Zeitlupe in the Roots of Wild Tobacco Regulates Jasmonate-Mediated Nicotine Biosynthesis and Resistance to a Generalist Herbivore

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The jasmonate (JA) phytohormone signaling system is an important mediator of plant defense against herbivores. Plants deficient in JA signaling are more susceptible to herbivory as a result of deficiencies in defensive trait expression. Recent studies have implicated the circadian clock in regulating JA-mediated defenses, but the molecular mechanisms linking the clock to JA signaling are unclear. Here, we report that wild tobacco (Nicotiana attenuata) plants rendered deficient in the clock component ZEITLUPE (ZTL) by RNA interference have attenuated resistance to the generalist herbivore Spodoptera littoralis. This effect can be attributed in part to reduced concentrations of nicotine, an abundant JA-regulated toxin produced in N. attenuata roots and transported to shoots. RNA interference targeting ZTL dramatically affects the root circadian clock and reduces the expression of nicotine biosynthetic genes. Protein-protein interaction experiments demonstrate that ZTL regulates JA signaling by directly interacting with JASMONATE ZIM domain (JAZ) proteins in a CORONATINE-INSENSITIVE1- and jasmonoyl-isoleucine conjugate-independent manner, thereby regulating a JAZ-MYC2 module that is required for nicotine biosynthesis. Our study reveals new functions for ZTL and proposes a mechanism by which a clock component directly influences JA signaling to regulate plant defense against herbivory.

Plants evolved under relentless attack from diverse insect herbivores, which drove the development of sophisticated defense responses (Wu and Baldwin, 2010). These include thousands of defense-related secondary metabolites or proteins that can deter or poison particular herbivores or attract their natural enemies (Howe and Jander, 2008; Schuman and Baldwin, 2016). These defensive traits may be constitutively or inducibly produced in plants, often in a tissue-specific manner (Li et al., 2016). In several tobacco species, the alkaloid nicotine is synthesized in roots and immediately transported into shoot tissues (Baldwin et al., 1994a; Hashimoto and Yamada, 1994). Nicotine is a potent neurotoxin that constitutively accumulates in tobacco (Nicotiana spp.) and increases in response to wounding (Baldwin, 1999; Steppuhn and Baldwin, 2007). Jasmonate (JA) phytohormone signaling is required for both constitutive and induced nicotine biosynthesis and, generally, to mount plant defenses against herbivores (Baldwin et al., 1994b, 1997; Howe and Jander, 2008; Shoji et al., 2008; Shoji and Hashimoto, 2011; Fragoso et al., 2014).

JAs include several phytohormones that regulate many physiological processes, including growth, fertility, and defense (Wasternack and Hause, 2013). Changes in de novo JA biosynthesis occur in response to environmental factors or at particular developmental stages. JAs are synthesized from α-linolenic acid (18:3) via the octadecanoid pathway to form the core compound jasmonic acid and its derivatives (Wasternack and Hause, 2013; Wasternack and Feussner, 2017). Among the derivatives, the jasmonoyl-isoleucine conjugate (JA-Ile) is reported to be the most bioactive form (Kang et al., 2006; Fonseca et al., 2009). The protein CORONATINE-INSENSITIVE1 (COI1) is the F-box component of a SKIP-CULLIN-F-box complex that binds with specific JASMONATE ZIM domain (JAZ) proteins and cofactors to perceive JA-Ile, leading to the degradation of JAZ (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). JAZ proteins repress JA-responsive transcription factors (TFs), and the degradation of JAZ results in the activation of JA signaling. Many TFs targeted by JAZ proteins have been identified. These TFs, such as the bHLH TF family members MYC2 and its homologs MYC3, MYC4, and MYC5, may bind directly to the promoters of many JA-mediated defense-related genes (Fernández-Calvo et al., 2011; Schweitzer...
et al., 2013; Song et al., 2017). MYC2 has been shown to regulate the synthesis of defense-related secondary metabolites in many species, including glucosinolates, nicotine, and volatile terpenes (Shoji and Hashimoto, 2011; Hong et al., 2012; Schweizer et al., 2013; Li et al., 2014). Remarkably, the accumulation of glucosinolates and the emission of terpenes is rhythmic, peaking at midday and decreasing at night (Loughrin et al., 1994; Martin et al., 2003; Goodspeed et al., 2013b; Joo et al., 2018).

Many rhythmic events in biology are mediated by the circadian clock, which coordinates metabolism and behavior with photoperiod, environmental changes, and diurnal events in most and perhaps all cells (Edgar et al., 2012). Disruption of the plant circadian clock can alter plant growth, development, responses to abiotic factors, and biotic interactions, reducing plant fitness (Greenham and McClung, 2015; Sanchez and Kay, 2016; Hubbard et al., 2018). Emerging evidence shows that the circadian clock plays a role in plant defense, which is better characterized in plant-pathogen than in plant-herbivore interactions (Lu et al., 2017). Arabidopsis (Arabidopsis thaliana) plants are more resistant to several pathogens, including the fungus Botrytis cinerea, the bacterium Pseudomonas syringae, and the oomycete Hyaloperonospora arabidopsidis, when inoculated at a particular time of the day (Bhardwaj et al., 2011; Wang et al., 2011; Zhang et al., 2013; Ingle et al., 2015). Disruption of the circadian clock in Arabidopsis differentially affects resistance to pathogens. Interestingly, mutants of clock genes expressed in both morning and evening are more susceptible to P. syringae and H. arabidopsidis. Manipulation of the single clock gene CIRCADIAN CLOCK ASSOCIATED1 (CCA1) renders Arabidopsis plants more susceptible to H. arabidopsidis when it is mutated and more resistant when it is overexpressed. In contrast, plants become more susceptible to P. syringae when CCA1 is overexpressed and when it is mutated together with its homolog LATE AND ELONGATED HYOCOTYL (LHY; Bhardwaj et al., 2011; Wang et al., 2011; Zhang et al., 2013). In Arabidopsis, a CCA1-overexpressing line and ARRHYTHMO (LUX) mutants had abrogated circadian rhythms and were more susceptible to herbivory by larvae of the generalist Trichoplusia ni (cabbage looper) when plants and larvae were entrained to the same diurnal cycle (Goodspeed et al., 2012, 2013a). Furthermore, Goodspeed et al. (2012) discovered that T. ni larvae fed rhythmically under laboratory conditions and that shifting the rhythms of plants and larvae by imposing opposite entrainment regimes increased the susceptibility of the plants. By contrast, when the circadian rhythm was shifted in wild tobacco (Nicotiana attenuata) plants, they remained resistant to the specialist herbivore Manduca sexta, which does not feed rhythmically (Herden et al., 2016). Thus, disrupting a plant’s circadian clock does not have predictable effects on susceptibility to pathogens or herbivores. However, one study with the pathogen B. cinerea showed greater susceptibility of Arabidopsis at night compared with dawn, in part due to basal fluctuations in JA signaling (Ingle et al., 2015). These fluctuations also may explain the circadian regulation of resistance to T. ni in Arabidopsis (Goodspeed et al., 2012, 2013a).

In general, the plant circadian clock participates in defense by regulating hormone signaling pathways (Atamian and Harmer, 2016). Defense responses mediated by JA signaling are subject to circadian regulation in several ways, but by unknown mechanisms. First, the basal biosynthesis of jasmonic acid in Arabidopsis peaks at midday (Goodspeed et al., 2012). Second, the oscillating protein levels of a master regulator of the JA pathway, MYC2, are most abundant at midday (Shin et al., 2012). Third, some defense-related metabolites regulated by JA signaling, including glucosinolates and terpenes, also oscillate and are abundant at midday in several plant species, at least under diurnal conditions (Loughrin et al., 1994; Martin et al., 2003; Joo et al., 2018). These fluctuations may coincide with the behavior of some herbivores or the herbivores’ natural enemies (Goodspeed et al., 2012; Joo et al., 2018). However, the mechanism by which the circadian clock regulates JA-mediated defense is unknown. A recent study found that the circadian evening complex comprising LUX, EARLY FLOWERING3 (ELF3), and ELF4 suppresses JA-mediated senescence in Arabidopsis by binding directly to the promoter of MYC2 (Zhang et al., 2018), indicating that MYC2 may be a direct target of clock regulation.

The ZEITLUPE (ZTL) gene encodes a circadian F-box protein containing a LIGHT, OXYGEN, OR VOLTAGE (LOV) domain that perceives blue light and is integral to its circadian function (Pudasaini et al., 2017). ZTL regulates the internal clock by targeting TIMING OF CAB EXPRESSION1 (TOC1) and PSEUDO-RESPONSE REGULATION5 for degradation in the dark (Kim et al., 2003; Más et al., 2003; Kiba et al., 2007). In N. attenuata, previous studies demonstrated that silencing of NaZTL abrogates floral rhythms, such as flower opening, volatile emission, and vertical movement (Yon et al., 2016). Here, we report that NaZTL-silenced plants are more susceptible to attack from the generalist herbivore Spodoptera littoralis. By analyzing defense-related secondary metabolites and protein-protein interactions of ZTL, we investigated how ZTL affects herbivore resistance. Our results indicate that basal nicotine biosynthesis is regulated by ZTL targeting a JAZ-MYC2 module in N. attenuata.

RESULTS

ZTL Contributes to Plant Resistance to the Herbivore S. littoralis

Previous phylogenetic and functional analyses revealed that NaZTL in N. attenuata is the ortholog of the Arabidopsis ZTL. First, silencing NaZTL (ir-ztl) by RNA interference significantly influences the expression of circadian clock genes in
plants; second, ir-ztl plants have a longer period in free-running conditions; third, hypocotyl length is increased in the seedlings of ir-ztl plants compared with empty vector (EV) plants; and finally, NaZTL interacts directly with NaTOC1 (Somers et al., 2004; Yon et al., 2012, 2016). To determine whether protein levels of NaZTL oscillate, we generated an anti-ZTL antibody. The specificity was evaluated using samples of GFP-NaZTL transiently expressed in Nicotiana benthamiana leaves. GFP-NaZTL was detected in crude extracts and extracts after immunoprecipitation with GFP-Trap beads (Supplemental Fig. S1A), indicating that the anti-ZTL antibody recognizes the NaZTL protein in vivo. ZTL protein levels were evaluated in leaves of rosette-stage wild-type N. attenuata plants. The results showed that the accumulation of the ZTL protein had a diurnal rhythm that peaked after dark and decreased in the light (Supplemental Fig. S1B), a pattern similar to that found in Arabidopsis (Kim et al., 2003). The ir-ztl lines were tested for their susceptibility to the generalist herbivore S. littoralis and compared with that of EV control plants. S. littoralis larvae gained more weight on ir-ztl lines than on control plants (Fig. 1), indicating that ZTL silencing reduced plant resistance to S. littoralis.

ZTL Regulates the Accumulation of Nicotine in Leaves

Plants produce abundant and diverse secondary metabolites that can mediate resistance against herbivores (Mithöfer and Boland, 2012). In N. attenuata, the most efficient and abundant defense metabolites include nicotine, phenylpropanoid-polyamine conjugates, and 17-hydroxygeranyllinalool diterpene glycosides (Baldwin, 1998; Steppuhn et al., 2004; Heiling et al., 2010, 2016; Kaur et al., 2010). To determine whether the attenuated resistance to S. littoralis in ir-ztl lines could be attributed to a deficiency in secondary metabolites, the levels of these compounds were measured in leaves. Both basal and herbivore-induced nicotine levels were significantly lower in ir-ztl lines compared with control EV plants (Fig. 2A). However, the levels of the two most abundant phenylpropanoid-polyamine conjugates, caffeoylputrescine and dicafeoylspermidine, did not differ between ir-ztl and EV plants (Fig. 2, B and D). The basal levels of nicotianoside II, the most abundant 17-hydroxygeranyllinalool diterpene glycoside measured, was increased slightly in one ir-ztl line (ir-ztl-318), but the inducible levels did not differ among the lines (Fig. 2D). These results suggested that reduced nicotine levels could mediate the susceptibility of ir-ztl plants to S. littoralis. To test this hypothesis, a nicotine complementation experiment was performed. When the petioles of excised ir-ztl-314 leaves were submerged in 1 mm nicotine solution, the nicotine contents of leaf lamina increased ~36% after 4 h of accumulating nicotine from the transpiration stream (Supplemental Fig. S2; Baldwin, 1989). S. littoralis performance was then measured on the excised leaves of EV and ir-ztl-314 in different solutions. Consistent with data from intact plants, S. littoralis grew larger on the leaves of ir-ztl-314 than on excised EV leaves whose petioles were incubated in water (Fig. 2F). However, this difference was reduced greatly when excised ir-ztl-314 leaves were incubated in a nicotine solution (Fig. 2E). Thus, exogenous nicotine supplementation via the transpiration stream (i.e. the normal means of nicotine transport from root to shoot in intact plants) partially restored S. littoralis resistance in ir-ztl leaves (Baldwin, 1989). To further test the effect of reduced nicotine in ir-ztl, the performance of a specialist herbivore, M. sexta larvae, which is highly resistant to nicotine, was measured on ir-ztl-314 and EV plants. After 9 d of feeding, the mass of M. sexta larvae did not differ significantly between those fed on ir-ztl and EV plants (Supplemental Fig. S3).

To determine how ZTL modifies the accumulation of root-synthesized nicotine in leaves, the function of ZTL in roots was investigated by a micrografting experiment. Hypocotyls from EV seedlings were grafted onto epicotyls of ir-ztl-314 lines (heterografts: EV/ir-ztl), and grafts between hypocotyls and epicotyls from the same genotype (homografts) were used as controls (EV/EV and ir-ztl/ir-ztl; Supplemental Fig. S4). The basal nicotine concentration was reduced by similar amounts (30%) in the leaves of EV/ir-ztl and ir-ztl/ir-ztl compared with EV/EV (Fig. 2F). These data are consistent with the hypothesis that ZTL’s function in roots is sufficient to alter the accumulation of nicotine in leaves. Moreover, the concentration of nicotine in the roots of ir-ztl was much lower than that of EV roots (Supplemental Fig. S5). Because nicotine is synthesized in roots, we inferred that root-expressed ZTL may directly regulate nicotine biosynthesis.

Figure 1. Silencing of ZTL attenuates plant resistance to S. littoralis. S. littoralis performance on ir-ztl plants versus EV controls is shown. S. littoralis larval mass ± se (n = 30) was measured at 6, 8, 9, and 10 d during feeding on intact plants. The inset shows an S. littoralis larva feeding on an N. attenuata leaf (photograph by Danny Kessler). Asterisks indicate significant differences in ir-ztl plants compared with EV control plants (**, P < 0.01, Student’s t test).
Silencing of ZTL in *N. attenuata* Alters the Circadian Clock in Roots

Misexpression of ZTL in plants dramatically affects the circadian clock in leaves and flowers (Somers et al., 2004; Yon et al., 2016). To evaluate if ZTL also regulates the circadian clock in roots, the transcript accumulation of circadian clock genes was examined in the roots of ir-ztl and EV plants under diurnal (LD) and constant light (LL) conditions. Transcripts of ZTL did not fluctuate in the root, which is the same as in leaves and flowers (Fig. 3, A and B). However, ZTL transcript abundance was more than 90% lower in ir-ztl lines at all measured time points. Next, the transcript abundance of four circadian clock genes was analyzed, including one morning clock component (*LHY*) and three evening clock components (*TOC1, LUX*, and *ELF4*). The transcript levels

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**Figure 2.** ZTL contributes to nicotine accumulation in leaves. A to D, Mean nicotine (A), caffeoylputrescine (B), dicaffeoylspermidine (C), and nicotianoside II (D) levels ± se (*n* = 6) in the leaves of ir-ztl and EV plants. Treated leaves were harvested 2 d after wounding and treatment with *S. littoralis* regurgitant, and controls were similar leaves from untreated plants. Asterisks indicate significant differences in ir-ztl plants compared with EV control plants (*, *P* < 0.05 and **, *P* < 0.01, Student’s *t* test). E, *S. littoralis* performance on differently treated leaves. Leaves of ir-ztl-314 and EV plants were excised and put into water or 1 mM nicotine solution. *S. littoralis* larvae were immediately placed on leaves. *S. littoralis* larval mass ± se (*n* = 10–20) was measured at 6 and 8 d after feeding on the indicated leaves. Letters indicate significant differences among different treatments (*P* < 0.05, Duncan’s multiple range test). For nicotine concentrations of the +nicotine treatment, see Supplemental Figure S2. F, Mean basal nicotine levels ± se (*n* = 5–6) in the leaves of different grafts. The inset illustrations of *N. attenuata* plants indicate different grafts. Letters indicate significant differences among different grafts (*P* < 0.05, one-way ANOVA followed by Duncan’s multiple range test). FM, Fresh mass.
of all these clock genes oscillated in EV plants under both LD and LL conditions with similar patterns in leaves and roots, indicating that these genes also act in the root clock (Fig. 3, C–J). In ir-ztl lines, the transcripts of the four clock genes remained rhythmic under LD but became arrhythmic in LL (Fig. 3, E–I).

_LHY_ transcripts were significantly lower in ir-ztl versus EV plants under both conditions (Fig. 3C). In LD, the peak transcript abundance of all three evening genes broadened in ir-ztl (Fig. 3, E, G, and I). Collectively, these data show that ZTL regulates the circadian clock in roots by affecting the transcript abundance of the circadian clock components.

**ZTL Regulates JA-Mediated Herbivore Defense**

To investigate whether the biosynthesis of nicotine is regulated by ZTL, the transcripts of nicotine biosynthesis-related genes were measured in ir-ztl and EV plants under LD and LL. ERF189 belongs to the NIC2-locus ethylene TFs (ERFs) cluster in _Nicotiana tabacum_ that directly regulates nicotine biosynthesis (Shoji and Hashimoto, 2011). The transcript abundance of _ERF189_ in _N. attenuata_, an ortholog of _NtERF189_, has a diurnal rhythm of expression in EV plants, with peak abundance at midday (Fig. 4A), while under LL, _ERF189_ transcripts did not show a clear rhythm (Fig. 4B). The transcript abundance of three nicotine biosynthesis pathway...
genes, *ornithine decarboxylase* (ODC), *N*-putrescine methyltransferase (PMT1.2), and *isoflavone reductase*-like protein (A622), displayed a similar diurnal pattern to *ERF189* in EV plants, but rhythmicity was dampened under LL (Fig. 4, C–H). The accumulation of *ERF189*, *ODC*, *PMT1.2*, and *A622* transcripts still oscillated in ir-ztl lines under LD, but the overall amplitude was reduced compared with EV plants (Fig. 4, E and F). Consistently, relative to EV plants, the transcript abundance of these genes was reduced in ir-ztl under both LD and LL conditions. Due to the diurnal expression of these genes in roots, we then asked if the accumulation of nicotine in leaves also oscillates. The concentration of nicotine was measured in leaves of EV and ir-ztl plants under LD and LL. The nicotine content was stable in EV leaves under both LD and LL (Fig. 4, I and J). Relative to EV plants, the concentration of nicotine was consistently lower in ir-ztl under both conditions. Nicotine is not degraded, and levels in leaves result from its allometric accumulation (Baldwin et al., 1998). Thus, we could not rule out the possibility that the biosynthesis of nicotine oscillates while the accumulated levels do not.

Figure 4. Transcripts of nicotine biosynthesis-related genes in roots and nicotine accumulation in leaves are attenuated in ir-ztl plants under both LD and LL conditions. For LD conditions, plants grown under a 12/12-h (light/dark) cycle were sampled over 2 d; white and black bars indicate light and dark periods. For LL conditions, plants grown under a 12/12-h (light/dark) cycle were transferred to LL conditions and sampled over 2 d; white and gray bars indicate light and subjective dark periods. A to H, Mean transcript abundance ± se (n = 4–5) of *ERF189* (A and B), *ODC* (C and D), *PMT1.2* (E and F), and *A622* (G and H) in the roots of ir-ztl and EV plants under LD or LL conditions. I and J, Mean nicotine concentrations ± se (n = 5) in the rosette leaves of ir-ztl and EV plants under LD (I) and LL (J) conditions. FM, Fresh mass.

Due to the diurnal expression of these genes in roots, we then asked if the accumulation of nicotine in leaves also oscillates. The concentration of nicotine was measured in leaves of EV and ir-ztl plants under LD and LL. The nicotine content was stable in EV leaves under both LD and LL (Fig. 4, I and J). Relative to EV plants, the concentration of nicotine was consistently lower in ir-ztl under both conditions. Nicotine is not degraded, and levels in leaves result from its allometric accumulation (Baldwin et al., 1998). Thus, we could not rule out the possibility that the biosynthesis of nicotine oscillates while the accumulated levels do not.
ZTL Does Not Significantly Affect JA Biosynthesis

The JA signaling pathway positively regulates the biosynthesis of nicotine (Shoji and Hashimoto, 2011; Fragoso et al., 2014). To determine if ZTL is involved in JA signaling, the accumulation of JAs was first evaluated in ir-ztl and EV plants. Leaves from each plant line were elicited by wounding and the addition of *S. littoralis* regurgitant to the puncture wounds. JAs were maintained at very low levels without treatment and showed no difference between ir-ztl and EV plants. After *S. littoralis* treatment, jasmonic acid and JA-Ile levels peaked around 45 min, while other JA metabolites peaked later (Fig. 5). The peak concentration of JA-Ile was significantly lower (by 15%–30%) in both ir-ztl lines compared with EV plants 45 min after treatment, but basal levels and levels 90 min after treatment were not different (Fig. 5B). For jasmonic acid, 12-hydroxy-JA-Ile, and Val-conjugated jasmonic acid, an increased or decreased level was observed only in one line of ir-ztl compared with EV plants under some treatments (Fig. 5, A, D, and F). Levels of 12-hydroxy-JA and dicarboxy-JA-Ile accumulated equally in ir-ztl and EV under all treatments (Fig. 5, C and E). These results suggested that ZTL slightly affects the accumulation of JAs after *S. littoralis* attack.

ZTL Interacts Physically with JAZs

Next, we asked if ZTL is involved in JA signal transduction. The fact that ZTL is an F-box protein prompted us to test whether ZTL could interact with JAZ proteins like COI1, an F-box protein mediating JA perception (Kim et al., 2003; Thines et al., 2007). The interactions between ZTL and all 13 JAZs in *N. attenuata* were screened by yeast two-hybrid (Y2H) assay. Surprisingly, ZTL interacted strongly with seven of 13 JAZ proteins (JAZa, JAZb, JAZd, JAZe, JAZj, JAZk, and JAZl) in yeast, and the JA ligand (e.g. coronatine) was not required for these interactions (Fig. 6A; Supplemental...
Fig. S6). To confirm these interactions, in vitro pull-down assays and in vivo communoprecipitation assays were performed. Most MBP-His-JAZs were pulled down by GST-ZTL, except for MBP-His-JAZb and MBP-His-JAZm (Fig. 6B), whereas no signal was observed when GST-ZTL was replaced by GST (Supplemental Fig. S7). The in vivo interaction between JAZb and ZTL was tested further by transient expression in N. benthamiana, as JAZb showed a strong interaction with ZTL in both yeast and pull-down assays. Myc-JAZb was immunoprecipitated by GFP-ZTL but not by GFP alone (Fig. 6C), indicating that the interaction between JAZb and ZTL also occurs in vivo.

JA-Ile is required as a molecular glue for the interaction between COI1 and JAZs (Thines et al., 2007; Li et al., 2017) but not for the interaction between ZTL and JAZ (Fig. 6), suggesting a different binding mechanism. To address how ZTL binds to JAZ, the interaction domains in ZTL and JAZ were screened by Y2H assay. The canonical JAZ protein harbors two important interaction domains: the N-terminal TIFF/YXG (TIFY) domain and the C-terminal JA-ASSOCIATED (Jas) domain. The Jas domain is responsible for COI1/ JA-Ile binding (Sheard et al., 2010). Interestingly, the TIFY domain-containing N-terminal sequence was sufficient for full-length ZTL binding, but not the Jas domain (Fig. 6D). On the other hand, the LOV domain of ZTL was sufficient to bind JAZb (Fig. 6E). These results were further confirmed by the interaction between the TIFY and LOV domains in yeast.

To determine if the stability of JAZ is affected by ZTL, an in vitro degradation assay was performed. ZTL protein levels accumulate at high levels during the evening, which, in turn, enhances the degradation of targets in the dark (Pudasaini et al., 2017). Thus, the samples from ZTL16 were chosen for the degradation assays. The His-JAZb recombinant protein was degraded more slowly after incubation with the crude protein extracts from ir-ztl than from EV plants, indicating that the stability of JAZb is partly ZTL dependent (Fig. 6, F and G; Supplemental Fig. S8).

A JAZ-MYC2 Module Regulates Nicotine Biosynthesis in N. attenuata

In N. tabacum and N. benthamiana, the TF MYC2 regulates nicotine biosynthesis by binding directly to the promoter regions of nicotine biosynthetic genes, and the activity of MYC2 is suppressed by JAZ proteins (De Boer et al., 2011; Shoji and Hashimoto, 2011). There are two MYC2 TFs in N. attenuata, and both MYC2a and MYC2b interact with a flower-expressed JAZi (Li et al., 2017). Here, we tested all JAZ-MYC2 interaction combinations in yeast. The majority of JAZs in N. attenuata could bind to MYC2a and MYC2b in yeast (Fig. 7, A and B).

To evaluate the role of MYC2a and MYC2b in nicotine biosynthesis, virus-induced gene silencing (VIGS) assays, which allowed for the silencing of two MYC2 homologs either singly or together, were performed. The transcript accumulation of MYC2a and MYC2b was first examined in the roots of MYC2a VIGS, MYC2b VIGS, and cosilenced plants (MYC2 VIGS). MYC2a and MYC2b transcripts were decreased dramatically in MYC2a VIGS and MYC2b VIGS plants compared with those of EV plants, respectively (Supplemental Fig. S9). Interestingly, relative to EV plants, MYC2a transcripts were reduced by 30% in MYC2b VIGS plants and MYC2b transcripts were reduced by 20% in MYC2a VIGS plants. It is possible that MYC2a and MYC2b regulate each other’s expression in roots, as the silencing constructs only contained sequences unique to the targeted gene. In MYC2 cosilenced plants, transcripts of both MYC2a and MYC2b were ~70% lower than those in EV. The transcript abundance of ERF189 and PMT1.2 in the roots and nicotine accumulation in the leaves were measured and shown to be affected slightly by silencing MYC2b but were affected strongly by silencing MYC2a or cosilencing both MYC2 homologs. This pattern indicates that nicotine biosynthesis is regulated by both MYC2a and MYC2b, with MYC2a having a stronger effect (Fig. 7, C–E).

To evaluate if the expression of MYC2 and JAZ is affected by ZTL silencing, the transcripts of MYC2a and JAZe were measured under LD conditions. The transcripts of MYC2a have a diurnal rhythm of expression in EV plants, with peak abundance at midday (Supplemental Fig. S10A). However, the phase of MYC2a transcripts is shifted in ir-ztl, resulting in lower expression at midday. JAZe is highly expressed in roots and shows strong interactions with ZTL in yeast (Li et al., 2017). The transcriptional abundance of JAZe has a mild diurnal pattern in EV, but it shows no difference between EV and ir-ztl plants (Supplemental Fig. S10B).

COI1 also is known to regulate nicotine biosynthesis by the JAZ-MYC2 module (Paschold et al., 2007; Shoji et al., 2008). To identify the relationship between COI1 and ZTL in regulating nicotine biosynthesis, an inverted repeat COI1 line (ir-coi1) and ir-ztl were crossed (Supplemental Fig. S11A, A and B). Basal nicotine levels in the leaves of ir-coi1 are 38% lower than in control leaves. Remarkably, leaves of the ir-ztl × ir-coi1 cross had ~50% less basal nicotine than EV plants (Supplemental Fig. S11C), suggesting that both ZTL and COI1 contribute to and perhaps function independently in the regulation of the JAZ-MYC2 module.

DISCUSSION

Plant defense against herbivory can be dissected into several layers, including the perception of herbivore attack, early signaling transduction, phytohormone signaling, and activation of defensive traits (Schuman and Baldwin, 2016). In most cases, these layers are characterized after plants are attacked by herbivores, which is referred to as an inducible defense. Recent studies have revealed the importance of the plant circadian clock in regulating constitutive defenses that also contribute to herbivore resistance (Goodspeed...
Figure 6. NaZTL interacts directly with different JAZ proteins. A, Interaction between ZTL and JAZ proteins by yeast two-hybrid assays. Binding domain (BD)-ZTL and activation domain (AD)-JAZs were cotransformed into yeast strain Y2Hgold. BD-ZTL and AD-cotransformed yeast were used as a control (left). Transformants were grown on QDO (SD-Ade/-His/-Leu/-Trp) plates with 40 mg L⁻¹ X-α-gal. B, In vitro pull-down assay. Two micrograms of GST-ZTL protein was used to pull down 2 μg of His-MBP fusion protein. Immunoblotting was performed using anti-His antibody to detect the associated proteins. Membranes were stained with Coomassie Brilliant Blue to monitor input protein amount. C, In vivo coimmunoprecipitation assays. Protein extracts of *N. benthamiana* leaves expressing YFP/JAZb-myc or YFP-ZTL/JAZb-myc were immunoprecipitated with GFP-Trap_A beads. Input proteins and the immunoprecipitates (IP) were detected with an anti-myc antibody. D, Screening of the ZTL-interacting JAZ domain in yeast. AD-JAZb or AD-JAZb derivatives were cotransformed with BD-ZTL into yeast strain Y2Hgold. E, Screening of the JAZb-interacting ZTL domain in yeast. BD-ZTL or BD-ZTL derivatives were cotransformed with AD-JAZb or AD-JAZb derivatives into yeast strain Y2Hgold. Transformants were grown on QDO plates with 40 mg L⁻¹ X-α-gal. F, In vitro JAZb degradation assays. Purified His-JAZb was incubated with total crude protein extracts from EV and ir-ztl lines. His-JAZb was detected using an anti-His antibody at the indicated incubation time points. Coomassie Brilliant Blue (CBB) staining is shown as a protein loading control. Numbers below the immunoblots indicate relative protein abundance using ImageJ grayscale analysis. G, Mean relative abundance ± se (n = 3) of His-JAZb protein levels in different treatments. Relative protein abundance was analyzed by ImageJ software. Asterisks indicate significant differences in ir-ztl compared with EV plants (*, P < 0.05 and **, P < 0.01, Student's t test).
et al., 2012, 2013a). Such constitutive defenses may encompass at least two layers. The JA phytohormone signaling pathway is circadian regulated, corresponding to a rhythmic production of JA-mediated defense compounds that, in turn, may be constitutive or inducible (Loughrin et al., 1994; Martin et al., 2003; Goodspeed et al., 2013b; Joo et al., 2018). In this study, we demonstrated a direct connection between the circadian clock gene \(ZTL\) and JA signaling in plant defense against herbivores. The results revealed that the F-box-containing evening component \(ZTL\) interacts directly with JAZ proteins, which, in turn, regulate basal nicotine accumulation and resistance against \(S.\) littoralis. Since nicotine is synthesized in roots, the function of \(ZTL\) in JA signaling to regulate nicotine biosynthesis was investigated mainly in the root. Consistent with the results from other tobacco species, a JAZ-MYC2 module was found to regulate nicotine biosynthesis in \(N.\) attenuata. Moreover, the transcript abundance of several nicotine biosynthetic genes showed diurnal rhythms, which may result from the ZTL-JAZ interaction.

Similar to its function in leaves, \(ZTL\) silencing also strongly influences the circadian clock in roots (Fig. 3). Given that \(ZTL\) protein levels oscillate, we inferred that the ZTL-JAZ-MYC2 interaction confers a circadian rhythm to JAZ-suppressed JA responses such as nicotine biosynthesis, as indicated by the fluctuation of nicotine biosynthesis-related gene transcripts (Fig. 4). Silencing of \(ZTL\) or MYC2s decreased the biosynthesis of nicotine, while \(JAZ\)-silenced plants (e.g. \(JAZd\)) had enhanced nicotine biosynthesis (Oh et al., 2013), suggesting that \(ZTL\) functions in the derepression of JAZ proteins. Most of the JAZ proteins in \(N.\) attenuata interact with MYC2a and MYC2b (Fig. 7). It is known that JAZ suppresses the activity MYC2 by recruiting the corepressor TOPLESS or competing with the mediator complex MED25 (Pauwels et al., 2010; Fernández-Calvo et al., 2011; Zhang et al., 2015). \(ZTL\) interacts directly with and affects the stability of JAZs (Fig. 6), suggesting
that JAZs could be the substrate of ZTL-mediated ubiquitination by the 26S proteasome pathway. Alternatively, ZTL could compete with NINJA to bind to the TIFY domain of JAZ proteins. All the JAZs in *N. attenuata* must recruit a NINJA-TOPELESS repressor complex to repress downstream TFs (Li et al., 2017). Furthermore, ZTL and NINJA share the same TIFY domain for JAZ binding (Fig. 6D; Pauwels et al., 2010). There is no clear rhythmicity of the transcripts of nicotine biosynthesis-related genes under LL conditions. In addition to mechanical wounding and JA treatments, other abiotic stresses, like salt and heat, are known to induce nicotine biosynthesis in cultivated tobacco (Chen et al., 2016; Yang et al., 2016). Constant light is known to be an abiotic stress that also may affect nicotine biosynthesis, leading to the arrhythmic expression of nicotine biosynthesis-related genes (Velez-Ramirez et al., 2014).

Both COI1 and ZTL interact with JAZ proteins and regulate nicotine biosynthesis, although they may function in different ways. While JA-Ile is essential for COI1-JAZ interactions, it is not required for the interaction between ZTL and JAZs (Supplemental Fig. S6; Thines et al., 2007; Li et al., 2017). The basal levels of JA-Ile are low or below the detection limit in leaves and roots of *N. attenuata*. Following elicitations (e.g. wounding and treatment with herbivore elicitors), high levels of JA-Ile accumulate rapidly, which, in turn, promote the binding of COI1 to JAZs with the resulting activation of JA responses. Thus, the regulation of the JAZ-MYC2 module by COI1 is regarded as a central component of induced defense. Indeed, the lower levels of nicotine in COI1-silenced plants compared with those of control plants were more pronounced when plants were treated with methyl jasmonate or mechanical wounding (Shoji et al., 2008). Our study shows that the high basal levels of nicotine in *N. attenuata* are at least partially regulated by a ZTL-mediated JAZ-MYC2 module, consistent with the notion that the circadian clock participates in regulating constitutive plant defenses against herbivores. Given that both JA signaling and ZTL regulate many traits across all plant tissues, we hypothesize that the ZTL-JAZ interaction also regulates phenomena in leaves or flowers. For example, the accumulation of trypsin proteinase inhibitors (TPIs) in leaves has a diurnal rhythm that peaks at midday (van Dam et al., 2001). Moreover, the biosynthesis of TPIs is regulated by JA signaling (Wang et al., 2008). Interestingly, TPI and nicotine can function synergistically as defenses against generalist herbivores not adapted to nicotine (Steppuhn and Baldwin, 2007). Further work is required to address whether basal levels of TPI in leaves also are regulated by ZTL. However, relative to nicotine, the basal levels of TPI and other defensive compounds, like caffeoylputrescine or dicaffeoylspermidine, accumulate at very low levels in leaves, implying a more important role in induced defense.

In Arabidopsis, both the evening complex (LUX, ELF3, and ELF4) and the clock-related gene *TIME FOR COFFEE* (TIC) act as suppressors of MYC2 (Shin et al., 2012; Zhang et al., 2018). Interestingly, the expression of LUX and ELF4 in *N. attenuata* ir-ztl lines is higher than that of control plants at midday (ZT4), which is the peak time for the expression of nicotine biosynthesis-related genes (Figs. 3 and 4). These data imply that ZTL also may indirectly regulate MYC2-mediated nicotine biosynthesis through the action of other clock components. However, TIC transcripts do not oscillate in the roots of *N. attenuata* and do not differ between ir-ztl and EV plants (Supplemental Fig. S10C), suggesting a different role of TIC in *N. attenuata* compared with Arabidopsis. Further experimental work with mis-expression of LUX or ELF4 in *N. attenuata* could illuminate the relative importance of direct and indirect functions of ZTL in the regulation of nicotine biosynthesis and other JA-related responses.

In summary, we revealed how ZTL regulates nicotine biosynthesis in the roots of *N. attenuata*, increasing plant resistance against a generalist herbivore. ZTL regulates basal nicotine biosynthesis by interacting with JAZ proteins but does not strongly affect JA biosynthesis and accumulation. This study provides evidence that a circadian clock component activates plant defenses by directly controlling JA signaling.

**MATERIALS AND METHODS**

**Plant Materials**

The 31st inbred generation of *Nicotiana attenuata* originating from seeds collected at the Desert Inn Ranch in Utah in 1988 was used as the wild type. Transgenic *N. attenuata* plants with silenced ZTL (ir-ztl-314 and ir-ztl-318) and COI1 (ir-coi1 and A-04-249-A-1) were obtained and screened as described previously (Paschold et al., 2007; Yon et al., 2016). EV plants were used as controls. Wild-type *Nicotiana benthamiana* plants were used for commonomopreservation experiments. For micrografting experiments, all grafts were prepared as described (Fragoso et al., 2014). Seeds were germinated on Gamborg B5 medium, and seedlings were transferred 10 d later into substrate as described previously (Herden et al., 2016). Plants for VIGS experiments, transient experiments, and micrografting experiments were grown in climate-controlled chambers at 20°C to 22°C under 16 h of light (400–1,000 μmol m⁻² s⁻¹). Plants for rhythm analyses were grown in the same chambers at 26°C under different light cycles. Plants for all other experiments were grown in a greenhouse with a 16-h-light (26°C–28°C)/8-h-dark (22°C–24°C) cycle under natural daylight supplemented by Master Sun-T PIA Agro 400 or 600 high-pressure sodium lamps (Philips).

**Insect Bioassay**

Caterpillars of *Spodoptera littoralis* (Egyptian cotton leafworm) were a generous gift from Syngenta Crop Protection. One second-instar *S. littoralis* larva was allowed to feed on each individual plant. To prevent larvae from escaping, the leaf with the caterpillar was confined in two ventilated plastic cups. A leaf was changed once it had been consumed by 80% or more. Larvae mass was measured on days 6, 8, 9, and 10. For nicotine complementation experiments, leaves of ir-ztl-314 and EV plants were excised and petioles were placed into water or 1 mM nicotine solution. Then, an *S. littoralis* larva was immediately placed on the leaf, which was confined in two plastic cups. The larvae were changed once 80% of the area was consumed by the caterpillar. *S. littoralis* larvae mass (n = 10–20) was measured at 6 and 8 d after feeding on the indicated leaves. *Manduca sexta* larvae was obtained from in-house colonies at the Max Planck Institute for Chemical Ecology. One freshly hatched *M. sexta* larva was allowed to feed on each individual plant. Twenty-five ir-ztl and EV plants were used. Larvae mass was recorded on day 9.

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ZTL Protein Analysis

Leaf material was ground (~1 mL) and homogenized with 0.5 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% [v/v] Tween 20, 10% [v/v] glycerol, 20 mM β-mercaptoethanol, and 20 μM MG132). A total of 50 μg of total protein was used for SDS-PAGE analysis. After electrophoresis by a 6% (w/v) separating gel, the gels were subjected to immunoblot analysis using a ZTL-specific antibody or stained by Coomassie Brilliant Blue. The ZTL antibody was generated by immunizing rabbits with the peptide SLVKEPTRLKDYRC and purified by GenScript.

Plant Treatments and Sample Collection

For S. littoralis regurgitant treatments, 20 μL of regurgitant (diluted 1:5 in distilled water) was rubbed into freshly produced puncture wounds created by rolling a fabric pattern wheel across the leaf lamina with a clean gloved finger. Leaves were collected at the indicated time points. Nontreated leaves and roots were collected as controls. Six to seven replicates were used for each experiment. For plants under LL treatment, light intensity was reduced to 25% of LD conditions in the climate-controlled chamber. The first sample was collected after 20 to 24 h of LL.

Nicotine Analysis

Approximately 100 mg of leaf material (precise mass was recorded) was ground and extracted with 1 mL of extraction solution (60% [v/v] methanol with 0.05% [v/v] acetic acid). After vortexing, extracts were centrifuged twice at 132,000 rpm at 4°C for 20 min. Samples were analyzed as described (Fragoso et al., 2014).

Secondary Metabolite Analysis

Approximately 50 mg of leaf material (precise mass was recorded) was ground and extracted with 0.8 mL of 80% [v/v] methanol solution. Extracts were analyzed by an electrospray ionization-time of flight mass spectrometer (Bruker Daltonic) as described previously (Li et al., 2017).

JA Analysis

Approximately 50 mg of leaf material (precise mass was recorded) was ground and extracted with 0.8 mL of ethyl acetate containing the internal standards (10 ng of D6-JA and 10 ng of D6-JA-ile). Extracts were analyzed by ultra-high-performance liquid chromatography-heated-electro-spray-ionization-tandem mass spectrometry (Bruker Daltonic) as described previously (Li et al., 2017).

Reverse Transcription-Quantitative PCR

Total RNA was isolated using the RNaseasy Plant Mini Kit (Qiagen), and 800 ng of total RNA for each sample was reverse transcribed using the PrimeScript RT-spCR Kit (TaKaRa). Three to eight independent biological samples were collected and analyzed. Reverse transcription-quantitative PCR was performed on the Stratagene Mx3000P using SYBR Green reaction mix (Eurogentec). The primers used for mRNA detection of target genes by reverse transcription-quantitative PCR are listed in Supplemental Table S1. The samples were collected after 20 to 24 h of LL.

Y2H Assay

Y2H assay was performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech) according to the manufacturer’s instructions. The indicated AD and BD control or fusion constructs were cotransformed into yeast strain Y2Hgold and plated on SD/-Leu/-Trp selective dropout medium. The transformants grew on QDO plates in the presence of 40 mg L⁻¹ X-gal at 30°C. After incubation for 3 d, the plate was photographed.

In Vitro Pull-Down Assay

Pull-down assays were performed as described previously (Li et al., 2017), and 2 μg of GST or GST-tagged ZTL and 2 μg of His-MBP-tagged JAZs were used. The samples were analyzed by SDS-PAGE. After electrophoresis, the gels were stained with Coomassie Brilliant Blue or subjected to immunoblot analysis using the anti-His antibody.

In Vitro JAZ Degradation Assay

His-JAZb degradation assays were performed as described (Li et al., 2017). Leaves sampled at ZT16 were used for crude protein extraction. The incubation was conducted at room temperature. Three biological replicates were performed.

Comminoprecipitation Assay

N. benthamiana leaves were coinfiltrated with Agrobacterium tumefaciens that contained genes encoding JAZb-Myc/YFP-ZTL or JAZb-myc/YFP. The immunoprecipitation was performed as described previously (Li et al., 2017). A total of 20 μL of immunoprecipitant was separated by SDS-PAGE and immunoblotted using the anti-Myc antibody.

Constructs

Full-length open reading frames encoding JAZa-m, ZTL, MYC2a and MYC2b without a stop codon, JAZb, or ZTL derivatives were amplified by PCR using Pfu DNA polymerase (Thermo Scientific) with the primers listed in Supplemental Table S1. For Y2H assays, each JAZ, ZTL, and ZTL derivative was cloned into pGKT7 to generate BD-fused constructs; each JAZ, JAZb derivative, MYC2a, and MYC2b was cloned into pGADT7 to generate AD-fused genes. For transient expression experiments, JAZb was cloned into pBa6-myc to generate the C-terminal myc-fused gene; ZTL was cloned into pEarlyGate 104 to generate the N-terminal YFP-fused gene. Constructs were transformed into A. tumefaciens GV3101. For prokaryotic expression, JAZa-m and ZTL were cloned into pDEST-N112-MBP and pDEST15 to generate His-MBP- and GST-fused genes, respectively. Constructs were transformed into Escherichia coli BL21 (DE3). For VIGS experiments, ~300-bp mRNA sequences of MYC2a and MYC2b were amplified by PCR using Pfu DNA polymerase (Thermo Scientific) with the primers listed in Supplemental Table S1. Fragments of NaMYC2a and NaMYC2b were combined by another round of PCR using the forward primer of MYC2a and the reverse primer of MYC2b. The DNA fragments were cloned into pTRVO and transformed into A. tumefaciens GV3101.

Accession Numbers

Sequence data from this work can be found in GenBank under the following accession numbers: NaZTL (LOC109213338), NaJAZa (LOC109220914), NaJAZb (LOC109231462), NaJAZc (LOC109233155), NaJAZd (LOC109266585), NaJAZE (LOC109229347), NaJAZf (LOC109206743), NaJAZg (LOC109219395), NaJAZh (LOC109215786), NaJAZi (LOC109240311), NaJAZj (LOC109205671), NaJAKZ (LOC109241858), NaJAKZj (LOC109220335), NaZTAl (LOC109211099), NaMYC2a (LOC1092252914), NaMYC2b (LOC109205493), NaERF189 (LOC109240873), NaERF2 (LOC109206599), NaERDC1 (LOC109209642), NaMETTL1 (LOC109236317), NaTOC1 (LOC10921941), NaHLY (LOC10921023), NaELF4 (LOC10920698), NaULX (LOC109226779), and NaTIC (LOC109213826).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Characterization of ZTL.

Supplemental Figure S2. Nicotine concentrations in ir-ztl leaves supplemented with 1 mM nicotine solution.

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Supplemental Figure S3. M. sexta performance on ir-ztl and EV plants.
Supplemental Figure S4. ZTL transcripts in different grafts.
Supplemental Figure S5. Nicotine levels in the roots of ir-ztl and EV.
Supplemental Figure S6. Interaction between ZTL and JAZ proteins by yeast two-hybrid assays with and without coronatine.
Supplemental Figure S7. Controls for in vitro pull-down assay show that GST alone does not interact with His-JAZs.
Supplemental Figure S8. In vitro His-JA2B degradation assays.
Supplemental Figure S9. Transcripts of MYC2a and MYC2b in MYC2 VIGS plants.
Supplemental Figure S10. Transcripts of MYC2a, JAZs, and TIC in the roots of ir-ztl and EV plants under LD conditions.
Supplemental Figure S11. Silencing efficiency of ZTL and CO1 and nicotine levels in EV, ir-ztl, ir-co1, and ir-ztl × ir-co1 plants.
Supplemental Table S1. DNA primers used in this study.

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