em50 data logger, Decagon Devices).

The activity of *T. notatus* was recorded on *N. attenuata* plants growing in the natural habitat of our field station (Lytle Preserve, Utah, USA) between 17 and 21 May 2012. Insects were observed with a camera setup consisting of a Logitech C920 HD Pro or Logitech Pro 9000 webcam connected to a laptop and controlled by the webcam software YAWCAM 0.3.1 (www.yawcam.com). The computer was placed in a white box to protect against sunlight and dust. To enable recording during the night, we removed the infrared filter from the camera and placed an infrared spotlight (850 nm) next to the plant, which was automatically turned on at darkness by a photosensor. The camera monitored the lower third of flowering *N. attenuata* plants and recorded images every 50 s. We monitored plants for a total time of about 24 hr. If technical or environmental problems caused an observation period of <24 hr we combined analyses from several days to cover a full 24 hr range for each plant. All pictures were then combined into a time-lapse video using Yawcam. Time-lapse videos were divided into sections of 1 hr and every section of 1 hr was rated on a scale from 0 to 5 for the activity of *T. notatus* in the respective hour (steps of 0.5). An activity level of 0 means no visible *T. notatus* activity and 5, the highest recorded activity observed in all videos. An average activity level was calculated for each hour of the 24 hr timeframe.

2.4 | Simulated herbivore treatment

To simulate herbivory, regurgitant (R) was collected and pooled from 3rd to 5th instar *M. sexta* larvae from an in-house colony at the Max Planck Institute for Chemical Ecology in Jena, Germany, fed on WT *N. attenuata* plants. Before use, R was diluted 1:5 with distilled water. For each leaf treatment, three rows of holes were made on either side of the midvein using a pattern wheel and 20 μl of 1:5 diluted R was added and gently rubbed into wounds using a clean, gloved finger as previously described (e.g. Schuman et al., 2015). This treatment has been shown to cause a response similar to that caused by *M. sexta* larval feeding (Halitschke et al., 2001).

2.5 | Sampling and analysis of volatiles from plants in the field and glasshouse

Silicon tubing (ST) preparation, volatile sampling from leaves, and TD-GC-MS sample analysis (Shimadzu, Kyoto, Japan) followed Kallenbach et al. (2014), Kallenbach, Veit, Eilers, and Schuman (2015) with the following minor modifications: STs were collected from plants in the field every 4 hr for 3 days from 17 May 2013 (1 day before and 2 days after W + R treatment) from the same single mature, non-senescent leaves (1/plant), and each exchange of STs after a 4 hr sampling took a total of 0.5 hr. The standardized time interval of 4 hr was chosen to optimize the sampling of both the more volatile GLVs and the less volatile sesquiterpenes (Kallenbach et al., 2014) and to correspond to the activity measurements of *M. sexta* and *Geocoris* spp.; the time scale of 24–48 hr is explained under Quantification of *Geocoris* spp. abundance and predation activity.

For plants in the glasshouse, STs were collected from headspace around leaves (second fully expanded leaf, −2) which were either treated with W + R or left untreated as controls, and enclosed in two ventilated 50 ml polyethylene tetraphthalate cups (www2.huhtamaki.com) lined on the edges with foam to protect leaves. STs were exchanged every 4 hr for 36 hr and each exchange of STs after a 4 hr sampling took a maximum of 0.5 hr. The standardized time interval of 4 hr was chosen corresponding to field sampling.

Silicon tubing samples were stored in tightly sealed screw-cap 1.5 ml vials at −20°C freezer prior to analysis, and were at room temperatures only during sample transport from Utah, USA to Jena, Germany (<1 day), which is known not to influence the analysis (Kallenbach et al., 2014, 2015). For both field and glasshouse ST samples, identification of volatiles by spectral libraries, relative retention and comparison to standard compounds, relative quantification using the Shimadzu software, and background correction based on samples of ambient air were done as previously described (Kallenbach et al., 2014). Chromatograms and extracted ion traces from day samples collected in the 24 hr before treatment were visually checked and no GLVs or sesquiterpene HIPVs were detected, thus no data are shown in Figure 2 for the pre-treatment collection. Pictures were taken of leaves with a size standard and background-corrected peak areas were additionally normalized to leaf areas calculated in pixels and converted to cm^2 (SIGMASCAN, www.systat.com).

In addition, we conducted an untargeted analysis as described in the Supplemental materials and methods.

2.6 | Headspace supplementation and quantification of predation rates

For headspace supplementation experiments, we placed cotton swabs with lanolin paste containing synthetic GLV blends near leaves to which we attached *M. sexta* eggs to quantify predation (described above and in Kessler & Baldwin, 2001); as a control, we placed cotton swabs with lanolin containing only solvents (Schuman et al., 2012). Empty vector plants were in triplets (n = 8 triplets) with individuals at least 1 m apart and triplets separated by at least 2 m for 2014 (between 8 and 11 June). Treatments were randomly arranged within triplets (1 plant/treatment/triplet). We also conducted similar headspace supplementation experiments in natural populations (n = 10 plants/treatment) with at least 1 m distance among treatments for 2015 (between 25 and 30 May); treatments were randomly distributed in populations and plants were matched across treatment groups for developmental stage and size. For the assay in native populations, basal predation rates were checked prior to supplementation by monitoring *M. sexta* eggs glued to a standardized leaf position as previously described (Kessler & Baldwin, 2001) to determine that predators were actively feeding on *M. sexta* eggs in these populations. We made standard blends to reflect both the relative abundance and ratios of GLVs in headspace samples from leaves treated with W + R at dusk or dawn using commercially available pure GLVs diluted in lanolin paste

(Sigma-Aldrich, St. Louis, MO, USA; Table 1), and placed these blends on cotton swabs adjacent to leaves on plants onto which *M. sexta* eggs were glued, as previously described (Halitschke et al., 2008; Schuman et al., 2012). *Geocoris* spp. predation was assayed using *M. sexta* eggs as described under Quantification of *Geocoris* spp. abundance and predation activity. Because ST sampling is equilibrium-based it provides a robust relative, but not absolute quantitative measurement with our sampling procedure (described above) (Kallenbach et al., 2014, 2015). Thus we used ST samples to determine ratios but used total concentrations similar to absolute values which were previously calculated for *N. attenuata* leaves after W + R treatment (Allmann & Baldwin, 2010). Blend compositions are given in Table 1.

2.7 | Quantification of *Geocoris* spp. predation on EV, irLOX2, and irLOX2/3 plants

For quantification of predation rates on EV, irLOX2, and irLOX2/3 plants after dawn or dusk treatments on 16 to 18 May 2014, plants were arranged in triplets (n = 10 triplets each for dawn and dusk treatment) with individuals at least 1.5 m apart. Each triplet was separated by at least 2 m and dawn- and dusk-treated triplets were distributed randomly across the experiment. Leaves with eggs were treated with wounding and regurgitant (W + R) of *M. sexta* to elicit HIPV emission. *Geocoris* spp. predation was assayed using *M. sexta* eggs as described under Quantification of *Geocoris* spp. abundance and predation activity.

Prior to this assay, on 11 and 12 May, all plants had been baited with three *M. sexta* larvae on a low stem or rosette leaf to provide an easy visual cue for *Geocoris* spp., and five *M. sexta* eggs were glued to a nearby leaf, so that *Geocoris* spp. could learn to associate all plants with prey (see source data file for Figure 6 and training period). This approach was taken for this predation assay only for two reasons: (i) this assay was conducted 2–4 weeks earlier in the season than the assays described above, at which point *Geocoris* spp. were less likely to have experienced a *Manduca* spp. oviposition event and thus less likely to feed on *M. sexta* eggs (Allmann & Baldwin, 2010; Schuman et al., 2012, 2013) and (ii) this was the only assay utilizing plants deficient in components of their HIPV blends and we wanted to rigorously test the role of these HIPVs after specifically timed elicitation in the main experiment, so we aimed to disrupt any preceding preference *Geocoris* spp. might have formed for foraging on HIPV-emitting plants. Predation of eggs and larvae were monitored after 24 hr, at which point eggs and larvae were replenished to three larvae and five eggs; predation was monitored after an additional 24 hr, at which point eggs and larvae were removed. Plants were elicited for the main assay at least 4 days later to allow any HIPV emissions from this "training period" to deplete. *Geocoris* spp. abundance and activity from this period were used together with data shown in figures to draw the hypothesized dynamics in Figure 6.

2.8 | Statistical analyses

To compare predation rates in different treatments, we conducted Fisher's exact tests. Diurnal rhythms of herbivore feeding activities were analysed by ANOVAs and correlation with temperature using Spearman's rank correlation. Relative comparison between morning GLV blends and evening blends were analysed by t-tests. Statistical analyses were performed using R version 3.3.1 and RStudio version 0.99.903 (2015), with significance set at α = 0.05.

3 | RESULTS

3.1 | Activity patterns of herbivores of *N. attenuata* and their predators

We measured feeding activity and abundance of the predator *Geocoris* spp. and two of *N. attenuata*'s specialist herbivores, *M. sexta* and *T. notatus*, on which the predator feeds, in their native habitat (Kessler & Baldwin, 2004) and which are known to elicit similar HIPVs (Kessler & Baldwin, 2001) (Figure 1).

To measure *Geocoris* spp. activity, we quantified the proportion of experimental bait consumed by *Geocoris* spp. as well as numbers of *Geocoris* spp. adults on focal plants, over the course of a day in a

TABLE 1 Composition of synthetic morning and evening GLV blends

	(1) Mimicking dawn and dusk ratios and relative amounts (ng/dose)		(2) Keeping amounts the same between dawn and dusk and only changing ratios (ng/dose)	
Compound name	Dawn	Dusk	Dawn	Dusk
3(Z)-hexenal	0.0	57.9	0.0	23.0
2(E)-hexenal	1000.0	3000.0	1000.0	1190.0
3(Z)-hexenol	1504.0	3959.3	1504.0	1570.5
2(E)-hexenol	1705.6	5691.5	1705.6	2257.6
3(Z)-hexenyl acetate	260.7	52.5	260.7	20.8
3(Z)-hexenyl isobutanoate	488.5	140.0	488.5	55.5
3(Z)-hexenyl butanoate	420.2	32.5	420.2	12.9
1-hexanol	966.3	2926.8	966.3	1161.0
Total GLVs	6345.3	15860.6	6345.3	6291.4

GLV, green leaf volatile.

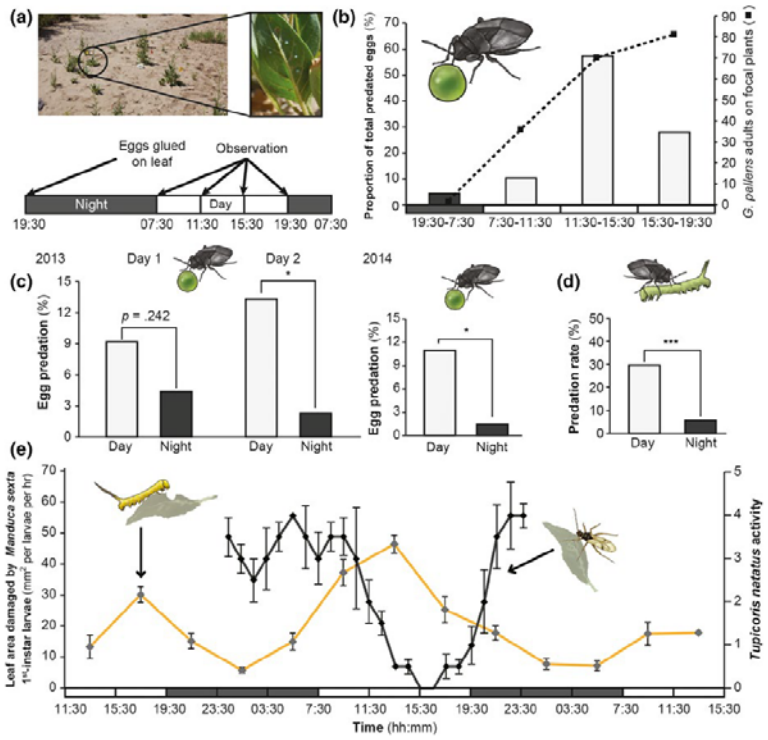

FIGURE 1 Diurnal activity of the specialist herbivores *Manduca sexta* and *Tupiocoris notatus* and their native predators, *Geocoris* spp. in nature. (a) Insect activities were measured in the Great Basin Desert, Utah (photo). To measure *Geocoris* spp. activity, we glued *M. sexta* eggs to the underside of one leaf per plant and counted predated eggs every 4 hr. (b) Activity of *Geocoris* spp. measured as percentage of eggs predated (*M* ± *SEM*, sums to 100%, left axis) or number of adults observed (right axis) (n = 35 plants, 175 eggs). (c and d) *Geocoris* spp. were consistently day-active over 2 years of observations, and more *M. sexta* larvae were predated during the day than during the night (cumulative percentage predation). (e) Activity of *M. sexta* (orange line) measured by leaf area damaged per time interval (*M* ± *SEM*, n = 18 larvae). Activity of *Tupiocoris natatus* (black line) were measured every 1 hr and their activities rated on a scale from 0–5 for the activity of *T. notatus* in the respective hour (steps of 0.5). An activity level of 0 means not visible *T. notatus* activity and five the highest recorded activity observed in all videos. $^{*}p < .05$; $^{***}p < .001$; p-values from Fisher's exact test and one-way ANOVA analysis

field plantation (Figure 1a,b). Interestingly, predation rates were higher on *M. sexta* larvae than on eggs (Figure 1c,d: note differences in *y*-axis scale). Predation activity and abundance of *Geocoris* spp. was mostly restricted to daylight hours and showed a strong diurnal rhythm with predation rates peaking at midday (Figure 1a,b). During these assays we observed only *G. pallens* on plants. However, we cannot exclude that some egg predation may have been due to the co-occurring *G. punctipes*.

Manduca sexta larvae are most vulnerable to *Geocoris* spp. attack in the first two larval instars and while moulting (Schuman et al., 2013). Feeding activity of 1st-instar *M. sexta* larvae also peaked at midday, although less sharply, with feeding bouts at other times of day; and also displayed a diurnal rhythm (Figure 1e, first day ANOVA: $F_{5,29}$ = 15.674, $p < .001$; second day ANOVA: $F_{5,29}$ = 24.824, $p < .001$). *M. sexta* feeding activity generally increases as larvae grow (Herden et al., 2016), but on the third day feeding activities were lower than on the second day because larvae began to moult (Figure 1e). Feeding activity of *M. sexta* larvae was strongly temperature-dependent (Figure S1a,c, Spearman's rho = 0.543, $p < .001$). In contrast, the activity of *T. notatus* was distinctly nocturnal (Figure 1e, Figure S2).

As a comparison, we assayed the naturally occurring generalist lepidopteran *S. exigua*, although we have not observed *Geocoris* spp. feeding on *S. exigua* in nature. *S. exigua* did not display a diurnal rhythm in feeding activity at a later (3rd) instar in which they can adapt to feeding on *N. attenuata* (Diezel, von Dahl, Gaquerel, & Baldwin, 2009), and their feeding behaviour appeared to be largely temperature-independent (Figure S1b,d, Spearman's rho = 0.017, p = .898).

Tupiocoris notatus move over large areas on the same plant when active, whereas 1st-instar *M. sexta* larvae remain on one leaf, and *S. exigua* intermittently hide in the soil or move between plants. *T. notatus* activity was therefore observed using cameras monitoring large portions of individual plants, whereas feeding assays with larvae were conducted in containers with freshly cut leaves to minimize effects of plant or predator responses and to permit precise quantification of feeding damage. Thus for day-active larvae, behaviours such as hiding (Shiojiri, Ozawa, & Takabayashi, 2006) which may only occur on intact plants were excluded by the conditions of the analysis.

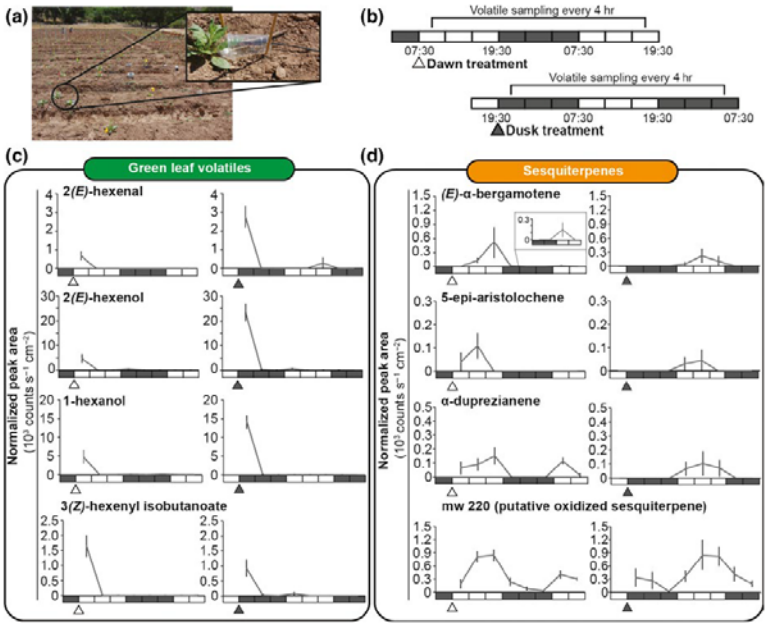

FIGURE 2 *Nicotiana attenuata* emits green leaf volatiles (GLVs) and sesquiterpene herbivore-induced plant volatiles (HIPVs) with different rhythms and composition in response to elicitation by *Manduca sexta sexta* at dawn or dusk. (a) Empty vector control (EV) plants were grown in a field plot in their native environment and single leaves of rosette-stage plants (+1 position) were elicited with wounding and *M. sexta* regurgitant (W + R) and sampled by silicone tubing pieces (STs) and analysed by TD-GC-MS (n = 7–10 plants). (b) W + R treatment occurred at dawn (white triangle) or dusk (dark grey triangle) and STs were exchanged every 4 hr over 48 hr. (c) Examples showing the pattern of emission for GLVs ($M \pm SEM$); full blends are shown in Figure 6. (d) Patterns of emission for all four sesquiterpenes detected at quantifiable levels including the HIPVs (*E*)-α-bergamotene and 5-epi-aristolochene ($M \pm SEM$)

3.2 | Herbivore elicitation at dawn vs. dusk changes composition of green leaf volatiles and post-elicitation dynamics of sesquiterpenes

Based on the variation we observed in feeding activity between herbivore species and among *M. sexta* feeding bouts, we decided to measure plant volatile emissions after either a dawn or a dusk elicitation of field-grown plants. These time points occur at the extreme ends of the period of *Geocoris* spp. activity and mark the points of greatest overlap between more day vs. night-active herbivores, and have relatively similar light intensity for timepoints separated by half a day (166.13 and 114.78 μmol s^{-1} m^{-2}, respectively, Figure S3).

Previously, we developed a technique for plant volatile sampling in the field using silicone tubing pieces (STs) (Kallenbach et al., 2014), and we used this approach to sample plant volatiles in 4 hr intervals from plants in a field plantation immediately before, or after a standardized herbivory elicitation treatment (wounding and addition of *M. sexta* regurgitant to wounds, W + R) at dawn or dusk (Figure 2a,b). Since *N. attenuata* emitted similar volatile blends in response to *T. notatus* and *M. sexta* attack (Kessler & Baldwin, 2001), the dawn and dusk treatments can represent HIPVs in response to day-herbivore and night-herbivore attack respectively. From these samples, we analysed the dynamics of all GLV and terpenoid HIPVs which could be quantified consistently across replicates in terms of their relative abundance in extracted ion chromatograms. GLVs and sesquiterpene HIPVs have been shown to increase predation of herbivores from *N. attenuata* plants by *Geocoris* spp. (Allmann & Baldwin, 2010; Halitschke et al., 2008; Kallenbach et al., 2014; Kessler & Baldwin, 2001; Schuman et al., 2012) and we expected these HIPVs to display extreme differences in their emission dynamics (Allmann & Baldwin, 2010; Halitschke, Kessler, Kahl, Lorenz, & Baldwin, 2000). We detected 2(*E*)-hexenal, 2(*E*)-hexenol, 1-hexanol, 3(*Z*)-hexenyl isobutanoate, (*E*)-a-bergamotene, and 5-epi-aristolochene (Figure 2c,d); none of these HIPVs were quantifiable in headspace samples from the same plants during the day prior to elicitation. In addition, the sesquiterpene α-duprezianene and a putative sesquiterpene oxide, which are constitutively emitted from *N. attenuata* (Schuman et al., 2009), and the monoterpenoid α-terpineol, which is repressed by *M. sexta* R (Gaquerel et al., 2009), were present in quantifiable levels in many samples (relative quantification; Figure 2d and source data file for Figure 2c,d).

Background controls dispersed throughout the experiment concurrently sampled ambient air, and we used these to subtract maximum background peak areas from all target peaks to correct for any environmental contamination, prior to further data analysis. We also conducted an untargeted analysis of total ion chromatograms from the same samples, which did not identify any other plant volatiles consistently detected above background levels in these samples.

Green leaf volatile emission was detected primarily in the first 4 hr after treatment, and GLV aldehydes and alcohols were more abundant after dusk than dawn treatment (Figure 2c). In contrast, emission of sesquiterpenes became more abundant at later timepoints post-treatment, persisted over a longer time period, and showed a strong diurnal rhythm; this was true both of the sesquiterpene HIPVs (*E*)-α-bergamotene and 5-epi-aristolochene as well as the constitutively emitted α-duprezianene and putative sesquiterpenes oxide (Figure 2d). Interestingly, after dusk elicitation, *N. attenuata* did not emit sesquiterpenes until the next daylight period (Figure 2d).

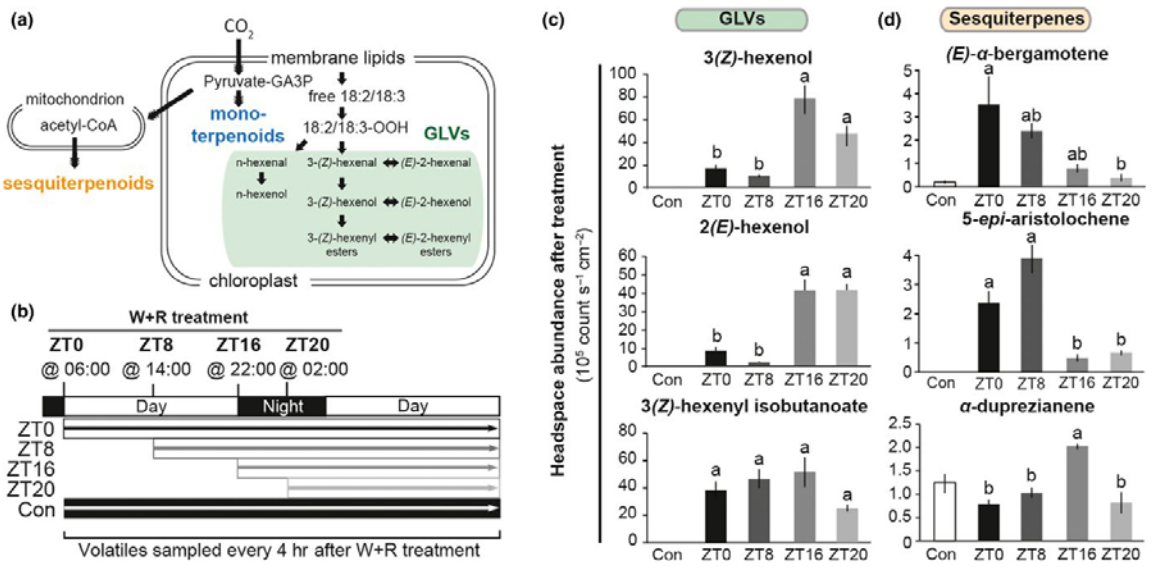

FIGURE 3 Herbivore-induced plant volatile (HIPV) profiles depend on the timing of herbivore attack. (a) The Utah wild-type (WT) genotype of *Nicotiana attenuata* primarily emits green leaf volatiles (GLVs) and sesquiterpenes in response to herbivore damage. (b) Glasshouse-grown WT plants were elicited with wounding and regurgitants from *Manduca sexta* larvae (W + R) to mimic herbivore damage at different times: dawn (Zeitgeber time 0, ZT0), day (ZT8), dusk (ZT16), and night (ZT20), or left undamaged (control). Plant volatiles were sampled using silicone tubing pieces (STs) and analysed by TD-GC-MS ($M \pm SE$, $n = 5$). (c) GLV alcohols and aldehydes were emitted in higher relative abundances after dusk or night time treatments, but GLV esters were emitted in similar abundances throughout the day. (d) Among the sesquiterpenes, (*E*)-α-bergamotene and 5-epi-aristolochene were strongly induced by the treatment. ZT, zeitgeber. Different letters indicate significant differences ($p < .05$) using one-way ANOVA with Tukey post hoc tests

3.3 | Induction time alters composition and post-induction dynamics, but not diurnal patterns, of GLV and sesquiterpene emissions

To determine whether timing of herbivore attack can alter the emission profile of HIPVs (biosynthetic overview in Figure 3a) in a more sensitive analysis under controlled conditions, we elicited WT plants grown in a glasshouse with W + R at different times of day: ZT0 (dawn), ZT8 (midday), ZT16 (dusk) and ZT20 (midnight) (Figure 3b) and used STs to sample plant volatiles every 4 hr after W + R treatments. Relatively low levels of volatile compounds were sampled simultaneously from undamaged (control) plants. Monoterpenoids, e.g. α-terpeneol, α-pinene and β-myrcene, were most abundant in undamaged samples and did not change in response to induction (Figure S4). Each group of plant volatiles had different emission patterns (Figure S4). Green leaf volatiles were detected immediately and transiently after induction. Green leaf volatile aldehydes and alcohols were detected in higher abundance at night, but GLV esters were similarly abundant regardless of induction time with the exception of 3(*Z*)-butanoate and 3(*Z*)-caproate (Figure 3c and Table S1). Among the sesquiterpenes, the emission of (*E*)-α-bergamotene and 5-epi-arisolechene was induced by W + R treatments and lasted longer than GLV emission, whereas emission of α-duprezianene was constitutive but slightly altered by induction (Figure 3d). The abundance of these three sesquiterpenes depended on induction time as well as time of day (Figure 3d). Farnesene, and elemene which likely results from a thermal Cope rearrangement of germacrene A during GC analysis, were detected constitutively, but not inducibly in the WT (Table S1) (de Kraker, Franssen, de Groot, Konig, & Bouwmeester, 1998; Schuman et al., 2015).

3.4 | *Geocoris* spp. predators prefer morning- to evening-typical GLV blends

We tested whether predators of *N. attenuata*'s herbivores can distinguish GLV blends from a dawn vs. a dusk elicitation in nature. These morning- vs. evening-typical GLV blends (sampled after dawn or dusk elicitation, respectively) differed not only in the amount, but also in the ratios of individual GLV structures: aldehydes and alcohols were relatively less abundant in comparison with esters in the morning- vs. the evening-typical blend (Figure 4a). We hypothesized that if predators showed a preference, then day-active predators would prefer the morning to the evening blend. Alternatively, predators might prefer the more abundant evening blend. Since both abundance of individual compounds and blend composition could determine predator responses (Dicke & Baldwin, 2010), we set up our tests to distinguish the role of ratios of individual GLV structures vs. total GLV abundance.

We first surveyed the response of day vs. night predators to the morning and evening GLV blends. We supplemented GLV emission for EV plants by placing cotton swabs with lanolin paste containing synthetic

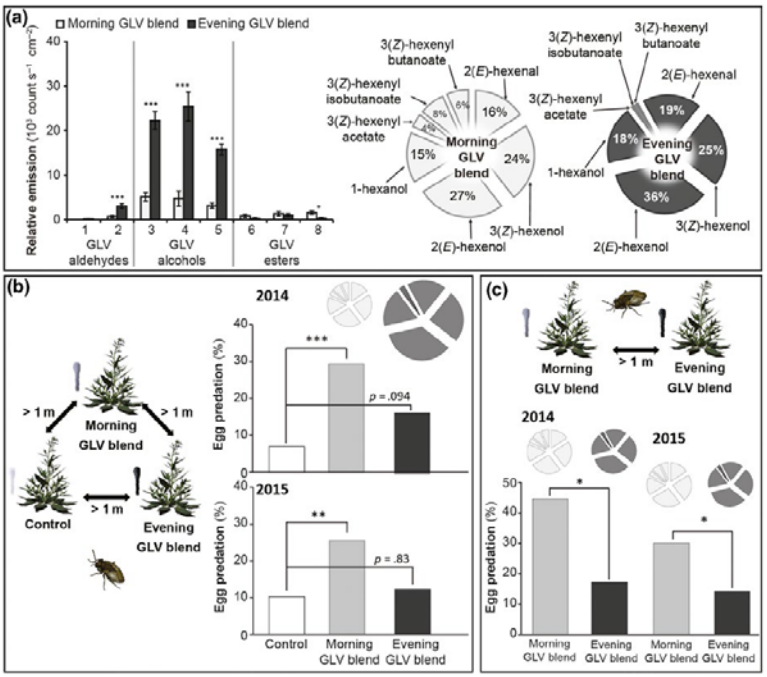

FIGURE 4 Composition is more important than quantity of green leaf volatiles (GLVs) for *Geocoris* spp. predation in nature. (a) Composition of the GLVs sampled from the headspace (*M* ± *SEM*) when plants were treated at dusk (evening blend) vs. those sampled at dawn (morning blend, left). Pie diagrams depicting both the abundance (size of pie) and the proportion of GLV aldehydes, alcohols and esters in the morning vs. the evening blends (right). (b) For predation assays, plants were at least 1 m apart within a replicate (n = 8–10), and replicates were separated by at least 2 m (left). Rates of egg predation by *Geocoris* spp. from plants supplemented with a synthetic GLV blend mimicking both the composition and abundances of the morning or evening blends, or a solvent control are shown (cumulative percentage, right). (c) Rates of egg predation by *Geocoris* spp. in a similar set-up in which two equal-concentration GLV blends were tested which differed only in their relative composition so as to reflect the composition of the morning and evening blends (cumulative percentage, right). *p < .05, **p < .01, ***p < .001 in Fisher's exact tests for predation assays and Student's t-tests for total GLV abundance. The labels in (A) represent the following: 1, 3(*Z*)-hexenal; 2, 2(*E*)-hexenal; 3, 3(*Z*)-hexenol; 4, 2(*E*)-hexenol; 5, 1-hexanol; 3(*Z*)-hexenyl acetate; 6, 3(*Z*)-hexenyl butyrate; 7, 3(*Z*)-hexenyl-isobutyrate. Pie graphs represent each GLV blend

GLV blends near leaves to which we attached *M. sexta* eggs to quantify predation (described above and in Kessler & Baldwin, 2001); as a control, we placed cotton swabs with lanolin containing only solvents (Schuman et al., 2012); and plants in triplets were at least 1 m apart (Figure 4b). We used a previous study in *N. attenuata* to estimate absolute amounts of GLVs (Allmann & Baldwin, 2010), and determined relative abundance in each blend using ratios measured in the field (Table 1).

Night predation rates were too low to test the effectiveness of different GLV blends (Figure S5, ≤5%), but day predators preferred the morning GLV blend (Figure 4b); most eggs predated during the day could be clearly attributed to *Geocoris* spp. based on markings typical of *Geocoris* spp. predation. Since the evening GLV blend had three times the amount of GLVs as the morning GLV blend, it was not clear how important the total amount was, vs. the differing ratio of esters to aldehydes and alcohols. Thus we repeated the assay using a second evening GLV blend with the same total amounts of GLVs as the morning blend, but in the evening-typical ratio. When the difference in abundance was eliminated, plants supplemented with the morning blend still received higher predation rates from day predators (Figure 4c).

3.5 | GLVs increase *Geocoris* spp. predation rates in the 24 hr after a dawn elicitation, and sesquiterpenes increase predation rates in the 24 hr after a dusk elicitation

Lastly, we asked predators about the relevance of the combination of transient GLV emission and diurnal, persistent sesquiterpene emission contained in *N. attenuata*'s HIPV profile (Figures 2 and 3, Figure S4). To disentangle the functional roles of GLVs and sesquiterpenes, we used an RNAi line deficient in the GLV biosynthetic gene *NaLOX2* (Allmann et al., 2010), and a line deficient in both *NaLOX2* and *NaLOX3* and thus in both GLVs and sesquiterpene HIPVs (irLOX2/3) (Schuman et al., 2015) (Figure 5a). We planted these lines in triplets with EV plants, with plants *c.* 1.5 m apart. To ensure that *Geocoris* spp. predators had the opportunity to learn that prey was available even on plants deficient in HIPVs, we first baited all plants with 1st-instar *M. sexta* larvae and eggs on rosette or low stem leaves, where larvae can provide a visual cue for *Geocoris* spp. hunting from the ground (Schuman et al., 2012, 2013). During this period, egg predation reached a maximum of

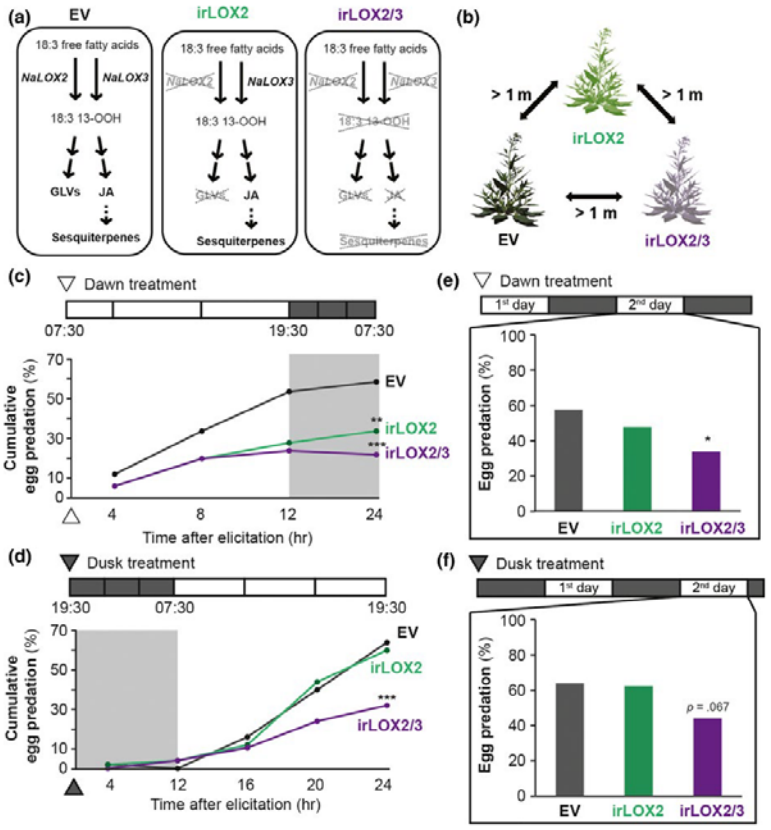

FIGURE 5 *Geocoris* spp. predation activity on empty vector (EV) plants can be attributed to green leaf volatile (GLV) emission after a dawn elicitation and to sesquiterpenes after a dusk elicitation. (a) To disentangle the functional role of each group of volatiles in different time periods, we assayed predation by *Geocoris* spp. predators from transgenic plants deficient in GLVs only (inverted repeat LIPOXYGENASE 2, irLOX2), or GLVs and terpenoids (irLOX2/3). (b) Plants were at least 1 m apart within a replicate (n = 10 plants or 50 eggs), and replicates were separated by at least 2 m in the field. At least 4 days prior to this assay, all plants were baited with *Manduca sexta* larvae and eggs low to the ground to provide visual cues for *Geocoris* spp., resulting in predation rates of 40–70% on larvae and *c.* 15% on eggs across genotypes (see Section 3 text); thus results shown here indicate *Geocoris* spp. responses to the experimental elicitation more than existing preferences for certain genotypes. (c and d) Predation rates (cumulative percentage) were counted every 4 hr for 24 hr after an elicitation with wounding and *M. sexta* oral secretions at dawn (white triangle) or dusk (grey triangle). (e and f) All eggs were replaced and cumulative predation rates were again counted for the second daytime period after treatment on the same plants: lower predation rates on irLOX2/3 indicate that sesquiterpenes are required to increase predation rates on the second day after elicitation. *p < .05,**p < .01, ***p < .001; p-values from Fisher's exact tests

17% and predation of larvae, a maximum of 67% in a 24 h period (see source data for Figure 6).

We waited at least 4 days after removing all larvae and eggs to allow any elicited volatile emissions to deplete. We then elicited half of the replicates at dawn, and the other half at dusk with W + R (Figure 5b), and allowed predators to find new *M. sexta* eggs we had glued to leaves. Within 24 hr after dawn elicitation, irLOX2 and irLOX2/3 plants exhibited similarly low predation rates in comparison to EV plants (Figure 5c). In contrast, within 24 hr after dusk elicitation, irLOX2 plants experienced predation rates similar to EV (Figure 5d), whereas predation rates from irLOX2/3 remained significantly lower (Figure 5d). Interestingly, on the second day after dawn elicitation, irLOX2/3 plants still showed lower predation rates than EV (Figure 5e, p < .05), whereas irLOX2 plants had similar predation rates to EV (Figure 5e, p = .321). In the second day after dusk elicitation, irLOX2/3 plants showed lower predation than EV (Figure 5f, p = .067) and irLOX2 plants showed similar predation rates as EV plants (Figure 5f, p = .99). Notably, predation rates on eggs were higher than during the training period, reaching ca. 60% within 24 hr on EV plants.

4 | DISCUSSION

Our study demonstrates that HIPV-mediated indirect defence can help predators find herbivores with variable feeding times by combining fast (GLV) and slow (sesquiterpene) components. Furthermore, plant responses to the timing of elicitation result in HIPV blends differing in their quantity and quality at different times of day, and these

time-typical blends can be differentiated by predators: *Geocoris* spp. ate more *M. sexta* eggs from plants supplemented with a morning- vs. evening-typical GLV blend, and this was due to blend composition—the higher relative abundance of GLV esters—rather than GLV abundance.

4.1 | HIPVs function as indirect defences by increasing apparency of herbivores

Herbivores may adjust their behaviour to avoid predation, but to function as indirect defences, HIPVs must make herbivores more apparent to predators. In fact, herbivores can use HIPVs as *zeitgeber* to time behaviours: laboratory studies showed that *Mythimna separata* larvae use plant volatiles rather than light to time day-typical hiding behaviour which likely functions to avoid predation in nature (Shiojiri et al., 2006). This may be an example of an herbivore adapting to plant indirect defence using diurnal plant volatile emission patterns to avoid predators which are attracted to the same volatiles. However, the function of *M. separata*'s behavioural response to HIPVs was not studied.

Higher predation rates on *M. sexta* larvae than on eggs in our study system (Figure 1c,d) may result from the overlap in timing of *M. sexta* and *Geocoris* spp. activity (Figure 1b,e), since mobile larvae may be more easily identified as prey by *Geocoris* sp. (Eubanks & Denno, 2000). Furthermore, actively feeding larvae elicit HIPV emission, whereas our artificially oviposited eggs do not (Kessler & Baldwin, 2001). Indeed, exposing *Geocoris* spp. predators to plants baited with both *M. sexta* eggs and larvae seemed to increase predation rates later for eggs on the same plants in this study. Interpreting predation of larvae is complicated by the influence of plant defensive compounds on larvae's ability to defend themselves from predation (Schuman et al., 2012; Shiojiri et al., 2006). Thus we decided to dissect the contribution of HIPV emission to predation rates apart from herbivore movement and behaviour. We first asked whether differently timed feeding bouts by the same herbivore could result in distinct HIPV emission profiles, and if so, to what extent these emission profiles appeared to synchronize with predator activity patterns.

We chose dawn vs. dusk elicitation because these time points have similar environmental conditions (Figure 2 and Figure S3), but dawn immediately precedes day while dusk precedes night. Thus we expected to have the best chance to detect differences in volatile profiles due to elicitation time rather than confounding factors. Furthermore, dawn and dusk elicitation are ecologically relevant: although *M. sexta* feeding bouts correlate to daytime temperature, larvae also have dawn and dusk feeding bouts and display ensuing long periods of inactivity while moulting (Figure 1e), during which time larvae are immobile but vulnerable to predation (Adams, 2003). Other common herbivores of *N. attenuata* such as *S. exigua* (Figure S1) as well as the specialist mirid *T. notatus* have other feeding rhythms. *T. notatus* in particular is night-active (Figure 1e) but, when HIPVs are collected over an entire day, appears to elicit a similar HIPV profile as *M. sexta* (Kessler & Baldwin, 2001). Interestingly, the dawn and dusk GLV blends are similar to midday and midnight GLV blends, respectively, and thus more broadly representative of day–night differences (Figure 3). Plants may produce common HIPVs to attract more generalist carnivores in response to different herbivore damage (Hare & Sun, 2011b; McCormick, Unsicker, & Gershenzon, 2012).

4.2 | GLV blends encode relevant temporal information for predators

Nicotiana attenuata produced more abundant GLVs after the dusk treatment, outside the window of most predator activity (Figures 1 and 4). However, we showed that the morning GLV blend is more attractive to generalist predators than the evening GLV blend even when the evening blend was more concentrated (Figure 4). These data suggest that although absolute abundance of GLVs does not synchronize with predator activity, the difference in blend composition is informative. Although minor compounds also can have large effects in plant–insect interactions (McCormick et al., 2014), abundance of minor GLVs, especially GLV esters, did not explain differences between the morning GLV and the evening GLV blend (Figure S6)—rather, the ratios of components were important.

Quantitative differences in HIPV emission certainly can matter when the difference is presence vs. absence (Halitschke et al., 2008; Rasmann et al., 2005; Schuman et al., 2012). The quantity of HIPV emissions is sometimes correlated with carnivore attraction, but the parasitoid *Cotesia marginiventris* has also been shown to disregard quantity and respond to HIPV blend quality across *Zea mays* accessions (Hoballah, Tamò, & Turlings, 2002). Many herbivorous insects also use specific blends of plant volatiles to find their host plants, likely because it permits greater flexibility in their odour-coding systems (Bruce & Pickett, 2011). Our data suggest that GLVs are qualitatively synchronized with predator activity in the sense that predators find morning blends more attractive than evening blends regardless of total abundance, and these blend preferences are important for plant indirect defence in nature.

4.3 | The combination of "fast" and "slow" HIPVs makes indirect defence robust to different herbivore feeding times

Specific HIPV blends can only function as indirect defences if they are both available at a time relevant for carnivores, and predict the presence of prey. GLVs were transiently emitted immediately after elicitation (within 4 hr), but sesquiterpenes were emitted only in the light period following elicitation, and for more than one day (Figure 2). This is consistent with previous studies of *N. attenuata* (Allmann & Baldwin, 2010; Halitschke et al., 2000) and similar to observations from lima bean that GLV emission rapidly follows damage regardless of elicitation time, whereas monoterpene emission is delayed after a dusk elicitation until the following light period (Arimura et al., 2008). GLVs and terpenoid volatiles are assumed to have different functional roles for carnivore attraction (Hoballah et al., 2002). We supposed that important differences could result from their different temporal dynamics.

Transient GLVs are likely more reliable indicators of herbivory, since their presence indicates active feeding; but relying exclusively on transient indicators may allow herbivores to escape notice by feeding

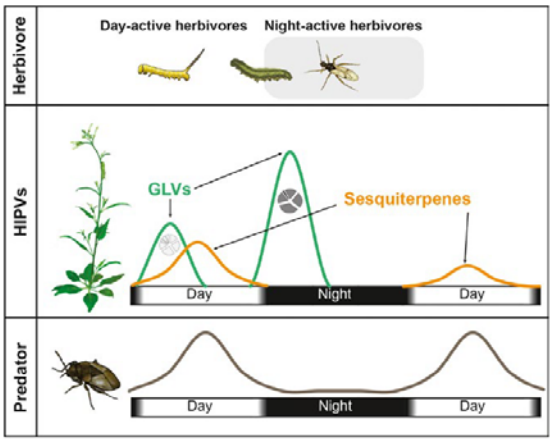

FIGURE 6 A schematic model depicting the hypothesized synchronization of plant indirect defence signals and predator activities. Abundance of herbivore-induced plant volatiles (HIPVs) emitted from *Nicotiana attenuata* leaves are differently synchronized with native predators. Sesquiterpenes are quantitatively synchronized and green leaf volatiles (GLVs) are qualitatively synchronized with predator activity. A robust and effective indirect defence that may increase a plant's Darwinian fitness emerges from the combination of different groups of HIPVs which provide sufficient information about the feeding activity of herbivores to inform the predatory behaviour of carnivores regardless of the timing of herbivore activity

when carnivores are inactive (Shiojiri et al., 2006) like *T. notatus* does. Persistent emission could reveal attempts at stealthy feeding: rhythmicity of persistently emitted volatiles could coincide with predator activity even if herbivore activity does not. Here we show that rapid GLV emission contributed to increased predation rates when plants were elicited at daytime prior to peak *Geocoris* spp. activity, while delayed but persistent sesquiterpene emission mattered more for plants elicited at dusk when *Geocoris* spp. activity is waning (Figures 1b, 5c,d). However our observations also suggest that even after a dawn elicitation, sesquiterpene HIPVs contribute to increase predation rates at later time points after elicitation (24–48 hr, Figure 5e,f).

We observed a tendency that plants elicited at dusk experienced higher predation rates overall (Figure 5), which we cannot explain by the dynamics of GLVs or sesquiterpene HIPVs (Figures 2 and 5) and may be due to other blend components. It is not clear that a difference in predation rates after dawn vs. dusk elicitation is advantageous for plants, but perhaps this difference, if common, is a concession to circadian- or otherwise-imposed physiological constraints. For the maintenance of HIPVs as indirect defences, it may be sufficient that they predictably increase predation rates regardless of herbivore elicitation time, even if they work better after some elicitation times than others.

4.4 | Conclusions and outlook

This study represents a first step in understanding the functions of temporal dynamics of HIPVs in nature and the ecological consequences of temporal heterogeneity in HIPV emissions. Dynamics at a higher level of temporal resolution than we achieved here may well be important for HIPV function. For example although GLVs can be broadly classified as transiently emitted, each compound has different emission dynamics (seconds or minutes) in *Arabidopsis thaliana* (D'Auria, Pichersky, Schaub, Hansel, & Gershenzon, 2007). Along similar lines, longer time-spans than we covered in this study are likely also relevant to HIPV function, most notably developmental time. In fact, developmental changes in plant volatile emission alter plant–parasitoid interactions in *Brassica rapa* (Desurmont, Laplanche, Schiestl, & Turlings, 2015), and may help to resolve ecological conflicts between HIPV-mediated carnivore attraction, and floral volatile-mediated pollinator attraction, when plants reach the flowering stage (Kessler, Halitschke, & Poveda, 2011). To directly test the hypothesis that temporal dynamics of HIPVs enhance their defensive function requires manipulations that change temporal dynamics of HIPV emission without altering average emission levels. Many factors can affect plant volatile emissions. For example terpenoid volatiles in plants are produced using substrates from two pathways, one of which is up-regulated (MEP) and one of which is downregulated by light (MVA) (Pokhilko, Bou-Torrent, Pulido, & Rodr, 2015; Rodríguez-Concepción, Campos, Ferrer, & Boronat, 2013). The plant circadian clock can regulate the emission of floral volatiles, including monoterpenes, and it was recently shown that monoterpene emission from leaves is regulated by a combination of the internal clock and light (Dudareva et al., 2003; Pokhilko et al., 2015). In addition, hydroperoxide lyase (HPL), which is a rate-limiting biosynthetic gene of GLVs, has a circadian rhythm peaking in the late evening (Pan et al., 2009). In an added layer of complexity, it was recently shown that the emission of plant volatiles can be an actively regulated process (Adebesin et al., 2017; Widhalm, Jaini, Morgan, & Dudareva, 2015). The temporal dynamics of HIPVs thus likely emerge from the combination of internal and external signals. Spatiotemporal regulation of gene expression is coded in the *cis*-regulatory region of genes, so the mutation of the right *cis*-regulatory elements could provide a way to test this hypothesis.

In conclusion, GLVs and sesquiterpene HIPVs emitted from *N. attenuata* leaves provide two ways of conveying timely information to native predators: sesquiterpenes are persistent and temporally synchronized with predator activity, whereas GLVs are transient but convey relevant temporal information as a result of qualitative blend differences (Figure 6). Robustness is a ubiquitous characteristic of biological systems which allows organisms to survive under unpredictable environments (Kitano, 2004); phenotypic plasticity is one important "fail-safe" mechanism through which biological systems obtain robustness (Hatakeyama & Kaneko, 2015; Whitacre, 2012). We conclude that temporal plasticity in HIPV emissions maintains robustness of plant indirect defence in response to temporally heterogeneous herbivore communities.

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AUTHORS' CONTRIBUTIONS

Conceptualization and Supervision: S.K., I.T.B. and M.C.S. Project Administration: I.T.B. Funding Acquisition: I.T.B. Methodology: Y.J., S.K., I.T.B. and M.C.S. Investigation: Y.J., J.K.G., S.K., F.Y. and M.C.S. Formal Analysis, Data Curation and Validation: Y.J., J.K.G. and M.C.S. Visualization: Y.J. Writing—Original Draft: Y.J. and M.C.S. Writing—Review & Editing: I.T.B.

DATA ACCESSIBILITY

Source data for this study are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.s5q8f (Joo et al., 2017).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Supporting Information

Supplemental materials and methods

Untargeted analysis of field headspace samples

We conducted an untargeted analysis using features extracted by XCMS/CAMERA (Smith *et al.* 2006; Tautenhahn *et al.* 2008; Benton *et al.* 2010; Kuhl *et al.* 2012) from total ion current data files (CDF) from field-grown plant samples. Peak-picking, removal of contaminants and de-duplication to yield one m/z feature per extracted peak has been previously described (Qi *et al.* 2016) and the R script for XCMS/CAMERA is provided in the source data file. Using data from simultaneously sampled and processed background controls, we then adjusted the peak areas for the extracted and de-duplicated m/z features by removing signal which could be attributed to background contamination, as described by Kallenbach and colleagues (Kallenbach *et al.* 2014). Due to missing samples in some treatment groups, up to two samples per group were removed which either had no detectable signal after background removal, or were randomly chosen, so that all groups contained 8 replicates. We excluded features which were not present above background levels in at least 50% of the replicates of at least one treatment group. The remaining features were checked individually against chromatograms.

Supplemental Figures and Legends

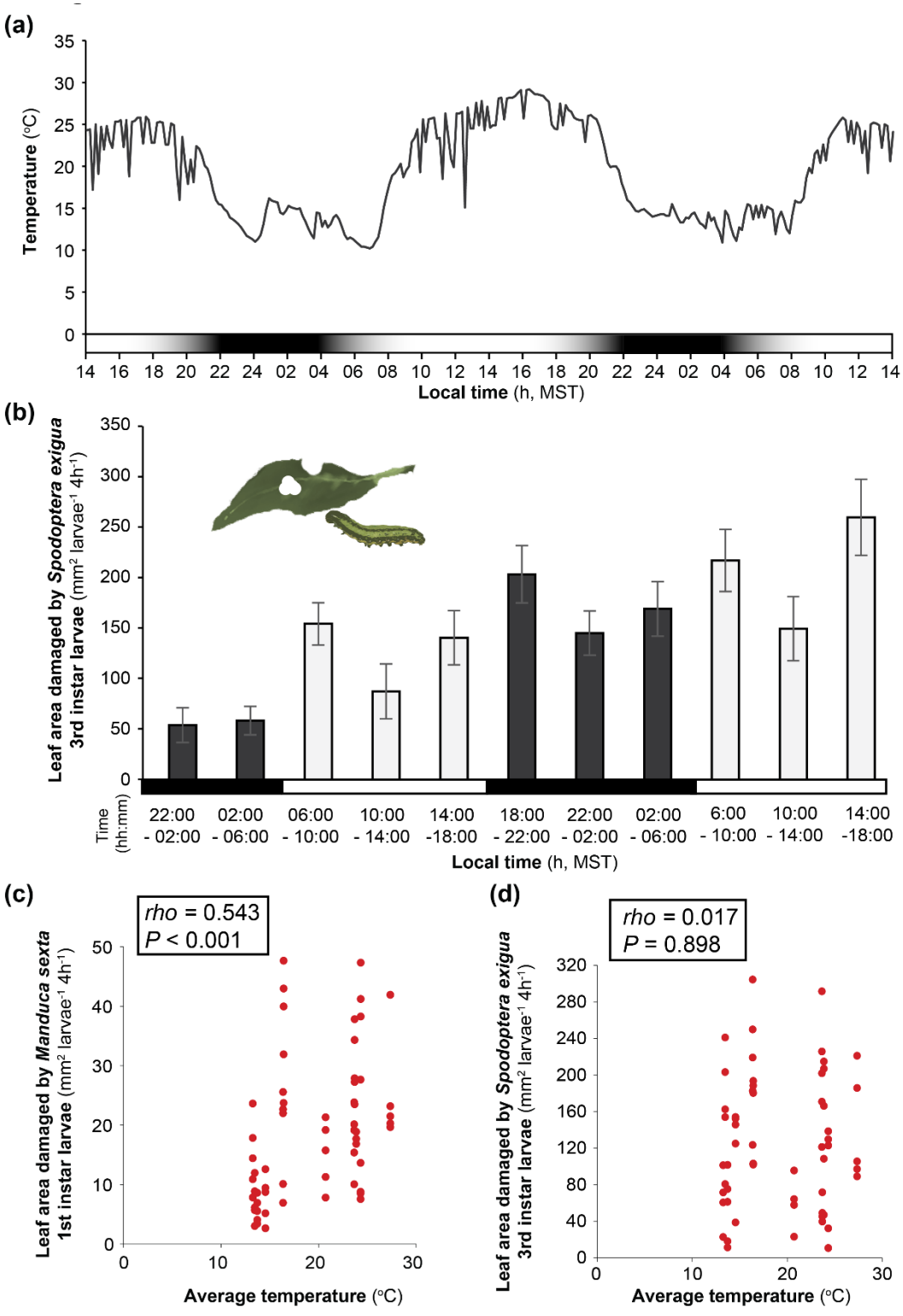

Fig. S1. *Spodoptera exigua* and *Manduca sexta* have different feeding activity patterns in the field. (a) Temperature logged every 10 min in the field. (b) Activity of *S. exigua* measured by leaf area damaged per time interval (mean ± SEM, n = 18 larvae). (c, d) Spearman's rank correlations were calculated for feeding activity versus temperature for each

herbivore.

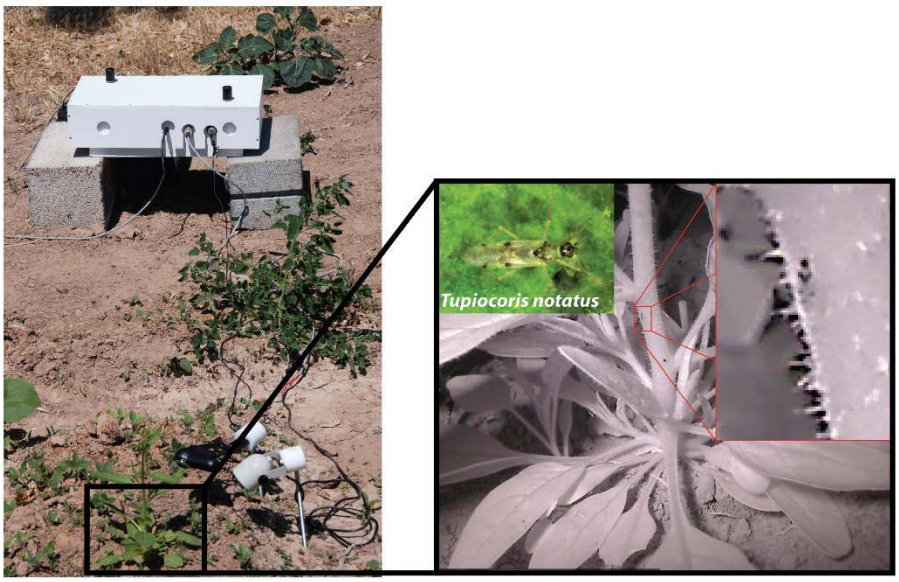

Fig. S2. Experimental setup for activity of *Tupiocoris notatus* on *Nicotiana attenuata* in their natural habitat.

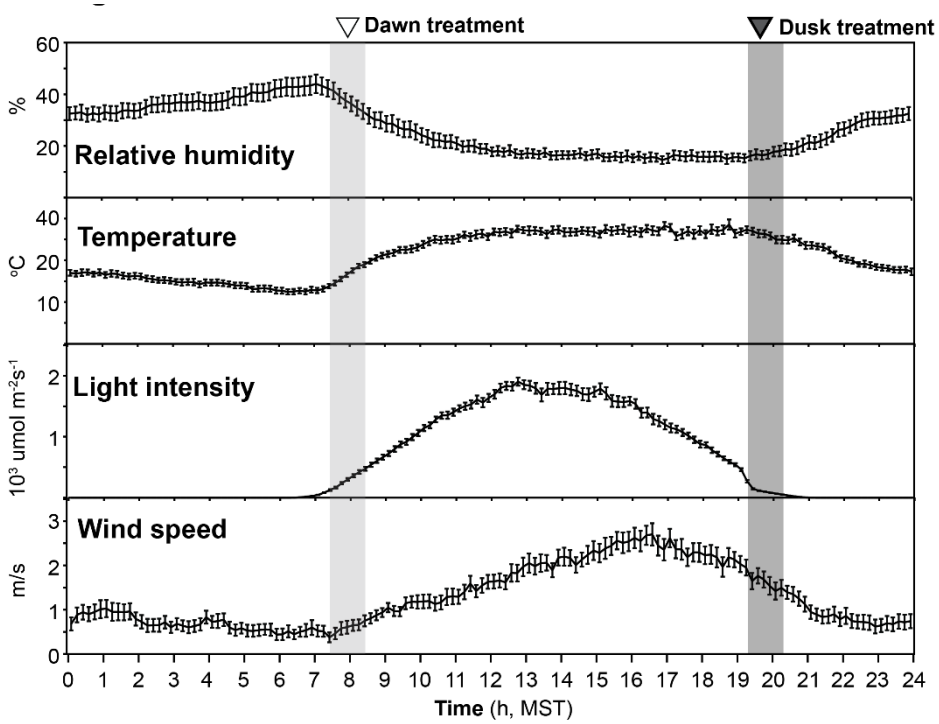

Fig. S3. Environmental factors at the field site. Relative humidity, temperature, light intensity, and wind speed logged every 10 min in the field during the May, 2013. Averaged values (± SE) presented in the figure.

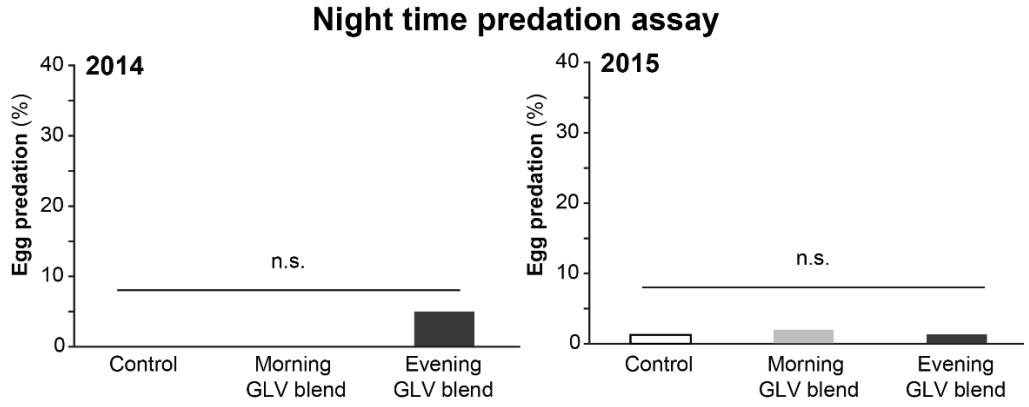

Fig. S5. Evening GLV blend does not increase nighttime egg predation rate. For predation assays, plants were at least 1 m apart within a replicate (n = 8-10 plants, 5 eggs/plant), and replicates were separated by at least 2 m. GLV blends were supplemented as shown in Figure 3 at dusk and cumulative egg predation rates were quantified at dawn.

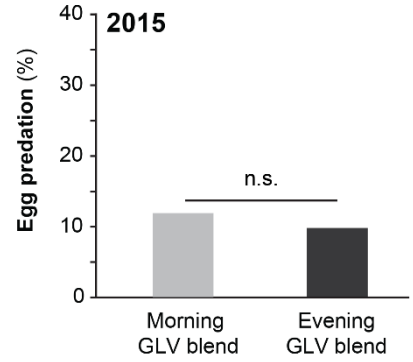

Fig. S6. Effects of GLV ester compounds in temporal variation of GLV blends for *Geocoris* spp. predation in nature. For predation assays, plants were at least 1 m apart within a replicate (n = 10), and replicates were separated by at least 2 m. Rates of egg predation by *Geocoris* spp. from plants supplemented with a synthetic blend mimicking the morning ester or evening ester blends, or a solvent control are shown (cumulative percentage).

Supplemental Tables

Table S1. Inducibility and time-dependent inducibility of plant volatiles after differently timed elicitations. Note that one or more elemene isomers likely result from the thermal rearrangement of germacrene A (11,50). $^{**}P < 0.01$; $^{***}P < 0.001$; *P*-values from one-way ANOVA analysis.

	Compound name	Inducibility	Time-dependent inducibility (one-way ANOVA)		
			Degree of freedom	F-value	p-value
Green leaf volatile	*2(E)*-hexenal	O	3, 16	36.31	***
	3*(Z)*-hexenol	O	3, 16	11.54	***
	2(E)-hexenol	O	3, 16	49.71	***
	3*(Z)*-hexenol acetate	O	3, 16	1.71	0.21
	3*(Z)*-hexenyl butanoate	O	3, 16	17.87	***
	3*(Z)*-hexenyl isobutanoate	O	3, 16	2.36	0.11
	3*(Z)*-hexenyl isovalerate	O	3, 16	2.85	0.07
	3*(Z)*-hexenyl caproate	O	3, 16	8.32	**
Sesquiterpene	*(E)*-*α*-bergamotene	O	3, 16	4.32	*
	5-*epi*-aristolochene	O	3, 16	17.88	***
	α-duprezianene	O	3, 15	20.66	***
	(E)-*β*-farnesene	X			
	Elemene isomers	X			
Monoterpene	*α*-terpeneol	X			
	α-pinene	X			
	β-myrcene	X			
	Ocimene	X			
	Linalool	X			
	Geraniol	X			

Table S2. Composition of synthetic morning and evening GLV blends.

	(1) Mimicking dawn and dusk ratios and relative amounts (ng/dose)		(2) Keeping amounts the same between dawn and dusk and only changing ratios (ng/dose)	
compound name	**dawn**	**dusk**	**dawn**	**dusk**
3(Z)-hexenal	0.0	57.9	0.0	23.0
2(E)-hexenal	1000.0	3000.0	1000.0	1190.0
3(Z)-hexenol	1504.0	3959.3	1504.0	1570.5
2(E)-hexenol	1705.6	5691.5	1705.6	2257.6
3(Z)-hexenyl acetate	260.7	52.5	260.7	20.8
3(Z)-hexenyl isobutanoate	488.5	140.0	488.5	55.5
3(Z)-hexenyl butanoate	420.2	32.5	420.2	12.9
1-hexanol	966.3	2926.8	966.3	1161.0
Total GLVs	**6345.3**	**15860.6**	**6345.3**	**6291.4**

Manuscript III

The circadian clock in *Nicotiana attenuata* times accumulation, but not emission, of herbivore-induced plant volatiles that function as indirect defenses

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Abstract

The plant circadian clock controls constitutive rhythms of chemical defense in apparent anticipation of herbivore feeding. However, many chemical defenses are not constitutively active but rather induced, i.e., produced or activated following herbivory. Herbivore-induced plant volatiles (HIPVs) mediate defense via their association with herbivory by making herbivores more apparent to foraging carnivores. Here we test alternative hypotheses for the role of the circadian clock in induced defense mediated by HIPVs. Using the wild tobacco *Nicotiana attenuata*, we show that the relative headspace abundance of HIPVs depends on elicitation time, not on internal circadian rhythm. However, among *N. attenuata*'s HIPVs, GLVs are transiently emitted after damage and thus rhythmicity of their emission cannot be measured with precision. We therefore measured internal GLV pools, which accumulate constitutively but are modified and released upon herbivory. Under diurnal and free-running conditions, abundant GLV aldehyde pools peaked at night and at subjective night, respectively, following abundance of transcripts for the biosynthetic enzyme HYDROPEROXIDE LYASE (*NaHPL*). Plants rendered deficient by RNAi in the morning clock element LATE ELONGATED HYPOCOTYL (irLHY) had altered accumulation of *NaHPL* transcripts and rhythmicity of GLV aldehyde pools. irLHY plants produced fewer GLVs in response to herbivore damage during the day, and day-active *Geocoris* spp. preyed on fewer herbivores from wild plants when plants were supplemented with irLHY-typical versus WT-typical GLV headspace blends. Thus the circadian clock controls the timing of production for GLVs, but herbivory controls their release, connecting hard-wired internal rhythms to herbivory-associated inducible defense responses.

Key words

circadian clock, diurnal rhythm, herbivore-induced plant volatiles (HIPVs), green leaf volatiles (GLVs), plant indirect defense, *Nicotiana attenuata*, *Manduca sexta*, *Geocoris pallens*

Introduction

Most animals are herbivores, and plants produce a plethora of secondary metabolites to defend themselves against herbivory (Vogel, 2012; Schuman and Baldwin, 2016) such as glucosinolates, alkaloids, phenolics, and proteinase inhibitors (Mithöfer and Boland, 2012). These metabolites may be constitutively produced but may also change or be activated or synthesized upon induction by herbivore attack (Wu and Baldwin, 2010). The plant inducible defense is cost efficient defense and it demonstrated in more than 100 plant species (Jander, 2012; Herden et al., 2016). Some of these metabolites are released into the environment in response to herbivory, including herbivore-induced plant volatile (HIPV) compounds (Dicke and Baldwin, 2010; Hare, 2011). HIPVs can be perceived by many organisms, and this bouquet of volatiles affects many different aspects of the ecological community (Hare, 2011; Schuman et al., 2012). For instance, HIPVs can attract natural enemies of herbivores, e.g. predators and parasitoids (De Moraes et al., 1998; Kessler and Baldwin, 2001; Halitschke et al., 2008; Allmann and Baldwin, 2010), thereby increase plant Darwinian fitness in nature (Schuman et al., 2012; Gols et al., 2015) in a phenomenon termed indirect defense.

Diurnal rhythms of HIPV emission have been described in several plant species (Loughrin et al., 1994; Turlings et al., 1998; De Moraes et al., 2001; Arimura et al., 2004; Arimura et al., 2008). Rhythmic HIPVs affect insect behavior. For example, night-time HIPVs induced by *Heliothis virescens* repel oviposition by conspecific females (De Moraes et al., 2001) and *Mythimna separata* larvae use plant volatiles to time their feeding and hiding activity (Shiojiri et al., 2006). Different biosynthetic groups of HIPVs have different characteristic emission patterns and in response to herbivore attack and “lag time” between attack and emission (Loughrin et al., 1994; De Moraes et al., 2001; Arimura et al., 2008). The composition of volatile blends rather than the presence or absence of individual HIPVs often determines their effects (Allmann and Baldwin, 2010; Webster et al., 2010; Kessler et al., 2013), and since timing of HIPV emission dynamically alters blend composition it also may be important for the effectiveness of plant indirect defense (Joo et al., in review).

The circadian clock allows plants to anticipate environmental changes (Greenham and McClung, 2015). This occurs in part through the coordination of plant metabolisms to synchronize with environmental rhythms (Wijnen and Young, 2006) and in fact around 30% of expressed genes in *Arabidopsis thaliana* (Brassicales: Brassicaceae) have circadian

expression patterns (Harmer et al., 2000; Covington et al., 2008; Pan et al., 2009). Previous studies with the polyphagous herbivore *Trichoplusia ni* (Lepidoptera: Noctuidae) and *A. thaliana* or post-harvest fruits and vegetables also suggest that the plant circadian clock orchestrates anticipation of herbivore attack by synchronizing basal defense metabolite accumulation with insect feeding activity (Goodspeed et al., 2012; Goodspeed et al., 2013). However, inducible responses in plant defense have been reported in more than 100 plant species (Agrawal and Karban, 1999) and the plant transcriptome and metabolome change dramatically after herbivore attack (Schuman and Baldwin, 2016). For interactions in which inducible responses are important, their effects may override any effects of basal metabolic fluctuation on the outcome of plant-herbivore interactions (Herden et al., 2016). The circadian clock modulates responsiveness to external stimuli in a phenomenon known as gating (Greenham and McClung, 2015), but clock functions in plant inducible defense responses are largely unknown.

Here, we use the wild tobacco *Nicotiana attenuata* (Solanales: Solanaceae) and its native herbivore *Manduca sexta* (Lepidoptera: Sphingidae) and associated native predators, *Geocoris* spp. (Hemiptera: Geocoridae), as an ecological model system in which to investigate the role of the plant circadian clock in the production and emission of HIPVs and their role in plant indirect defense. First, we asked whether the magnitude or composition of HIPV emissions depended on time of induction under diurnal conditions, since such changes might affect the natural role of HIPVs in indirect defense. We then analyzed rhythms of HIPVs under free-running conditions in order to identify clock-regulated HIPVs. Lastly, we quantified differences in HIPV emissions between WT and clock-deficient plants under natural conditions and compared the effectiveness of WT versus clock-shifted blends for plant indirect defense in nature.

Results

GLVs and sesquiterpenes have time-dependent induction in response to herbivory

The genotype of *N. attenuata* we are working with has a vegetative volatile profile comprising pirmarily green leaf volatiles (GLVs), sesquiterpenes, and a few monoterpenes (Schuman et al., 2009) in addition to methanol and ethylene (Schuman et al., 2016), which can only be detected using alternative methods and are thus not investigated here. In

particular, GLV and sesquiterpene HIPVs have been shown to mediate indirect defense in this genotype (Kessler and Baldwin, 2001; Halitschke et al., 2008; Schuman et al., 2012). To determine whether timing of herbivore attack can alter the emission profile of HIPVs (see also Joo et al., in review), we treated plants with wounding and regurgitant of *M. sexta* (W+R) at different times of day: ZT0 (dawn), ZT8 (midday), ZT16 (dusk), and ZT20 (midnight). Previously, we developed a technique for plant volatile sampling using silicone tubings (STs) (Kallenbach et al., 2014; Kallenbach et al., 2015), and we used this approach to sample plant volatiles every 4 h after W+R treatments. Relatively low levels of volatile compounds were sampled simultaneously from undamaged (control) plants. Monoterpenoids, e.g. *α*-terpeneol, *α*-pinene, and *β*-myrcene, were most abundant in undamaged samples and did not change in response to induction. Each group of plant volatiles had different emission patterns. GLVs were detected immediately and transiently after induction. GLV aldehydes and alcohols were detected in higher abundance at night, but GLV esters were similarly abundant regardless of induction time with the exception of 3*(Z)*-butanoate and 3*(Z)*-caproate (**Figures 1A**). Among the sesquiterpenes, the emission of *(E)*-*α*-bergamotene and 5-epi-arisolechene was induced by W+R treatments and lasted longer than GLV emission while emission of *α*-duprezianene was constitutive but slightly altered by induction (**Figure 1B**). The abundance of these three sesquiterpenes depended on induction time as well as time of day (**Figure 1B**). Farnesene and elemene were detected constitutively, but not inducibly in the wild-type, which is consistent with previous studies (Schuman et al., 2009).

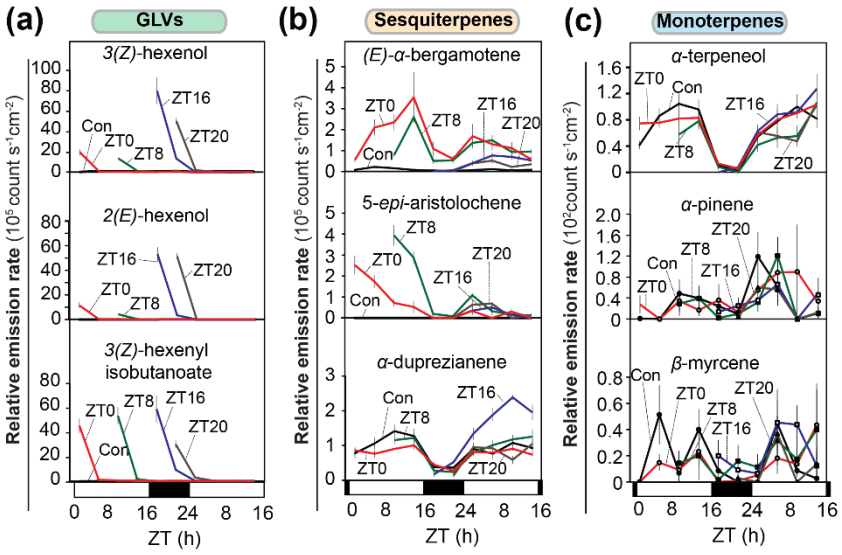

Figure 1. Rhythms of selected plant volatile emissions after different elicitation times. Glasshouse-grown WT plants were elicited with wounding and regurgitant from *Manduca sexta* (W+R) to mimic herbivore damage at different times: dawn (*zeitgeber* time 0, ZT0, red), day (ZT8, green), dusk (ZT16, blue), and night (ZT20, dark brown), or left undamaged (control, black). Plant volatiles were sampled using silicone tubings (STs) and analyzed by TD-GC-MS (mean ± SE, n = 5). (A) GLV production peaked in the first 4 h after W+OS and was no longer detectable after 8-12 h. GLV alcohols and aldehydes were emitted in higher relative abundance after dusk or nighttime treatment, but GLV esters were emitted in similar abundance throughout the day. (B) Among the sesquiterpenes, *(E)*-α-bergamotene and 5'-epi-aristolochene were strongly induced by the treatment and all sesquiterpene emissions showed a similar diurnal pattern. (C) Monoterpenes were not induced by W+OS at any time point.

HIPV emissions have diurnal, but not circadian rhythms

Although emission patterns were strongly different between GLVs and sesquiterpenes, the inducibility of HIPVs was highly affected by timing of induction. We therefore tested whether HIPV emission is regulated by the circadian clock in *N. attenuata*. To test circadian regulation of HIPVs, 2 groups of plants were grown under 12h day/12h night (LD) and one group was transferred to a continuous light environment (LL) (**Figure 2A**). As most GLVs are only transiently emitted after a single elicitation (**Figure 1**), GLVs were abundant in the first day under both LD and LL, but scarce on the second day (**Figure 2B**). Herbivore-induced sesquiterpenes have strong diurnal rhythm and rhythm persist in the second day in LD, except 5-epi-arisolechene (**Figure 2C**). However, induced-sesquiterpenes lost the rhythmicity under LL (**Figure 2C**). We also measured monoterpenes and only α-pinene had a slight circadian rhythm (p = 0.07, **Figure S1**). These data suggest that HIPV emissions are mainly determined by other factors, such as induction and light/dark transition, rather than the circadian clock. irLHY plants emitted lower amounts of total GLVs than EV plants but other clock components, *NaTOC1* and *NaZTL*, did not affect to the emission of GLVs (**Figure 3**).

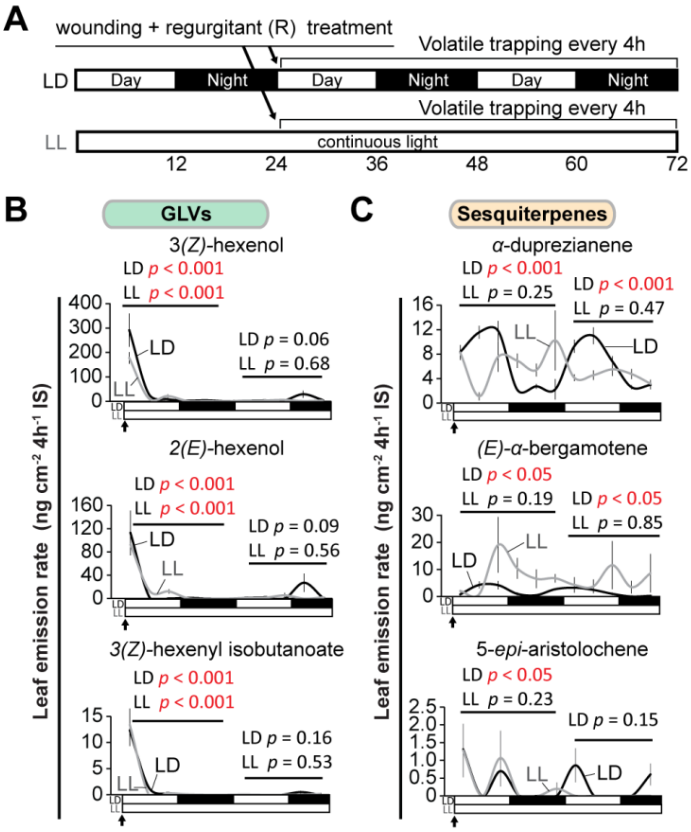

Figure 2. The circadian clock does not strongly control emission of green leaf volatiles or sesquiterpenes. (A) WT plants were grown in a climate chamber to identify circadian-regulated HIPVs. WT plants were entrained in a day/night cycle (LD) and transferred to continuous light (LL). Plant volatiles were sampled by Poropak-Q filters and analyzed by TD-GC-MS (mean ± SE, n = 5 for LD and 6 for LL samples). (B) Most GLVs were transiently and strongly emitted after the W+OS elicitation with reduced emission during the night of the second day, but emission was too transient for rhythmicity to be evaluated. (C) Emission of *α*-duprazianene and *(E)-α*-bergamotene was strongly diurnal under LD which continued for the second day after elicitation, but under LL, none of the sesquiterpenes were rhythmically emitted. *P*-values from one-way ANOVA analysis.

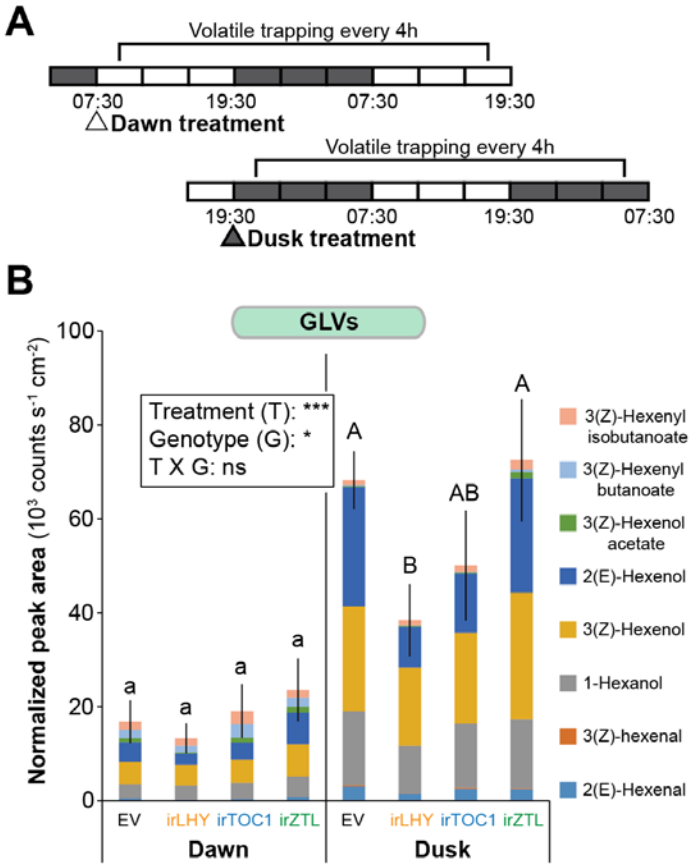

Figure 3. Emissions of herbivore-induced plant volatiles are altered in the clock transgenic plants in nature. All plants were grown in a field plot in their native environment and single leaves of rosette-stage plants (+1 position) were elicited with wounding and *M. sexta* regurgitant (W+R) and sampled by silicone tubes (STs) and analyzed by TD-GC-MS (n = 6 plants). (A) W+R treatment occurred at dawn (white triangle) or dusk (dark gray triangle) and STs were exchanged every 4 h over 48 h. (B) Composition of the GLVs sampled from the headspace (mean ± SE) when plants were treated at dusk versus those sampled at dawn. Different letters indicate significant differences ($p < 0.05$) using ANOVAs with Tukey post hoc tests.

Production of internal GLV aldehydes pool is regulated by the circadian clock

Although HIPV emission did not follow circadian patterns (**Figure 2**), transient GLV emission was affected by silencing the clock component *NaLHY* (**Figure 3**). GLVs are normally released from storage and rapidly metabolized (or activated) like glucosinolates in *A. thaliana* (Paré and Tumlinson, 1997; Matsui et al., 2012). Volatile emission is a complex process, so there are often discrepancies between production and emission (Widhalm et al., 2015). Therefore, we further hypothesized that the internal pools of GLVs are regulated by

the clock. To determine the diurnal rhythm of GLVs, we quantified internal GLV pools using a sorbent extraction method employing silicone tubings (STs) in intact leaf tissues from plants grown in LD (Childers et al. in preparation). The internal GLV pool in intact leaves comprises fewer compounds than the set of GLVs emitted from damaged leaves: 3*(Z)*-hexenal, 2*(E)*-hexenal, 1-hexenol, 3*(Z)*-hexenol, and *3(Z)*-hexenal isobutanoate were detected in the internal pool (**Figures 4A**). In addition, GLV aldehydes were the most abundant GLVs in intact leaf tissue whereas GLV aldehydes and alcohols are similarly abundant in emissions from damaged leaves (Allmann and Baldwin, 2010). In EV plants, the initial product of HYDROPEROXY LYASE (HPL) activity, *3(Z)*-hexenal and its isomer *2(E)*-hexenal had strong diurnal rhythms (**Figure 4A**, both $p < 0.001$), but internal GLV alcohols and esters did not (**Figure S2**, $p = 0.052$ and $p = 0.07$, respectively).

We monitored internal GLV pools under LD and LL conditions to determine whether internal pools had circadian regulation. Interestingly, internal pools of 3*(Z)*-hexenal and 2*(E)*-hexenal maintained their rhythmic accumulation under free-running (LL) conditions (**Figure 4A**, $p < 0.01$ and $p < 0.05$, respectively). To test whether a specific circadian clock component regulates internal GLVs accumulation, we quantified internal GLV pools in LHY-silenced plants (irLHY) in LD and LL conditions. Pools 3-*(Z)*-hexenal and *(E)*-2-hexenal in irLHY plants exhibited an earlier peak time than in EV plants under LD conditions; between ZT 4 and ZT 8 (**Figure 4B**, $p < 0.05$ and $p < 0.01$, respectively). Furthermore, GLV pools in irLHY plants exhibited two peaking times per day under LL: in the middle of day and middle of night, following transcript abundance of *NaHPL* in irLHY plants (**Figure 4B**). Transcript abundance of *NaHPL* were highly correlated with endogenous production of GLVs (**Figure S3A**) and the internal pool of GLV aldehydes in *NaHPL*-silenced plants strongly decreased (**Figure S3B**).

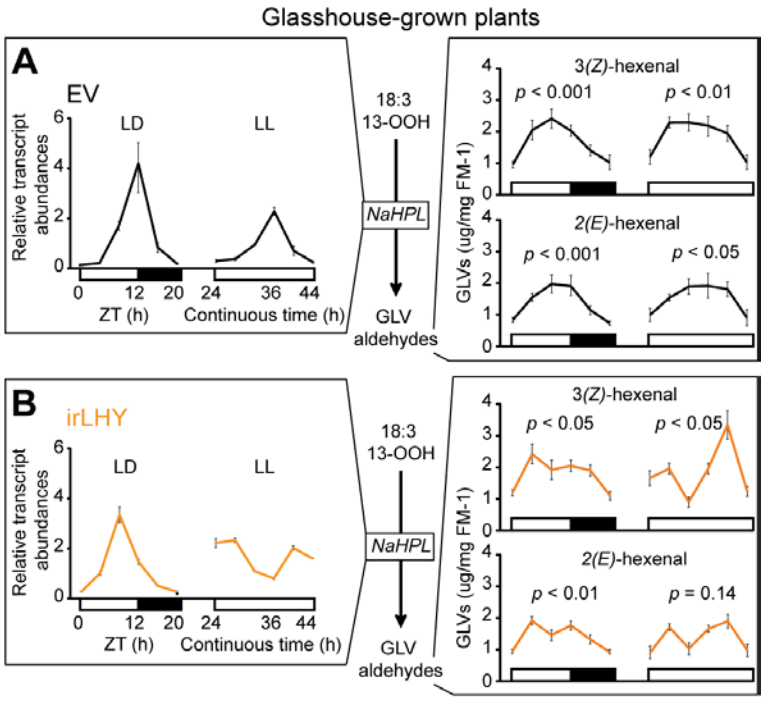

Figure 4. Internal GLV pools accumulate and decrease with a circadian rhythm.

Single, mature, non-senescent leaves were collected from rosette-stage EV and *irLHY* plants to measure the accumulation of GLV pools and transcript abundance of *NaHPL* in in LD and continuous light (LL) conditions. Two groups of plants were entrained LD and one of these was transferred to LL one day before sample collection. Mean (± SE, n = 5) accumulation of *3(Z)*-hexenal and *(E)-2*-hexenal were sampled from aqueous leaf extracts on STs and analyzed by TD-GC-MS. (A) In EV plants, both GLV aldehydes maintained strongly rhythmic fluctuations in abundance under LL. (B) In LHY-deficient plants, transcript abundances of *NaHPL* had double peaks on the second day under LL. GLV aldehyde accumulation was similar to the transcript abundance of *NaHPL*. Black lines indicate LD and gray indicate LL. *P*-values from one-way ANOVA analysis.

Transcripts of the GLV biosynthetic gene *NaHPL* show circadian patterns of accumulation

We measured transcript abundance for several GLV biosynthetic genes: *NaLOX2*, *NaHPL* and *NaADH*, every 4h for 3 days in LD. Transcript abundance of all three genes peaked around dusk (black line, **Figure 5**). To determine whether the circadian clock regulates these accumulation patterns, we measured transcript accumulation for 72 h under free-running conditions. All seedlings were entrained in LD and released to LL. *NaADH* lost rhythmicity under LL, and the phase of *NaLOX2* transcripts was not consistent from day to day under LL with peaking times on the 1st, 2nd, and 3rd days at ZT20, ZT36, and ZT60, respectively (**Figure 5A**). However, *NaHPL* transcript abundance exhibited a strong circadian rhythm and peaked consistently at subjective dusk under LL (**Figure 5B**). Furthermore, transcript levels

of *NaLOX2* gradually under LL condition, so that the maximum transcript level on the 3rd day was more than 4-fold higher than the maximum on the 1st day (**Figure 5A**). In addition, although transcript abundance of *NaHPL* decreased in plants rendered deficient in *NaLOX2* by RNAi (irLOX2), the rhythmic pattern of *NaHPL* transcript accumulation remained similar in EV and irLOX2 plants (**Figure S4**), suggesting that rhythmic transcript abundance of *NaHPL* is *NaLOX2*-independent.

To determine whether *NaLHY* was involved in regulating circadian transcript abundance of *NaHPL*, we used *NaLHY* silenced plants which have earlier peaking time of *NaCAB2* transcripts and early floral behaviors (Yon et al., 2016). Relative transcript abundancesof *NaHPL* in EV and irLHY plants had a rhythmic expression in both in LD and LL (**Figure 5D**). Under LD conditions, *NaHPL* still had a ca. 24 h period, but the peaking time in irLHY was around 4 h earlier than EV (**Figure 5D**). Interestingly, amplitude of the *NaHPL* rhythm was strongly attenuated in irLHY compared to EV plants in LL (**Figure 5D**). This suggests that *NaHPL*, but not *NaLOX2* or *NaADH*, is a circadian-regulated gene.

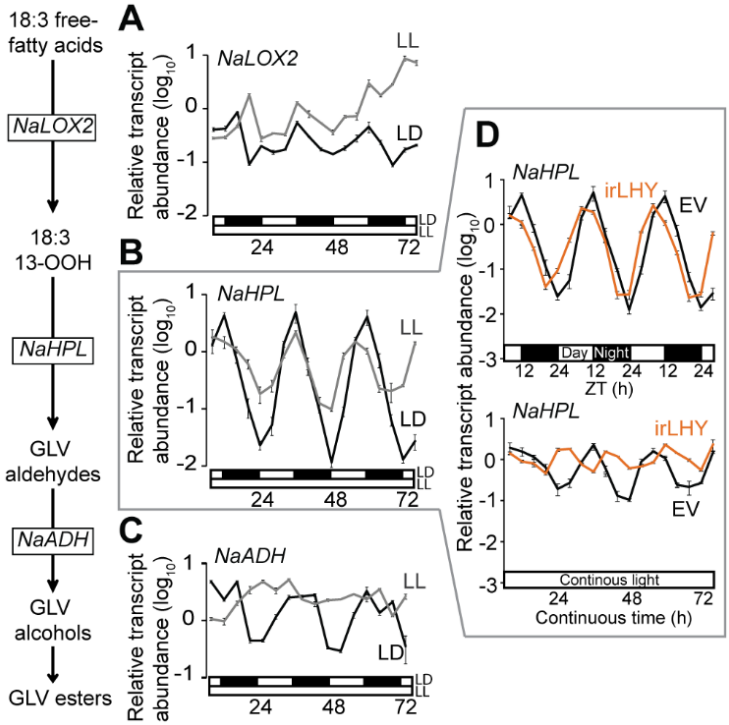

Figure 5. Transcript accumulation of the GLV biosynthetic gene *NaHPL* has a strong circadian rhythm.

Mean (± SE, n = 3) relative transcript abundance of *NaLOX2*, *NaHPL*, and *NaADH* were measured in *N. attenuata* seedlings grown under 12 h light/12 h dark conditions (LD) and

under constant light conditions (LL). Seedlings were harvested every 4 h for three days. (A-C) Transcripts of GLV-biosynthetic genes oscillated diurnally in abundance, but only *NaHPL* oscillation was maintained under free-running conditions. (D) Transcript abundance of *NaHPL* in *irLHY* plants showed early shifted phase in LD (a) and shortened period and amplitude in LL compared with EV plants. Values are shown on a $\log_{10}$ scale. White bars: day; black bars: night; *EF1$_\alpha$*, *elongation factor 1α*; *LOX2*, *lipooxygenase 2*; *HPL*, *hydroperoxide lyase*; *ADH*, *alcohol dehydrogenase*; *LHY*, *late elongated hypocotyl*; *irLHY*, inverted repeat *NaLHY*; EV, empty vector control.

The irLHY-typical GLV blend is less attractive to *Geocoris* spp. in nature

Since native habitats have more complex *zeitgebers*, clock regulation can be different in nature. So we checked transcript abundance of *NaHPL* and internal GLV pools in the field and found that differences between irLHY and EV identified under laboratory conditions were robust in the field (**Figure 6A**). To test the clock's role in plant indirect defense, we conducted headspace supplementation experiments using synthetic GLV mixtures representative of GLVs sampled from the headspace of EV or irLHY plants. We tested the attractiveness of these mixtures to *Geocoris* spp. in a natural population of *N. attenuta* in the Great Basin Desert of Utah. We glued five eggs under a lower stem leaf at a standardized position for 10 pairs of plants at the morning (7 am, MST) and counted predated eggs at the evening (7 pm, MST) for 3 days. GLV supplementation treatments (n = 10 pairs) were randomly distributed in populations and plants were matched across treatment groups for developmental stage and roughly for size (**Figure 6C**). Predation of *M. sexta* eggs by *Geocoris* spp. was lower on plants supplemented with the irLHY-typical GLV blend than those supplemented with the EV-typical GLV blend (**Figure 6D**).

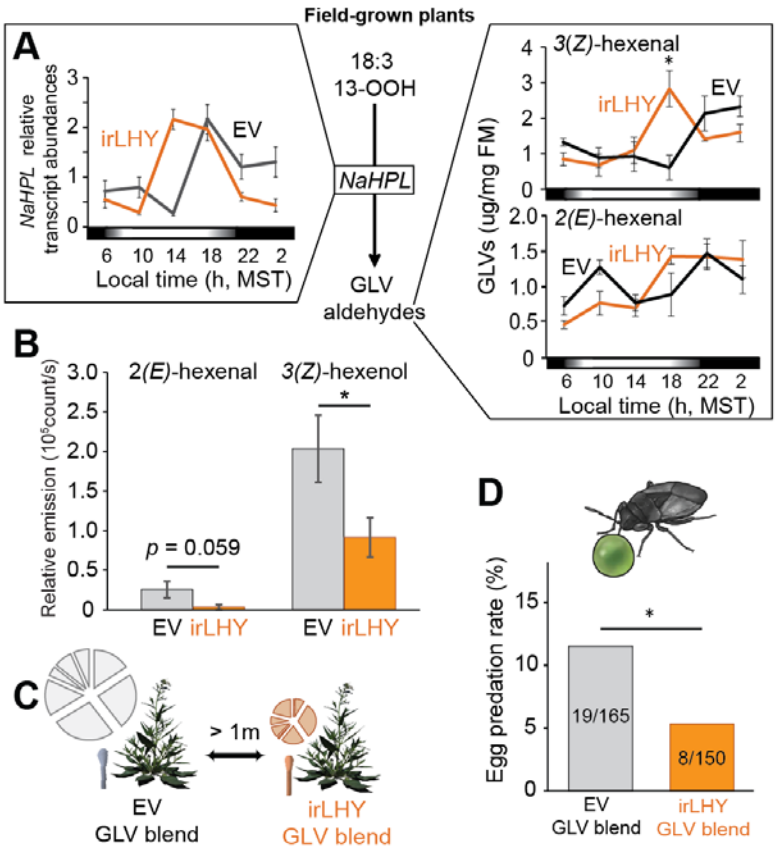

Figure 6. LHY-deficient plants emit relatively fewer GLVs in nature due to a shift in timing of internal accumulation, resulting in a blend less attractive to *Geocoris* spp. carnivores.

Single, mature, non-senescent leaves were collected from rosette-stage EV and *irLHY* plants to measure endogenous GLVs and transcript abundance of *NaHPL* in EV and *irLHY* plants in the field. (A) A similar difference was observed in the mean (± SE, n = 3) relative transcript abundance of *NaHPL* for EV and irLHY (line 406-3) plants grown in their native habitat, and GLV aldehyde abundance peaked earlier in irLHY plants in nature (n = 6). (B) Relative emission of GLVs was reduced in irLHY plants (Mean ± SE, n = 5-6). (C, D) *Geocoris* spp. tended to predate more eggs from wild plants supplemented with the EV blend than plants supplemented with the irLHY GLV blend. *$p < 0.05$; p-values from Fisher's exact test and Student's t-test. MST, Mountain Standard Time

Discussion

In previous literature, Goodspeed *et al.* (Goodspeed et al., 2012; Goodspeed et al., 2013) proposed that plant defenses are synchronized with herbivore feeding activity by anticipating with plant circadian clock. However, not every defense response in plant can be explained by the anticipatory hypothesis because the inducible defenses in plants require external stimuli for clock regulations (Greenham and McClung, 2015). To explain circadian-regulated plant

inducible defenses, which is very common in many plant-herbivore interactions (Jander, 2012; Herden et al., 2016), we investigated HIPV emission and showed that circadian clock function in plant indirect defense is modulating responsiveness (="*gating*") of the GLVs emission. Although there is no circadian-regulated synchronization between *Nicotiana attenuata* and *Manduca sexta* (Herden et al., 2016), the clock enhances plant indirect defense by regulating the production of GLVs. Up to our knowledge, this is the first study to show that the circadian clock regulates plant inducible defense.

Not only variations of the endogenous oscillator itself in plants (Takata et al., 2008), different plant species also have different regulations in outputs of the circadian clock (Poire et al., 2010). The oxylipin pathway produced various acyclic and cyclic products which are important for plant defense and development in plants (Creelman and Mulpuri, 2002). There are two branches (jasmonic acids and green leaf volatiles) in the oxylipin pathway in many plants (Allmann et al., 2010; Mochizuki et al., 2016). In *N. attenuata*, GLVs-biosynthetic genes (*NaLOX2* and *NaHPL*) show dusk-specific rhythm, but JA-biosynthetic genes (*NaLOX3* and *NaAOS*) genes show day-specific rhythm **(Figures 4 and S5).** Several genes in the oxylipin pathway, e.g. LOX, AOS, and AOC, exhibited circadian-regulated patterns in *Arabidopsis thaliana* (Pan et al., 2009). Also, LOX10 has a circadian rhythm in *Zea may* (Christensen et al., 2013). But, in *N. attenuata*, only *NaHPL* have a circadian rhythm among other biosynthetic genes. Moreover, final products are also species-specific; e.g. accumulation of JA in *A. thaliana* show a circadian-regulated pattern (Goodspeed et al., 2012), but JA and JA-Ile do not have significant diurnal rhythm in the leaf of *N. attenuata* **(Figure S5)**. It suggested circadian clock-regulated metabolisms in jasmonates are species-specific. However, it needs to be further tested about the functional consequence of species-specific circadian regulations in the oxylipin pathway.

Both GLVs and sesquiterpenes had time-dependent inducibilities, but only total production of GLVs is regulated by the circadian clock through *NaHPL*. Inducible defense compounds can be divided into *de novo* synthesized metabolites and activated metabolites after herbivore attack (Howe and Jander, 2008). For instance, phenolamides are inducible defense compounds after because biosynthesis is induced by herbivore attack (Onkokesung et al., 2012), in contrast glucosinolate and 2, 4-dihydroxy-1, 4-benzoazin-3-one (DIBOA) are stored in an inactive form and enzymatically activated to produce toxic compounds after herbivore attack (Howe and Jander, 2008). Among HIPVs, sesquiterpenes are synthesized *de*

novo and GLVs are more likely to store and to be activated or metabolized in response to insect feeding (Paré and Tumlinson, 1997; Matsui et al., 2012). Although flux of isoprenoids, which is precursors of sesquiterpenes, also have circadian rhythms in many species (Dudareva et al., 2005; Pokhilko et al., 2015), MEP pathway is also affected by post translational regulation of DXS (1-deoxy-D-xylulose 5-phosphate synthase) (Pokhilko et al., 2015). The alternation/activation of MEP pathway by herbivore may disrupt the circadian regulation of herbivore-induced sequiterpenes. Therefore, the circadian clock may directly regulate plant defense by activating metabolites, e.g. glucosinolate (Goodspeed et al., 2013) and GLVs (**Figure 3**), but not *de novo* synthesizing metabolites, e.g. sesquiterpenes, among plant inducible defense metabolites.

The circadian regulation of GLVs for plant indirect defense may be also ecologically relevant than that of sequiterpenes. *Geocoris* spp. are general predators, so more general chemical cue can increase plant indirect defense more efficiently (Hare and Sun, 2011; Clavijo McCormick et al., 2014). In *N. attenuata*, jasmonate-regulated sesquiterpenes are more variable than GLVs in different natural accessions (Schuman et al., 2009). Moreover, not every herbivore has a circadian rhythm and plants are normally under attacks from different herbivore that have different active time in nature, so differential regulation in HIPVs may enhance more plasticity in plant indirect defense. For example, sesquiterpenes did not have circadian rhythms, but they have strong diurnal rhythms and play a role as a long-term signal to a day-active predator (Joo *et al.* in review).

We have shown clear evidence of clock-regulated production of HIPVs, but the clock function in whole of HIPV emissions remains an open question. Rhythmic pattern of GLV production is clearly shifted, but overall production is not significantly lower during the day LHY-silenced plants. However, GLV emission was significantly decreased in irLHY plants. The current literature provides interesting intuitions in this context. In contrast with HIPVs (Zhang et al., 2010), many of floral volatiles are regulated by the clock (Kolosova et al., 2001; Dudareva et al., 2003; Dudareva et al., 2005; Fenske et al., 2015; Yon et al., 2016). Even the same compound, β-ocimene show circadian rhythm in the flower but not in the leaf (Dudareva et al., 2003; Arimura et al., 2008). Plant volatile need to cross membranes and stomata to be emitted, which means emission process of plant volatile can be largely divided in the producing step and the releasing step (Cna'ani et al., 2015; Widhalm et al., 2015). Therefore, each step of the volatile emission, e.g. stomatal opening and metabolic transport

process, may be differently affected by the circadian clock (Harmer et al., 2000; Dodd et al., 2005).

In conclusion, the circadian clock also expects the timing of predator and modulates the responsiveness to herbivore. Plants have faced different herbivore community every year and many herbivores have different feeding behaviors (Joo *et al.* in review). It may not reasonable to make all different circadian rhythms of plant secondary metabolites to survive in complex and heterogeneous herbivore community. Plants can use the circadian clock to temporally restrict inducible defense, so circadian regulated and inducible HIPVs may minimize fitness costs of plants (Greenham and McClung, 2015). Therefore, differential clock regulations in plant volatiles allow the plant to enhance plasticity to defend from complex and heterogeneous insect community and increase the robustness of plant indirect defense.

Materials and Methods

Plants and growth condition

Seeds of wild type *Nicotiana attenuata* originated from Utah in 1988. Seeds were sterilized by sterilization solution for 5 minutes. Sterilized seeds incubated for an hour in 1mM GA3 and 1:50 diluted liquid smoke for breaking the seed dormancy. We germinated treated seeds on Gamborg's B5 medium (Duchefa) with 86 mg/L hygromycin for 10 days in Percival chamber with light (16h, 27°C) / dark (8h, 24°C) as described in Krügel *et al.*, (2002). For the diurnal and continuous light treatment, petri dishes with seedlings are transferred to two growth chambers (Microclima 1000, Snijders Scientific, Netherlands) which were maintained at same environmental conditions. For glasshouse experiments, seedlings were transferred to small pots (TEKU JJP 3050 104 pots, Poeppelmann GmbH & Co. KG, Lohne, Germany) in the glasshouse. Another 10 days after, plants were transferred to 1 L pots. Growth conditions of the glasshouse are at 24–26°C, 16 h light (supplemental lighting by Philips Sun-T Agro 400 W and 600 W sodium lights) and 55% humidity. For climate chamber experiments, seedlings were transferred to 1 L pots and place grew them in the climate chambers. Growth conditions of the climate chambers are at 26°C, 16 h light (supplemental lighting by Philips Sun-T Agro 400 W and 600 W sodium lights) and 60%

humidity.

To grow *N. attenuata* plants in the field, seedlings were transferred into previously hydrated 50-mm peat pellets (Jiffy 703, Always Grows, Sandusky, OH, USA) 14 days after germination. The seedlings were gradually exposed to the native environments to adapt them to the high sunlight and low relative humidity of the Great Basin Desert habitat. Adapted size-matched seedlings were transplanted into a field plot at the Lytle Ranch Preserve, which is located at latitude 37.146, longitude 114.20 (Santa Clara, UT, USA). Seedlings were watered every other day until roots had established. The three different transformed genotypes were randomly planted in rows with 1.5 m among plants.

A specific fragment of *NaLHY, NaTOC1, NaZTL*, *NaLOX2* and *NaHPL* was independently inserted into the pRESC8, pSOL8, pSOL8, and pSOL3 binary vector, respectively; these vectors were transformed into *N. attenuata* WT plants to silence *NaLHY* (gene ID)*, NaTOC1* (gene ID)*, NaZTL* (gene ID), *NaLOX2* (gene ID) and *NaHPL* (gene ID) transcript levels. All transgenic lines were previously fully characterized (Halitschke et al., 2008; Allmann et al., 2010; Yon et al., 2012; Yon et al., 2016). As a control, we used an empty vector (EV) line A-04-266-3 transformed with pSOL3NC, which is known to be completely comparable to wild-type plants (Bubner et al., 2006; Schwachtje and Baldwin, 2008).

Plant tissue sampling

Mature, non-senescent, and non-damaged rosette leaf were collected both in the glasshouse and the filed for further analysis. Leaves were cut at the petiole without mid-vein and wrapped with aluminum foil. Glasshouse-harvested leaf samples were immediately frozen by liquid nitrogen. Field-harvested leaf samples were put on dry ice and stored at – 20 ^{o}C freezer until transport to Jena. Before analysis, all samples were ground with a mortar and pestle and transferred to 2 ml or 15 ml tube for storage.

Treatment with herbivory elicitors

To measure herbivore-induced plant volatiles (HIPVs) in a comparable manner, plant were treated with wounding and diluted regurgitant (R) of *Manduca sexta* by mimicking feeding damage of *Manduca spp*. Pure regurgitant of *M. sexta* was collected glasshouse-grown *M. sexta*, which is from later 3rd instar to 4th instar of larvae. *M. sexta* only were fed on WT or EV plants. Collected pure R were diluted right before the experiments with distilled

water (1: 5 = R: DW). For both glasshouse- and field-grown plants, a similar, mature, non-damaged, and non-senescent 1st or 2nd stem leaf were chosen from each plant. The chosen leaves were wounded by a pattern wheel; the wheel run over 6 times on the adaxial side of the leaf. And diluted OS were rubbed over the damaged areas gently with gloved fingers.

Relative quantification of HIPVs in the plant headspace

The silicone tubings (STs) preparation and volatile collection method have been described in detail by Kallenbach *et al*. (Kallenbach et al., 2014). We installed open plastic cups (600 ml of PET cups) after treatment and immediately put a piece of STs inside of the plastic cup during the trapping period. To avoid cross contamination among treatments, extra empty plastic cups are installed near treated plants to capture background level of plant volatiles. STs samples collected every 4h after treatment. Samples were analyzed in a TD-20 thermal desorption unit (Shimadzu) connected to a quadrupole GC-MS-QP2010Ultra (QP-5050, Shimazu). Peak areas were integrated and the concentration calculated based on each standard of green leaf volatiles.

As STs headspace volatile trapping method did not work in the climate chamber, we also used cumulative volatile collecting method by the Poropak-Q filters. 1st stem leaf was treated and whole plants were enclosed immediately with a plastic bag. Activated charcoal filter attached in incoming air flow and self-packed Poropak-Q filters, which are containing 20 mg of Poropak, were connected in pulling part. Likes STs samples, we collected every 4h after treatment. After elution, eluents from all filters were analyzed in the liquid injector to a quadrupole GC-MS-QP2010Ultra (QP-5050, Shimazu). Peak areas were integrated and the concentration calculated based on each standard of green leaf volatiles.

Stir bar sorptive extraction for the internal pool of GLVs

To measure volatiles in intact leaf tissues, whole single rosette leaf (+1) were harvested and immediately put into liquid nitrogen. Frozen samples are ground and aliquoted in glass vials. We directly added 1ml of saturated CaCl2 solution, which is spiked by 4-*(Z)*-hexenol to 1 ug/ul, to inactivate enzymes activities. We further add a piece of STs and incubate overnight (8h) on desktop shaking incubator at 600 rpm. Rinsing processes for STs were followed by Childers et al. (in preparation). PDMS samples were analyzed in a TD-20 thermal desorption unit (Shimazu) connected to a quadrupole GC-MS-QP2010Ultra (QP-5050, Shimazu). Peak areas were integrated and normalized by fresh mass and internal

standard. For quantification, aqueous solutions of each compound added to each PDMS tubing with different concentrations. Each compound is quantified based on response curves of each compound to the internal standard.

Transcript abundances

Total RNA was extracted from *N. attenuata* using Plant RNeasy Extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was quantified using NanoDrop (Thermo Scientific, Wilmington, USA). The cDNA was synthesized from 500ng of total RNA using RevertAid H Minus reverse transcriptase (Fermentas) and oligo (dT) primer (Fermentas). qPCR was performed in Mx3005P PCR cycler (Stratagene) using SYBR GREEN1 kit (Eurogentec). The EF gene was used as a control.

Predation assay

To test whether different GLV blends have different ecological functions, we exposed different GLV blends by the headspace supplementation and conducted predation assay in a natural population of *N. attenuata* in Great Basin desert of southwest Utah in 2015. We selected similar size plants at the late elongated stage. To test whether *Geocoris* were able to sense different GLV blends, different GLV mixes and lanolin control were tested as a single group (n=8-10) for 3 days. Each pair of plants was separated by an average distance of more 1 m. 0.05 ml of lanolin paste containing different GLVs mixtures were spread to a cotton swab and placed adjacent to the each plant. We glued 5 frozen *Manduca sexta* eggs per each plant. The number of *Geocoris* ssp. and predated eggs counted and changed GLVs mixture every 12h either to distinguish day predation rates and night predation rates.

Statistical analyses

Time effects of inducibilities in HIPVs, rhythmicity of HIPV emissions, and internal pool of GLVs were assessed using ANOVAs. Repeated measures ANOVAs followed by Bonferroni *post hoc* tests were employed to compare treatments across time. Student's *t*-test was used for simple comparisons and predation assay were analyzed by Fisher's exact test. All statistical analyses were performed using the statistical package R. Significance level was set at $\alpha = 0.05$.

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Supporting Information

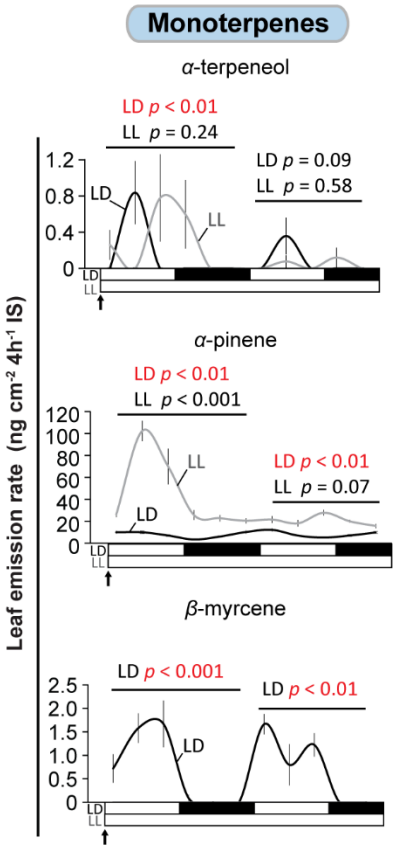

Fig. S1. Monoterpenes have strong diurnal rhythm, but not circadian rhythm after treatment. WT plants were grown in a climate chamber to identify circadian-regulated HIPVs. WT plants were entrained in LD and transferred to LL. Plant volatiles were sampled by Poropak-Q filters and analyzed by TD-GC-MS (mean ± SE, n = 5 for LD and 6 for LL samples). The release of *α*-pinene remained rhythmic under LL, but *α*-terpeneol and *β*-myrcene did not. *p*-values from one-way ANOVA analysis. Black arrow represent treatment time. IS, internal standard; LD, light/dark cycle (12h day / 12h night); LL, free-running condition (24h day)

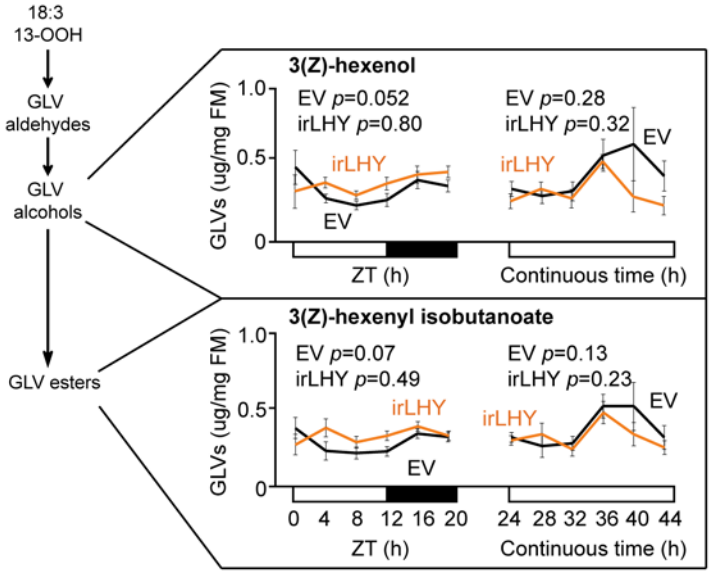

Fig. S2. Internal pool of GLV alcohol and esters do not have circadian rhythms. Mean (± SE, n = 5) accumulation of 3(Z)-hexenol and 3(Z)-hexenyl isobutanoate were sampled from aqueous leaf extracts on STs and analyzed by TD-GC-MS. Both EV and irLHY plants did not have significant rhythms of 3(Z)-hexenol and 3(Z)-hexenyl isobutanoate under LD and LL. Black lines indicate LD and gray indicate LL. Mean ± SE. *P*-values from one-way ANOVA analysis.

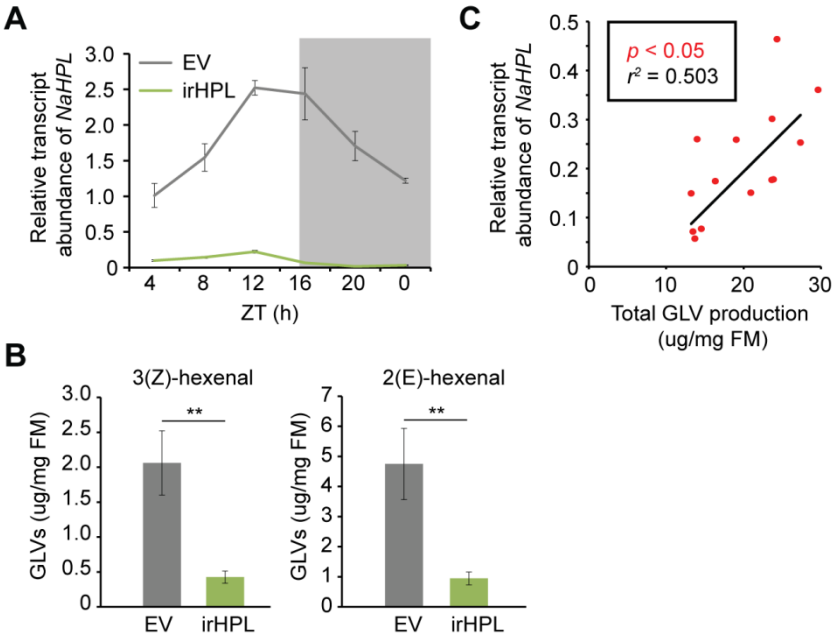

Fig. S3. *NaHPL* modulates internal pool of GLV aldehydes. Single, mature, non-senescent leaves (n=6) were collected from rosette-stage EV and *irHPL* plants to measure the accumulation of internal GLV pools and transcript abundance of *NaHPL*. (A) Transcript abundances of *NaHPL* strongly decreased in irHPL plants. (B) irHPL produced less internal

pool of GLV aldehydes at ZT16. (C) Total production of GLVs was significantly correlated with transcript abundances of *NaHPL*. Mean ± SE. **$p < 0.01$; p-values from Student's t-test.

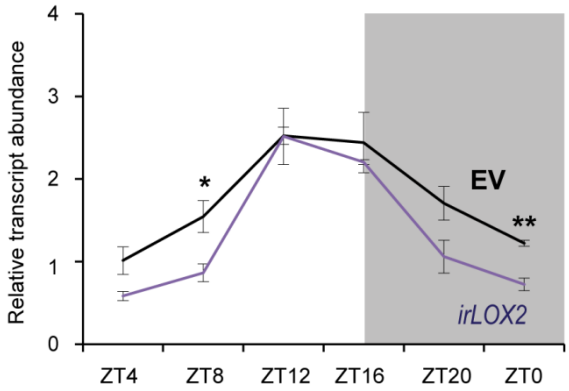

Fig. S4. Rhythm of transcript abundance of *NaHPL* is NaLOX2-independent. Single, mature, non-senescent leaves (n=6) were collected from rosette-stage EV and *irHPL* plants to measure transcript abundance of *NaHPL*. Transcript abundances of *NaHPL* in irLOX2 plants were lower than that in EV plants at ZT8 and ZT0. However, *NaHPL* kept strong rhythmic expression in irLOX2 plants. Mean ± SE; *, $p < 0.05$; **, $p < 0.01$; p-values from Student's t-test.

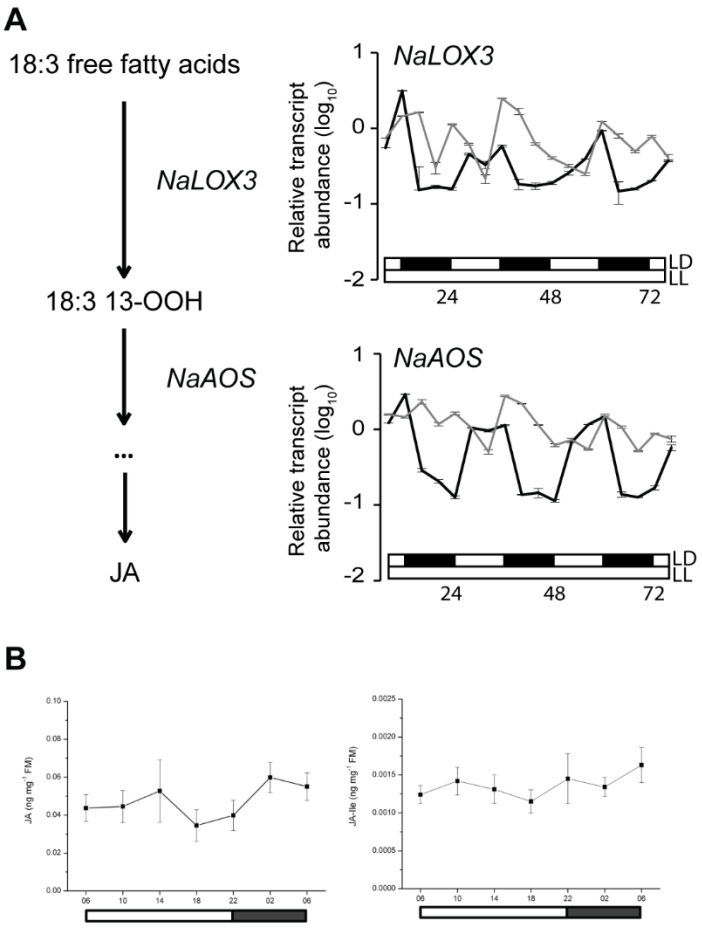

Fig. S5. JA-biosynthetic genes and JA do not have circadian rhythms. (A) Mean (± SE, n = 3) relative transcript abundance of *NaLOX3* and *NaAOS* were measured in *N. attenuata* seedlings grown under 12 h light/12 h dark conditions (LD) and under constant light conditions (LL). Seedlings were harvested every 4 h for three days. Transcript abundances of *NaLOX3* and *NaAOS* had diurnal rhythms and peaked during the day, but they lost their rhythms under free-running condition. (B) Basal level (mean± SE, n = 6) of JA and JA-Ile in *N. attenuata* did not have diurnal rhythms.

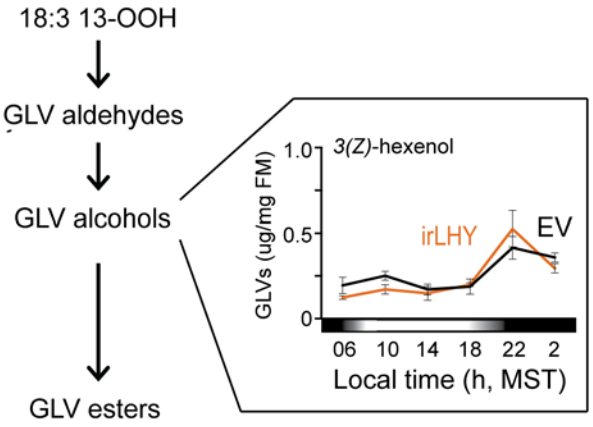

Fig. S6. *NaLHY*-silenced plants produce a similar amount of GLV-alcohol with EV plants in nature. Single, mature, non-senescent leaves were collected from rosette-stage EV and *irLHY* plants to measure endogenous GLVs and transcript abundance of *NaHPL* in EV and *irLHY* plants in the field. Mean ± SE; n = 6; MST, Mountain Standard Time.

Manuscript IV

Research

New Phytologist

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

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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

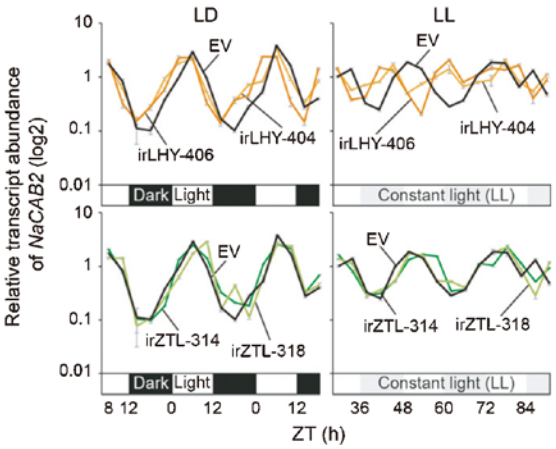

Fig. 1 Effect of silencing *NaLHY* and *NaZTL* on the internal rhythms in seedlings. Mean (± SE) transcript accumulation of *CAB2* in *Nicotiana attenuata* seedlings of empty vector, irLHY-406, irLHY-404, irZTL-314 and irZTL-318 grown under 12 h : 12 h, light : dark (LD) conditions, and seedlings in 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.

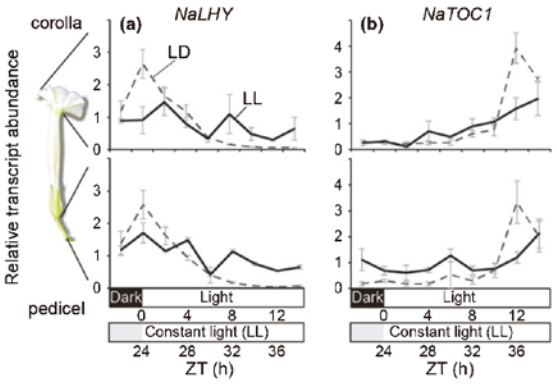

Fig. 2 *NaLHY* and *NaTOC1* transcript expression in corolla limbs and pedicels. Mean (± SE) levels of transcript accumulation of (a) *NaLHY* and (b) *NaTOC1* in corolla limbs and pedicels of *Nicotiana attenuata* under 16 h : 8 h, light : dark (LD) and constant light (LL) conditions. The relative transcript 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

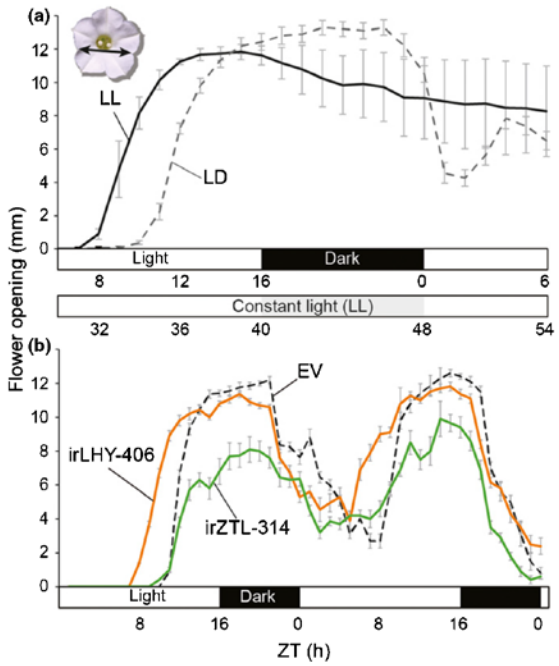

Fig. 3 Silencing of *NaLHY* and *NaZTL* alters flower opening. (a) Mean (± SE) distance between petal junctions on corolla limbs of wild-type *Nicotiana attenuata* plants under 16 h : 8 h, light : dark (LD) conditions and constant light (LL) conditions. We exposed LD-grown flowering plants to LL conditions 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.

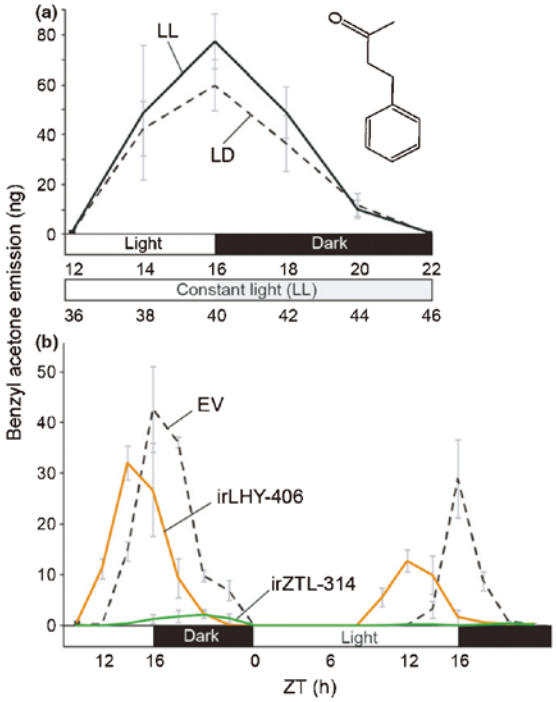

Fig. 4 Silencing of *NaLHY* and *NaZTL* alters the emission of attractive floral volatile, benzyl acetone (BA), from flowers. (a) Mean (± SE) levels of BA emission from *Nicotiana attenuata* wild-type plants under 16 h : 8 h, light : dark (LD) and constant light (LL) conditions. We exposed LD-grown flowering 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).

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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).

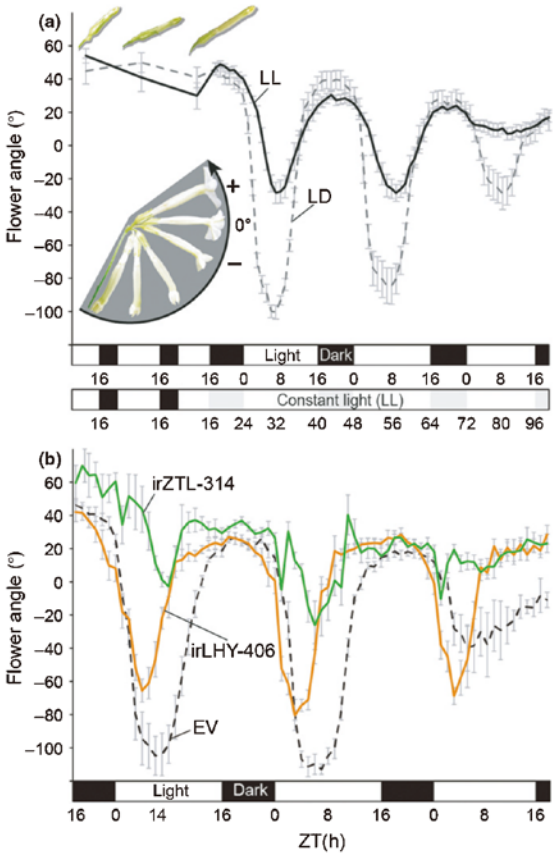

Fig. 5 Silencing of *NaLHY* and *NaZTL* alters vertical movement in flowers. (a) Mean (± SE) angles of flowers in *Nicotiana attenuata* wild-type plants under 16 h : 8 h, light : dark (LD) and constant light (LL) conditions. Flower movement is initiated in the morning of the first opening day and repeated 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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Science Program (RGP0002/2012) and the Max Planck Society. There are no conflicts of interest among the authors of this work.

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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***New Phytologist* Supporting Information Figs S1–S6 and Table S1**

Article title: Silencing *Nicotiana attenuata LHY* and *ZTL* alters circadian rhythms in flowers

Authors: Felipe Yon, Youngsung Joo, Lucas Cortés Llorca, Eva Rothe, Ian T. Baldwin and Sang-Gyu Kim

Article acceptance date: 24 August 2015

The following Supporting Information is available for this article:

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

Fig. S2 Protein alignment of 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 LL and LD conditions.

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Table S1 Insertion fragments of irLHY and irZTL silenced lines

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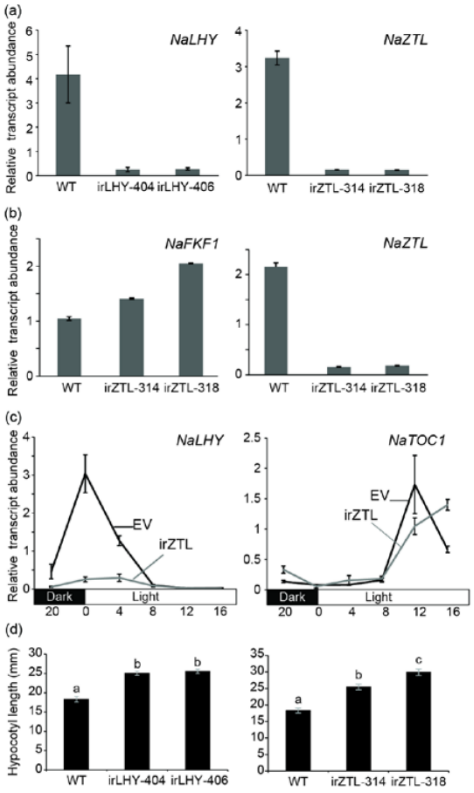

Fig. S1 Silencing efficiency and hypocotyl length of irLHY and irZTL lines. (a) Mean (± SE) levels of transcript accumulation of *NaLHY* and *NaZTL* in irLHY and irZTL lines, respectively. Plants were grown under 16 h : 8 h, light : dark conditions, and leaf samples were collected at ZT0 for irLHY, at ZT12 for irZTL lines. (b) Mean (± SE) levels of transcript accumulation of *NaFKF1* in irZTL lines collected at ZT12. (c) Mean (± SE) levels of transcript accumulation of *NaLHY* and *NaTOC1* in irZTL-314. Plants were grown under 16 h : 8 h, light : dark conditions, and leaf samples were harvested every 4 h for 1 d. (d) Mean (± SE) length of hypocotyl in EV, irLHY-406, and irZTL-314 seedlings grown under the dim light conditions. Different letters indicate significant differences in hypocotyl length among the lines (P <0.05, one-way ANOVA with Bonferroni *post hoc* test).

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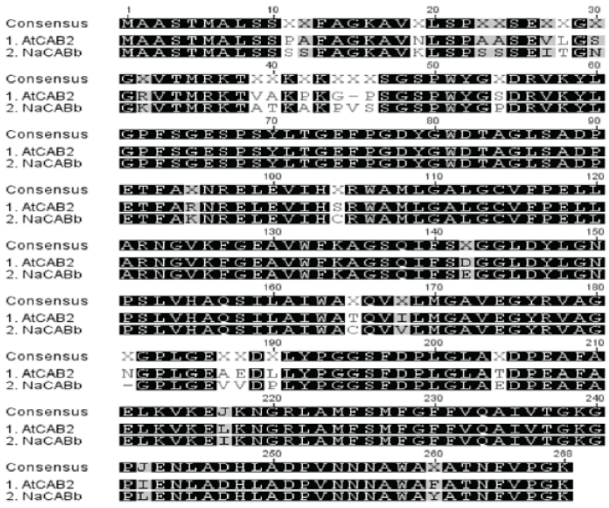

Fig. S2 Protein alignment of CAB2 orthologs in *Nicotiana attenuata* and *Arabidopsis thaliana*. Full-length amino acid sequences were aligned using the Geneious software V5.7.7 (www.geneious.com). TAIR accession number of *A. thaliana* CAB2 (AtCAB2) is At1g29920. CAB2, CHLOROPHYLL A/B BINDING PROTEINS 2.

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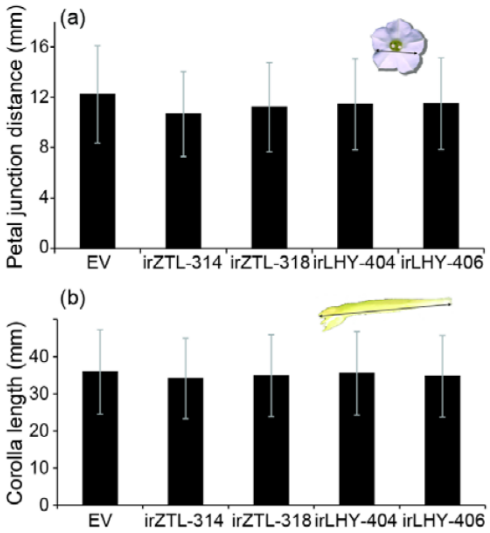

Fig. S3 Flower morphology of the clock-silenced lines. (a) Mean (± SE) distance between petal junctions on corolla limbs of EV, irLHY-406, irLHY-404, irZTL-314, and irZTL-318 flowers. (b) Mean (± SE) corolla length of EV, irLHY-406, irLHY-404, irZTL-314, and irZTL-318 flowers.

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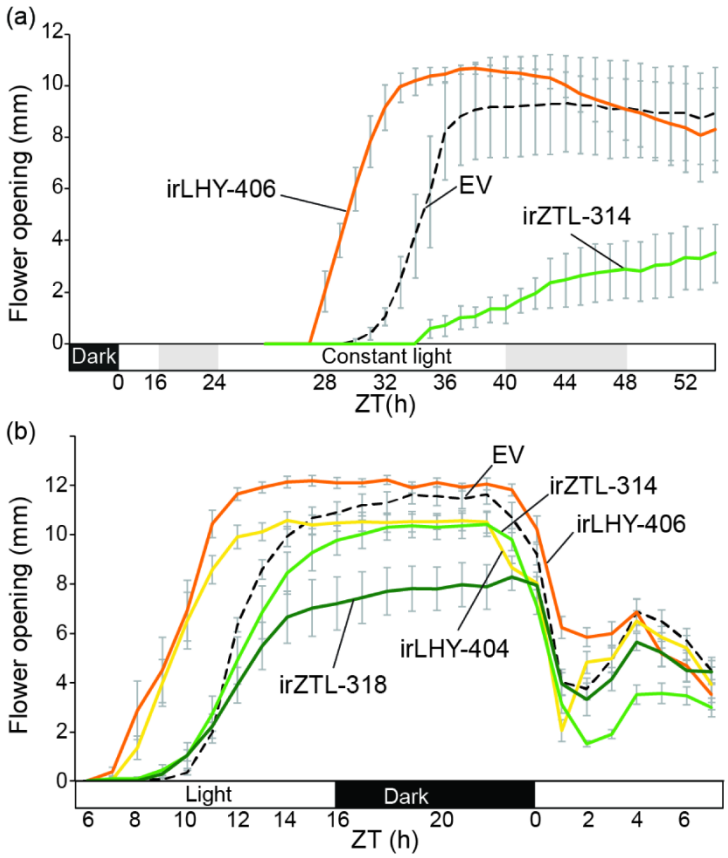

Fig. S4 Flower opening in the clock-silenced lines under LL and LD conditions. (a) Flower opening and closing of *N. attenuata* clock-silenced lines under LL conditions. Mean (± SE) distance between petal junctions on corolla limbs of EV, irLHY-406, and irZTL-314. We exposed LD-grown flowering plants to LL conditions for 24 h and then measured flower opening/closing. A gray box indicates the subjective dark period under LL conditions. (b) Mean (± SE) distance between petal junctions on corolla limbs of EV, irLHY-406, irLHY-404, irZTL-314, and irZTL-318 plants grown under LD conditions. LD, 16 h : 8 h, light : dark; LL, constant light. ZT, zeitgeber time.

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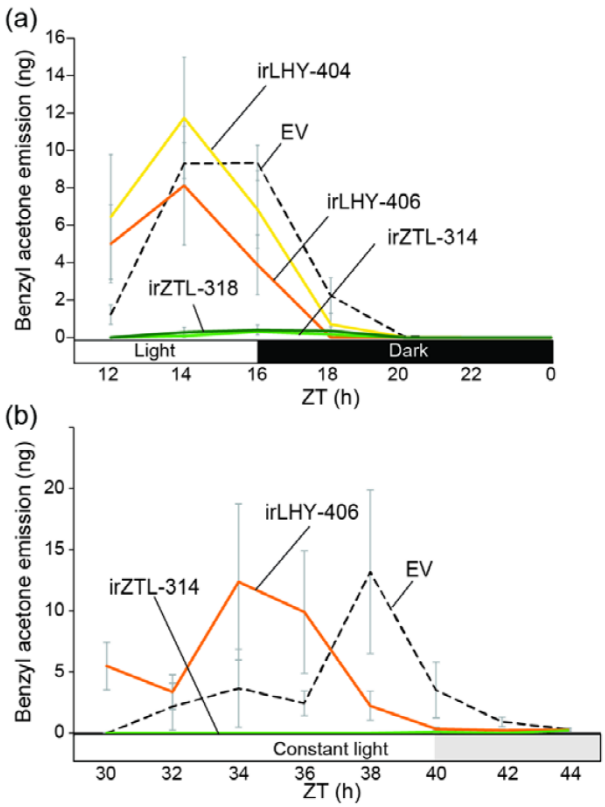

Fig. S5 Benzyl acetone emission in the clock-silenced flowers. (a) Mean (± SE) levels of BA emission of flowers in EV, irLHY-406, irLHY-404, irZTL-314 and irZTL-318 under LD conditions, BA emission measured using a z-Nose^{TM} instrument for real time measurements. (b) Mean (± SE) levels of BA emission of flowers in EV, irLHY-406, and irZTL-314 under LL conditions. We exposed LD-grown flowering plants to LL condition for 24 h and measured BA emission using a PDMS trapping and TDU-GC-MS instrument. LD, 16 h : 8 h, light : dark; LL, constant light. ZT, zeitgeber time.

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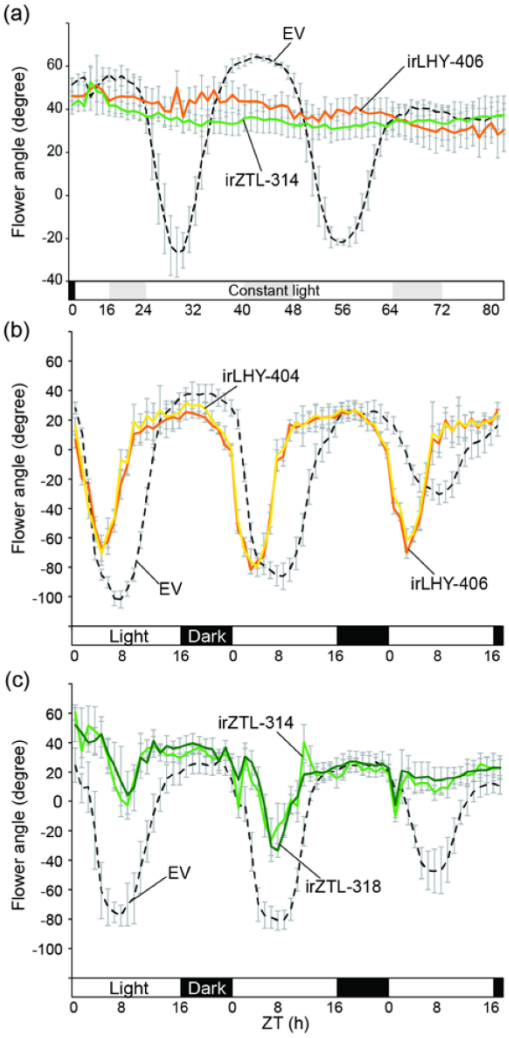

Fig. S6 Vertical movement in the clock-silenced flowers under LL and LD conditions. (a) Mean (± SE) angle of flowers in EV, irLHY-406, and irZTL-314 under LL conditions. We exposed LD-grown flowering plants to LL condition for 24 h and measured flower movement. A gray box indicates the subjective dark period of LL conditions. Mean (± SE) angle of flowers in (b) *NaLHY*-silenced lines and (c) *NaZTL*-silenced lines under LD conditions. LD, 16 h : 8 h, light : dark; LL, constant light. ZT, zeitgeber time.

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Table S1 Insertion fragments of irLHY and irZTL silenced lines

Target gene	NCBI accession	Inserted fragment for silencing construct
LHY	JQ424913	GGTAAAGAAGAGCCTCAAGAACCTAATGTTAACCTTCTAGCTGGAGATGCTGG GAATCGGCGTGGTAGGAATTGCATCAGTCCAAATGATTCTTGGAAAGAAGTCT CCGAAGGGGGACGGATAGCGTTCCAGGCTCTTTTCACCAGAGAGAAGTTGCCT CAAAGCTTTTCTCCTTCAAATGATCCGAAAAATAAGGGAACAATCAATCTTGA AAACGTTAAGCAAAAGCCAGACGAGAAAGGTCTAAGTGGATCGCAGTTAGAC CTTAATGATCAGGCATCCGACATCTGTTCCAGTCATCAAGCAGTGGAAGATAA TGTGTTAGTAATTGGC
ZTL	JQ424912	GCGAGGAAGAACCATGCTGGAGATGTGTTACAGGAAGTGGAATGCCTGGTGCC GGAAATCCTGGAGGTGTTGCTCCTCCACCAAGGCTTGATCACGTGGCAGTAAG TCTCCCTGGTGGCAGAATTCTGGTCTTTGGTGGGTCCGTTGCTGGTCTCCACTC AGCATCTCAGCTCTACATTTTGGATCCAACAGAAGAGAAGCCTACATGGAGGA TATTGAATGTACCTGGTCGGCCTCCAAGATTTGCTTGGGGACATAGCACATGTA TTGTTGGAGGAACTAGAGCAATAGTCCTCGGAGGTCAAACTGGTG

Specific fragments used for the inverted repeated constructs to silence NaLHY and NaZTL. NCBI accession numbers of each gene sequence.

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High-Impact Article

Fitness consequences of altering floral circadian oscillations for *Nicotiana attenuata*[FA]

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Abstract Ecological interactions between flowers and pollinators are all about timing. Flower opening/closing and scent emissions are largely synchronized with pollinator activity, and a circadian clock regulates these rhythms. However, whether the circadian clock increases a plant's reproductive success by regulating these floral rhythms remains untested. Flowers of *Nicotiana attenuata*, a wild tobacco, diurnally and rhythmically open, emit scent and move vertically through a 140° arc to interact with nocturnal hawkmoths. We tethered flowers to evaluate the importance of flower positions for *Manduca sexta*-mediated pollinations; flower position dramatically influenced pollination. We examined the pollination success of phase-shifted flowers, silenced in circadian clock genes, NaZTL, NaLHY, and NaTOC1, by RNAi. Circadian rhythms in *N. attenuata* flowers are responsible for altered seed set from outcrossed pollen.

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INTRODUCTION

In the eighteenth century, Carl Linnaeus noticed that many flowers open at specific times of the day, and designed a garden known as the "flower clock" in which flower behavior revealed the time of day (Somers 1999). These diurnal rhythms in flowers that include floral opening/closing and scent emissions, likely coevolved with the activity times of pollinators to maximize outcrossing (Fründ et al. 2011). The scent profiles of some flowers, such as *Petunia axillaris* and *P. parodii* are under the control of internal clocks and synchronize emissions with the active time of the flower's pollinators (Hoballah et al. 2005). In the wild tobacco, *Nicotiana attenuata*, floral scent emission, as well as nectar production depend on daily rhythms (Euler and Baldwin 1996; Kessler et al. 2012); both have been shown to be important mediators of pollinations and essential for maximizing this plant's fitness (Kessler et al. 2015). In the genus *Aquilegia*, flowers have evolved fixed orientations to match the particular active periods of their main pollinators and so radiated by floral isolation (Fulton and Hodges 1999; Hodges et al. 2004); in a dynamic way, *N. attenuata* flowers adjust their upward or downward orientations (Yon et al. 2016) in synchrony with the active periods of their main pollinators.

To examine the ecological relevance of the diurnal rhythms of flowers, it is essential to choose a model system that offers the possibility of physical and/or genetic manipulations of floral rhythms (Resco et al. 2009). Several studies conducted under normal light/dark or constant light conditions have produced results consistent with the notion that the internal circadian clock regulates diurnal rhythms in flowers (Sweeney 1963; Hoballah et al. 2005; Fenske et al. 2015; Yon et al. 2016). Components of the circadian clock have been identified in the model plant, *Arabidopsis thaliana* (Nagel and Kay 2012), and these clock components are highly conserved across many plant species (McClung 2013). The main oscillator is composed of two morning components, *LATE ELONGATED HYPOCOTYLE* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), and two evening components, *TIMING OF CAB EXPRESSION1* (*TOC1*) and

ZEITLUPE (*ZTL*). The clock functions through several negative feedback loops: LHY/CCA1 repress the accumulation of *TOC1* transcripts; TOC1 protein also represses transcript accumulation of *LHY/CCA*; and ZTL ubiquitinates TOC1 for degradation (Kim et al. 2007). Altering the expression of clock genes produces arrhythmic or dysrhythmic plants, which display many developmental (Adams and Carré 2011) and metabolic (Wang et al. 2011; Goodspeed et al. 2012) defects. However, most of the plant species used to unravel the genetic mechanisms of the clock are poor models for the study of flower-pollinator interactions due to their domestication histories or pollination systems.

The wild tobacco, *N. attenuata* inhabits the Great Basin Desert of the USA, produces opportunistically out-crossing self-compatible flowers (Bhattacharya and Baldwin 2012) that interact with different pollinators: nocturnal hawkmoths (e.g. *Manduca sexta* and *Manduca quinquemaculata*) and diurnal hummingbirds (e.g. *Archilochus alexandri*) (Kessler et al. 2010). *N. attenuata* flowers display numerous diurnal rhythms that likely influence outcrossing success. Most flowers open at night when they produce nectar and emit benzyl acetone (BA), the main floral volatile compound that attracts nocturnal hawkmoths (Kessler et al. 2008), and close again by the next morning. A relatively small proportion of flowers remain closed and scentless on their first night and partially open in their first morning with reduced BA emissions and fully open during the next night (Kessler et al. 2010). These morning-opening flowers (MOF), which require a burst of jasmonate signaling for their maturation, are produced in greater frequency on herbivore-attacked plants and are visited by diurnal pollinators (Kessler et al. 2010). Additionally, *N. attenuata* flowers move vertically during the day – flowers face downward during the midday and upward during the night (Video S1) (Yon et al. 2016). These floral rhythms, the vertical movement, scent emissions, opening, and nectar secretions repeat for the entire lifespan of the flower (2–3 d). So, why do *N. attenuata* flowers show rhythmic upward vertical movement? Both the type of flight and the construction of the proboscis of *M. sexta* moths (Sprayberry and Suver 2011), may constrain how readily the moth can access the nectar reward, and so we hypothesized that flower orientations affect the success of cross-pollinations mediated by *M. sexta*.

Previously, we identified the homologous genes of the core clock components, *Arabidopsis LHY*, *TOC1*, and *ZTL* in *N. attenuata* (Yon et al. 2012). To manipulate floral rhythms, we independently silenced these clock genes in *N. attenuata* by transformation with gene-specific inverted-repeat (ir) constructs and found that silencing the clock genes alters the plant's floral rhythms (Figure 1) (Yon et al. 2012, 2016). Here we investigate the consequences of these floral rhythms for plant fitness by using these "time-altered" plants, as well as mechanical modifications of the floral angle under glasshouse conditions. Outcrossed pollination mediated by nocturnal *M. sexta* hawkmoths was assessed by measuring capsule and seed production from antherectomized flowers, as an estimate of plant fitness. By physically constraining, or genetically altering the floral rhythms, we show that the circadian clock can establish a time-dependent pollination strategy that may help the plant to optimize outcrossing rates.

RESULTS

Nicotiana attenuata flowers maintain an approximately 40° upward orientation from horizontal during their development. In the morning of the first day of opening, flowers move downward to approximately 90° below horizontal; these flowers return to upright orientation before dusk, open and release floral scents from their corolla limbs (Figure 1). By the next morning, flowers face down again and close their corollas. This vertical movement of flowers is repeated for 2–3 days under long day (LD, 16 h light and 8 h dark) conditions, with diminishing amplitude on the third day (Yon et al. 2016). Similarly, the accumulation of nectar continues for 2 days in flowers of *N. attenuata*. The secretion of nectar occurs mainly during evening and night hours, and thus in the upright orientation of a flower's first 2 days; nectar volume decreases on the third day (Kessler 2012).

Hand-pollinated antherectomized flowers restrained at the three angles (+45°, 0°, −45°) all produced a full complement of capsules and seeds ($F = 0.87$, $P = 0.43$). However, when pollinated by naïve *M. sexta* moths, the antherectomized flowers tethered at 45° and 0° produced 13 (65%) and seven (35%) capsules, respectively, and no capsules and hence no seeds were produced when flowers were tethered at −45° (Figure 2B).

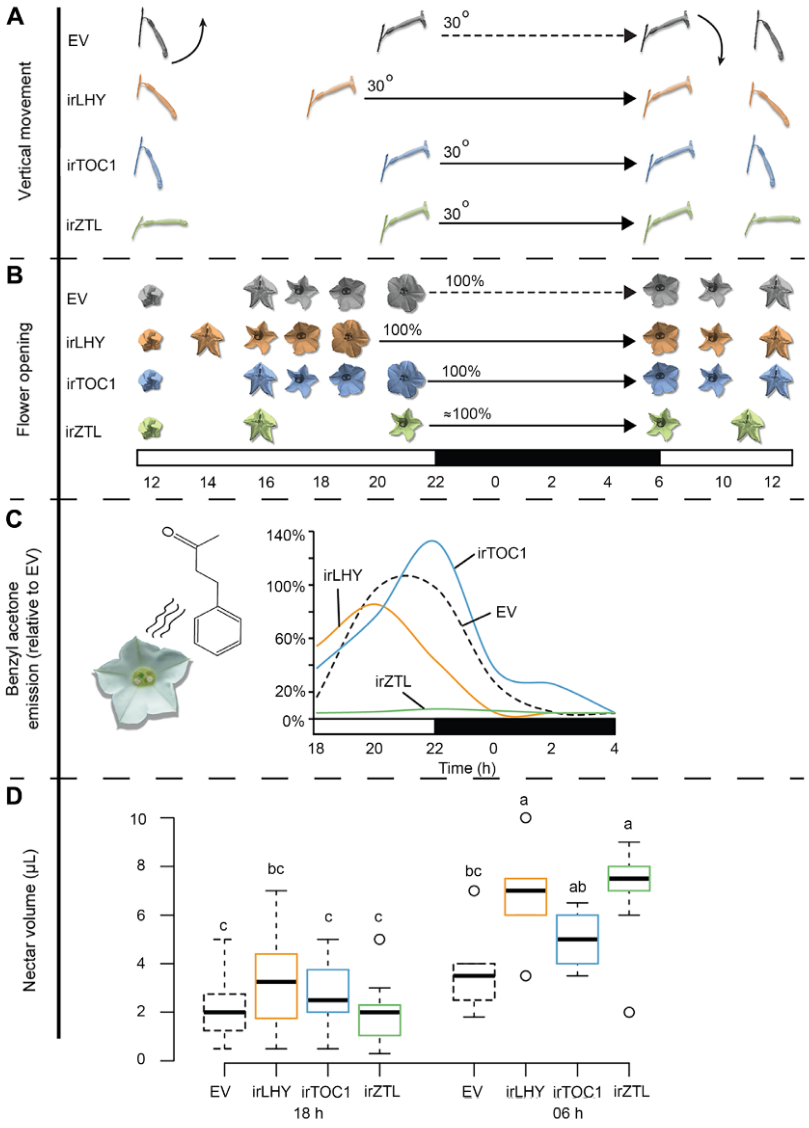

Figure 1. The circadian clock regulates floral rhythms in *Nicotiana attenuata*

Clock silenced genes: irLHY (LHY, LATE ELONGATED HYPOCOTYL); irTOC1 (TOC1, TIMING OF CAB EXPRESSION1); irZTL (ZTL, ZEITLUPE); and EV, control plant transformed with the empty-vector used to generate transgenic lines. Representations of **(A)** vertical movement and **(B)** aperture of the clock gene-silenced flowers on the first opening day. **(C)** Benzyl acetone (BA) emission trends in relative percentage to the maximum amount of BA emission from controls (*i.e.* EV flowers) from z-Nose^{TM} measurements. Inset figure depicts BA molecule. The data are the summary of previous research (Yon et al. 2016) and Figure S1. **(D)** Standing nectar volume in absence of pollinators, measured in microliters by pulse centrifugation extraction. All plants were grown under long day conditions (16 h light: 8 h dark). Each line is color coded: black-EV, orange-irLHY, blue-irTOC1 and green-irZTL.

Pearson's χ test for goodness-of-fit (hypothesis test described in Methods) and χ^2 test of independence between the flowers at 45° and 0° ($\chi^2 = 0.038$, d.f. = 2, $n = 20$, $P < 0.02$) indicated significant differences in hawkmoth-mediated cross-pollination success that depended on the angle of the flowers. The average number of seeds per capsule (± *SE*) was also strongly influenced: 144 ± 27 seeds in the flower at 45°, and 41 ± 18 seeds at 0° ($t = 3.49$, $P < 0.01$) (Figure 2A). Clearly, hawkmoths provide superior pollination services when flowers are at 45° and deliver more pollen to the stigma when flowers are oriented at 45° compared to 0° with respect to the horizontal.

With this experimental evidence of the importance of floral orientation, we used the clock gene-silenced plants to evaluate the ecological significance of floral rhythms in glasshouse assays that quantified outcrossing rates mediated by *M. sexta* pollinators. In previous research, we found that silencing circadian clock genes in *N. attenuata* alters diurnal rhythms in flowers; the flowers of irLHY plants opened, moved and released scent 2 h earlier compared to EV plants; flowers of irZTL plants partially opened, released no scent, and moved weakly (Figure 1). In contrast, irTOC1 flowers displayed all rhythms comparably to those of EV flowers (Figures 1, S1). Nectar in wild type plants is produced mainly between 6 p.m. and 4 a.m. (Kessler 2012). The nectar volume was not different among lines at 6 p.m., but differences were seen at 6 a.m. of the following day. The average nectar volume of irLHY (6.8 µL) and irZTL (6.9 µL) was larger and significantly different from EV (3.5 µL), but not from irTOC1 (5.0 µL) (Figure 1D); the nectar volumes of EV and irTOC1 flowers did not differ.

The differences between EV and irLHY, as well as irZTL plants are likely associated with the different oscillation times of these plants. Flowers of irLHY plants move 2 h earlier to an upright position than EV flowers,

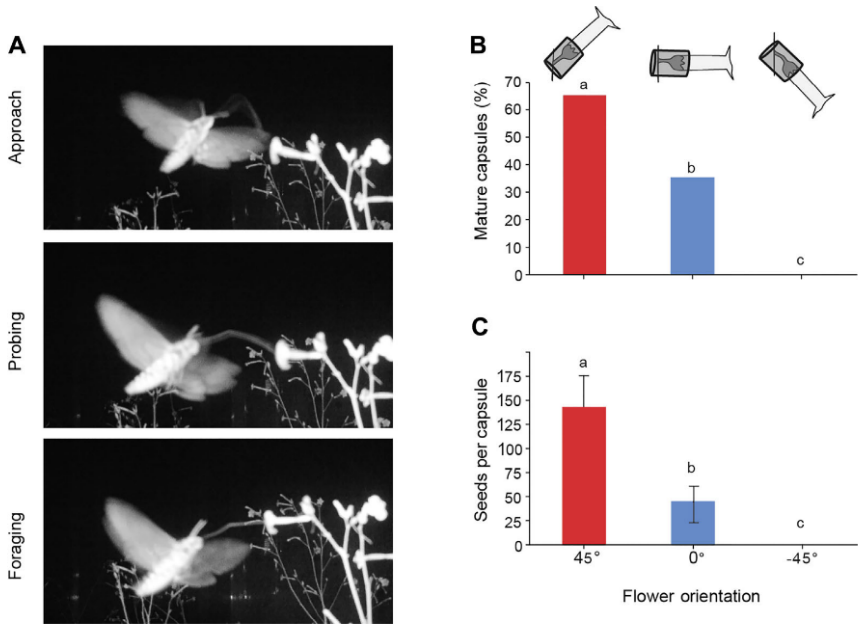

Figure 2. Flower angles determine rates of outcrossing mediated by *Manduca sexta* moths
(A) *M. sexta* approaching, probing and foraging on *N. attenuata* EV flower facing naturally upwards. Photo sequence taken at approximately 22 h with a wild-camera Snapshot Mini (Dörr, Germany), equipped with a PIR sensor camera and IR flash. Percentages of **(B)** mature capsules and **(C)** mean (± *SE*) number of seeds per capsule in emasculated WT flowers which were fixed at each of three positions (−45°, 0°, 45°). Different letters indicate significant differences among the flower positions as determined by Pearson's χ^2 test ($P < 0.05$) for capsule formation and one-way ANOVA followed by Tukey *post-hoc* test ($P < 0.01$) for seeds produced per capsule.

and accumulate nectar earlier than do EV flowers, as seen in the nectar volume trend at 6 p.m. The irZTL flowers have the larger nectar volume in the morning, which does not significantly differ from that of irLHY flowers.

We used both single (no competition) and paired (competition with EV) experimental designs to evaluate how *Manduca* could provide cross-pollination services to the different clock gene-silenced lines (Figure 3). For the no-competition experiment using a single line, we antherectomized a total of 25 flowers (five flowers/plant) on EV, irLHY, irTOC1, or irZTL plants and placed plants of a single line on the table with two naïve *M. sexta* moths and 10 WT pollen-donor plants for one night (Figure 3A). After 2 weeks, 60% of EV flowers had matured capsules, and similar pollination rates were observed in irLHY (60%) and irTOC1 (56%). However, irZTL (44%) tended to produce fewer capsules than did flowers from EV plants. Seed numbers per capsule did not differ among the lines (Figure 3A).

In contrast, when clock gene-silenced plants competed with EV plants for the *Manduca*-mediated pollination services, out-crossing pollination success differed significantly. irZTL plants produced half the number of capsules than did EV plants (Figure 3B, $t = 3.1$, d.f. $= 4.4$, $P < 0.05$) and this difference in capsule number was larger than the difference found between EV and irZTL plants when pollinated singly (Figure 3A, B). EV plants in the EV-irZTL competition

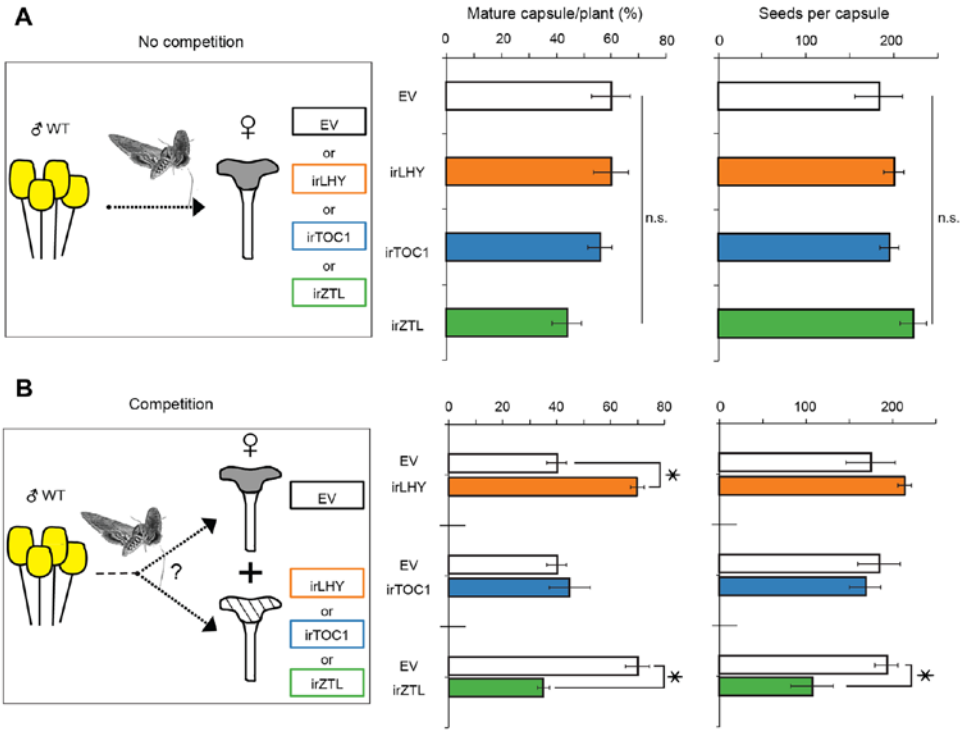

Figure 3. The circadian clock coordinates pollination success in *N. attenuata*

(**A**) Flowers of EV, irLHY, irTOC1, and irZTL plants were emasculated and individually exposed to two *M. sexta* moths for one night with 10 WT pollen donor plants (no competition design). (**B**) In the competition experiments, each of the clock gene-silenced lines were individually paired with EV plants and their flowers competed for the pollination services of two *M. sexta* moths which also had access to 10 WT plants as pollen donors. Mean (± *SE*) percentage of mature capsules per plant and mean (± *SE*) number of seeds per capsule resulting from pollinating emasculated flowers by *M. sexta* moths. Asterisks represent significant difference between EV and clock gene-silenced lines (** = $P < 0.01$, Student's *t*-test).

pairs produced 75% more capsules than in other experimental pairs. In addition, the irZTL line produced significantly fewer seeds per capsule ($t = 4.01$, d.f. $= 9.6$, $P < 0.05$) than the EV pair (Figure 3B). EV-irTOC1 pairs did not differ either in capsule formation or in number of seeds per capsule (Figure 3B). Unexpectedly, in the EV-irLHY pairs, irLHY plants produced almost twice as many capsules ($t = 3$, d.f. $= 5.6$, $P < 0.05$) as did EV, an outcome opposite of the results of the EV-irZTL pairs (Figure 3B). There was no significant difference in the numbers of seeds per capsule in the EV-irLHY pairs ($t = 1.34$, d.f. $= 8.1$, $P = 0.21$).

DISCUSSION

Ecological consequences of flower orientation

Several hypotheses have been proposed about the role of flower orientation in outcrossing success. For example, the horizontal or downward orientations of flowers increase pollen transfer because pollinators are in contact with flowers for a longer time due to an increase in handling time to obtain the reward (Fenster et al. 2009). In contrast, the upward orientation of flowers could facilitate visual recognition from multiple directions by pollinators despite a reduction in pollen transfer (Ushimaru and Hyodo 2005; Fenster et al. 2009). Here we show that the upward orientation of *N. attenuata* flowers at night improves the outcrossing success mediated by naïve *M. sexta* moths, allowing an easier access to the nectar at the bottom of the flower given the stiffness of the moth's proboscis. The downward facing flowers produced no capsules, and this result is consistent with those of studies with *Hyles lineata* and *Aquilegea* plants, in which pendent flowers were rarely visited by hawkmoths (Fulton and Hodges 1999). This vertical movement of flowers might be a particular adaptation of *N. attenuata*, as closely related *Nicotiana* species are not known to move their flowers rhythmically downward and at most only bend them upwards. These results suggest that the plant's circadian clock entrains flower orientation to facilitate the recognition, probing, and foraging of *M. sexta* (Figure 2), and hence has a different function than that proposed for the clock in stem circumnutation and leaf movement (Stolarz 2009).

Downward orientation of flowers can provide several advantages: It reduces susceptibility to florivores (Ashman and Schoen 1994) and nectar desiccation caused by solar radiation (Kessler 2012), and it could filter out the visits of diurnal pollinators (Fenster et al. 2004). Nectar accumulation in *N. attenuata* flowers decreases, while the sugar concentrations increase during the day, presumably as a consequence of the strong solar irradiance in the Great Basin Desert (Kessler and Baldwin 2007; Kessler et al. 2012), even though flowers face downward and close during the day. If flowers would face upward and remained open during the day, this nectar concentration effect could be much greater.

However, the vertical movement is not likely to protect flowers from nectar robbery by carpenter bees, which collect nectar by puncturing the corolla tube base at dawn and dusk (Kessler et al. 2008); the upward orientation is attained before nightfall when the robbers are active, suggesting that flower movement alone would not prevent damage by these opportunistic nectar robbers. However, the downward orientation of the corolla may reduce its visibility to robbers or florivores.

Ecological implication of the circadian clock

A fundamental assumption in chronobiology is that the circadian clock increases the fitness of organisms. Therefore, we predicted that dysrhythmic/arrhythmic traits in the clock-altered flowers would reduce their out-crossing pollination success. This is what was observed for the flowers of irZTL plants but not for the flowers of irLHY plants (Figure 3B).

As expected, cross-pollination rates in irZTL plants were reduced when irZTL plants competed with EV plants for the pollination services of naïve *M. sexta* moths (Figure 3B). However, irLHY flowers had higher night pollination rates when they competed with EV flowers (Figure 3B). At first, the clear advantage of irLHY flowers in competing for the *Manduca*'s pollination services appears to be consistent with their earlier moving, opening, and scent-emission phenotype, but given that *M. sexta* moths were most active much later in the evening (10–11 p.m.), this is not likely the correct explanation as at this later time the flower angles, opening, and scent emissions were similar to those of EV flowers, providing no particular time advantage to be visited first. Similarly, the small increase in nectar

volume of irLHY flowers at 6 p.m. is not likely to explain its greater success in the trials. An alternative hypothesis is that other unmeasured floral traits, such as minor floral scent compounds, or UV floral pigments, may be altered in irLHY flowers. We predict that early advertisements in nature may decrease pollination success and increase the visitation of unfavorable insects, such as florivores, suboptimal pollinators or nectar robbers (Kessler and Baldwin 2011).

We expect that foraging moths will learn to associate particular flower traits with a nectar reward, but as we used naïve moths for all experiments, this associational learning would be expected to occur during the experiment itself and this is reflected in our results. To avoid having the results confounded by prior associations, non-competition experiments with single lines tested at a given time were used in all experiments reported here.

Circadian rhythms in flowers have evolved in response to interactions with mutualists and antagonists and also to synchronize with the environmental rhythms in their native habitats. Their main function is presumably to ensure outcrossing services by attracting pollinators at the right time (Jones and Little 1983; Harder and Barrett 2006). *N. attenuata* is an interesting model species with which to study the function of floral rhythms for plant-pollinator interactions, because its three floral rhythms – aperture, vertical movement, and BA emission – can function to attract different types of pollinators (Kessler et al. 2010). The mechanical and genetic manipulation of floral rhythms clearly reveals that altering circadian rhythms in flowers affects pollination success.

It is thought that the circadian clock helps plants to anticipate abiotic factors, such as preparing the photosynthetic machinery at dawn to anticipate the rising of the sun (Green et al. 2002) or to increase its pollination service (Vandenbrink et al. 2014). The clock has been argued to help plants anticipate attack from herbivores (Bhardwaj et al. 2011; Wang et al. 2011; Goodspeed et al. 2012; Zhang et al. 2013), an inference which has not found support in the *N. attenuata* system (Herden et al. 2016). Based on evolutionary considerations, beneficial biotic interactions, rather than antagonistic interactions, are more likely to be usefully anticipated by a circadian clock, because antagonists can readily counter a plant's clock-mediated anticipation by changing the timing of their attack. The evidence provided here, demonstrates that *N. attenuata*'s clock exquisitely prepares its flower for pollination by *M. sexta* moths and in doing so, exhibits a botanical version of synchronized dancing.

MATERIAL AND METHODS

Plant growth conditions

We used *Nicotiana attenuata* Torr. Ex. Wats (Solanaceae) plants (30th inbred generation) and isogenic transformed plants that all originated from the same accession in Utah. Seeds were sterilized and germinated on Petri dishes and grown under long-day conditions (LD, 16 h light/ 8 h dark) in a growth chamber for 10 d until being transferred to 1 L pots in a glasshouse located in Jena, Germany, as described in Krügel et al. (2002).

Floral traits of the clock-gene silenced lines in *N. attenuata*

NaLHY (NCBI accession number JQ424913), NaTOC1 (JQ424914) and NaZTL (JQ424912) were independently silenced by transformations with gene-specific inverted repeat (ir) constructs driven by the CaMV 35S promoter (Yon et al. 2012, 2016). Two independently transformed T_2 and T_3 homozygous lines (irLHY-404, irLHY-406, irTOC1-205, irTOC1-212, irZTL-314, and irZTL-318) were used to characterize the diurnal rhythms in flowers (Yon et al. 2016) and all pollination experiments described in this study were conducted with the fully characterized irLHY-406, irTOC1-205, and irZTL-314 lines (Yon et al. 2016). Empty-vector (EV) plants were used as controls for the silenced lines in the pollination experiments, and the 31st inbred generation wild-type (WT) plants from the same accession from Utah were used as pollen donors. The floral traits of the clock-altered lines used in this study are summarized in Figure 1A–C from data previously published (Yon et al. 2016), except for irTOC1 for which the primary data is presented in Figure S1 and acquired as described in Yon et al. (2016).

Benzyl acetone emission from the first opening flowers of irTOC1 was measured in real time using a portable gas chromatograph, z-Nose^{TM} 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 approximately

9,000 mm^3. A single hole was made in a cap through which the sampling needle of the z-Nose sampled the headspace of flowers.

Nectar volume was measured by collecting flowers at 6 p.m. of the first opening day and at 6 a.m. of the following day, and placing them in ice-cold Eppendorf tubes (0.5 mL) for a short centrifugation of approximate 2 s. Nectar was removed from the Eppendorf tubes and quantified with a calibrated pipette tip with 0.5 μL calibrations.

Cross pollination experiments

To measure cross pollination rates in the clock gene-silenced lines, stem vertical supported plants with antherectomized flowers were transferred to a table covered with a green mesh tent (1.8 m height × 1.6 m width × 6 m length) in a glasshouse cabin. Fully-developed flowers from LD-grown plants were emasculated in the early morning to prevent self-pollination. We chose experimental days when there were no other flowering *N. attenuata* plants in the glasshouse cabin. We manipulated the flower orientation by fixing them mechanically at three different angles: +45°, 0° and −45°, with soft plastic straws surrounding the flower pedicels and wire. Flowers move through a 140° arc each day and the three experimental angles represent the two extreme and the middle positions of this arc. Three emasculated flowers on 10 WT plants were fixed at each experimental angle, and 20 WT plants were placed as pollen donors. Three male naïve *M. sexta* moths were released at 20 h, to function as the sole pollinators for one night. On the next morning, the *M. sexta* moths and flower tethers were removed and the experimental plants remained on another table in the glasshouse to mature seed capsules in case of successful outcrossing; flowers that received no outcrossing aborted their flowers during this time. After capsule maturation, the number of capsules and seeds per capsule were counted. The experiment was repeated twice with the same number of emasculated plants and pollen donor plants. To control for possible fertility differences due to angle, a hand-pollination conducted under the same experimental conditions served as a control for the experimental manipulations. The small nectar volume of flowers (Figure 1D) and the narrow corolla tube prevents nectar from draining from flowers at any angle.

To measure outcrossing rates in the clock gene-silenced lines, plants with antherectomized flowers were transferred to the same table with a tent cover as described above. The experiment was conducted twice: once in which EV and all clock gene-silenced lines competed with EV plants for the pollination services of two naïve moths and once when each line was evaluated separately. Possible *M. sexta* learning effects can be excluded in these experiments, given that naïve moths were used, and these moths were exposed to only one line of flowers. In this way, the experiments avoided any confounding effects of a moth's previous experience. For the no-competition experiment, five flowers on five plants per each line (EV, irLHY, irTOC1, and irZTL) were antherectomized in the morning and provided with the pollination services of two naïve *M. sexta* moths, for one night in separate experiments. Moths obtained pollen while nectaring on 10 WT plants which were placed in the arena in parallel to the target pollen receiver plants, and thus served as pollen donors. For the paired-competition experiments, five flowers on four EV plants and five flowers on four plants of each of the clock gene-silenced lines (irLHY, irTOC1, and irZTL) in separate paired-experiments were emasculated in the morning while all other flowers were removed. These plants competed for the pollination services of two naïve *M. sexta* moths for one night. Again 10 WT plants placed in addition to the competing transgenic lines into the experimental arena, served as pollen donors. Each of the three clock gene-silenced lines competed against EV plants in separate experiments. EV and clock gene-silenced plants were arranged in pairs, with 30 cm distance between plants. The number of matured capsules and seeds were counted after ripening.

Statistical analysis

To evaluate the results from the flower angle manipulations on the discrete seed capsule formation, three different null hypothesis models were tested with a Pearson's χ test for goodness-of-fit: the first hypothesis was that cross-pollination by the hawkmoth does not differ among the three positions; the second was that pollination at flowers fixed at 45° and 0° angles was not different; the third was that flowers fixed at 45° are preferentially pollinated. The first and second null hypotheses were rejected ($P < 0.001$ and $P < 0.01$, respectively), and the third hypothesis (preference at 45°) was not rejected ($P > 0.3$). χ^2 test of independence was used between the flowers at 45° and 0°. The results from capsule number and seed number were

statistically analyzed using Student's *t*-tests and one-way ANOVAs, all were performed with R 2.15.3 (http://www.r-project.org/). Plants were used as replicates; in other words, matured capsules from a plant were averaged, and this plant average was used as the replicate, to avoid pseudo-replication. Nectar volumes from both time points were analyzed via generalized linear model, using time and transgenic line as factors with interaction terms. Each line per time was tested using *post-hoc* HSD. All tests were performed in R, the *post-hoc* HSD test was performed using the agricolae v.1.2-3 package.

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AUTHOR CONTRIBUTIONS

F.Y., S.K., and D.K. designed and performed experiments and wrote the manuscript. L.C.L. and Y.J. performed experiments. F.Y. and S.K. screened and characterized the transgenic lines. I.T.B. and S.K. conceived the study, coordinated and wrote the manuscript. All authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12511/suppinfo

Video S1. Vertical movement and opening of the *Nicotiana attenuata* flower

Figure S1. Effect of silencing TOC1 on *Nicotiana attenuata* floral traits in comparison to *empty-vector* (EV)

(**A**) Mean (± SE) flower angles. (**B**) Mean (± SE) distance between petal junctions on corolla limbs. (**C**) Mean (± SE) levels of BA emission measured using a z-Nose™ portable GC and BA concentrations were calculated using a standard curve of BA dilutions. Plants grown under 16 h : 8 h, light : dark (LD) conditions.

Manuscript VI

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What happens in the pith stays in the pith: tissue-localized defense responses facilitate chemical niche differentiation between two spatially separated herbivores

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SUMMARY

Herbivore attack is known to elicit systemic defense responses that spread throughout the host plant and influence the performance of other herbivores. While these plant-mediated indirect competitive interactions are well described, and the co-existence of herbivores from different feeding guilds is common, the mechanisms of co-existence are poorly understood. In both field and glasshouse experiments with a native tobacco, *Nicotiana attenuata*, we found no evidence of negative interactions when plants were simultaneously attacked by two spatially separated herbivores: a leaf chewer *Manduca sexta* and a stem borer *Trichobaris mucorea*. *T. mucorea* attack elicited jasmonic acid (JA) and jasmonoyl-L-isoleucine bursts in the pith of attacked stems similar to those that occur in leaves when *M. sexta* attacks *N. attenuata* leaves. Pith chlorogenic acid (CGA) levels increased 1000-fold to levels 6-fold higher than leaf levels after *T. mucorea* attack; these increases in pith CGA levels, which did not occur in *M. sexta*-attacked leaves, required JA signaling. With plants silenced in CGA biosynthesis (irHQT plants), CGA, as well as other caffeic acid conjugates, was demonstrated in both glasshouse and field experiments to function as a direct defense protecting piths against *T. mucorea* attack, but not against leaf chewers or sucking insects. *T. mucorea* attack does not systemically activate JA signaling in leaves, while *M. sexta* leaf-attack transiently induces detectable but minor pith JA levels that are dwarfed by local responses. We conclude that tissue-localized defense responses allow tissue-specialized herbivores to share the same host and occupy different chemical defense niches in the same hostplant.

Keywords: tissue-specific defense, localized defenses, *Manduca sexta*, *Trichobaris mucorea*, *Nicotiana attenuata*, chlorogenic acid, niche differentiation.

INTRODUCTION

Single plant species often interact with a wide variety of herbivores, and a plant specifically responds to different feeding guilds of herbivores (Erb *et al.*, 2012; Kant *et al.*, 2015). The ecological consequences of multiple herbivore interactions on a single hostplant depend largely on their temporal and spatial characteristics, e.g. the sequence of arrival and localization relative to the attacked tissues on the same hostplant, as well as on their feeding guilds (Erb *et al.*, 2011a,b; Tack and Dicke, 2013; Kant *et al.*, 2015; Lortzing and Steppuhn, 2016).

Plants can also shape terrestrial community composition by mediating herbivore interactions indirectly (van Zandt and Agrawal, 2004; Stam *et al.*, 2014; Poelman and Kessler, 2016). As many herbivores are highly mobile, they can easily escape from 'defended parts' to 'undefended parts' of the plant. Many plant species have developed systemically induced defense responses and signals that amplify and activate defense genes so that induced defense responses spread from the attacked tissues to distal, unattacked tissues (Heil and Ton, 2008). Jasmonates (JAs) play a central role in these systemic resistances (Howe and Jander, 2008). This spread of induced resistance is often constrained by the vascular connectivity of damaged and undamaged tissues (Orians, 2005). To overcome vascular constraints, many plant species also activate systemic responses via airborne signaling (Karban *et al.*, 2006; Kessler *et al.*, 2006; Heil and Silva Bueno, 2007). These systemic responses increase the chance of competitive interactions

among herbivores, even if they colonize different tissues, with the consequence of homogenizing the different ecological niches of a plant.

However, the plant-mediated effects are not always symmetric (Kaplan and Denno, 2007); plant-mediated herbivorous insect interactions also can be synergistic or neutral (Kaplan *et al.*, 2008; van Dam and Heil, 2011; Erb *et al.*, 2015). Facilitation generally happens among herbivores of different feeding guilds, e.g. chewing herbivores and sap-sucking herbivores, because different feeding styles, or the different resistance mechanisms elicited by them, trigger different phytohormones that can benefit one of the herbivores (Soler *et al.*, 2013). For instance, antagonistic interactions of JAs and salicylic acid (SA) are frequently evoked to explain interactions among herbivores (Rayapuram and Baldwin, 2007; Pieterse *et al.*, 2009; Ali and Agrawal, 2014; Kroes *et al.*, 2016). However, the hormonal signaling pathways important for other less-studied feeding guilds of herbivores are relatively less known, e.g. leaf folders, stem borers and gall-inducing herbivores (Erb *et al.*, 2012). The outcome of plant-mediated herbivore interactions can also be affected by other systemic changes in growth/stress-related hormones and nutritional quality (Erb *et al.*, 2011a, b; Tytgat *et al.*, 2013). The herbivore interactions in the same hostplant are mainly determined by the characteristics of multiple systemic signals (Soler *et al.*, 2013). Therefore, a holistic view of plant primary/secondary metabolism and the multiple hormonal signaling pathways that modulate these changes is required for an understanding of how the plant shapes interactions among different herbivores that share a host.

Most studies of plant-mediated herbivore interactions have focused on interactions among different folivores (Kessler and Baldwin, 2004; Ali and Agrawal, 2014; Desurmont *et al.*, 2016; Kroes *et al.*, 2016) or between above- and below-ground herbivores (Masters and Brown, 1992; Wäckers and Bezemer, 2003; Kaplan *et al.*, 2008; Erb *et al.*, 2009; Huang *et al.*, 2014). Although important for structural support and transport (Fordyce and Malcolm, 2000), how the stem responds to stem-feeding herbivores remains less explored (Dafoe *et al.*, 2013; Liu *et al.*, 2016), and how folivores and stem herbivores affect each other is largely unexplored.

To investigate the interactions of spatially separated herbivores, we used *Nicotiana attenuata* and its native herbivores as a study system. *N. attenuata* is attacked by a wide range of herbivores from different feeding guilds in its native habitat. Here, we examined the interactions between the well known leaf herbivore, *Manduca sexta* and the poorly-studied stem herbivore, *Trichobaris mucorea*. In their above-ground interactions, these herbivores are spatially separated but temporally coexisting on the same host. *T. mucorea* adults oviposit in the basal stem, when plants are in their early elongating stage and the larvae develop into pupae in the stem through plant senescence (Lee *et al.*, 2016) while *M. sexta* moths prefer to oviposit on leaves which position from third leaf position from the stem in *N. attenuata* when plants are in their elongating stage and the larvae commonly feed on vegetative tissues of reproductive plants (Kessler and Baldwin, 2002). Usually, only one *T. mucorea* larva is found per plant, and larvae spend their entire development in the stem tissues (Diezel *et al.*, 2011; Lee *et al.*, 2016). *M. sexta* adults normally oviposit less than two eggs per plant in nature (Kessler and Baldwin, 2002; Kessler, 2012). Recently, we developed the ability to rear *T. mucorea* in laboratory colonies (Lee *et al.*, 2016), which facilitated the comparisons of defenses elicited by this herbivore with those elicited by the leaf-chewing herbivore, *M. sexta* to understand how these two herbivores manage to co-exist on plants in nature.

RESULTS

Stem herbivore attack does not influence levels of leaf herbivore damage

To understand the interaction between leaf and stem herbivores, we compared naturally occurring leaf herbivore damage on *N. attenuata* plants between *T. mucorea*-infested plants and control plants in their native habitat, the Great Basin Desert of southwestern Utah, USA. To standardize *T. mucorea* damage and infestation rates, we used a previously described egg inoculation method (Figure 1a; Lee *et al.*, 2016). We measured the damage from leaf herbivores twice during the 2014 field season and found no significant differences in leaf resistance between *T. mucorea*-infested plants and control plants (Figure 1b). Moreover, *T. mucorea* larval biomass was not significantly correlated with total canopy damage by any herbivores in native populations during the 2015 field season (Figure 1c).

The lack of a significant interaction between *T. mucorea* and leaf herbivores may have resulted from variable environmental conditions in the field, leading us to conduct experiments in the glasshouse to directly examine the reciprocal impact between temporally co-occurring and spatially separated pairs of specialist herbivores (*M. sexta* and *T. mucorea*). We divided treatments into three groups of plants which were inoculated: (1) only with a *T. mucorea* egg into the basal stem; (2) only with two *M. sexta* neonates on stem leaves; and (3) both with a *T. mucorea* egg and two *M. sexta* neonates (Figure 1d). The spatial-temporal differences in herbivore occurrence mimicked their co-occurrence in nature. We measured larval biomass simultaneously after 7 and 13 days for *M. sexta* feeding and after 14 and 20 days for *T. mucorea* feeding. The average biomass of *M. sexta* caterpillars was not statistically different between *T. mucorea*-attacked and non-attacked

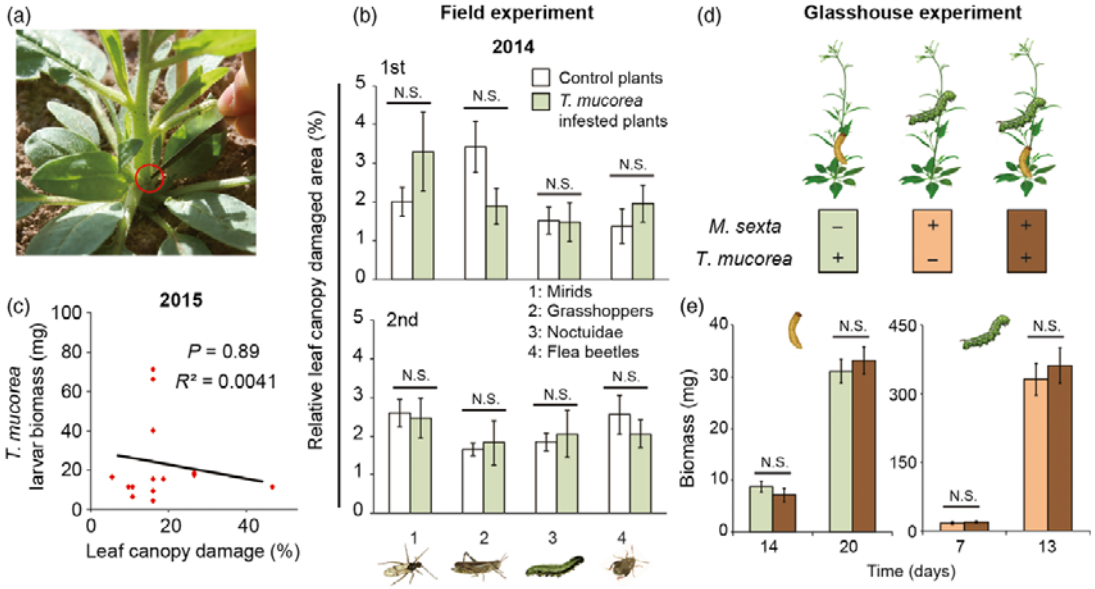

Figure 1. Spatially separated *Nicotiana attenuata* herbivores in nature and glasshouse show no signs of host-mediated negative interactions.
(a) To test reciprocal effects between the leaf resistance and the stem defense, (a) *Nicotiana attenuata* plants were grown in their native habitat (the Great Basin Desert of southwestern Utah, USA) and experimentally infested with one *T. mucorea* larvae per plant (infested plants; $n = 14$, uninfested plants $n = 25$ plants) in 2014.
(b) *T. mucorea*-infested plants suffered a similar degree of leaf canopy damage from the native herbivore community (mirids, grasshoppers, noctuidae and flea beetles) as did uninfested control plants (one-way ANOVA followed by Tukey's HSD).
(c) Individual leaf canopy damage from the native herbivore community was not significantly correlated with the biomass of *T. mucorea* larvae developing in the stems of the same plants ($n = 14$) in native population, 2015.
(d) The potential reciprocal effects of leaf and stem herbivores were further tested with the glasshouse-grown plants attacked by the leaf chewer, *M. sexta* larvae and the stem borer, *T. mucorea*.
(e) Larval mass of *T. mucorea* and *M. sexta* from the different treatments were consistent with the field data, in that no evidence of plant-mediated negative interactions were found (one-way ANOVA followed by Tukey's HSD; $n = 8$–13 for *T. mucorea*, $n = 19$–23 for *M. sexta*). NS indicates no significant difference between groups.

plants. Also, the average biomass of *T. mucorea* larvae was not statistically different between *M. sexta*-attacked and non-attacked plants (Figure 1d). Therefore, the results from this reciprocal bioassay in the glasshouse were consistent with the field observations.

T. mucorea larva attack induces tissue-specific responses in the pith

Plant-mediated herbivore interactions are highly dependent on the particular phytohormone signals used by host-plants in mediating defenses against attack from each of their herbivores (Soler *et al.*, 2013). As we did not find significant reciprocal effects between leaf and stem herbivores, we hypothesized that different phytohormones were being elicited by their attack. To evaluate which phytohormones and secondary metabolites were related to *T. mucorea* attack, we compared the pith chemistry of *T. mucorea* egg-inoculated plants with that of non-inoculated plants (Figure 2a). *T. mucorea* larval attack elicited high levels of JA and JA-Ile (jasmonoyl-L-isoleucine) in the pith (Figure 2b, c; $P < 0.01$), while levels of SA were unchanged (Figure S1; $P = 0.52$). Levels of ABA were also increased in the pith of plants that had been attacked, but ABA induction was JA-dependent (Figure S1). Among the measured secondary metabolites, chlorogenic acid (CGA) levels were highly elevated in the attacked pith (Figure 2d; $P < 0.001$); the CGA levels in the pith of control plants were only 1.9 ± 0.41 μg g^{-1} FM. At the same time, levels of nicotine and rutin were not changed (Figure S2; $P = 0.980$ and $P = 0.33$, respectively). Regarding the transcript levels of biosynthetic genes for each metabolite, levels of the *hydroxycinnamoyl quinate CoA transferase* (*NaHQT*) gene, a key enzyme involved in CGA synthesis, were also strongly induced in attacked pith (Figure 2e, $P < 0.001$). While nicotine levels in the pith of attacked plants were similar to those in the pith of controls, transcript levels of *putrescine* N-*methyltransferase* (*NaPMT*), a key enzyme in nicotine biosynthesis, were significantly induced in the attacked pith (Figure S2; $P < 0.001$). Other defense-related secondary metabolites in *N. attenuata*, e.g.

caffeoylputrescine (CP), dicaffeoylspermidine (DCS), and 17-hydroxyheranyllinalool diterpene glycosides (HGL-DTGs) were not detected in the pith.

To evaluate if JA signaling regulates the induction of CGA levels in the pith, we measured the amount of CGA in transgenic lines impaired in JA biosynthesis (silencing-*allene oxide cyclase*, irAOC), JA-Ile conjugation (silencing-*jasmonate resistant4/6*, irJAR4×6), or JA-Ile perception (silencing-*coronatine-insensitive protein 1*, irCOI1) 3 weeks after *T. mucorea* egg inoculation. Pith CGA levels of irAOC, irJAR4×6, and irCOI1 lines attacked by *T. mucorea* larvae were significantly lower than those of attacked empty-vector transformed wild-type *N. attenuata* (EV) plants [Figure 2f; $P < 0.001$, one-way analysis of variance (ANOVA)]. Although other factors than JA signaling alone, can mediate CGA induction by the *T. mucorea* attack, these results suggest that JA and JA signaling are essential for CGA induction in the pith of attacked *N. attenuata* plants.

To test whether JA or JA signaling affects the performance of *T. mucorea* larvae, we inoculated eggs into irAOC, irJAR4×6, and irCOI1 transgenic plants. Larval mass and larval developmental stages were measured 3 weeks after egg inoculation. *T. mucorea* larvae performed better in JA- or JA signaling-deficient plants than in EV plants (Figure 2g; $P < 0.05$, one-way ANOVA). In addition, most of the larvae fed on JA signaling-impaired plants reached the pre-pupal stage, while larvae fed on EV plants had only reached the second- or third-instar stages (Figure S3; $P < 0.05$, one-way ANOVA).

CGA metabolites in the pith decrease performance of *T. mucorea* larvae

To evaluate the defensive value of CGA against this stem-boring weevil, we fed *T. mucorea* first-instar larvae artificial diets spiked with different amounts of CGA: 0 mM, 0.85 mM (average CGA levels in the leaf), and 8.46 mM (similar to the maximum CGA levels measured in the attacked pith). Fourth-instar larvae fed artificial diets containing 8.46 mM CGA gained significantly less mass than those fed on 0 mM and 0.85 mM CGA (Figure S4).

We next generated CGA-deficient plants (irHQT-153 and irHQT-121) by silencing the *NaHQT* gene, the final enzyme in the CGA biosynthesis pathway (Figure 3a), using *Agrobacterium*-mediated transformation, as previously described (Krügel *et al.*, 2002; Gase *et al.*, 2011). The full coding sequence of *NaHQT* shared high similarity with *HQT* genes in potato and tomato (Figure S5). Levels of *NaHQT* transcripts in irHQT leaves were 95% lower than those in EV leaves (Figure S6), and CGA levels in the leaves of irHQT-153 and irHQT-121 plants were significantly lower than the levels in EV leaves (Figure S6; $P < 0.001$, one-way ANOVA). CGA levels in the pith of unattacked *N. attenuata* EV and irHQT plants were barely detectable (Figure 3b). Attack by *T. mucorea* larvae elicited

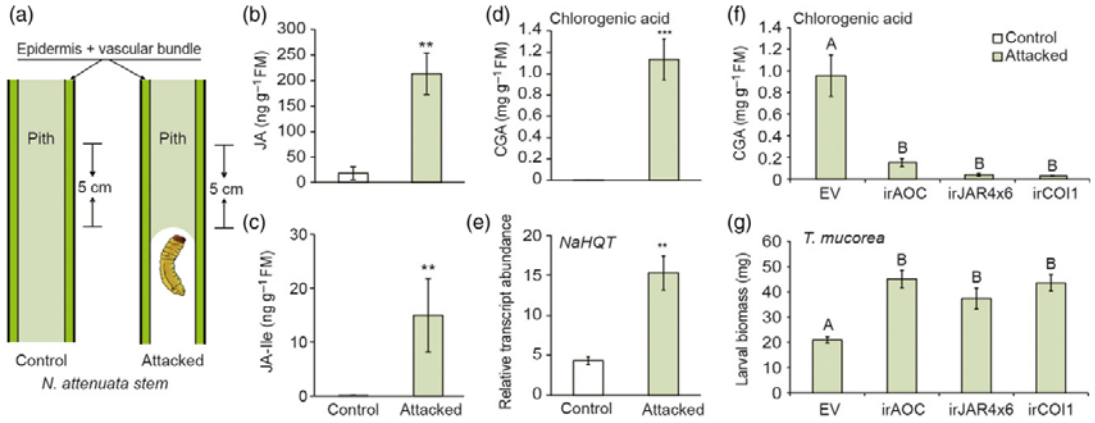

Figure 2. Pith inducible defense of *N. attenuata* against *T. mucorea* larva attack.
(a) Schematic diagram of the pith sampling from control and attacked plants.
(b, c) Three weeks after egg inoculation, approximately 5 cm of pith sections above the attacked area by *T. mucorea* larva and control pith were analyzed for mean (±SE) levels of jasmonic acid (JA) and jasmonoyl-L-isoleucine (JA-Ile). *T. mucorea* larva attack strongly increased JA and JA-Ile levels ($n = 6$).
(d) Mean (±SE) levels of chlorogenic acid (CGA) were highly increased in the attacked pith ($n = 6$).
(e) Mean (±SE) transcript levels of the biosynthetic gene of CGA, *hydroxycinnamoyl quinate CoA transferase, NaHQT* in control and attacked pith. Transcript abundance of *NaHQT*, a key enzyme involved in CGA synthesis was also strongly induced in the attacked pith by *T. mucorea* larva ($n = 3$).
(f) Mean (±SE) levels of CGA in JA biosynthesis (irAOC), JA-Ile conjugation (irJAR4×6), or JA-Ile perception (irCOI1) transgenic plants treated *T. mucorea* egg inoculation. CGA levels in the pith of attacked irAOC, irJAR4×6, and irCOI1 plants were significantly lower than CGA levels in the pith of attacked EV plants ($n = 6$). The levels of CGA in attacked pith are elicited in a JA-dependent manner.
(g) *T. mucorea* larvae perform better in the stems of JA- or JA signaling-deficient irAOC, irJAR4×6, and irCOI1 plants after *T. mucorea* egg inoculation. (one-way ANOVA followed by Tukey's HSD; $n = 20$). one-way ANOVA; $^{**}P < 0.01$; $^{***}P < 0.001$; different letters indicate statistically significant differences; $P < 0.05$.

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dramatic increases in CGA levels in the pith of EV plants. However, induced levels of CGA in the pith of irHQT plants were much lower than those in the pith of EV plants (Figure 3b; $P < 0.001$, one-way ANOVA). Levels of nicotine, rutin and HGL-DTGs were similar between EV and irHQT plants, but most of caffeic acid conjugates decreased in the pith of irHQT plants compared with EV plants (Figure S7). Larvae fed the two irHQT lines gained significantly more mass than larvae fed on EV plants (Figure 3c; $P < 0.05$, one-way ANOVA), demonstrating that plants use CGA, as well as other caffeic acid conjugates, to defend their pith against attack from *T. mucorea* larvae.

To examine whether CGA also functions as a defense compound in the field, we inoculated *T. mucorea* eggs into EV and irHQT-153 plants grown under field conditions as previously described in 2014 (Figure 3d). For this experiment, we selected 20 plants per genotype in the early stages of stalk elongation, when *T. mucorea* adults normally select plants for oviposition; there was no growth difference between EV and irHQT-153 (and see Figure S6). Three weeks after egg inoculation, we counted the number of surviving larvae in egg-inoculated EV and irHQT-153 plants. The survival rate of larvae was significantly higher in irHQT-153 than in EV plants (Figure 3e; $P < 0.05$). We also monitored herbivore damage from naturally occurring leaf herbivores attacking EV and irHQT-153 plants that had not been inoculated with *T. mucorea* eggs. Interestingly, leaf herbivore damage on EV plants did not differ statistically from that of irHQT-153 plants (Figure 3f). These results suggest that CGA, as well as other caffeic acid conjugates, produced by *N. attenuata* plants is more critical for the plants' defenses against stem-boring insects than against leaf herbivores.

Localized inducible jasmonates and defense metabolites accumulated differently in each tissue after stem–herbivore or leaf–herbivore attack

Unexpectedly, although there is no significant negative interaction effect between *M. sexta* and *T. mucorea* colonizing the same hostplant, *N. attenuata* uses JA signaling

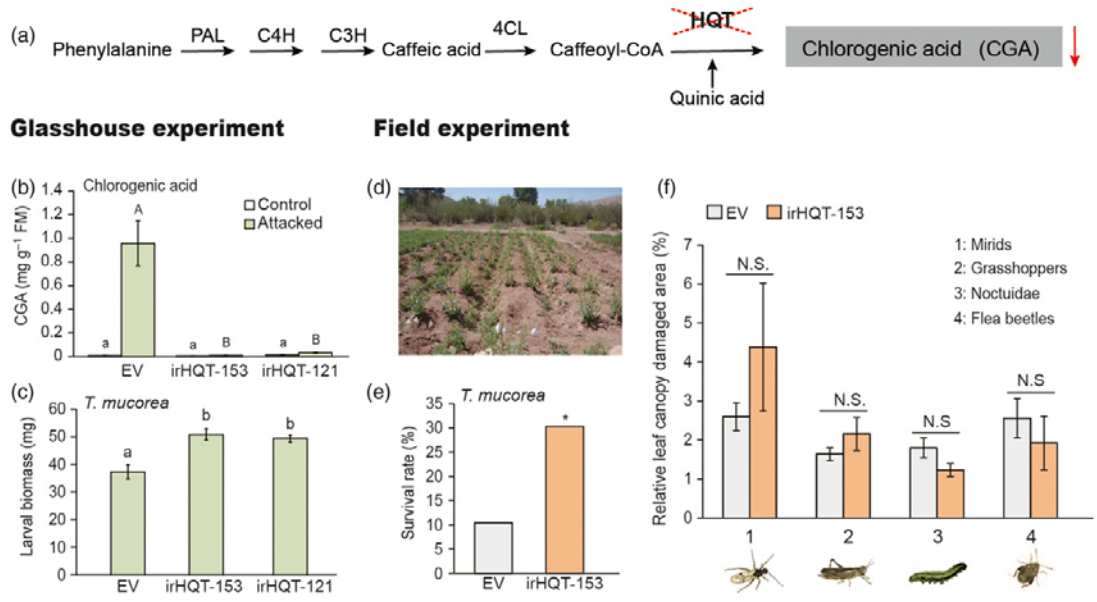

Figure 3. Effects of silencing a pith inducible phenolic defense in the glasshouse and nature.
(a) Simplified biosynthetic pathway of CGA in *N. attenuata* plants (from Kaur *et al.*, 2010). PAL, pheammonia lyases; C4H, cinnamic acid-4-hydroxylase; C3H, cinnamic acid-3-hydroxylase; 4CL, 4-coumaroyl-CoA: ligase; HQT, hydroxycinnamoyl-CoA quinate transferase. We used the CGA-deficient plants silenced in the *NaHQT* gene, the key enzyme in the CGA biosynthesis pathway.
(b) In glasshouse experiments, the induced levels of CGA in the pith of irHQT plants (lines 153 and 121) were significantly lower than the levels in the piths of attacked EV plants (one-way ANOVA followed by Tukey's HSD; mean ± SE, $n = 6$).
(c) Silencing *NaHQT* transcripts increased the performance of *T. mucorea* larvae (one-way ANOVA followed by Tukey's HSD; $n = 20$).
(d) In field experiments, conducted in field plots in the Great Basin Desert, southwestern Utah, USA, size-matched EV and irHQT plants were experimentally inoculated with eggs, as in the glasshouse experiment. Three weeks after egg inoculation, the number of surviving larvae and leaf damage from natural herbivore community were assessed.
(e) Survival rate of *T. mucorea* larvae hatched from eggs inoculated into irHQT-153 stems was significantly higher than in EV plants (Fisher's exact test; $n = 9$–17).
(f) Relative leaf area damaged of EV and *NaHQT*-silenced plants (−153) from the native herbivore community did not differ statistically (one-way ANOVA followed by Tukey's HSD; $n = 13$–25). $^*P < 0.05$; different letters indicate statistically significant differences; NS indicates no significant difference between groups.

in both tissues to activate inducible defenses against these spatially separated herbivores. From these results, we inferred that JA-mediated inducible defenses were not systematically activated from the leaf to the pith and *vice versa*. To test this hypothesis, we analyzed leaf and pith jasmonates and secondary metabolites in response to *M. sexta* and *T. mucorea* attack, respectively. We used wounding and regurgitant (R) treatments to mimic *M. sexta* attack (Halitschke *et al.*, 2001). CGA levels were highly induced in *T. mucorea*-attacked piths but not in the leaves of the same plants (Figure 4a; $P < 0.001$ and $P < 0.905$, respectively). Other secondary metabolites, such as CP, DCS, nicotine, rutin and HGL-DTGs, were not induced by *T. mucorea* attack in the leaf nor in the pith (Figures 4a and S8). In response to wounding with R of *M. sexta*, CP was strongly induced in the attacked leaf; although CP was not detected in the pith of either treated or untreated plants (Figure 4b; $P < 0.001$). These results demonstrate that tissue-specific inducible defenses are highly localized between the leaf and the pith.

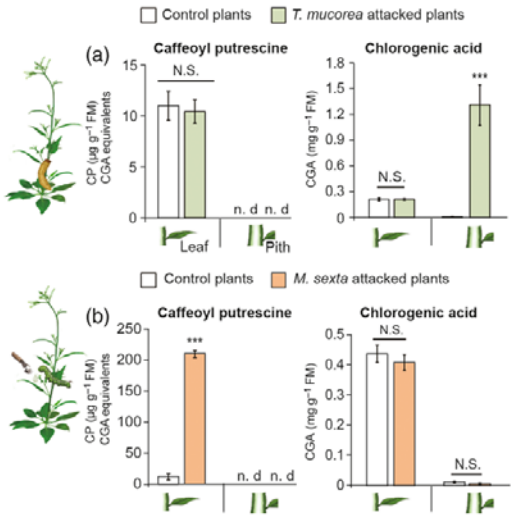

Figure 4. Accumulations of inducible defense metabolites in response to leaf- or stem herbivore attack.
(a) Mean (±SE) levels of CP and CGA in leaf and pith after attack from the stem herbivore, *T. mucorea* larvae. *T. mucorea* eggs were inoculated at the basal stem (1/plant) of early stage elongating plants. Pith and systemic stem leaves (S2 or S3) of control and attacked *N. attenuata* plants were collected after three weeks. The level of CGA was highly induced in *T. mucorea*-attacked pith but not in the leaves from the same plants. The level of CP was not induced by *T. mucorea* attack in leaves or pith ($n = 6$).
(b) Mean (±SE) levels of CP, and CGA in elicited leaves and pith after leaf wounding and treatment with regurgitant (R) from the leaf herbivore, *M. sexta*. Elicited leaves and pith 3 days after W+R elicitation ($n = 6$) were analyzed and CP levels were strongly induced in R-elicited leaves, but not in the adjacent pith. The level of CGA was not increased in R-elicited leaves and the adjacent pith ($n = 6$). One-way ANOVA; ***$P < 0.001$; NS, indicates no significant difference between groups; n.d., not detected.

We further investigated whether localized inducible defenses were also mediated by localized JA signaling. When *T. mucorea* larvae fed on *N. attenuata* stems, JA and JA-Ile were both highly induced in the attacked pith. However, neither JA nor JA-Ile was induced in the leaves (Figures 5a and S9). In contrast, when we performed wounding treatments with the R of *M. sexta* in leaves, JA and JA-Ile levels were induced in local leaves, and systemic JA signaling was also activated in both systemic leaves and the pith (Figure 5b). We also have measured other phytohormones (SA and ABA), soluble sugars, and free amino acids to evaluate whether another systemic signal was also localized from the pith to leaf by *T. mucorea* attack. SA, ABA and nutritional levels in the leaf were also not systematically affected by *T. mucorea* attack in the pith (Figure S10).

DISCUSSION

Plant-mediated interactions among herbivores are generally negative when they share the same phytohormonal pathway (Soler *et al.*, 2013). However, this study has shown that plant-mediated interactions between leaf chewers and a stem borer are not negative, even though *N. attenuata* uses JA signaling to elicit defense responses against both groups of specialist herbivores. Our results also demonstrated that *N. attenuata* plants elicit different JA-dependent chemical compounds in response to attack from these two spatially separated specialist herbivores, and that JA-mediated inducible defenses are localized. Taken together, these results provide an example of how plants facilitate chemical niche differentiation of two spatially separated specialist herbivores at the plant level through localized tissue-specific defenses (Figure 6).

How plants shape tissue-specific JA-mediated inducible defenses

Plants produce various toxic metabolites in order to defend themselves against herbivores (Schuman and Baldwin, 2016). These chemicals can reduce herbivore performance, survival rates, or reproductive success (Howe and Jander, 2008). Here, we show that high levels of CGA, as well as other caffeic acid conjugates, in the *N. attenuata* pith reduce the growth of *T. mucorea* larvae, but have little effect on attack by leaf-chewing or -sucking insects (Figure 3). CGA can be oxidized to form chlorogenoquinones, which are electrophilic molecules that bind to free amino acids and proteins, and thus reduce the activity of digestive enzymes in insect guts (Felton *et al.*, 1989; Felton and Duffey, 1991). CGA is known to increase the resistance of corn to herbivore attack by the fall armyworm (*Spodoptera frugiperda*), corn earworm (*Helicoverpa zea*), leaf beetles, and leafhoppers (Gueldner *et al.*, 1992; Dowd and Vega, 1996; Ikonen, 2002; Jassbi, 2003). However, CGA has little effect on the larval growth of the tobacco hornworm, *M.*

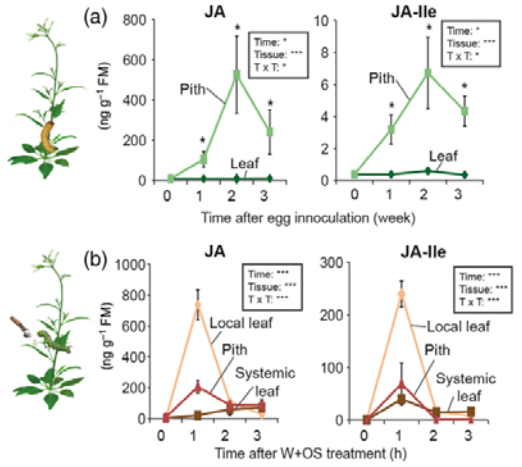

Figure 5. Systemic induction of jasmonates is asymmetrically activated depending on site of elicitation.
(a) Mean (±SE) levels of JA and JA-Ile were highly induced in the attacked pith after egg inoculation, but JA and JA-Ile levels in the leaf were not changed ($n = 6$).
(b) Mean (±SE) levels of JA and JA-Ile were rapidly induced in the local leaf within 1 h after W + R elicitation. Jasmonate signaling in the pith was systematically activated by W + R elicitation of the leaf ($n = 6$). one-way ANOVA; $^{*}P < 0.05$; different letters indicate statistically significant differences; $^{*}P < 0.05$; $^{***}P < 0.001$.

sexta, or on the tobacco budworm, *Heliothis virescens* (Eichenseer *et al.*, 1998; Johnson and Felton, 2001). Therefore, herbivore-specific effects of CGA on herbivore resistance as demonstrated by these results are consistent with the literature.

Interestingly, CGA levels in *N. attenuata* are differentially regulated in the leaf and the pith. Methyl jasmonate treatment has little effect on the level of CGA in *N. attenuata* leaves (Oldham and Baldwin, 2001), and treatment with oral secretions from the specialist herbivore *M. sexta*, which induces high levels of JA and JA-Ile in *N. attenuata* leaves, does not induce CGA levels in leaves, but rather decreases them (Onkokesung *et al.*, 2012). Similarly, silencing JA biosynthesis or signaling does not alter CGA levels in *N. attenuata* leaves (Paschold *et al.*, 2007; Demkura *et al.*, 2010). In contrast, CGA levels were highly induced in the pith, and their regulation was tightly coupled with JA production and JA signaling (Figure 2). Also, other caffeic acid conjugates, e.g. putative methyl caffeate and putative dicaffeoyl quinic acid, are induced only in the pith in a *NaHQT*-dependent manner by *T. mucorea* attack (Figure S7b), so it may be interesting for future work to test their roles in the interaction between *T. mucorea* and *N. attenuata*. These results suggest that the connection between JA signaling and defense metabolites can vary between different tissues.

Differential JA signaling can contribute to tissue-specific regulation of CGA. The COI1 protein is a JA-Ile receptor found in several plants (Feys *et al.*, 1994; Li *et al.*, 2004; Paschold *et al.*, 2007). The COI1-JA-Ile complex initiates the degradation of proteins from the jasmonate ZIM domain (JAZ) family that repress the expression of JA-responsive genes (Chini *et al.*, 2007). Interestingly, JAZ proteins, which are repressors of jasmonate signaling, are differentially expressed in the pith in comparison with the leaf (Figure S11; Oh *et al.*, 2012). In Arabidopsis, JAZ genes in shoot and root tissues are also differently

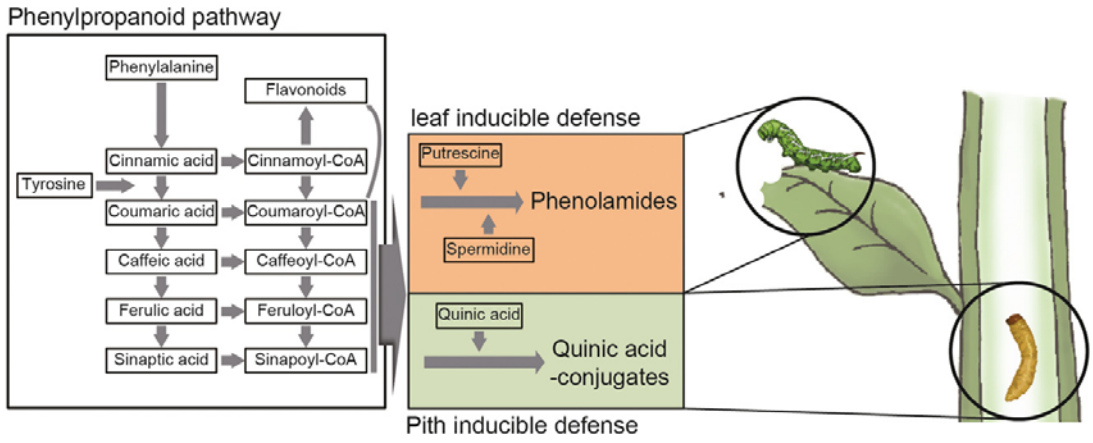

Figure 6. A schematic diagram depicting tissue-specific inducible defense in *Nicotiana attenuata* that allows for the differentiation of chemically distinct feeding niches between two herbivores that feed on leaves and pith, respectively.
The accumulation of phenolamides and quinic acid conjugates derived from the phenylpropanoid pathway differ in response to attack from these spatially separated herbivores that feed on the same hostplant (Table S1). Tissue-specific defense response induced by JA signaling in leaf and stem tissues allow a hostplant to create different chemical niches for its attacking herbivores.

expressed in response to JA treatment (Tytgat *et al.*, 2013). The *N. attenuata* genome contains at least 12 JAZ proteins; these proteins are involved in several JA-dependent signaling pathways; specifically, JAZd regulates JA and JA-Ile levels in flowers, and JAZh is involved in leaf defense responses when the specialist herbivore *M. sexta* attacks (Oh *et al.*, 2012, 2013). We did not observe differences in the larval mass of *T. mucorea* that fed on irJAZd or irJAZh plants when compared to larvae fed on control plants (Figure S11). This suggests that tissue-specific inducible defenses can also be mediated by tissue-specific jasmonate signaling; in particular, other JAZ proteins could regulate CGA levels in the pith of *N. attenuata*, and this hypothesis should be tested.

How plants shape localized JA-mediated inducible defenses in each tissue

Our data suggest that JA-mediated inducible defenses can be localized depending on the location of the attack. Pith JA signaling is highly localized to the pith, but leaf herbivore attack systematically activates attenuated JA signaling in the pith. Moreover, most of the other potential systemic signals in the pith were also highly localized: SA, ABA, and primary metabolites (Figure S10). Surprisingly, JA-dependent inducible defense compounds are strongly localized in each tissue. A recent review has proposed 'the squeeze cell hypothesis' (Farmer *et al.*, 2014) according to which, systemic responses require pressure differences in xylem tissues in order to activate JA signaling systemically. Pith damage in *N. attenuata* may not result in sufficient pressure changes to activate leaf JA signaling due to the histology of the parenchyma cells that dominate the pith (Fahn, 1982). Vascular bundles may not occur at a sufficient density or have the appropriate connectivity to transmit systemic signals from damaged pith to leaves in *N. attenuata*. Moreover, given the very different down-stream responses mediated by JA signaling in leaf and pith, there are clearly very interesting tissue-specific differences in signaling mechanisms that need to be explored in future research.

Specificity in inducible responses to herbivores can also contribute asymmetrically to the systemic induction of JAs and to localized inducible defenses. Plants can recognize different herbivores by damage type (mechanical cues) and through different elicitors (chemical cues) (Erb *et al.*, 2012). Although *N. attenuata* JA signaling is strongly induced after a single W + R treatment (Schuman and Baldwin, 2016), plants maintain high levels of JA for a longer period of time in response to both constant damage by *T. mucorea* attack in the pith as well as *M. sexta* attack in the leaf (Figure 5; Skibbe *et al.*, 2008). Interestingly, the ratio between JA and JA-Ile in the pith after *T. mucorea* attack is much lower than that in the leaf after *M. sexta* attack, although systematically-induced JAs in the pith by *M. sexta* R leaf treatments showed a similar JA to JA-Ile ratio as those found in the leaf. In addition, JA-Ile was more rapidly metabolized to its inactive forms in the pith in comparison to the leaf, e.g. OH-JA-Ile and COOH-JA-Ile (Figure S12). Therefore, localized defenses may not only be limited just by structural constraints, e.g. vascular connectivity; herbivore-specific signals may also be required to activate inducible defenses in the pith. This suggests that *N. attenuata* differently induces JA signaling in response to *M. sexta* and *T. mucorea*, and that herbivore-specific differential JA metabolism could contribute to the systemic induction of JA signaling in an asymmetric manner and the localized JA-dependent inducible defenses.

Localized JA-mediated tissue-specific inducible defenses optimize defenses for *N. attenuata*

Inducible defenses are thought to be metabolically more economical than constitutively expressed defenses (Karban *et al.*, 1997), but are still costly in terms of plant Darwinian fitness (Baldwin, 1998; Zavala *et al.*, 2004). For instance, nitrogen is a limited nutrient for plant growth and defense (Elser *et al.*, 2007) and herbivory to *N. attenuata* results in dramatic changes in resource allocation of this fitness limiting resource (Lynds and Baldwin, 1998); the total amount of nitrogen for proteins is significantly decreased, and increased for small *N*-containing defense metabolites (Ullmann-Zeunert *et al.*, 2013). According to optimal defense theory, the different fitness values among tissues, as well as their different probabilities of attack, determine the different investments in defensive metabolites (McKey, 1974; McCall and Fordyce, 2010). In response to *M. sexta* (leaf herbivore) attack, leaves of *N. attenuata* mainly induce nitrogen-containing phenolamide metabolites, while the pith mainly induces quinic acid-conjugated acids, which do not contain nitrogen (Table S1). Although recent studies demonstrated that indole-3-acetic acid (IAA)-mediated systemic responses to *M. sexta* attack increased phenolamides and anthocyanins in the stem, and suggested that these changes can alter the performance of stem borers (Machado *et al.*, 2016b), larval performance of *T. mucorea* was not improved when larvae infested MYB8-silenced plants, which are highly impaired in phenolamides production (Figure S13). One possible explanation for this difference is the specificity of sample collections. As *T. mucorea* feeds only on the pith of stems (Diezel *et al.*, 2011; Lee *et al.*, 2016), we specifically collected and analyzed only these pith tissues. Therefore, auxin-mediated systemic activation of phenolamides and anthocyanins in the epidermal parts of stems could be relevant defenses against mammalian herbivores, which are stem-peelers (Machado *et al.*, 2016a). As the free-living herbivore community of *N. attenuata* is more complex than the endophytic one in nature, *N. attenuata* may protect leaves with costly N-containing secondary metabolites and utilize more varied inducible metabolites to obtain an optimal defense pattern. We also

have shown that leaf herbivore damage and stem herbivore damage are not positively correlated (Figure 1), and that there was no significant difference in the amount of leaf damage from herbivores between control and CGA-silenced plants (Figure 4). These results are consistent with the hypothesis that plants localize their inducible defenses according to the probability of herbivore attack, and that the choice of localized defenses may also reflect an optimization of resource allocation to optimize Darwinian fitness (van Dam and Heil, 2011).

Here, we demonstrate that *N. attenuata* plants have tissue-specific defenses for spatially separated herbivores by deploying different metabolites from the phenyl propanoid pathway to different tissues: phenolamides for leaf herbivores, and quinic acid conjugates for stem herbivores (Figure 6 and Table S1). Also, tissue-specific inducible defenses in the leaf and pith are localized in each tissue. These tissue-specific localized defenses contribute to the differentiation of chemical niches in a single plant. To determine whether tissue-specific localized defenses increase a plant's Darwinian fitness, and therefore can be regarded as an adaptation, will require the elucidation of the genetic mechanisms required for these tissue-specific responses. Recent advances in our molecular understanding of the systemic induction of JA signaling will be very helpful in this regard (Chauvin *et al.*, 2013; Kiep *et al.*, 2015). Importantly, plant-mediated interactions between leaf and stem herbivores ultimately minimize the effect of each herbivore on their own performance in a shared hostplant. Localized tissue-specific defenses may facilitate chemical niche differentiation in a single hostplant, and could facilitate a diversification of plant insect interactions that include the full spectrum from antagonistic to mutualistic interactions.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

We used the 31st inbred generation of *N. attenuata* seeds originally collected from a native population at a field site located in Utah, USA. Stably silenced inverted repeat (ir) plants were used for secondary metabolite measurements and *T. mucorea* larvae performance: plants silenced in JAs synthesis (irAOC, line A-07-457; Kallenbach *et al.*, 2012), JA conjugation (irJAR4/6, line A-07-756; Wang *et al.*, 2008), JA-Ile perception (irCOI1, line A-04-249; Paschold *et al.*, 2007), transcription factor for phenylpropanoids pathway (irMYB8, A-07-810; Kaur *et al.*, 2010). *NaHQT*-silenced lines (irHQT) were produced by the published *Agrobacterium tumefaciens*-mediated transformation method (Krügel *et al.*, 2002) using pRESC8 binary vector containing the inverted repeat (ir) fragment of the *NaHQT* sequence (Figure S7) and the number of inserted T-DNA was determined by Mendelian segregation ratios of hygromycin resistance of selected irHQT lines and southern hybridization of genomic DNA using Dig high prime DNA labeling system (Dig system) (Figure S7). All further experiments were performed with stably transformed homozygous plants. Seeds were sterilized and germinated on Gamborg's B5 medium (Duchefa) as described previously (Krügel *et al.*, 2002). Seedlings were maintained at 26°C/16 h of 155 μmol m^{-2} sec^{-1} light; 24°C/8 h of dark cycle. After 10 days, young seedlings were planted individually in Teku plastic pots containing peat-based substrate. Ten days later, early rosette plants were transferred to soil in 1 litre pots and grown in the glasshouse with a day/night cycle of 16 h (26–28°C)/8 h (22–24°C) under supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600 high-pressure sodium lamps (Philips) with an automatic glasshouse watering system.

For field-grown plants, seeds of the transformed *N. attenuata* lines (EV, irHQT) were imported and released under US Department of Agriculture Animal and Plant Health Inspection Service (APHIS) notification numbers 13-350-101r (release of EV, irHQT in 2014). Seeds were germinated and seedlings were adapted to field conditions as previously described (Krügel *et al.*, 2002; Kessler *et al.*, 2015). Adapted size-matched seedlings were transplanted into a field plot at the Lytle Ranch Preserve Snow Ranch property, located at latitude 37.141°, longitude 114.03° (Santa Clara, UT, USA). Plants were watered using a trench irrigation system until roots had established, and then as needed.

Field herbivore assays

The field experiments were carried out in a field plot at the Lytle Ranch Preserve, Utah, USA in 2014 and 2015. To experimentally infest plants in the field, we used a previously described egg inoculation method (Lee *et al.*, 2016) with slight modifications. We used eggs freshly collected from newly emerged adults of *T. mucorea* from *Datura wrightii* plants near the field plot. We inoculated eggs into size-matched early elongation-stage EV plants (experiment for comparison between infested and uninfested plants) from 11 May 2014 to 13 May 2014. To test the effects of *T. mucorea* attack on leaf–herbivore resistance, total canopy damage from natural leaf herbivores was determined by estimating the percentage of canopy area damaged by each type of leaf herbivores (Schuman *et al.*, 2012; Gaquerel *et al.*, 2013). The percentage of total leaf area damaged by sucking herbivores (*Tupiocuris* spp. and *Empoasca* spp.), grasshoppers (*Trimerotropis* spp.), noctuidae (*Spodoptera* spp.), and flea beetles (*Epitrix* spp.) in the field plot in 2014 was calculated, and additionally in the 2015 field season, we also collected the *T. mucorea* larvae from natural populations of plants to measure their biomass. To test the effects of CGA on leaf and stem herbivores, we planted size-matched EV and irHQT plants in a fully randomized design in the field plot and used the above procedure to measure plant damage by natural herbivores. In addition, to test larval survival, we inoculated *T. mucorea* eggs into EV and irHQT plants on 16 May 2014 and measured the survival rate on 9 June 2014.

Leaf and stem herbivory treatment

For *T. mucorea* larvae experiments, adults were collected from their natural habitat, the Great Basin Desert, southwestern Utah during the 2013 field season. In a previous study, we reported procedures for the maintenance of laboratory colonies of *T. mucorea* and egg inoculation (Lee *et al.*, 2016) under laboratory conditions. Three weeks after egg inoculation, we slit stems lengthwise to collect larvae and transferred these to tubes containing artificial diet. The egg inoculation method was used in all experiments of *T. mucorea* larval performance and elicited pith characterization.

For *M. sexta* caterpillar experiments, two freshly hatched *M. sexta* neonates were placed on stem leaves (S2 or S3) of control or inoculated plants 1 week after *T. mucorea* egg inoculation when all plants were in the early elongation-stage *N. attenuata*. Another 7 and 13 days after *M. sexta* neonates placed (same time

as *T. mucorea* larvae, 14 and 20 days after egg inoculation), we measured the biomass of both herbivores.

For phytohormones and secondary metabolites analyses in which the timing of elicitation and sampling is critical, we applied regurgitants and oral secretions (R) of *M. sexta* larvae to freshly produced puncture wounds to simulate larval feeding. A fabric pattern wheel used to create standardized puncture wounds that were immediately treated with 20 µl of 1:5 (v/v) water-diluted R (W + R). Treated and untreated leaf and pith tissues were collected after 1–3 h after elicitation. *M. sexta* caterpillar R were collected from larvae reared on *N. attenuata* wild-type plants from neonates until the third to the fifth instar stages.

Details of *in vitro* bioassay of *T. mucorea* larvae are described in Method S1.

Analysis of primary and secondary metabolites

Approximately 100 mg of frozen pith and leaf materials were extracted by adding 1 mL of the extraction buffer (60% solution 1; 2.3 mL L^{-1} of acetic acid, 3.41 g L^{-1} ammonium acetate adjusted to pH 4.8 with 1 M NH_4OH, and 40% (v/v) methanol) with two steel beads as described in Heiling *et al.* (2010). The samples were homogenized by a Genogrider 2000 (SPEX Certi Prep) operated at 1200 strokes per min, for 60 sec. Supernatants were collected after 20 min of centrifugation at 16 100 *g* at 4°C. In total, 1 ml of particle-free supernatant (after additional centrifugation) was analyzed by HPLC (Agilent-HPLC 1100 series) and the analytes were detected with Photo Diode Array (PDA) and Evaporative Light Scattering (ELS), Varian detectors. Nicotine eluted at a retention time (RT) of 0.5 min (detected by UV absorbance at 260 nm); CP, CGA and DCS eluted at RTs of 2.6, 3.0, and 3.9 min, respectively (detected at 320 nm). Rutin eluted at RT 4.7 min and was detected at 360 nm. HGL-DTGs eluting between RT 7.0 and 8.5 min were detected by the ELS detector. The peak areas were integrated using the Chromeleon chromatographic software (version 6.8: Dionex), and the amounts of metabolites were calculated using serial dilution of external standard mixtures of nicotine, CGA and rutin. Details of primary metabolite analysis are described in Method S2.

Phytohormone analysis

Approximately 100 mg of frozen materials was homogenized with two steel beads in a Genogrider 2000 (SPEX Certi Prep) at 1200 strokes min^{-1}. Phytohormones (JA, JA-Ile, SA, and ABA) were extracted by vortexing for 10 min after the addition of ethyl acetate spiked with internal standards: 100 ng of [$^2H_2$] JA and 20 ng each of JA-[$^{13}C_6$] Ile, [$^2H_4$] SA, and [$^2H_6$] ABA. The extracted samples were centrifuged at 16 100 *g* at 4°C for 20 min, and the upper supernatant was transferred into another new tube. The samples were evaporated to near dryness in a vacuum concentrator (Eppendorf) at 30°C. The dried samples were dissolved in 500 µl 70% (v/v) methanol: water for analysis with the Varian 1200 LC-ESI-MS/MS system as described by Gilardoni *et al.* (2011). The phytohormones were detected in negative ESI mode and the detailed detection method followed Gilardoni *et al.* (2011). The resulting amounts of hormones were divided by the exact fresh mass of plant materials used in the extraction.

Gene expression analysis by RT-qPCR

To analyze transcript levels of *NaPMT, NaCHS, NaHQT* and *NaJAZs* genes in attacked and unattacked pith with biological replicates. Total RNA was extracted using RNeasy Plant mini kit (Qiagen, Hilden, Germany). The synthesis of cDNA was performed with 1 µg of total RNA using RevertAid™ H Minus Reverse Transcriptase kit (Fermentas, Schwerte, Germany) and oligo-dT primer (Fermentas). Quantitative real-time PCR (qPCR) was carried out with the synthesized cDNA from three biological replicated samples using the core reagent kit for SYBR Green I (Eurogentec, Seraing, Belgium) and gene specific primer pairs (Table S2) on a Stratagene MX3005P PCR cycler. Relative transcript levels were calculated from a dilution series of cDNA samples and normalized by the expression of the tobacco housekeeping gene, *Nicotiana tabacum* elongation factor-1α (*NtEF1α*).

Statistical analysis

Data analysis was conducted with Origin 8 SR1 (OriginLab Cop. Northampton, USA) or the publically available R package (version 3.1.2, http://www.r-project.org/). We used one-way ANOVA followed by Tukey's honestly significant difference (HSD) as *post hoc* test for multiple samples and, chi-squared test for survival rate analysis.

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AUTHORS' CONTRIBUTIONS

Conceptualization and Supervision: S.K. and I.T.B. Project Administration: I.T.B. Funding Acquisition: I.T.B. Methodology: I.T.B., G.L., and Y.J. Investigation: G.L. and Y.J. Formal Analysis, Data Curation and Validation: G.L. and Y.J. Visualization: G.L. and Y.J. Writing Original Draft: G.L., Y.J. and S.K: Rewriting Review & Editing: I.T.B.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Levels of ABA and SA in attacked pith.

Figure S2. Levels of nicotine, rutin and transcripts of their related major biosynthetic genes in piths attacked by *T. mucorea* larvae.

Figure S3. Effects of JA signaling on larval development of *T. mucorea*.

Figure S4. *T. mucorea* larval mass is negatively correlated with CGA concentrations in artificial diets.

Figure S5. Phylogenetic trees and protein alignment of HQT genes.

Figure S6. Generation of *NaHQT*-silenced *Nicotiana attenuata* plants.

Figure S7. Other secondary metabolites of leaf and pith in EV and irHQT plants.

Figure S8. Levels of DCS, nicotine, rutin and HGL-DTGs in leaves and piths.

Figure S9. Levels of JA and JA-Ile of leaf and pith tissue in control plants.

Figure S10. ABA, SA and primary metabolites in leaves and piths of *T. mucorea* larvae-attacked plants.

Figure S11. Transcript abundances of JAZ genes in the pith, and mass of *T. mucorea* larvae fed on irJAZd, irJAZh or EV plants.
Figure S12. Jasmonate metabolism in the leaf and pith in response to *M. sexta* elicitation.
Figure S13. Mass of *T. mucorea* larvae fed EV and irMYB8 plants.
Table S1. Comparison of phenolamide and quinate conjugate metabolites measured by UPLC-TOF-MS in leaf and pith tissue elicited by *M. sexta* R treatment and *T. mucorea* larval attack, respectively.
Table S2. Primers used in this study.
Method S1. Bioassay of *T. mucorea* larvae.
Method S2. Analysis of primary metabolites.

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Supporting Experimental Procedures

Method S1. *T. mucorea* Bioassay

To examine larval performance on artificial diets supplemented with chlorogenic acid (CGA; Sigma Aldrich), we used 1st instar larvae of *T. mucorea*, which were reared for one week in the stems of wild type *N. attenuata* plants. Bioassays were conducted with concentrations of 0mM, 0.85mM, and 8.46mM CGA added to the artificial diets. The concentration of 2.8mM CGA is the average level of CGA that is induced in the attacked pith by *T. mucorea* larva attack. A 0.85mM CGA is similar to the level of CGA in a leaf of *N. attenuata* grown in the glasshouse. An 8.46mM CGA level is similar to the maximum level of CGA that we measured in the attacked pith. All *in vitro* assays were performed in a growth chamber with a 16h light (26°C)/8h dark (24°C) cycle and 65% humidity (Snijders Scientific).

Method S2. Analysis of primary metabolites

We measured free amino acids and soluble sugars as described by Schäfer, Brütting, Baldwin, & Kallenbach, 2016. For extraction of the samples, about 100mg frozen ground plant material was aliquoted into 96-well biotubes (1.1mL individual tubes, Arctic White LLC, catalog number: AWTS-X22100) that added 2 steel balls to improve the homogenization during extraction and closed with strips of 8-plug caps (Arctic White LLC, catalog number: AWSM-T100-30). During aliquoting, the samples were kept in liquid nitrogen. And then we added to each sample 800uL pre-cooled (-20°C) acidifed MeOH [MeOH: H_2O: HCOOH 15:4:1: (v:v:v:)]. The tubes were tightly sealed with a seal mate, homogenized in a Genogrinder for 1min at 1150 strokes/min (Geno/Grinder 2000, SPEX SamplePrep) and incubated over night at -20°C. After incubation, the samples were homogenized using Genogrinder during 1min at 1150 strokes/min, centrifuged (1913xg for 20min at 4°C). And 10uL of the supernatant was transferred to a new vial. For analysis of free amino acids, we diluted 2uL of the supernatant in 98uL of a mixture of ^{13}C, ^{15}N-labeled amino acids (1ng/uL, Aldrich, catalog number: 487910) containing 949 fmol/uL $^{13}C_3$, $^{15}N_1$-Ala, $^{13}C_6$, 186 fmol/uL $^{15}N_4$-Arg, $^{13}C_4$, 1500 fmol/uL $^{13}C_4$, $^{15}N_n$-Asx-$_{Asn}$, 1209 fmol/uL $^{13}C_4$, $^{15}N_n$-Asx$_{Asp}$, 648 fmol/uL $^{13}C_5$, $^{15}N_n$-Glx$_{Gln}$, 516 fmol/uL $^{13}C_5$, $^{15}N_n$-Glx$_{Glu}$, 1465 fmol/uL $^{13}C_2$, $^{15}N_1$-Gly, 41 fmol/uL $^{13}C_6$, $^{15}N_3$-His, 196 fmol/uL $^{13}C_6$, $^{15}N_1$-Ile, 522 fmol/uL $^{13}C_6$, $^{15}N_1$-Leu, 216 fmol/uL $^{13}C_6$, $^{15}N_2$-Lys, 8.1 fmol/uL $^{13}C_5$, $^{15}N_1$-Met, 255 fmol/uL $^{13}C_9$, $^{15}N_1$-Phe, 240 fmol/uL $^{13}C_5$, $^{15}N_1$-Pro, 410 fmol/uL $^{13}C_3$, $^{15}N_1$-Ser, 404 fmol/uL $^{13}C_4$, $^{15}N_1$-Thr, 191 fmol/uL $^{13}C_9$, $^{15}N_1$-Tyr, 210 fmol/uL $^{13}C_5$, $^{15}N_1$-Val in water. For sugar analysis, we diluted 2uL of the supernatant in 998uL of a 500 pg/uL sorbitol solution in water. The analysis was performed on a UHPLC-HESI-MS/MS (ultra-high performance liquid chromatography, Bruker Elite EvoQ Tri-ple quad-mass spectrometer equipped with a heated electrospray ionization) ion source. The samples were analyzed in muti-reaction-monitoring. Source parameters and the settings were followed by Schäfer et al., (2016). Raw data was integrated and calibrated by Bruker MS workstation program, and normalized by internal standard.

Supporting Information Legends

Supplementary tables

Table S1. Comparison of phenolamide and quinate conjugate metabolites measured by UPLC-TOF-MS in leaf and pith tissue elicited by *M. sexta* OS treatment and *T. mucorea* larval attack, respectively.

The leaf column shows that the comparison between leaf control samples versus leaf elicited samples by R of *M. sexta* treatment. The pith column shows that the comparison between pith control samples versus pith attacked samples by *T. mucorea* larva. Leaf metabolites data was reproduced from Onkokesung et al., 2012. nd indicates that no detected; ns means there were no significant between groups.

Class	Compound	m/z	Ion type	Main /frag	Mode	Leaf	Pith
Phenolamide	N-Coumaroylputrescine (two isomers)	235.143	$[M+H]^+$	main	positive	***	nd
Phenolamide	N-Caffeoylputrescine isomer (multiple isomers)	251.138	$[M+H]^+$	main	positive	***	nd
Phenolamide	N',N"-Di-caffeoylspermidine (multiple isomers)	470.229	$[M+H]^+$	main	positive	ns	nd
Phenolamide	Unknown pyrescine metabolite (two isomers)	347.196	$[M+H]^+$	main	positive	**	ns
Phenolamide	N',N"-Caffeoyl,feruloylspermidine (multiple isomers)	484.244	$[M+H]^+$	main	positive	**	***
Phenolamide	Unknown spermidine metabolite (two isomers)	566.286	$[M+H]^+$	main	positive	ns	nd
Phenolamide	Unknown spermidine metabolite (multiple isomers)	580.301	$[M+H]^+$	main	positive	*	ns
Phenolamide	N-Feruloylspermidine (multiple isomers)	322.212	$[M+H]^+$	main	positive	***	nd
Phenolamide	N-Feruloylputrescine (two isomers)	265.153	$[M+H]^+$	main	positive	***	nd
Phenolamide	N'N"-Di-feruloyl-spemidine (multiple isomers)	498.259	$[M+H]^+$	main	positive	**	nd
Phenolamide	N-Coumaroylspermidine (multiple isomers)	292.202	$[M+H]^+$	main	positive	***	nd
Phenolamide	N-Coumaroyl,caffeoylspermidine (multiple isomers)	308.195	$[M+H]^+$	main	positive	ns	nd
Phenolamide	N-Coumaroyl,caffeoylspermidine (multiple isomers)	454.233	$[M+H]^+$	main	positive	*	nd
Quinate conjugate	O-Coumaroylqunic acid (multiple isomers)	339.106	$[M+H]^+$	main	positive	ns	***
Quinate conjugate	Chlorogenic and (O-Caffeoylquinic isomers)	355.103	$[M+H]^+$	main	positive	ns	***
Quinate conjugate	O-Feruloylquinic acid (multiple siomers)	339.119	$[M+H]^+$	main	positive	*	***

Table S2. Primers used in this study

Gene name	Forward primer sequence	Reverse primer sequence
NaPMT	TCATTGGACCAAGATCGAG	TGGAAATTATGATAATTACTGCAGA
NaCHS	TTCACGTTTCAAGGCCCAA	TGCTCCATCAGCGAAAAGG
NaHQT	CCTCCTTTGCCACCAGGTTA	ATGTCGGGCCACGGATTAAA
NaJAZa	ATGACGATATTCTACGGCGG	TAAGTGAAGCTCGTCTCGCA
NaJAZb	ACACCAAATGCATCCACAAA	GACGCCGTTCTTCTTCTTG
NaJAZc	TACCTGCCTCAGGTCATTCC	GGAACCGCTGCTGACATTAT
NaJAZd	ACCGCAGTTTTGAACCAACT	ATTTGCCTTAGCTGCTGGAA
NaJAZe	CGCACTACACGTCGACAACT	CAGCGCTGTTAGTTGGAACA

NaJAZf	AGAGCTCCATTTTGGCTGAA	GTTGCTTCTCTTCTGTGCCC
NaJAZg	AAGTCGTCCGTTCTCAGGAA	TCCAGCTGCTAGATCCAGGT
NaJAZh	TCGAATTTCGTGCAGACTTG	TACAGCACTCTGACGAACGG
NaJAZi	TCATTCTGTGGCATGTTCGT	TGAAACTGCAGAGATGGTGC
NaJAZj	AGCTCAGGCTTATGCCTCCT	TCTGAAATTGGTGACCGGAT
NaJAZk	GATGCAACTCCCAATCTGGT	AAAGGGAGCTTGAAGCAACA
NaJAZl	GCTGGCAATTTTACCAGGAA	TTTGGTTCAGCTTCTTTGGG
NaJAZm	TTGTGGCAAGGTGAATGTGT	GCATGAGCACCATAGCAGAA
NaEFa	CCACACTTCCCACATTGCTGTCA	CGCATGTCCCTCACAGCAAAAC

Supplementary figures

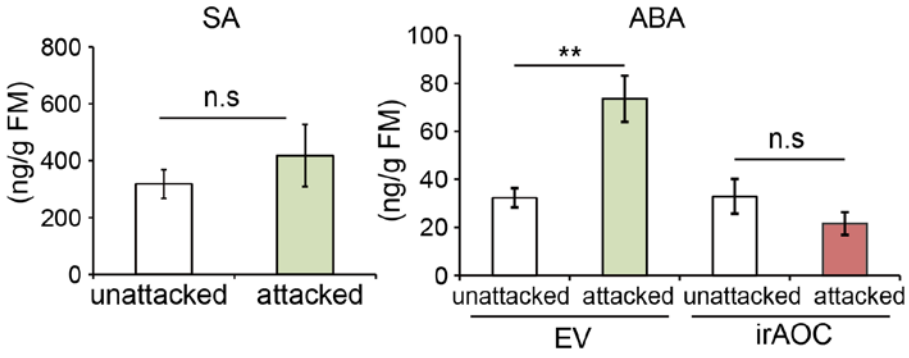

Figure S1. The levels of ABA and SA in attacked pith. Mean (± SE) levels of salicylic acid (SA) and abscisic acid (ABA) in control (unattacked) and attacked pith. SA levels in attacked pith were similar with those in control pith. ABA levels were significantly increased in the attacked pith. The level of ABA did not change between unattacked and attacked irAOC plants. (one-way ANOVA; **, $p < 0.01$; n=6). NS, not significant. EV, empty-vector transformed wild-type plants; irAOC, *allene oxidase cyclase* -silenced line.

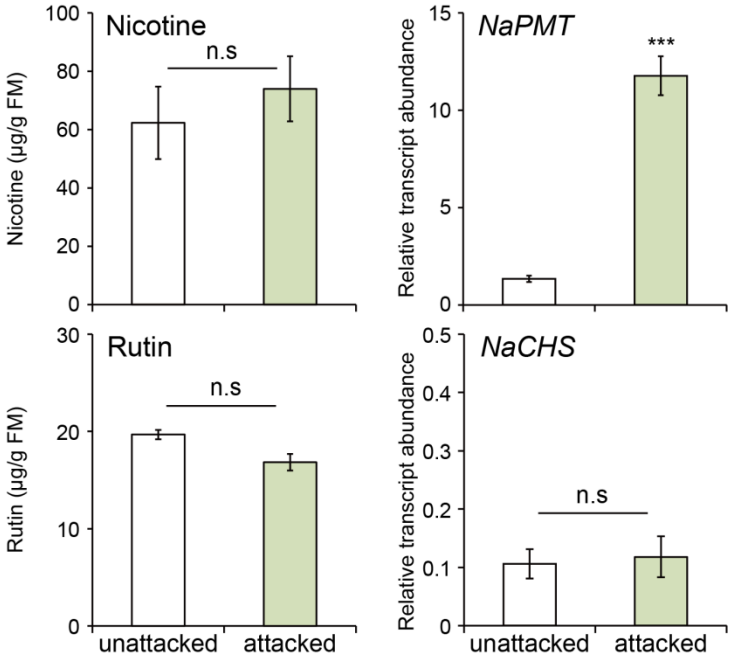

Figure S2. The levels of nicotine, rutin and transcripts of their related major biosynthetic genes in pith attacked by *T. mucorea* larvae. Mean (± SE) levels of nicotine, rutin and transcript abundance of *NaPMT* (putrescine N-methyltransferase), *NaCHS* (chalcone synthase), key biosynthetic enzymes for nicotine, and rutin in control and attacked pith three weeks after egg inoculation. Asterisks indicate significant differences among treatments (one-way ANOVA; ***, $p < 0.001$; n=6). FM, fresh mass; NS, not significant.

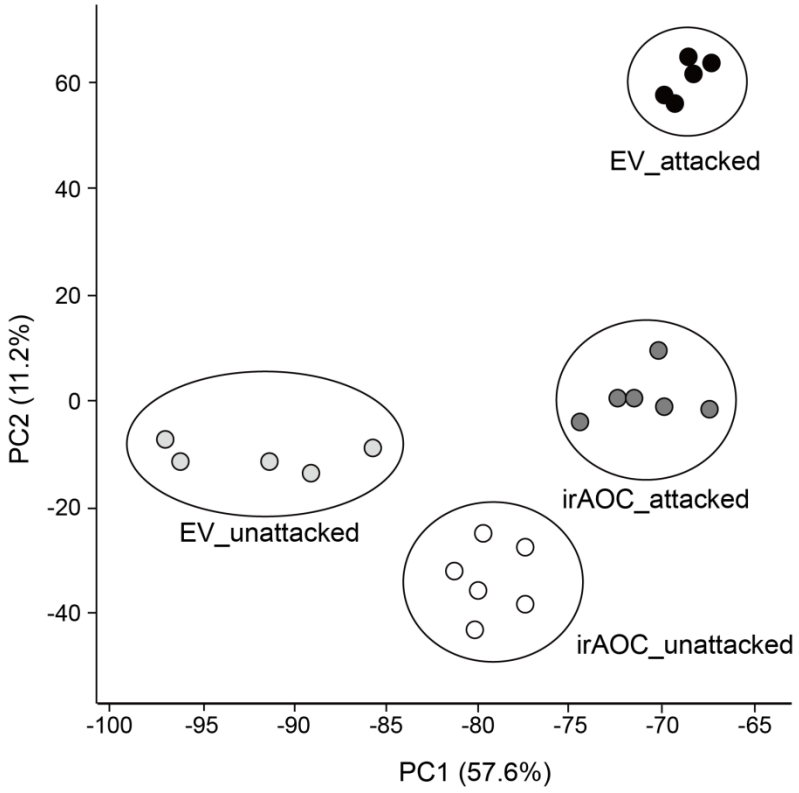

Figure S3. Principal component analysis (PCA) of untargeted metabolic profiles in control and attacked pith of EV and irAOC plants. We measured pith chemistry in EV and irAOC plants with and without egg inoculation. Samples were collected three weeks after continuous feeding after *T. mucorea* egg inoculation. We used 40% methanol-based extraction to extract pith metabolites and separated the metabolites using Dionex rapid separation liquid chromatography system. The separated metabolites were positively charged by electro spray ionization (ESI) and exact mass to charge ratio of ions was measured with a MicroToF (Time-of-Flight; Bruker Daltonics, Bremen, Germany). Raw data files were converted to netCDF format and processed by XCMS (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/Peak Alignment/xcms/) and CAMERA.Only peaks that were found in at least 75% of the replicates with absolute intensities higher than 5 megacounts s^{-1} were used in the analysis. Principal component analysis (PCA) was performed using MetaboAnalyst 3.0, following normalization by log transformation and Pareto scaling.

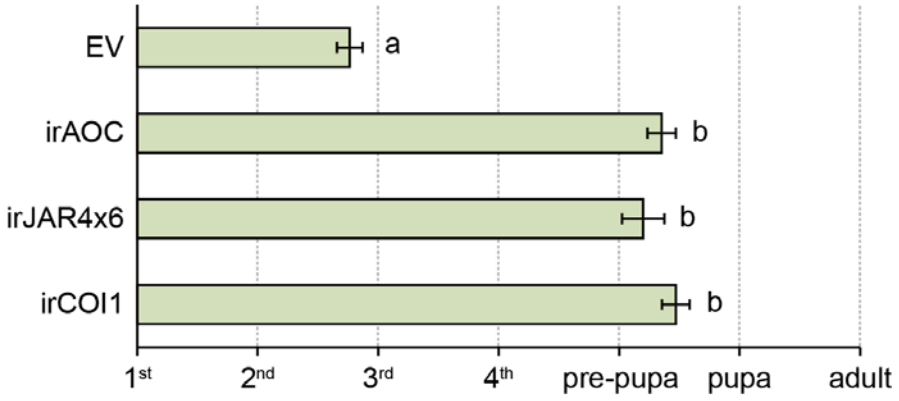

Figure S4. *T. mucorea* larvae perform better in the stems of transgenic plants impaired in JA-

biosynthesis, JA-Ile conjugation, or JA-perception than in EV plants. Developmental stages of larvae fed on EV, irAOC, irJAR4x6, or irCOI1 three weeks after the egg inoculation. *T. mucorea* larvae collected from irAOC, irJAR4x6 or irCOI1 developed faster than those in the stems of EV plants (one-way ANOVA followed by Tukey's HSD; $p < 0.05$; n=20). Different letters indicate significant differences among treatments.

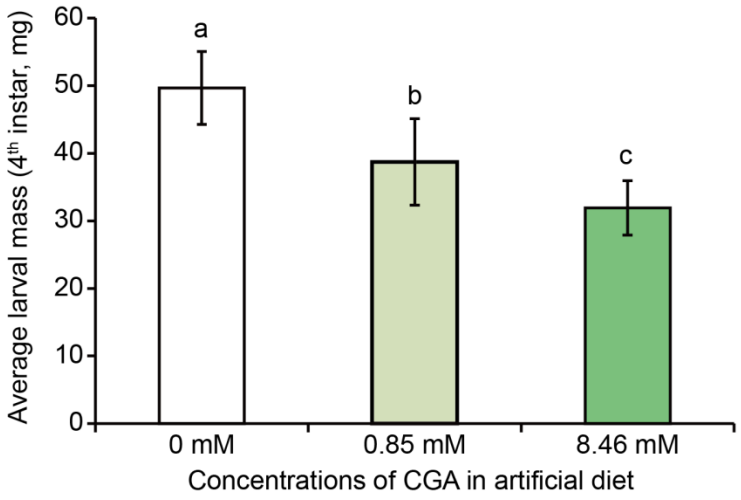

Figure S5. *T. mucorea* larva mass is negatively correlated with CGA concentrations in artificial diets Mean (±SE) mass of *T. mucorea* larvae fed artificial diet supplemented with different levels of CGA: 0mM, 0.85mM (typical levels of CGA in *N. attenuata* leaves), and 8.46mM (similar to the maximum CGA levels measured in attacked pith). Different letters indicate significant differences among treatments (one-way ANOVA followed by Fisher's LSD test; $p < 0.05$; n=20).

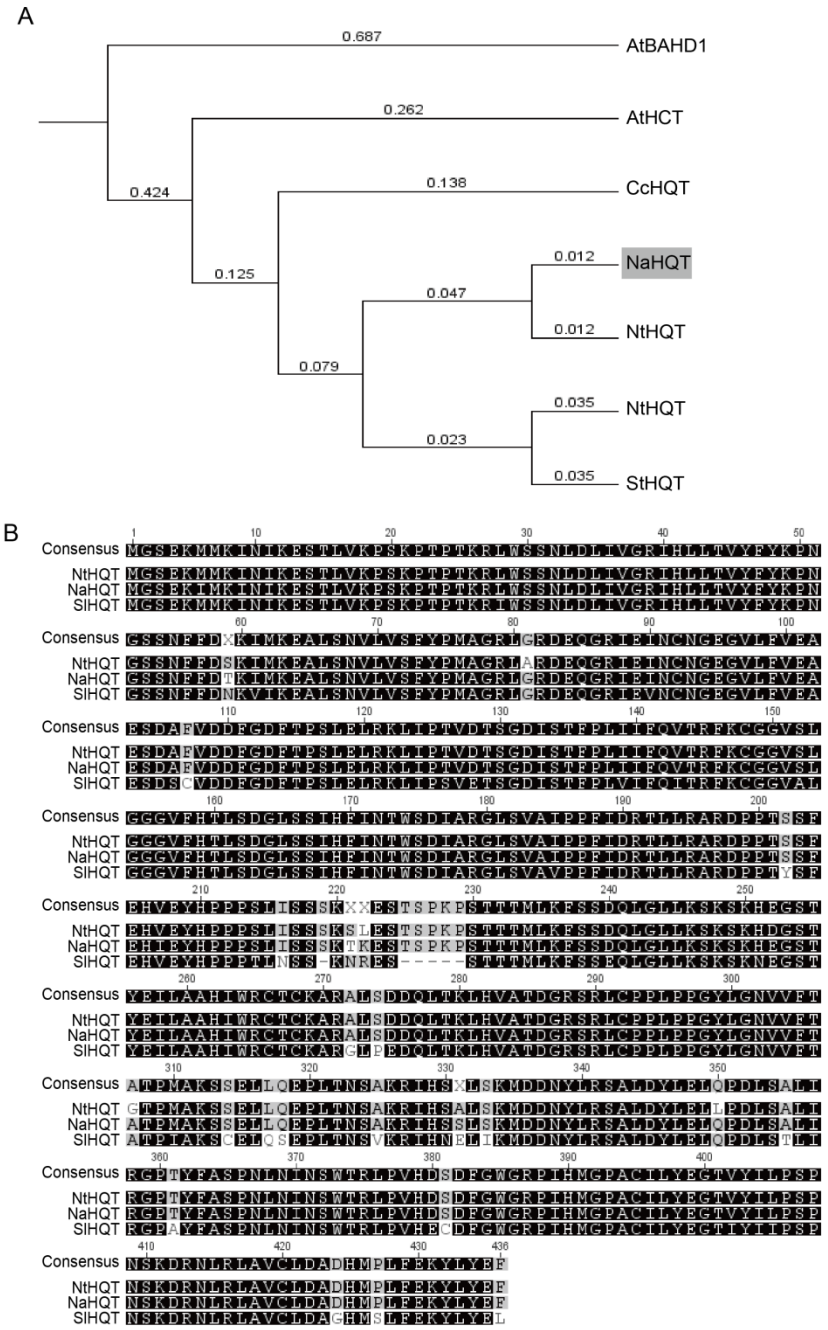

Figure S6. Phylogenetic trees and protein alignment of HQT genes. **(A)** Full-length amino acid sequences were aligned using the Geneious software. Unweighted Pair Group Method with the Arithmetic mean (UPGMA) was used to make the phylogenetic tree. The numbers represent the number of amino acid substitutions per site by applying the Jukes-Cantor model. *AtBAHD1*, another BAHD family acyltransferase, was used as the outgroup. *NaHQT* is highlighted in a grey background. **(B)** Full-length amino acid sequence was aligned using the Geneious software (At, *Arabidopsis thaliana;* Cc, *Coffea canephora;* Na, *Nicotiana attenuata;* Nt, *Nicotiana tabacum;* Sl, *Solanum lycopersicum;* St, *Solanum tuberisum*).

A

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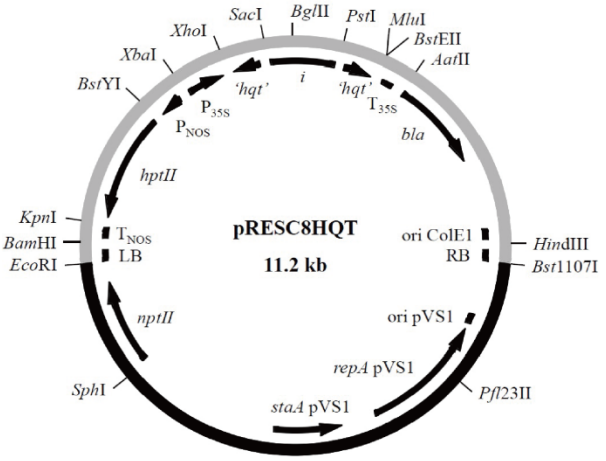
B

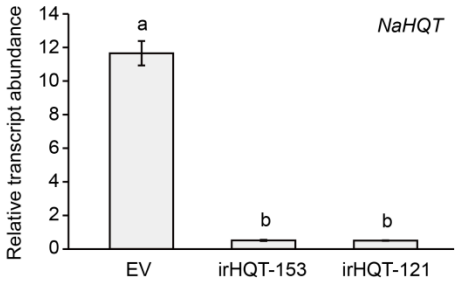


C

D

	A-13-153	A-13-121	Expected proportions
Hygromycin resistance	43	40	3
Hygromycin sensitive	17	12	1
p-value	0.551	0.749	

E



F

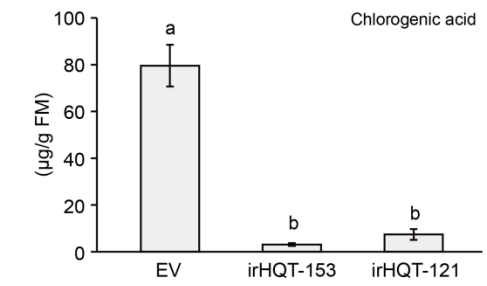


Figure S7. Generation of *NaHQT*-silenced *Nicotiana attenuata* plants. (A) Protein coding sequence of *NaHQT* gene. We used a 331bp region of the *NaHQT* gene for the gene silencing construct (red letters). **(B)** The pRESC8HQT vector containing inverted repeat elements of *NaHQT* gene used for *Agrobacterium tumefaciens*-mediated transformation and generation of stably silenced *N. attenuata* irHQT plants. **(C)** Southern blot analysis of stable transgenic irHQT line (irHQT-153) which was used for all experiments in this study. 10µg of genomic DNA was digested with BamHI and EcoRI enzyme and hybridized with a probe coding for the hygromycin resistance gene. **(D)** Two independent lines that showed 3:1 segregation ratios in the T1 generation were selected for the further experiments. **(E)** Relative transcript abundance of *NaHQT*, a key enzyme in CGA biosynthesis, in EV and two independent *NaHQT*-silenced lines (irHQT-153 and -121). Silencing efficiency of *NaHQT* gene was approximately 95% in the rosette-stage leaves (one-way ANOVA; $p < 0.05$; n=3). Leaves of rosette-

stage EV plants were used for RNA extraction. **(F)** Mean (±SE) levels of CGA in the leaves of EV, irHQT-153 and -121 plants (one-way ANOVA; $p < 0.05$; n=6). Different letters indicate significant differences among treatments.

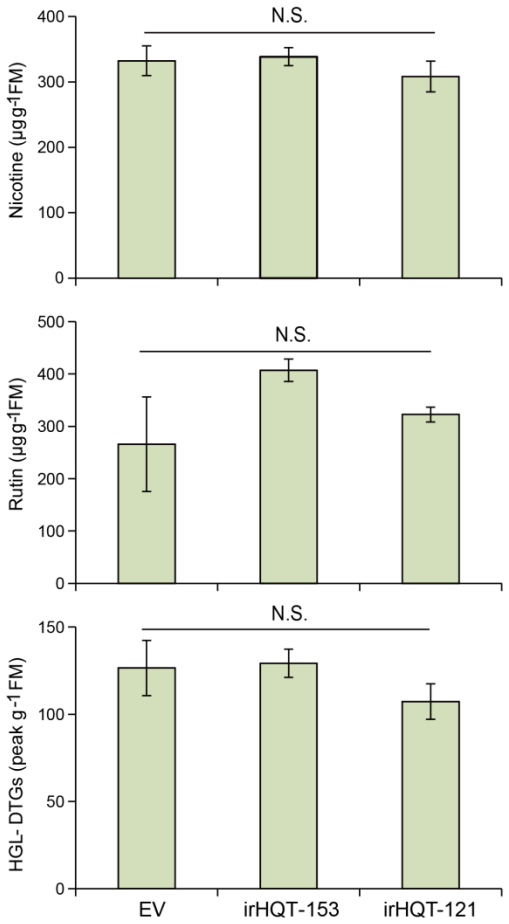


Figure S8. Levels of nicotine, rutin, and17-hydroxygerany linalool diterpenoid glycosides (HGL-DTGs) in irHQT plants. Silencing *NaHQT* does not affect the levels of nicotine, rutin, and HGL-DTGs in rosette-stage leaves. Leaves from EV, irHQT-153, and irHQT-121 plants were harvested without *T. muocrea* egg inoculation. Nicotine, rutin and HGL-DTGs did not differ in EV, irHQT-153, and irHQT-121 plants (one-way ANOVA; $p = 0.246$, $p = 0.144$; $p = 0.381$ respectively, n < 10). FM, fresh mass; NS, not significant.

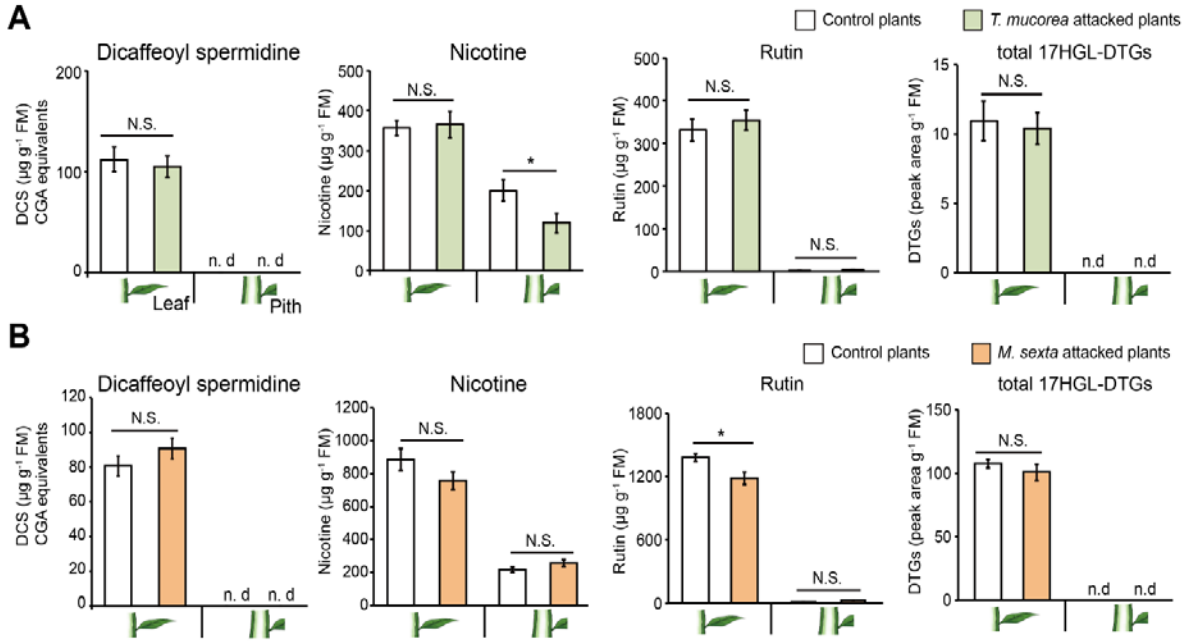


Figure S9. **Levels of dicaffeoyl spermidine (DCS), nicotine, rutin, and HGL-DTGs of leaf and pith from control and *T. mucorea* larva attacked plants or leaves elicited by wounding (W) + oral secretion (OS) of *M. sexta* caterpillar. (A)** Induced pith defense did not affect the levels of DCS, nicotine, rutin, and HGL-DTGs in systemic leaves by *T. mucorea* larva attack three weeks after egg inoculation (one-way ANOVA; $p = 0.986$; $p = 0.343$; $p = 0.794$; $p = 0.09$; respectively; n=6). The levels of nicotine, rutin, and HGL-DTGs of the pith also were not induced by *T. mucorea* larva attack. DCS and HGL-DTGs were not detected in the pith. The level of nicotine of *T. mucorea* larva attacked pith was lower than that of control plants, rutin did not change by *T. mucorea* larval attack (one-way ANOVA; $p = 0.01$; $p = 0.999$; respectively; n=6). **(B)** In case of *M. sexta* attack in the local leaf and pith after three days W+OS treatment, the level of DCS, nicotine and HGL-DTGs of local leaf did not change between *M. sexta* attacked plant and control plant (one-way ANOVA; $p = 0.741$; $p = 0.196$; $p = 0.06$; respectively; n=6).The level of rutin in *M. sexta* attacked leaf was lower than control leaf (one-way ANOVA; $p = 0.02$; n=6). Induced leaf defense by *M. sexta* did not significantly affect the levels of DCS, nicotine, rutin and HGL-DTGs (one-way ANOVA; $p = 0.913$; $p = 0.999$; respectively; n=6). NS indicates not significant; nd not detected.

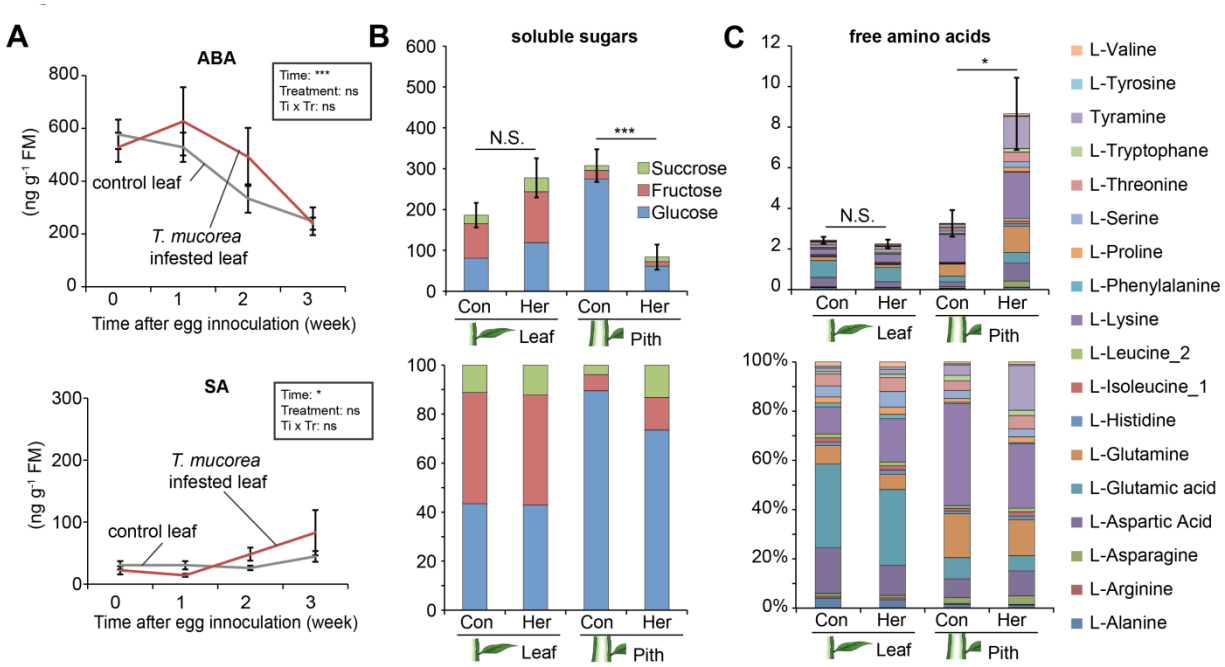


Figure S10. Phytohormones (ABA and SA) and primary metabolites (soluble sugars and free amino acids) in the leaf and the pith from *T. mucorea* larva attacked plants. (A) Mean (±SE) levels of ABA and SA in pith by *T. mucorea* larva attack after 1-3 weeks after egg inoculation (Her). Both levels of ABA and SA in the pith were not significantly induced by *T. mucorea* larva attack. **(B, C)** To evaluate systematic changes in nutrient levels after *T. mucorea* attack, we collected attacked pith and systemic leaves 3 weeks after the egg inoculation to analyze soluble sugars and free amino acid. **(B)** Total amounts and composition of soluble sugars (sucrose, fructose, and glucose) in leaf and pith part by *T. mucorea* larva attack after three weeks egg inoculation. Induced pith defense did not affect the composition and proportion of soluble sugars in systemic leaves. However, the composition and proportion of soluble sugars of *T. mucorea* attacked pith were significantly decreased compared to the pith of control plants. **(C)** Total amounts and composition of free amino acids in leaves and pith part of *T. mucorea* attacked plants three weeks after egg inoculation. Induced pith defense did not change composition and proportion of free amino acids in systemic leaves. In contrast, the composition and proportion of free amino acids in *T. mucorea* larva attacked pith were highly increased compared with those of control piths.

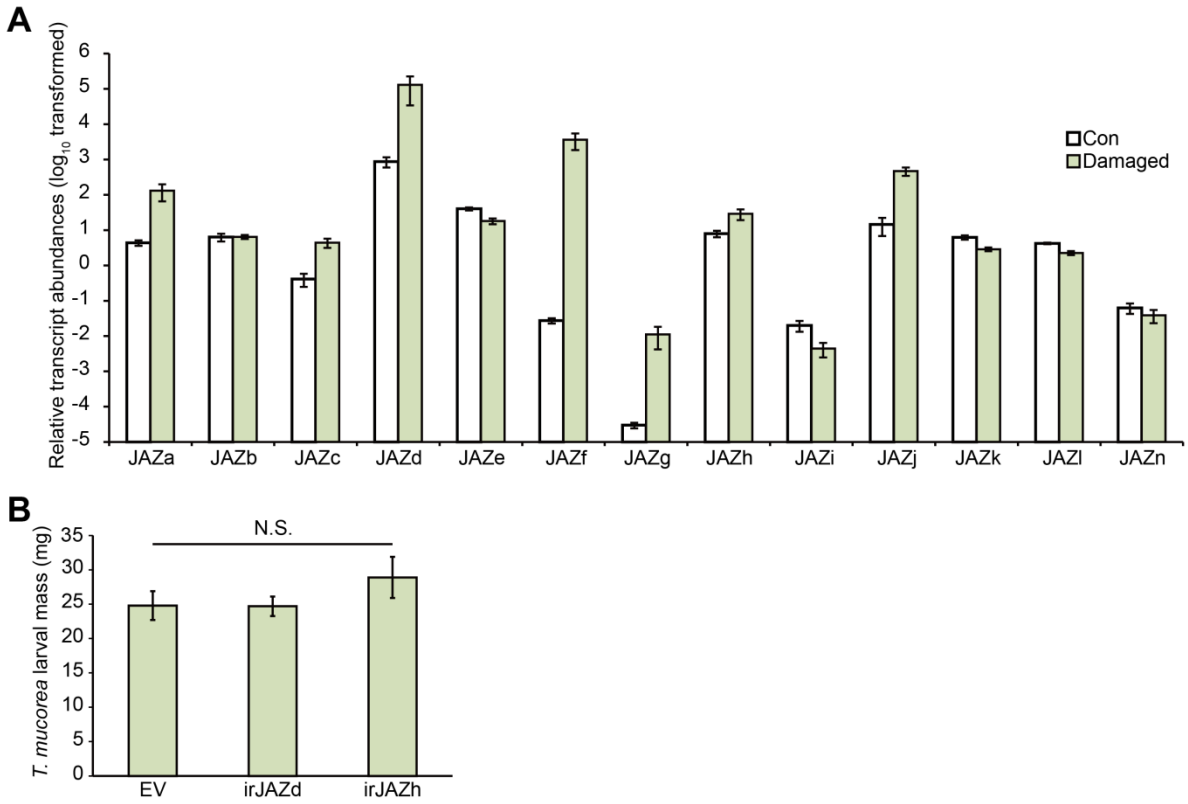


Figure S11. Transcript abundance of JAZs gene in the pith and mass of *T. mucorea* larvae fed irJAZd, irJAZh, or EV plants. (A) The expression of 13 JAZs genes in attacked pith by *T. mucorea* larva three weeks after egg inoculation (n=3). **(B)** Larvae were collected after three weeks of continuous feeding after egg inoculation in EV, irJAZd, and irJAZh plants. There were no significant differences between EV and irJAZs plants. NS indicates not significant (one-way ANOVA followed by Fisher's LSD test; $p = 0.330$; n=20).

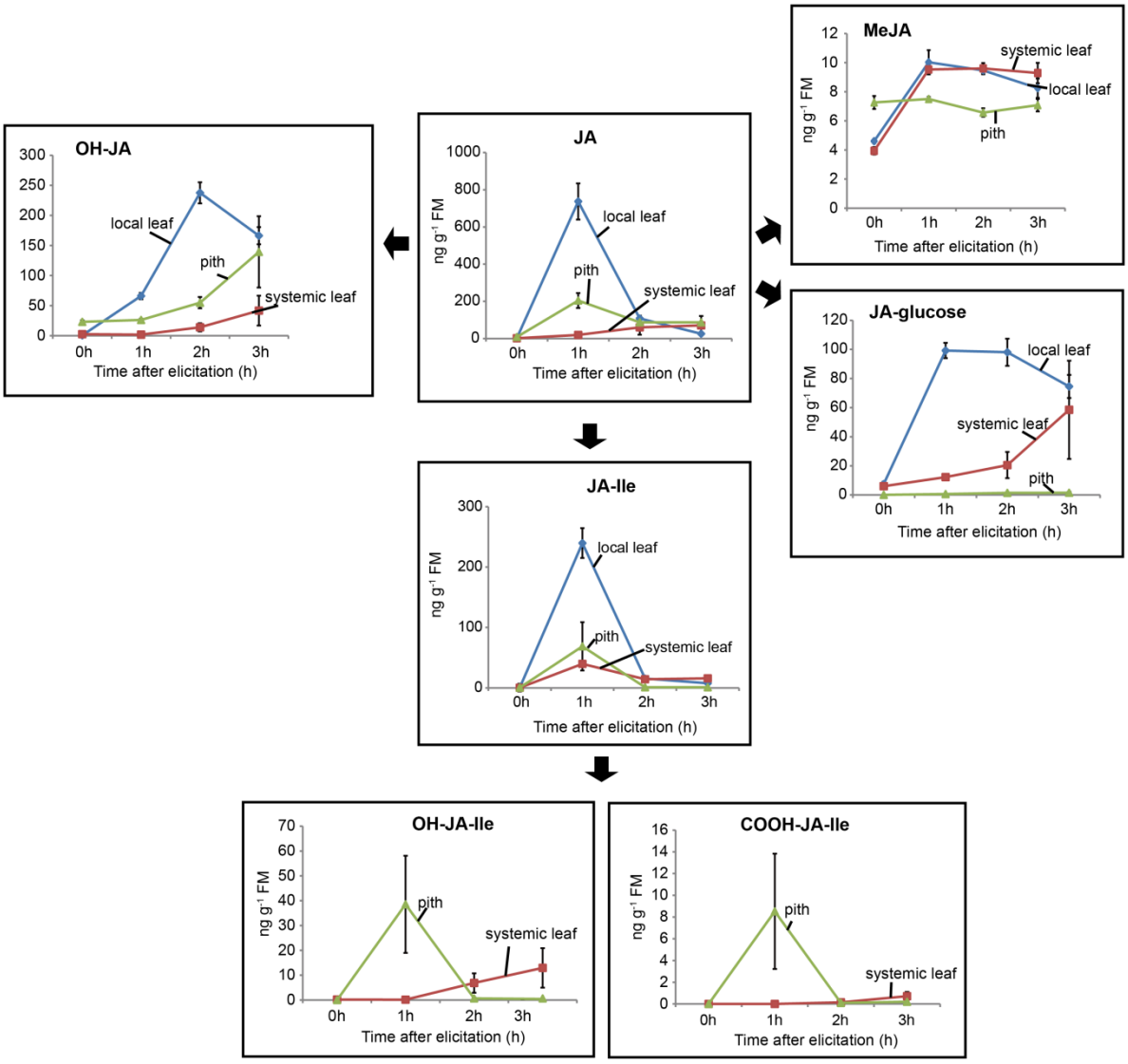


Figure S12. Jasmonate metabolism in the leaf and pith in response to *M. sexta* elicitation JA-Ile levels are also transiently activated in the pith as they are in leaves, but a lower degree. To understand the differential jasmonates metabolism between in the leaf and in the pith, we measured JA metabolites from the same samples as in **Figure 5b**. As expected, inactive forms of JA-Ile (OH- and COOH-JA-Ile) more rapidly increased in the pith than in systemic leaves. In addition, all other three jasmonate metabolites accumulated differently in the pith comparing with the leaf.

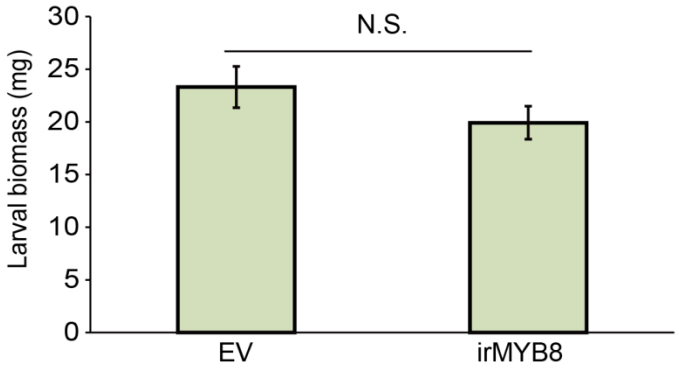


Figure S13. Mass of *T. mucorea* larvae fed EV and irMYB8 plants. Larvae were collected after three weeks of continuous feeding after the egg inoculation. *T. mucorea* larvae mass was not significantly different between EV and irMYB8 plants. NS indicates not significant (one-way ANOVA followed by Fisher's LSD test; $p = 0.334$; n=20).

Supporting References

Onkokesung, N., Gaquerel, E., Kotkar, H., Kaur, H., Baldwin, I.T. and Galis, I. (2012) MYB8 Controls Inducible Phenolamide Levels by Activating Three Novel Hydroxycinnamoyl-Coenzyme A:Polyamine Transferases in Nicotiana attenuata. *Plant Physiol.*, **158**, 389–407.

Schäfer, M., Brütting, C., Baldwin, I.T. and Kallenbach, M. (2016) High-throughput quantification of more than 100 primary- and secondary-metabolites, and phytohormones by a single solid-phase extraction based sample preparation with analysis by UHPLC-HESI-MS/MS. *Plant Methods*, **12**, 30. Available at: http://plantmethods.biomedcentral.com/articles/10.1186/s13007-016-0130-x.

4. General discussion

The main aim of my dissertation was to investigate the adaptive strategies of the plant under spatially and temporally variable environments. The plant circadian clock contributes to generating fine temporal plasticity, but the functional consequence of the circadian clock poorly described in nature. In the **manuscript I**, I have shown the function of the plant circadian clock in photosynthesis. The plant circadian clock has been expected to be important anticipating light coming during the dawn, but I found that the plant circadian clock played a role to ignore the light when they are not expected in the field. As well as abiotic factors, the insect communities also are temporally variable. I have shown that different emission patterns of sequiterpenes and GLVs can work as the fail-safe mechanism to maintain the robustness of the plant indirect defense under temporally various insect communities (**manuscript II**) and the plant circadian clock contribute time-dependent tri-trophic interactions (**manuscript III**). I further have investigated that the circadian rhythm of the floral volatile (**manuscript IV**) and the how plant circadian clock affects the plant-pollinator interactions (**manuscript V**). Since ecological interactions in different plant tissues are not homogeneous, the spatial plasticity of plant responses is also critical in nature. In **manuscript VI**, I have shown that plants have tissue-specific inducible defense in response to spatially-separated herbivores. Although plants use same JA signaling to defend the leaf and the stem herbivore, there is no negative effect between two spatially-separated herbivores because these tissue-specific defenses are localized each tissue.

4.1. Testing the functional consequence of temporal and spatial plastic response

The insect communities in the native habitat of *N. attenuata* are highly variable, and plants also have the temporal plasticity in response to herbivore attacks. Plants generate time-dependent responses using external cues (e.g. herbivore attack, light, and temperature) as well as internal factors (e.g. circadian clock) (**Figure 1A**). Temporal plasticity of plant behaviors is important for the plant-insect interactions (**manuscript II, III and V**), and it may ultimately increase the Darwinian fitness of plants (Loughrin *et al.*, 1994). To understand the ecological consequences of temporal plasticity in plant behaviors, here I have mainly used two different gene-silenced plants to test the functional impacts of the temporal plastic response of plants. First, I have silenced the biosynthetic genes of plant volatiles (**Figure 1B**). By combining

with time-series data of insect activities, plant volatiles, and the interaction between plant and insect, I have tested the time-dependent functionality of herbivore-induced plant volatiles. Interestingly, functions of each plant volatiles are not identical, and the different rhythmicity of plant volatiles work as "fail-safe system" in nature (**manuscript II**).

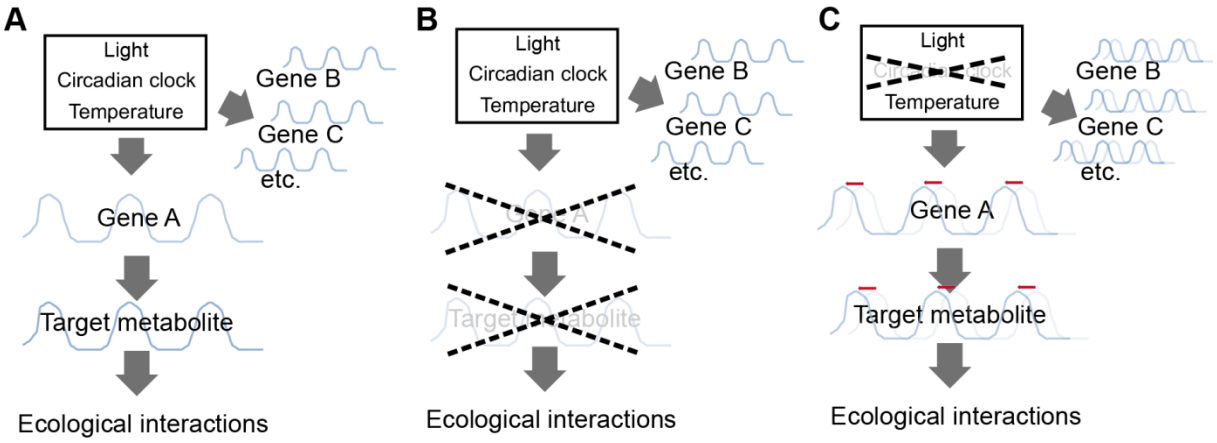


Figure 1. Methodological approaches to manipulate the rhythmicity of plant behaviors. (A) Schematic figure of wild-type regulation flow (B) Silencing the biosynthetic gene of the target metabolite (C) Silencing the circadian clock gene.

I further have tried to manipulate the rhythmicity of plant rhythmic behaviors. Many of diurnal responses are light-dependent. Thus, several photoreceptors play an important role for diurnal rhythms in the plant (Bae & Choi, 2008).The circadian clock also generates diurnal rhythms in the plant. For instance, *toc1* mutant has a short period, and *ztl* mutant has a longer period in *A. thaliana* under the free-running environment (Dodd *et al.*, 2005). Although both mutants have the same period and phase under the diurnal environment both in *A. thaliana* and *N. attenuata* (Dodd *et al.*, 2014; Yon *et al.*, 2016; Joo *et al.*, 2017), silencing *LHY* (irLHY) in *N. attenuata* also shows the phase-shifted phenotypes like *lhy/cca1* double mutant in *A. thaliana* under the diurnal environment (Alabadí *et al.*, 2002; Mizoguchi *et al.*, 2002; Yon *et al.*, 2016). It allows me testing the function of rhythmicity, especially the phase of the plant rhythm (**Figure 1C**). I have found that the gas exchange (**manuscript I**), accumulation of green leaf volatiles (**manuscript III**), and emission of benzyl acetone (**manuscript IV**) are regulated by the circadian clock; phases of their rhythms are shifted in irLHY plants. In further studies, I have tested the function of rhythmicity in plant volatiles

(**manuscript III and V**). However, since the circadian clock functions not only keeping the rhythmicity of plant metabolism and the manipulate the responsiveness environmental signals, the direct manipulating the circadian clock components are usually pleiotropic. Therefore, to investigate the fitness consequence of the particular temporal plasticity, it requires understanding the functionality of the circadian clock and alternative approaches for the manipulation.

4.2. Functions of the plant circadian clock: not just for the rhythmicity

I manipulated the transcript levels of the circadian clock genes to test the ecological consequence of temporal plasticity in plant metabolism. Although many circadian clock components have been identified and their physiological functions are relatively well-characterized, the functional consequence of nature was not well investigated. The functional consequence of the plant circadian clock is more beneficial in 24h T-cycle than other T-cycles (20T, 28T), because the circadian clock system has been evolved to match plant physiology with the 24h light-dark cycle (Ouyang *et al.*, 1999; Dodd *et al.*, 2005). However, many papers have characterized clock mutants under the free-running conditions, because rhythmic alterations of clock mutants are exaggerated without *zeitgeber*. This manipulation is useful to know how plant circadian clock maintains the rhythmicity (Sanchez & Kay, 2016), but those approaches cannot give the adaptive value of the clock regulation because *zeitgeber* is prevailing in nature (de Montaigu *et al.*, 2015; Joo *et al.*, 2017). Moreover, the phenotypes of animal clock mutants in nature also did not show the hypothesis which set up based on laboratory (Daan *et al.*, 2011; Vanin *et al.*, 2012). However, the function of the plant circadian clock in nature is largely untested in the native habitat.

Although we manipulate the circadian clock components to alter the rhythmicity of plant behavior, the circadian clock shapes complex regulatory systems (interlocked multiple feedback loops), and the systematic approach is required to understand how the plant circadian clock works (Fogelmark & Troein, 2014; Foo *et al.*, 2016). Without understanding the characteristics of the circadian clock system, it is almost impossible to investigate the function of the circadian clock. There are many different ways to generate the rhythmicity in the living organism, e.g. Goodwin oscillator, repressilator, amplified negative feedback oscillator, fussenegger oscillator, smolen oscillator, variable link oscillator, and metabolator

(Purcell *et al.*, 2010).

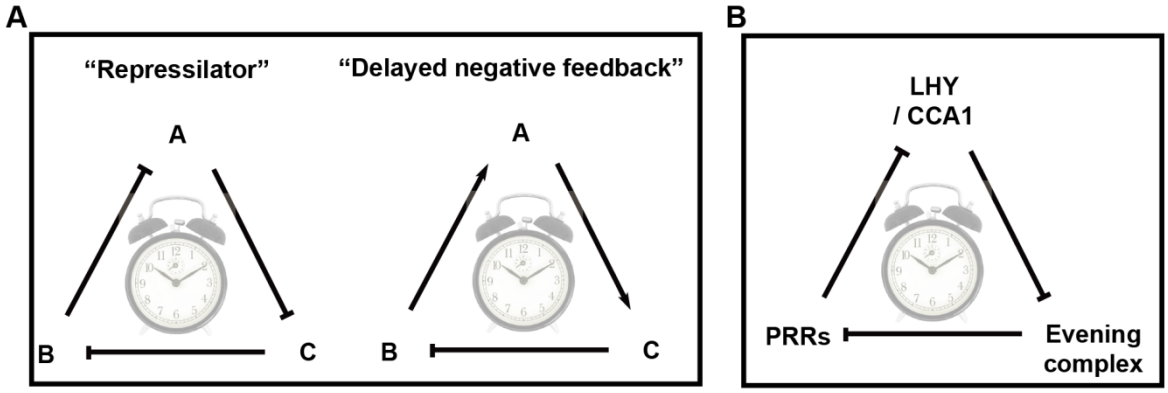


Figure 2. **Schematic models of the oscillators.** (A) A basic network structure of the transcription circuit of the circadian clock of the living organisms. (B) The core structure of the plant circadian clock is shown to include a repressilator, a three-inhibitor ring oscillator.

Many chronobiologists assumed that the circadian clock system conserves in the living organisms (Doherty & Kay, 2010), but the clock system in plants differs from animal clock system. The animal clock system was identified earlier than the plants, and the central oscillator of the animal clock consists of a morning element (a repressor) and an evening element (an activator), which formed the delayed negative feedback (**Figure 2A**) (Hogenesch & Ueda, 2011). In the plant circadian clock, the morning element (LATE HYPOCOTYL ELONGATED, LHY) was identified first (Schaffer *et al.*, 1998) and it worked as a repressor. Later, the evening element was identified (TIMING OF CAB2 EXPRESSION 1, TOC1). Initially, TOC1 described as an activator, because LHY transcript abundances in *toc1* mutant decrease and TOC1 transcript abundances in the *lhy* mutant increase (Alabadı *et al.*, 2001). However, TOC1 also works as a repressor (Huang *et al.*, 2012). Later, LHY reported as a potential activator of PRR genes, but a recent study showed that LHY did not play a role as an activator (Adams *et al.*, 2015). Although the few of activators in the plant circadian clock system have identified (Hsu *et al.*, 2013), the plant clock mainly uses the repressilator to keep the rhythmicity (**Figure 2B**) (Foo *et al.*, 2016).

Table 1. Circadian clock components in different organisms

Kingdom	species	Activator	Repressor	Ratio between repressor and activator
Plant	*Arabidopsis thaliana*	RVE8, NOX	LHY, CCA1, PRR5, PRR7, PRR9, ELF3, ELF4, GI, ZTL, LUX	6:1
Animal	*Mus musculus*	BMAL1, BMAL2, CLOCK NPAS2, DBP, TEF, HLF RORa, RORb, RORc	CRY1, CRY2, DEC1, DEC2, PER1, PER2, PER3, E4BP4, RevErbAa, RevErbAb	1:1
Animal	*Drosophila melangaster*	CYCLE, CLOCK	PERIOD, TIMELESS CRYPTOCHROME	3:2
Fungi	*Neurospora crassa*	WC1, WC2	FRQ, FRH	1:1
Bacteria	*Synechococcus* sp.	KaiA	KaiB, KaiC	2:1

Interestingly, the plant circadian clock contains fewer activators than the animal circadian clock, so the ratio of the number of activator to the number of repressors strongly differ between the plant and the animal (**Table 1**) (Hogenesch & Ueda, 2011; Foo *et al.*, 2016). Theoretically, positive feedback loops and negative feedback loops have different characteristics. A positive feedback loop **enhances** or **amplifies** changes (i.g. it makes a system away from its equilibrium state), but a negative feedback loop tends to dampen or **buffer** changes (i.g. it holds a system to some equilibrium state, so it makes the system more stable). Even in the same repressilator an additional positive feedback loop and a negative feedback loop significantly affect the robustness of the circadian clock system (Tsai *et al.*, 2008). It suggests that the characteristics of the plant circadian clock system are different compared to the animal clock, so the function of the plant circadian clock is potentially different.

Also, the major components of the circadian clock are not conserved in living organisms (Song *et al.*, 2010). The most reliable hypothesis of circadian clock evolution is that most of the living organism share similar clock system, rather than component, as transcriptional-translational feedback loops (Harmer, 2009) and it is most likely the consequence of convergent evolutions to generate the 24h of endogenous rhythm coping with rotating Earth. The fact that components are not preserved in the circadian clock of living organisms suggests that biological circadian clocks were not engineered but evolved through the process of evolutionary tinkering by natural selection and likely explains why the components of clocks differ among different taxa (Doherty & Kay, 2010; Rosenblum *et al.*,

2014). The circadian clock may increase temporal plasticity in response to an external and internal signal (or perturbations), and this temporal plasticity can improve the robustness of biological phenomenon (Kitano, 2007; Tsai *et al.*, 2008). Particularly, the plant circadian clock may play a role as "the filter (or buffer)" of various environmental cues because the plant circadian clock includes more repressors than the animal circadian clock.

Currently, the function of the circadian clock is known to "anticipating" or modulating responsiveness in response to external stimuli (= '*gating*') (**Figure 3**). The major difference between "anticipating" and "gating" is the presence or absence of the external stimuli. For the "anticipating," the circadian clock uses the external stimuli only as an entrainment cue, but the circadian clock also modulates the responses to external stimuli for the "gating." One of the interesting conclusions of my dissertation is "anticipating" and "gating" is difficult to disentangle in the plant clock function (**manuscript 1**). Therefore, I think it is necessary to use the broader terminology to describe the functionality of the plant circadian clock for explaining both gating and anticipating together. As I described above, the repressors are more dominant in the plant circadian clock, and the clock-regulated pattern is the cupidate wave which is different with the animal circadian clock (Foo *et al.*, 2016). These suggest that the plant circadian clock may "expect" the environmental changes not anticipate or gate.

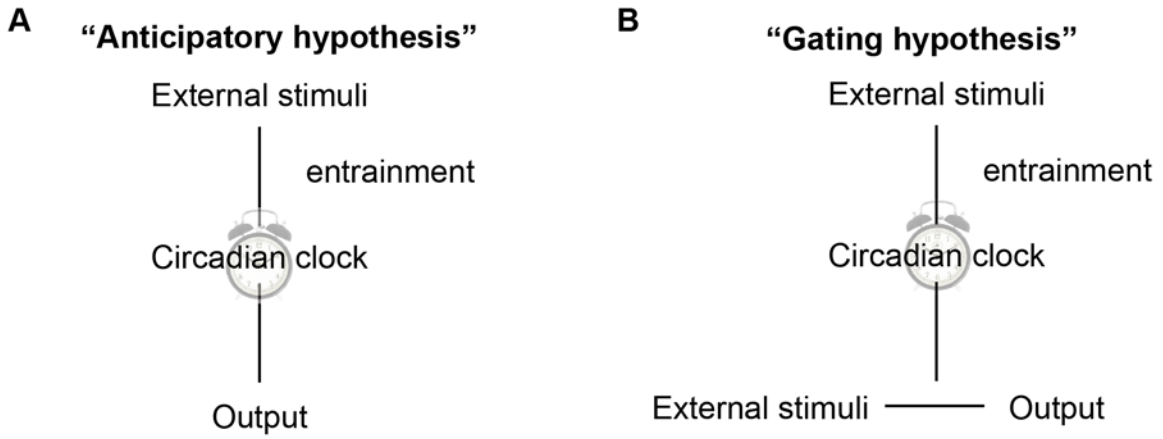


Figure 3. **Two hypothesis explaining how the circadian clock controls functional consequences.**

4.3. The function of the circadian clock is context-dependent

Variations (or heterogeneity) in ecological interactions are often described as context dependency (Chamberlain *et al.*, 2014). The light period is predictable, but light intensity is highly unpredictable (**manuscript I**). Plant-light responses can be different in the different context. Plant-insect interactions are even more complex. Therefore, the circadian clock should function differentially in every ecological interaction. Perhaps synchronization via the circadian clock is more common in mutualistic interactions; the pollinator activity well synchronized with the circadian-regulated floral volatile (**manuscript IV and V**), but the herbivore activity was not synchronized with the circadian-regulated herbivore-induced plant volatiles (**manuscript II and III**). The evolutionary strategy between plants and herbivores is a diffuse arms race: each side responds to selection pressure for counter-adaptation from the other side (Fox, 1981). For example, plants are thought to diversify their production of defensive metabolites in response to herbivore adaptation to the older defenses (Speed *et al.*, 2015). Thus, if plants have developed rhythmic traits to synchronize with herbivore behavior, the herbivore may experience selection pressure to change the behavior and escape the synchronization. However, if two species have mutualistic interactions (e.g. pollinator-plant), both sides could benefit from the synchronization, and this may be one way in which mutualistic interactions increase the stability of communities (Georgelin & Loeuille, 2014). Therefore, I would predict the clock regulations in plant-herbivore interactions are less stable than those in plant-pollinator interactions.

Although the clock regulation in plant-herbivore interactions is likely less stable in the evolutionary perspective, there are still clear evidence, showing that the circadian clock regulates plant resistance metabolites and their functional consequences (Goodspeed *et al.*, 2012, 2013). If feeding activities of major herbivores are predictable, circadian regulated plant defense responses could be beneficial. However, we need to interpret carefully about the circadian-regulated plant defense in the current literature. Although previous research has shown that basal level of the glucosinolates synchronized with the herbivore feeding activity, glucosinolate is not constituent defense metabolites. These group of metabolites needs to be activated by the enzyme after herbivore attack (Howe & Jander, 2008). Therefore, the functional consequence of the circadian clock in the plant-herbivore interaction also requires considering the changes in defense metabolites after the herbivore attack. However, it is still largely unknown whether induced (or activated) glucosinolates levels also synchronize with the feeding activity of herbivores. Moreover, the robustness of the plant circadian clock often is decreased by the environmental factors, e.g. cold (Bieniawska *et al.*, 2008), fungal

infection (Wang *et al.*, 2011). As phytohormones play important roles in response to the environmental signal, exogenous treatments of phytohormones also heavily decrease robustness of the plant circadian clock (Hanano *et al.*, 2006). Herbivory changes the levels of transcriptome and metabolome in plants (Halitschke *et al.*, 2001, 2003; Gaquerel *et al.*, 2009), so herbivory could affect plant circadian clock as well. I found that the herbivore damage strongly alters the transcript abundances of circadian clock components and clock-regulated genes (unpublished data). This result suggests that under the herbivore attack, plant circadian clock cannot work normally. Therefore, current assumptions (Goodspeed *et al.*, 2012, 2013) about the role plant circadian clock in plant direct defense may need to be strongly revised. The role of the circadian clock in the herbivore resistance can separate into three phases: before herbivore attack, early phase after herbivore attack, and late phase after herbivore attack. The role of the circadian clock may be greater at the first phase, and the importance may decrease as time goes by because the herbivore attack strongly disrupts the clock system.

4.4. Alternative manipulations to test the fitness consequence of the spatiotemporal plasticity

In **manuscript II, III** and **V**, I have examined the functional consequence of temporal plasticity in plant volatiles. Gene-based deletion mutants or transgenic plants haven been successfully used to understand the adaptive evolution of existing gene. However, there are some limitations in the gene-bases genetic manipulations. First, mutants and transgenic plants often cause pleiotropic effects, which is not related to the questions. For example, the circadian clock controls flower opening, flower movement, and floral scent emission in *N. attenuata* (**manuscript IV**) and these three floral behaviors were early-shifted in *NaLHY*-silenced plants. Therefore, I was not able to disentangle the effects of all these traits on the fitness consequences during my PhD course. Moreover, to test the adaptation, it requires more long-term experiments to measure the Darwinian fitness parameters, e.g. seed numbers. However, silencing the specific biosynthetic gene cannot disentangle whether the fitness consequence is caused by the particular metabolite itself or rhythmicity of that metabolites.

Then, how we can test the fitness consequence of spatiotemporal plastic responses of plants? The differential gene expression can mediate temporal and spatial plastic responses. Indeed, many genes are differentially expressed at temporal and spatial scales in a single

organism and it may for not wasting energy for making unnecessary proteins. Complex gene regulation programs depend on recognition of specific promoter sequences (*cis*-acting factors) by transcriptional regulatory proteins (*trans*-acting factors) which are regulator-gene interactions (Lee *et al.*, 2002). Although complex genetic regulations can be crucial for fine ecological interactions, the ecological consequences of the complex gene regulations are largely untested. Therefore, manipulating the "gene regulatory information" instead of the "gene itself" may allow testing the fine ecological questions, e.g. spatiotemporal plasticity of plant responses.

The number of genes was thought to be the proper indicator of the biological complexity (Szathmáry *et al.*, 2001). Before the whole genome sequencing projects started, many scientists expected that the mammalian species might have much more genes than other model species, e.g. *Drosophila melanogaster*, *Caenorhabditis elegans, Arabidopsis thaliana*, etc (Bird, 1995). This expectation based on the degree of the biological complexity; the natural selection does not directly guarantee this, but certain lineage increases the complexity (Szathmáry *et al.*, 2001). Unexpectedly, the nematode worm has 18,000 genes, and the human has a similar number of genes; currently, the scientist reports that human has 21,000 genes (Taft *et al.*, 2007). The many mammalian organisms do not contain the "expected high" numbers of genes, which has been termed the G-value paradox or N-value paradox (Hahn & Wray, 2002). To solve the paradox, many biologists have highlighted the importance of regulatory information in genome, e.g. epigenetics, alternative splicing events (Jaenisch & Bird, 2003) and promoter regulation (Carroll, 2001). It suggests that the biological complexity can be better explained by the variety of networks rather than total gene numbers. The complex regulations of the genes have been investigated in molecular biology field for a long time (Civelek & Lusis, 2013). But, the regulatory information is also relevant for ecological questions because most of the ecological interactions are highly context-dependent (Chamberlain *et al.*, 2014). Therefore, it is necessary to regard the regulatory module as the functional unit like the gene.

As described above, the temporal plastic responses cannot be tested by silencing the biosynthetic gene of the particular metabolite because temporal plasticity is the information about the regulation not the existence of the metabolites. Although the regulatory information in coded in the transcription factors, e.g. the plant circadian clock, the consequence of the knock-out or –down the transcription factors are too pleiotropic to test the adaptation. By

manipulating the information in the promoter region (*cis*-regulatory element) (**Figure 4A**), I can test the functional significance of the regulation. For instance, nicotine is well-studied plant resistance traits (**Figure 4B**) (Steppuhn *et al.*, 2004). Defensive roles of nicotine have been tested in different ways, e.g. genetic manipulations (Voelckel *et al.*, 2001; Steppuhn *et al.*, 2004), artificial diet (Parr & Thurston, 1972), etc. However, the nicotine production is highly herbivore-specific (**Figure 4C**) (Von Dahl *et al.*, 2007; Lee *et al.*, 2016). The specificity is the consequence of the complex regulation of nicotine biosynthesis which is mediated by JA and ethylene (Winz & Baldwin, 2001). The herbivore-specific induction of the nicotine may be the adaptive phenotypic plasticity through the resource allocation; nicotine is not induced by the attacks of nicotine-tolerant herbivores (*Manduca sexta* and *Trichobaris mucorea*), but nicotine is induced by the generalist herbivores (*Spodoptera* sp.). Recent study about the wild tobacco genomes shows that G-box motif (binding site for MYC2 transcription factor which is important for the JA signaling) and GCC motif (binding site for ERF IV which is important for the ethylene signaling) are highly enriched in the *Nicotiana* genus than other Solanaceae plants (Xu *et al.*, 2017). The herbivore-specificity in the nicotine biosynthesis is likely to be regulated by these *cis*-regulatory elements. Therefore, the hypothesis about whether the herbivore-specific inductions of nicotine biosynthesis are adaptive can be tested by the manipulation of the binding motifs in the promoter instead of the gene. Although it was difficult to manipulate the *cis*-regulatory element in the non-model organism, CRISPR/Cas9-mediated genome editing enable us to manipulate the *cis*-regulatory elements (Swinnen *et al.*, 2016).

In **manuscript VI**, I further have tested the ecological consequence of localized tissue-specific defense. The localized tissue-specific defense may allow differentiating the ecological niche in a hostplant to spatially-separated herbivores. However, it is still unknown whether the localized defense is an adaptation to survive under spatially various herbivore community. Therefore, to test this hypothesis, we need to know how plant shapes the localized defense to manipulate. However, our current knowledge still does not reach this stage. Recently, the molecular mechanism of JA-mediated systemic defense has been increased (Chauvin *et al.*, 2013; Farmer *et al.*, 2014; Kiep *et al.*, 2015), so it will be possible to investigate the fitness consequence of the JA-mediated systemic responses.

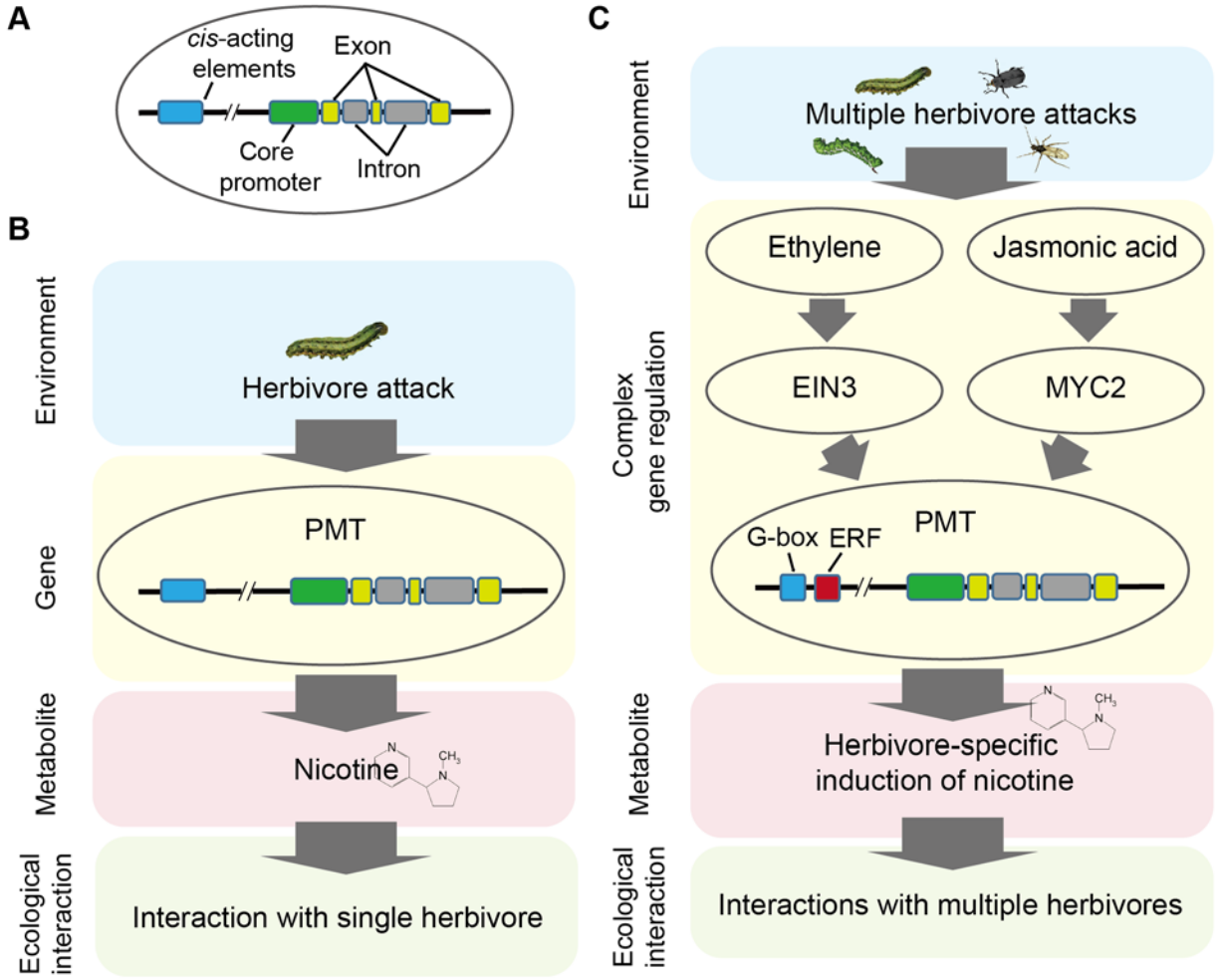


Figure 4. Manipulation of regulatory modules. (A) Schematic structure of the gene. (B) The genetic determinist views of ecological interactions. (C) The complex gene regulatory view of ecological interactions.

4.5. Conclusion

Since ecological interactions are highly context-dependent, temporal and spatial phenotypic plasticity is essential to survive in variable environments. Although the spatiotemporal variations of plant responses are well characterized, their functional consequences are less studied. In response to the environmental variations, the plant has developed unique plastic responses to maintain the ecological interactions. In this dissertation, I show examples of how plants cope with variable environments using analytical chemistry, molecular biology techniques, and natural history observations. However, future work will be

necessary to test the fitness consequence of temporal and spatial variations in plant responses and to confirm whether those differential responses are adaptive plasticity. I think the manipulation of variable *cis*-regulatory elements can be the direction to understand the fitness consequence of the plant plastic responses.

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5. Summary

Environmental heterogeneity is ubiquitous in nature and generates different ecological niches. Plants require evolving plastic responses over time and space to cope with the variable environment. Indeed, the ecological interactions between environmental factors and the plant depend largely on their temporal and spatial characteristics. It may be important to maintain the function under heterogeneous environments by having temporal plastic responses. However, the functional consequences of the plant temporal and spatial plastic responses in nature are largely unknown. In my dissertation, I have investigated the plant strategies to generate the plasticity and their functional consequences.

One of the most important systems to generate the temporal plasticity is the circadian clock. The output of the plant circadian period under free-running conditions provides valuable information to drive forward genetic screens of circadian clock genes in the laboratory environment. However, in nature, the plant's clock is always entrained to a 24 hours period, and hence in nature, the phase established by the clock is more important than the circadian period. In addition, the phenotyping screens in the laboratory are conducted in rarefied environments depleted of *zeitgebers*, such as circadian temperature cycles, that in addition to light cycles, can entrain endogenous clocks. As a consequence, when clock-mediated behaviors are studied in real-world settings, these studies frequently fail to show the behavior discovered in the laboratory. I tested under real world conditions with a native plant whether the circadian clock primes the photosynthetic machinery to anticipate light at dawn, and showed that it does not. I further found that the clock is more important for dusk anticipation and that it also does something more interesting: the clock tells a plant when to ignore the light, particularly, not to respond to the light in the middle of the night. By measuring photosynthetic rates under field and laboratory conditions, I conclude that the plant circadian clock allows plants to selectively pay attention to light signals rather than "when to wake up" in anticipation of the sunrise.

When attacked, plants emit herbivore-induced volatiles (HIPVs) as one avenue of defense. HIPVs can indirectly defend plants by attracting predators and parasitoids to prey on the attacking herbivores. This strategy can be fast and effective, increasing herbivore mortality and thus plant fitness. However, the success depends on HIPVs reliably attracting predators and parasitoids to their herbivorous prey. HIPVs usually comprise blends of compounds from different biosynthetic pathways. Here I showed characterized the varying

activity patterns of two specialist herbivores of the wild tobacco *Nicotiana attenuata*, which only partly overlap with the activity of their predators on field-grown plants. I monitored the green leaf volatiles (GLVs) and sesquiterpenes emitted by field-grown *N. attenuata* plants after precisely timed and standardized mock herbivore attack either at the beginning of the day, when predators are becoming active, or at the end of the day when predator activity ceases. I found striking differences in the composition of GLVs after morning versus evening attack, while sesquiterpenes are emitted mainly during light periods and peak in the early afternoon. Under controlled conditions, I showed that timing of herbivore attack has a greater effect on total HIPV blends than either light cues or the plant's internal clock, although these factors also play a role. Over two years of field studies, I found that day-active predators remove more prey from field-grown and wild plants when plants are supplemented with a GLV blend typical of dawn attack versus a blend typical of dusk attack. This can be attributed to different ratios of specific GLVs rather than differences of GLVs in these blends. Using transgenic plants lacking either GLVs or both GLVs and sesquiterpenes, I found that after a dawn attack, GLVs are required to increase predation rates, while sesquiterpenes do not contribute even though they are also emitted. However, sesquiterpenes increase predation rates in the day following a dusk attack and also contribute to a prolonged elevation of predation rates on the second day after an attack. In conclusion, the combination of transient GLV emission with diurnal, persistent sesquiterpene emission provides a robust indirect defense of plants under real-world conditions by the increased predation of herbivores. GLVs and sesquiterpene HIPVs provide two ways of conveying timely information to native predators: sesquiterpenes are persistent and temporally synchronized with predator activity, while GLVs are transient but convey relevant temporal information as a result of qualitative blend differences.

As well as the temporal plasticity, individual plants can provide space for various herbivore communities, and often several herbivores can colonize different parts of the same plant. Plants can, therefore, play an important role in shaping community composition in ecosystems by mediating interactions among herbivores. Plant-mediated interactions among different folivores or among above- and below-ground herbivores are relatively well understood. However, it is largely unknown how the stem responds to stem-feeding herbivores, and whether leaf- and stem-responses to herbivore attack are integrated. Unexpectedly, I found that jasmonic acid (JA) signaling is also important for resistance to the stem herbivore, and, that *N. attenuata* induced chlorogenic acid in the stem in the face of a

stem herbivore attack, though chlorogenic acid was not induced in leaves in response to a leaf herbivore attack. I also found that plant inducible defenses in the pith and the leaf are not systemically induced in other tissues, but systemic induction of JA signaling was asymmetric between the stem and the leaf. Herbivores are often highly specialized in a certain tissue, and so systemically acquired resistance might not always be an adaptive strategy for plants. I suggested that localized, tissue-specific defenses allow plants to differentiate chemical niches among their tissues, enabling them to respond specifically to the attack of various herbivores which can have different fitness consequences for the plant.

Plants cope with variable environmental factors by revealing an exciting temporal and spatial plasticity. Unique responses to environmental variations developed by plants which are important to maintain the ecological interactions comprise diurnal rhythm, plant circadian clock, and tissue-specific localized defense.

6. Zusammenfassung

Durch die Heterogenität natürlicher Lebensräume, entstehen verschiedene ökologische Nieschen. Um in ihrer Umwelt zu überleben mussten Pflanzen zeit- und ortsspezifisch plastische Reaktionen entwickeln. Daher sind ökologische Interaktionen zwischen Pflanzen und ihrer Umwelt stark durch ihre zeitlichen und räumlichen Charakteristika geprägt. Es könnte hierbei wichtig sein mithilfe zeitlich plastischer Reaktionen auch in heterogenen Umgebungen Funktionen aufrecht zu erhalten. Jedoch ist es bisher größtenteils unbekannt welche funktionellen Konsequenzen zeit- und ortsspezifisch plastische Reaktionen unter natürlichen Bedingungen haben. In meiner Dissertation habe ich die pflanzlichen Strategien untersucht die diese Plastizität hervorrufen, sowie deren funktionelle Konsequenzen.

Eins der wichtigsten Systeme zur Generierung zeitlicher Plastizität ist die circadiane Uhr. Die Informationsausgabe der circadianen Uhr unter freilaufenden Bedingungen ermöglicht wertvolle Rückschlüsse für sogenannte *forward genetic screenings* zur Identifizierung von circadianen Uhr Genen unter Laborbedingen. Unter natürlichen Bedingungen ist die circadiane Uhr hingegen immer auf eine 24h Periode eingestellt. Dadurch ist unter diesen Bedingungen, die durch die circadiane Uhr etablierte Phase, wichtiger als die circadiane Periode. Außerdem finden Phenotypische Analysen im Labor unter stark vereinfachten Bedingungen statt, in denen einige *Zeitgeber* wie z.B. circadiane Temperaturzyklen fehlen, die eigentlich in Ergänzung zum Licht die innere Uhr einstellen können. Eine Konsequenz davon ist, dass viele circadiane Verhaltensweisen die unter Laborbedingungen beobachtet wurden, unter natürlichen Bedingungen nicht auftreten. In dieser Arbeit habe ich untersucht, ob die circadiane Uhr der Photosynthese-Maschinerie zum Sonnenaufgang ermöglicht das Licht bereits vorauszuahnen. Die Tests wurden unter natürlichen Bedingungen mit einer einheimischen Pflanze durchgeführt und es hat sich gezeigt, dass dies nicht der Fall ist. Hingegen zeigte sich, dass die innere Uhr wichtiger ist, um den Sonnenuntergang vorherzusagen. Außerdem scheint sie für etwas noch interessanteres verantwortlich zu sein: die Uhr sagt der Pflanze wann sie Licht ignorieren soll, bzw. konkreter, dass sie in der Nacht nicht auf Licht zu reagieren soll. In unserer fortlaufenden Untersuchungen mit transgenen Pflanzen mit veränderter circadianer Uhr unter natürlichen Bedingungen zeigt sich immer wieder ein zentrales Thema: *die innere Uhr sagt der Pflanze wann sie Umweltsignale beachten soll und wann nicht*. Biologische circadiane Uhren wurden nicht konstruiert, sondern haben sich mithilfe des Prozesses der natürlichen

Selektion entwickelt. Durch die Ergebnisse der Photosynthesemessungen unter Feld- und Laborbedingungen schlussfolgern wir, dass die circadiane Uhr Pflanzen ermöglicht selektiv auf Lichtsignale zu achten, jedoch nicht um das Aufgehen der Sonne vorherzusagen.

Wenn Pflanzen angegriffen werden verströmen sie zu ihrer Verteidigung spezielle Herbivory induzierte Duftstoffe (HIPVs). HIPVs können als indirekte Verteidigung fungieren indem sie Räuber und Parasitoide anlocken die die angreifenden Pflanzenfresser attackieren. Diese Strategie kann sehr schnell und effektiv sein, indem sie die Sterblichkeitsrate der Herbivoren erhöhen, was wiederum die Fitness der Pflanze erhöht. Jedoch hängt der Erfolg dieser Strategie davon ab, dass HIPVs zuverlässig Räuber und Parasitoiden zu ihrer Beute führen. HIPVs bestehen gewöhnlich aus einer Mischung verschiedener Stoffe mit unterschiedlichem biosynthetischem Ursprung. Hier zeige ich die Aktivitätsmuster zweier spezialisierter Herbivoren des wilden Tabaks, *Nicotiana attenuata,* die sich in ihrer natürlichen Umgebung nur teilweise mit den Aktivitätszyklen ihrer Räuber überschneiden. Außerdem haben wir die von Feldpflanzen verströmten Grüne Blattduftstoffe (GLVs), sowie Sesquiterpene analysiert, nachdem wir diese vorher zu spezifischen Zeiten durch standardisierten, vorgetäuschten Herbivorbefall induziert haben. Die Behandlungen wurden hierbei zu Beginn des Tages, wenn die Räuber gerade aktiv werden, sowie am Ende des Tages, wenn deren Aktivität wieder nachlässt, durchgeführt. Wir haben deutliche Änderungen in der GLV Zusammensetzung nach morgendlicher und abendlicher Attacke feststellen können. Die Sesquiterpene hingegen werden überwiegend während der Lichtperiode verströmt, mit der höchsten Rate am frühen Nachmittag. Unter kontrollierten Bedingungen konnten wir zeigen, dass das Timing der Herbivorattacke einen größeren Einfluss auf die HIPVs Zusammensetzung hat als Licht Reize und die innere Uhr der Pflanze; wobei auch diese Faktoren eine Rolle spielen. Im Verlauf einer zweijährigen Feldstudie konnten wir zeigen, dass tagaktive Räuber mehr Beute auf Feldpflanzen machten, die vorher mit einem GLV Gemisch behandelt wurden, das charakteristisch für morgendlichen Angriffe durch Pflanzenfresser ist, als nach Behandlung mit einer für abendliche Angriffe spezifischen Zusammensetzung. Dies lässt sich auf die verschiedenen Verhältnisse der spezifischen GLVs, nicht aber auf deren Anwesenheit/Abwesenheit zurückführen. Mithilfe transgener Pflanzen, die entweder keine GLVs oder sowohl keine GLVs als auch keine Sesquiterpene besitzen, konnten wir zeigen, dass nach einem morgendlichen Herbivorangriff GLVs notwendig sind um die Prädationsrate zu erhöhen, während die ebenfalls verströmten Sesquiterpene keinen diesbezüglichen Beitrag leisten. Im Gegensatz dazu, erhöhen Sesquiterpene am Tag nach

einem abendlichen Angriff die Prädationsrate und tragen dazu bei, die erhöhte Prädationsrate auf den zweiten Tag nach der Attacke zu verlängern. Zusammenfassend kann man sagen, dass die Kombination aus kurzzeitigen GLV Emissionen und diurnalen, anhaltenden Sesquiterpene Emissionen eine robuste indirekte Verteidigung darstellt, mit der sich Pflanzen unter natürlichen Bedingungen schützen, indem sie die Prädationsrate ihrer Herbivoren erhöhen. GLVs und Sesquiterpene HIPVs vermitteln auf zweierlei Weise zeitliche Informationen an die heimischen Prädatoren: Sesquiterpene sind lang anhaltend und zeitlich mit der Aktivität der Prädatoren synchronisiert, während GLVs kurzlebig sind, aber durch qualitative Unterschiede in ihrer Zusammensetzung wichtige zeitliche Informationen vermitteln.

Neben den zeitlichen Faktoren, können einzelne Pflanzen auch räumlich getrennt Platz für verschiedene Pflanzenfresser-Gemeinschaften bereitstellen, die unterschiedliche Teile der Pflanze kolonisieren. Pflanzen können Interaktionen zwischen verschiedenen Herbivoren vermitteln und spielen daher eine wichtige Rolle indem sie die Zusammensetzung von Ökosystemen mitgestallten. Pflanzen vermittelte Interaktionen zwischen Blatt-fressenden Herbivoren, sowie zwischen überirdischen und unterirdischen Herbivoren sind relative gut verstanden. Für den Stamm hingegen ist es größtenteils unbekannt, wie er auf Stamm-fressende Herbivoren reagiert und ob Blatt und Stamm Reaktionen auf Herbivorbefall miteinander verknüpft sind. Überraschender Weise haben wir herausgefunden, dass der JA-Signalweg auch für die Verteidigung gegen Stamm-Herbivoren eine wichtige Rolle spielt. Außerdem beobachteten wir, dass in *N. attenuata* nach einem Angriff auf den Stamm die Ansammlung von Chlorogensäure induziert wird, obwohl dieses Metabolit in den Blättern nicht auf Angriffe durch Pflanzenfresser reagiert. Darüber hinaus kam es zu keiner systemischen Reaktion der induzierbaren Verteidigung des Marks und der Blätter durch das jeweilige andere Gewebe und eine asymmetrische Induktion des JA-Signalweges. Pflanzenfresser spezialisieren sich häufig auf bestimmte Pflanzengewebe, wodurch systemische Verteidigungsreaktionen vermutlich nicht immer die bestmögliche Anpassung für Pflanzen darstellen. Wir stellen die Vermutung auf, dass lokalisierte, gewebsspezifische Verteidigungsantworten es einer Pflanze ermöglichen verschiedene chemische Nischen innerhalb ihrer Gewebe zu etablieren, was ihnen erlaubt spezifisch auf Angriffe durch verschiedene Herbivoren zu reagieren, die jeweils einen sehr unterschiedlichen Einfluss auf die pflanzliche Fitness haben können.

Zeitlich und räumlich plastische Reaktionen erlauben es Pflanzen mit den variablen

Umweltfaktoren in ihrem Lebensraum fertig zu werden. Als Reaktion auf Variationen ihrer Umwelt haben Pflanzen spezifische plastische Reaktionen entwickelt, wie z.B. diurnale Reaktionen, die pflanzliche circadiane Uhr und gewebsspezifisch, lokalisierte Verteidigungsantworten, die wichtig sind um ihre ökologischen Interaktionen aufrecht zu erhalten.

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8. Erklärung

Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dis- sertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertig- stellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geld- werte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Jena,

Youngsung Joo

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufen hatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät ist mein erstes Promotionsverfahren überhaupt.

Jena,

Youngsung Joo

9. List of Publications

[11] **Joo, Y.**, Baldwin, I.T. (*in preparation*). The effects of plant endogenous rhythm in growth, phenology, and fitness consequence under competition.

[10] Yon, F., Kessler D., **Joo, Y.**, Kim, S.G., and Baldwin, I.T. (*submitted to* ***Journal of Integrative Plant Biology***). Fitness consequences of altered floral rhythms in *Nicotiana attenuata* in nature.

[9] **Joo, Y.**, Schuman, M.C., Baldwin, I.T., Kim, S.G., (*in preparation*). The circadian clock in *Nicotiana attenuata* times accumulation, but not emission, of herbivore-induced plant volatiles that function as indirect defenses.

[8] (Lee, G.[*], **Joo, Y.**[*]), Kim, S.G., and Baldwin I.T. (under review in ***The Plant Journal***). What happens in the pith stays in the pith; tissue-localized defense responses facilitate niche differentiation between two spatially separated herbivores. (* both author equally contributed)

[7] **Joo, Y.,** Schuman, M.C.[#], Goldberg, J.K., Kim, S.G., and Baldwin, I.T.[#] (under review in ***Functional Ecology***). Herbivore-induced volatile blends with both "fast" and "slow" components provide robust indirect defense in nature. (# Co-corresponding author)

[6] **Joo, Y.**, Fragoso, V., Yon, F., Baldwin, I.T.[#], Kim, S.G[#]. (2017) The circadian clock tells a plant when to respond photosynthetically to light. ***Journal of Integrative Plant Biology*** (# Co-corresponding author)

[5] Yon, F., Kessler D., **Joo, Y.**, Cortés Llorca, L., Kim, S.G., and Baldwin, I.T. (2017). Fitness consequences of altering floral circadian oscillations for *Nicotiana attenuata*. ***Journal of Integrative Plant Biology***. doi: 10.1111/jipb.12511

[4] Schuman, M. C., Valim, H., **Joo, Y.** (2016). Temporal dynamics of plant volatiles: mechanistic bases and functional consequences. In J. Blande, R. Glinwood (Eds.), *Deciphering Chemical Language of Plant Communication*. (pp. 3-34). **Springer**

[3] Lee, G., **Joo, Y.**, Diezel, C., Lee, E.J., Baldwin, I.T.[#], Kim, S.G[#]. (2016). *Trichobaris* weevils distinguish amongst toxic hostplants by sensing volatiles that do not affect larval performance. ***Molecular Ecology***. doi: 10.1111/mec.13686. (# Co-corresponding author)

[2] 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***. doi: 10.1111/ jipb.12458. 2015

[1] Yon, F., **Joo, Y.**, Cortés L.L., Rothe, E., Baldwin, I. T., Kim, S.G. (2015). Silencing *Nicotiana attenuata* LHY and ZTL alters circadian rhythms in flowers. ***New Phytologist***, 209(3), 1058-1066. doi: 10.1111/nph.13681.