RICE UNIVERSITY

# Plant Defense against Insect Herbivory is Mediated by the Circadian Clock

**by**

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A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

**Master of Arts**

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HOUSTON, TEXAS

April 2011

# Abstract

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Organisms on earth evolved a circadian clock that matches the planet's 24-hour rotation. The plant clock controls many behaviors and proper entrainment of the clock to the environment leads to a competitive overall growth advantage. Despite the finding that many wound-inducible genes are also circadian regulated, it was uncertain whether this regulation is important for plant defense against herbivorous insects. We found that plants entrained to light-dark cycles 12 hours out of phase with the predator, *Trichoplusia ni* (cabbage loopers), were more susceptible to *T. ni* herbivory than plants entrained in phase with *T. ni.* In contrast, arrhythmic clock and jasmonate-deficient mutants were equally susceptible to *T. ni* herbivory whether entrained in the same or reciprocal 12-hour light-dark cycles. These results suggest that the circadian rhythms, acting through jasmonate signals and the clock, add selective advantage to plants through enhanced anticipation of and defense against herbivory.

# Acknowledgments

I thank Stacey Harmer for providing the arrhythmic clock mutant seeds and Wassim Chehab for providing the jasmonate synthesis deficient mutants. I especially thank Janet Braam, John Liu, Kalie Van Ree, and Se Kim for their help in critiquing my manuscript.

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# List of Abbreviations

**AOC**..... **Allene Oxide Cyclase**

**AOS**..... **Allene Oxide Synthase**

**CCA1**..... **Circadian Clock Associated 1**

**COL**..... **Columbia**

**DD**..... **Constant Dark**

**DL**..... **Dark/Light Cycles**

**ELF**..... **Early Flowering**

***Hpa***..... ***Hyaloperonospora arabidopsis***

**JA**..... **Jasmonic Acid**

**JAR1**..... **Jasmonate Resistant 1**

**JAZ**..... **Jasmonate ZIM Domain**

**LD**..... **Light/Dark Cycles**

**LHY**..... **Late Elongated Hypocotyl**

**LL**..... **Constant Light**

**LOX**..... **Lipoxygenase**

**LUX**..... **LUX Arrhythmo**

**OPDA**..... **12-oxo-phytodienic acid**

**OPR3**..... **OPDA reductase 3**

***T. ni***..... ***Trichoplusia ni***

**TOC1**.....**Timing of CAB Expression 1**

# Chapter 1

# Introduction

## 1.1. Circadian clock

To gain a competitive advantage, organisms have evolved an endogenous clock that matches our planet's 24-hour cycle. For example, plants properly entrained to their environment increase photosynthesis, growth, and survival, resulting in a competitive advantage (Dodd et al., 2005). This endogenous circadian clock is found in nearly all eukaryotes and some prokaryotes (reviewed in Doherty and Kay, 2010; Harmer, 2009).

There are three criteria that define a circadian rhythm (reviewed in Harmer, 2009; Más, 2008). First, circadian rhythms persist with an approximately a 24-hour period, even under constant conditions. Second, environmental cues can reset and synchronize the rhythms in the organism. Third, the rhythm maintains its period over a wide range of temperatures thereby compensating for temperature fluctuations. The circadian clock also regulates or "gates" an organism's sensitivity to particular stimuli; for example plants show a stronger response to shading at dusk than at dawn (reviewed in Más, 2008; Salter et al., 2003).

At the heart of the circadian clock is an oscillator that is typically comprised of many interlocking feedback loops. A simplified description of the clock is that feedback

loops enable the oscillator to be self-sustaining (reviewed in Doherty and Kay, 2010). Temporal and rhythmic patterns of behavior constitute the clock output (reviewed in Harmer, 2009; Más, 2005; Más, 2008). Light and temperature are examples of inputs that can entrain the clock (reviewed in Harmer, 2009; Más, 2005; Más, 2008).

Although the architecture of circadian systems from different organisms share common themes, the components that make up the circadian oscillator vary among plants, insects, mammals, fungi, and prokaryotes (reviewed in Doherty and Kay, 2010). In *Arabidopsis thaliana* the core components of the circadian clock are *CIRCADIAN CLOCK ASSOCIATED1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY),* and *TIMING OF CAB EXPRESSION1 (TOC1)* (Yakir et al., 2009). CCA1 and LHY are homologous MYB-domain transcription factors that have partially overlapping functions and exhibit a robust circadian oscillation of both transcript and protein (Ni et al., 2009; Schaffer et al., 1998; Wang and Tobin, 1998). Both *CCA1* and *LHY* are positively regulated by *TOC1*, and in turn negatively regulate *TOC1* (Figure 1; Alabadi et al., 2001; Park et al., 1999; Strayer et al., 2000). Transcription of *CCA1* and *LHY* occurs just before dawn, which allows them to repress *TOC1* expression in the morning (Figure 1; Alabadi et al., 2001). In the evening, *TOC1* expression increases as CCA1 and LHY levels decrease; elevated TOC1 then activates *CCA1* and *LHY* (Figure 1; Alabadi et al., 2001). Even though this central

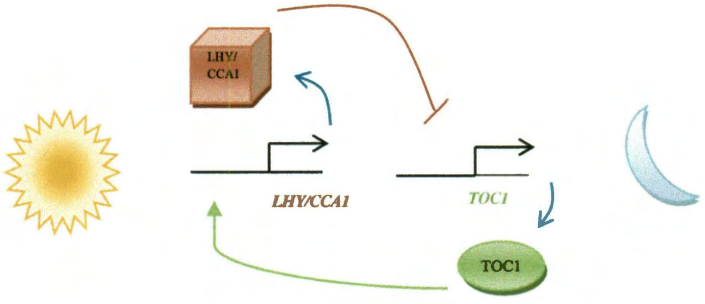


**Figure 1. Regulation of Key Components of Circadian Clock in Plants**
At night *TOC1* is expressed and activates *LHY/CCA1*. During the day, *TOC1* is repressed by *LHY/CCA1*.

oscillatory loop (*CCA1/LHY* and *TOC1*) is thought to be the main circuit in the *Arabidopsis* circadian clock, there are many other important players that are thought to operate in close conjunction to this central oscillator, forming multiple interlocking feedback loops.

The circadian clock controls the rhythmic expression of over 30% of *Arabidopsis* expressed genes (Covington et al., 2008; Michael and McClung, 2003). The rhythmic expression of clock controlled genes suggests that organisms may anticipate daily and seasonal changes in the environment (Covington and Harmer, 2007; Edwards et al., 2006; Green et al., 2002). Analysis of this circadian transcriptome is revealing which physiological processes are temporally controlled in plants and providing insight into how these processes may be controlled.

The plant's internal circadian clock regulates many functions, including gene expression, flowering time, tuberization, movement of cotyledons, petals and leaves, stomatal opening and closing, cold responses, photosynthesis rates, and light perception (Dodd et al., 2005; Más, 2008; Yakir, 2009). The role of photoperiod in controlling seasonal responses was linked to the circadian clock in 1936, and led to the external coincidence model stating that photoperiodic control of flowering results from the coincidence of external light levels and the endogenous clock controlled processes (reviewed in McClung, 2006). Furthermore, when an organism's circadian period matches that of the environmental light/dark cycle there is a competitive advantage conferred to the organism (Dodd et al., 2005; Ouyang, et al., 1998).

### 1.2. Plant defense

As sessile organisms, plants must endure many biotic and abiotic stresses because they cannot relocate to avoid stresses in their environment. To survive, plants have evolved mechanisms to respond to such stresses. A stress-related phytohormone involved in biotic stress response is jasmonic acid (JA) (Fujita et al., 2006; Schilmiller et al., 2007). JA accumulates in response to tissue damage and regulates plant defense against necrotrophic pathogens and insect herbivory (Boughton et al., 2005; Glazebrook, 2005; Schmelz et al., 2009).

JA is synthesized via the octadecanoid pathway. The initiation of JA synthesis occurs in the chloroplast (Howe and Jander, 2008), where linolenic acid is converted to 12-oxo-phytodienoic acid (OPDA) through a series of reactions by lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) (Schilmiller et al., 2007; Wasternack and Kombrink, 2009). OPDA is then transported to the peroxisome where it is reduced to OPC-8:0 by OPDA reductase 3 (OPR3) (Schilmiller et al., 2007; Wasternack and Kombrink, 2009). OPC-8:0 is then converted to its CoA derivative by OPC-8:0 CoA ligase and then undergoes 3 cycles of β-oxidation to yield JA (Schilmiller et al., 2007; Wasternack and Kombrink, 2009). JA is metabolized by JAR1 to its biologically active form, JA-isoleucine (Howe and Jander, 2008; Wasternack and Kombrink, 2009).

JAs are required for defense against herbivores. In response to attack by the generalist herbivore *Trichoplusia ni* (cabbage looper), tomato plants generate anti-digestive and anti-nutritive compounds in a JA-dependent manner (Scott et al., 2010).

JAs also enable plants to produce volatiles that attract predator wasps that feed on plant herbivores (Bruinsma et al., 2009).

Although plant defense against herbivores is critical for plant survival, to date there has been no evidence that the circadian clock has a role in herbivore defense. However, many rapid wound-responsive genes are circadian regulated (Walley et al., 2007). This gene expression regulation may indicate that plants use circadian rhythms to anticipate and defend against stresses plants experience during the course of the day and night (Walley et al., 2007). Genes induced in response to JA are enriched among genes identified as circadian regulated. 40% of the genes upregulated by JA and 48% of the genes downregulated by JA are under circadian regulation (Covington et al., 2008), but the physiological implications of circadian clock control on stress responses remain unknown.

The goal of this research is to test whether the circadian clock plays a role in how plants anticipate and respond to herbivore attack. Here we show that *T. ni* feed preferentially at dusk and more successfully feed on plants that are entrained in light-dark cycles that are offset by 12 hours from their own light-dark entrainment. The *T. ni* feeding on these out-of-phase entrained plants is lost when the *T. ni* feed on either arrhythmic clock mutants or JA-synthesis defective mutants. Our findings indicate that both the circadian clock and the JA pathway play integral roles in plant defense against herbivore attack and both are needed to confer the highest fitness level to *Arabidopsis* plants.

# Chapter 2

# Methods

## 2.1. Plant materials and growth conditions

Seeds were sterilized with 75% ethanol, then 90% ethanol, imbibed and plated on 3% sucrose 1X Murashige & Skoog (MS, Research Products International) plates. Seeds were stratified at 4 °C for 4 days and then moved to a 22 °C incubator with either a 12 hour Light/Dark (LD) cycle or a 12 hour Dark/Light (DL) cycle. Seedlings were grown on plates to the cotyledon stage (~ 5 days) and then transplanted to soil. Sixteen seedlings were transferred to each 4x4 inch pot. At 3weeks of age, plants were transferred to constant light (LL) for 24 hours and experiments commenced.

## 2.2. *Trichoplusia ni* growth

*T. ni* eggs from Benzon Research (Carlile, PA) were hatched at 25 °C and transferred with a fine brush to fresh vitamin-fortified insect media (Guy et al. 1985) and placed in an incubator under a 12 hour LD cycle at 22C. *T. ni* were entrained for 3 days and then moved to LL conditions. *T. ni* were moved to 3-week-old plants and confined in a plastic mesh from Lehle Seeds (Round Rock, TX).

### 2.3. No-choice *T. ni* studies

Five naïve *T. ni*, previously fed with only insect media, were placed in each pot of 16 plants. Pots were moved to LL and the *T. ni* were allowed to feed freely for 72 hours. Every 24 hours the level of *T. ni* feeding was documented by taking pictures with a digital camera. After 72 hours, the *T. ni* were removed from the plants, and fresh weights of *T. ni* were determined. Then final visual documentation of the plants was recorded. To statistically compare larvae weights, student t-tests were performed.

### 2.4. Choice *T. ni* studies

The potted plants were removed from their pots and moved into a flat so that the *T. ni* had a choice between wild type and clock mutants. The plant arrangement was alternated between wild type and clock mutants so that the back row had a configuration of mutant, wild type, mutant, and the front row had the configuration of wild type, mutant, wild type. Prior to experimentation, *T. ni* were grown in 12-hour LD conditions at 22C for 3 days then moved to LL for 24 hours. Five naïve *T. ni* were then placed in between each pair of plants to give the *T. ni* the choice between a wild type and mutant plant and allowed to feed freely on the plants for 72 hours. Every 24 hours the extent of plant tissue loss by *T. ni* feeding was documented by photographs. After 72 hours the *T. ni* were removed from the plants, and final pictures of the plants were taken. Fresh weights of the plants were determined and statistically compared with student t-tests.

### 2.5. Time of day feeding studies

Twenty 4-day-old *T. ni* entrained in 12-hour LD conditions were transferred to a petri dish with approximately 5 grams of insect media (food) on a piece of filter paper. The weight of each food slice was determined at the start of the experiment. Every 4 hours for 72 hours the food was removed and weighed and a new 5 gram aliquot was substituted. As a control, food aliquots were placed on filter paper in a petri dish and weighed at 4 hour intervals to measure the amount of evaporation.

# Chapter 3

# Results

### 3.1. Plants are less susceptible to *T. ni* infestation when appropriately entrained to their environment

We hypothesize that circadian clock-dependent entrainment enables plants to anticipate and more successfully defend themselves against insect herbivory. To test this hypothesis, I sought to compare herbivory damage on plants whose circadian rhythm was in phase with the predatory insect, *T. ni*, to plants whose circadian rhythm was out of phase with the herbivore. If matched entrainment phases for plants and herbivores are advantageous to the plants, then we would expect to see greater plant resistance for in-phase entrained plants than for plants whose circadian rhythm entrainment was out of phase to that of the insects. 3-week-old plants were entrained in either a 12 hour Light-Dark (LD) cycle, consisting of 12 hours of light then 12 hours of dark or a 12 hour Dark-Light (DL) cycle, consisting of 12 hours of dark followed by 12 hours of light (Figure 2A). I refer to these two sets of plants as "LD plants" and "DL plants", respectively. 4-day-old *T. ni* were entrained in a 12-hour LD cycle coincident with that of the LD plants. *T. ni*, LD plants, and DL plants were placed in constant light (LL) conditions for 24 hours

to uncouple the endogenous clock-dependent rhythms from diurnal responses. Five *T. ni* were then placed with either 16 LD plants or 16 DL plants. The *T. ni* were allowed to eat freely for 72 hours. Individual *T. ni* were collected from the plants and weighed, and the plants were photographed to record tissue loss. Plants (Figures 2B and C) in-phase entrained with *T. ni* showed less tissue damage than plants entrained out-of-phase with *T. ni* (Figures 2D and E). The *T. ni* that fed on the out-of-phase entrained plants weighed significantly more than the *T. ni* that fed on in-phase entrained plants, with an average weight of 9.19 mg and 5 mg, respectively (Figures 2F and G). In addition, the *T. ni* that fed on the out-of-phase entrained plants had a higher survival rate than the *T. ni* that fed on the in-phase entrained plants, 56.7% and 20% respectively (data not shown).

We next tested whether similar results are obtained under constant darkness (DD conditions). Plants that had been entrained in phase with the *T. ni* showed less tissue loss (3A) than plants entrained out of phase with the *T. ni* (3B). The *T. ni* that fed on the out-of-phase entrained plants weighed significantly more than the *T. ni* that fed on in-phase entrained plants, with an average weight of 3.74 mg and 0.68 mg, respectively (Figure 3C). These results indicate that *T. ni* more successfully eat out-of-phase entrained plants than in-phase entrained plants and support our hypothesis that when plants are properly entrained to the external environment they can better anticipate herbivore feeding times and activate defenses against such attacks.

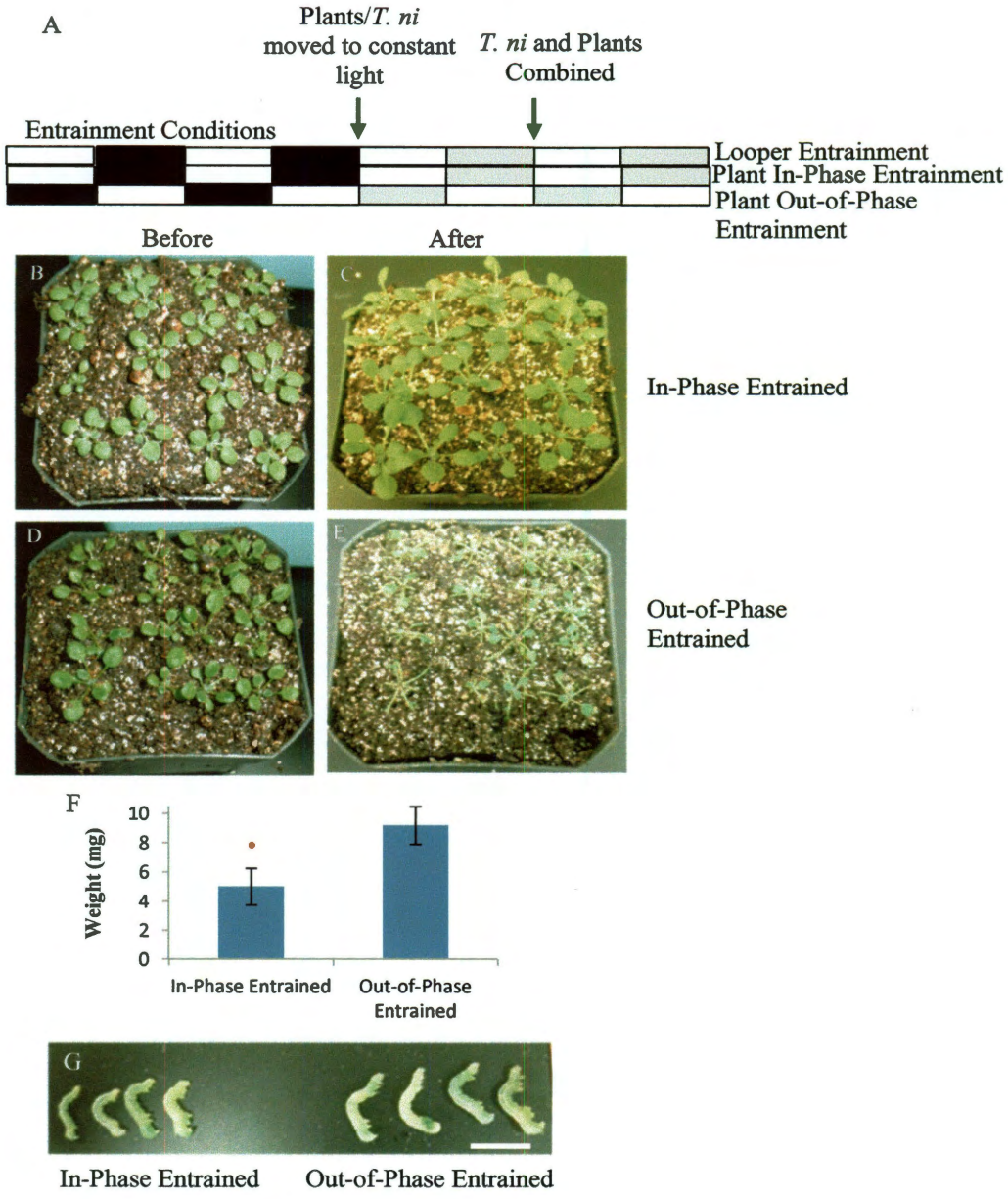


**Figure 2. Plants are less susceptible to insect herbivory when properly entrained to their environment**

Plants were grown for 3 weeks in either12-hour LD or 12-hour DL cycles at 22C. *T. ni* were entrained for 3 days in 12-hour LD cycle at 22C. Plants and *T. ni* were moved to LL conditions 24 hours to uncouple diurnal rhythms then *T. ni* were added to plants and allowed to feed freely for 72 hours in constant light. Six pots were used for each condition, 5 *T. ni* were added to each pot. (A) Illustration of light-dark cycle entrainment and experimental protocol. Open rectangles represent 12-hour light periods, black rectangles represent 12-hour dark periods, and gray rectangles represent 12-hour light periods that correspond to subjective dark periods. The timing of shifting LD and DL cycles to constant light is indicated by the first arrow. *T. ni* were placed with plants at the second arrow marked time point. (B-C) In-phase entrained plants before (B) and after (C) *T. ni* feeding. (D-E) Out-of phase entrained plants before (D) and after (E) *T. ni* feeding. (F) Mean *T. ni* weight at the end of the experiment. (error bars=standard error; asterisk= significance difference $P$= 0.045) (G) Photo of representative *T. ni* after experiment (scale bar=0.5 mm). Experiment was repeated at least 10 times with similar results.

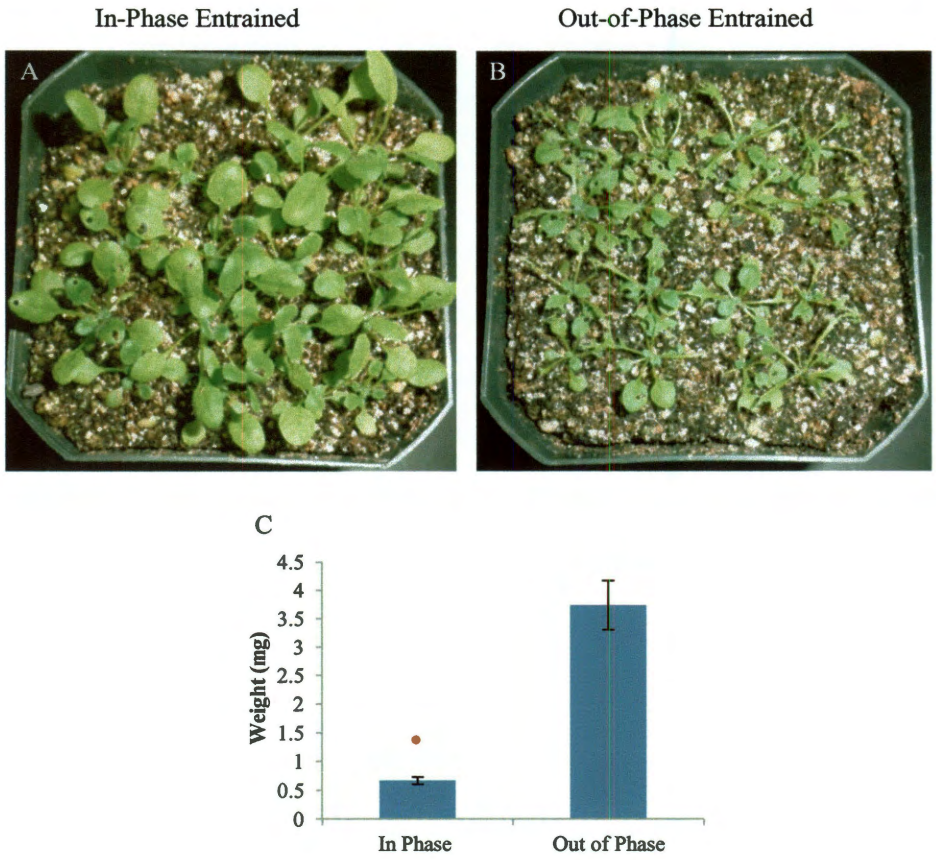


**Figure 3. Under constant dark conditions in-phase entrained plants are more *T. ni* resistant than out-of-phase entrained plants**

Plants were grown for 3 weeks in either12-hour LD or 12-hour DL cycles at 22C. *T. ni* were entrained for 3 days in 12-hour LD cycle at 22C. Plants and *T. ni* were moved to constant dark conditions 24 hours to uncouple diurnal rhythms then *T. ni* were added to plants and allowed to feed freely for 72 hours in constant dark. Six pots were used for each condition, 5 *T. ni* were added to each pot. (A) In-phase entrained plants after 72 hours. (B) Out-of phase entrained plants after 72 hours. (C) Mean *T.ni* weight at the end of the experiment. (error bars=standard error; asterisk=significant difference $P = 0.043$)

### 3.2. *T. ni* feed preferentially at dusk

Many insects have a circadian clock; for example, *Drosophila melanogaster* has a circadian clock that is well characterized and established as a tool for circadian clock studies (reviewed in Bradshaw and Holzapfel, 2010; Tomioka and Matsumoto et al., 2010). In *Drosophila*, feeding behavior is circadian regulated (Chatterjee et al., 2010; Xu et al., 2008). We hypothesize that *T. ni* also have a circadian clock that dictates their feeding time preferences. To determine if *T. ni* display periodic feeding behavior that may be linked to circadian rhythms, *T. ni* were entrained in 12-hour LD cycles for 3 days and then moved to either 12-hour LD cycles, constant dark (DD), or LL and were allowed to eat freely for 72 hours on insect media. Feeding activity, measured as food weight loss, was determined at 4-hour intervals, and fresh food was provided every 4 hours. *T. ni* maintained under 12-hour LD conditions ate more during the light periods, with peaks of food loss occurring at the light-dark transition ("dusk") (Figures 4A, 12, 36, 60 hour time points). The least food loss occurred at the dark-to-light transition ("dawn") (Figures 4B, 14, 48, 72 hour time points). These results indicate that under these conditions, *T. ni* prefer to eat more at dusk than dawn. Therefore, *T. ni* display a diurnal feeding rhythm. Under constant dark *T. ni* also displayed greatest feeding at peaks corresponding to subjective dusk and least feeding at subjective dawn (Figure 4B), strongly suggesting that *T. ni* feeding behavior is circadian regulated. Constant dark conditions dampened the feeding inhibition at dawn relative to behavior under light-dark cycles; more food was eaten at the 24, 48, and 72 hour time points in constant darkness

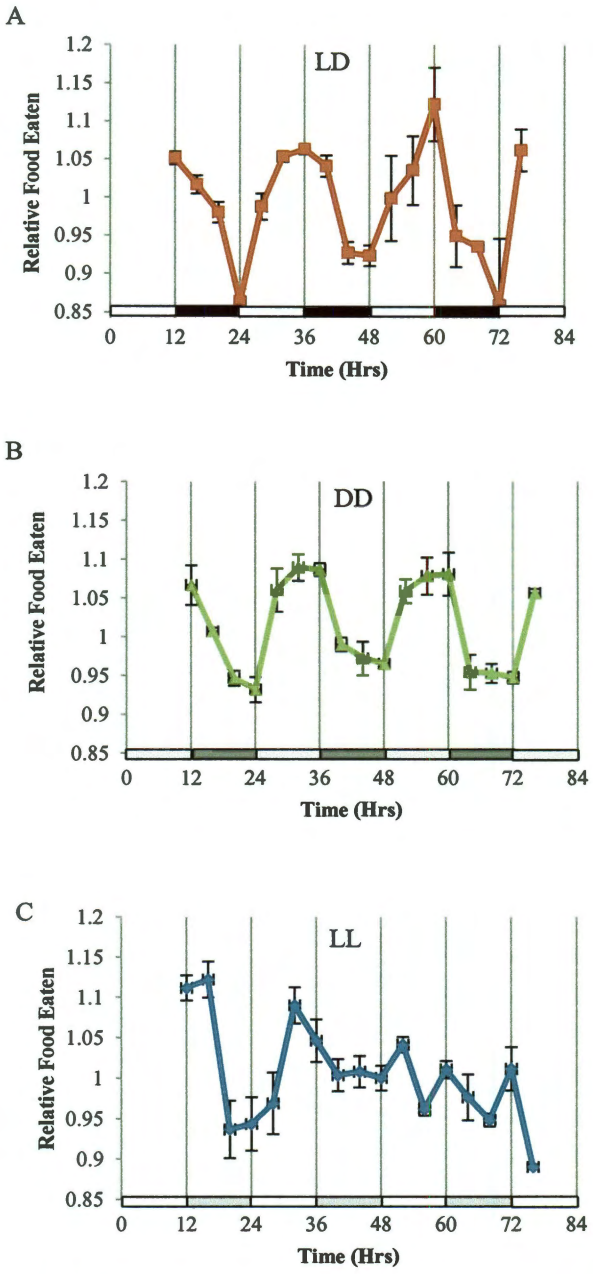


**Figure 4. *T. ni* feeding behavior is under circadian clock regulation with enhanced eating at dusk**

*T. ni* were entrained in 12-hour LD conditions at 22C and then moved to feeding dishes in (A) LD, (B) DD, or (C) LL conditions. Food quantity was determined every 4 hours for 72 hours. Three plates with 20 *T. ni* for each condition. (A) Relative food eaten under LD conditions. (B) Relative food eaten under DD conditions. (C) Relative food eaten under LL conditions. Error bars=standard error. "1" is when the ratio between before and after feeding with experimental food is equal to before and after with control food. Experiment was repeated 4 times with similar results.

than when light-dark cycling was maintained (compare Figures 4A and 3B, 24, 48, 72 hour time points). *T. ni* shifted from light-dark cycling to constant light (LL) maintained the pattern of decreased feeding during subjective night and increased feeding during subjective day for the first 24 hours (Figure 4C). Similarly to *T. ni* in constant darkness, *T. ni* in constant light fed more at subjective dawn than *T. ni* maintained in cycling light conditions (compare Figures 4A-C, 24 hour time point). However, after the first 36 hours in LL conditions, the *T. ni* feeding pattern became largely arrhythmic (Figure 4C, 40-76 hours). Other insects display dampened rhythms in constant light conditions (Tomoika and Matsumoto 2010; Bradshaw and Holzapfel 2010); therefore loss of rhythmic behavior under constant light may be a characteristic of insects. However, I found that under LL conditions, *T. ni* survival was affected (data not shown). *T. ni* generally prefer shaded conditions, and under the experimental conditions of this study, shade was not provided. It is also possible that the *T. ni* in LL conditions maintain circadian regulation but have a dampened rhythm that may be below our detection level. Taken together, these results support the hypothesis that *T. ni* feeding behavior is under circadian clock regulation with a preference to feed at dusk.

### 3.3. Arrhythmic clock mutants have decreased *T. ni* resistance

If the circadian clock enhances defense preparation against herbivory, then when plant circadian clock function is lost, plants should demonstrate no differential resistance dependent on in-phase or out-of-phase circadian entrainment. To test this hypothesis, we investigated whether arrhythmic clock mutants showed entrainment–dependent variance in defense resistance. *elf4-1* is a mutant with a loss of function in ELF (Early Flowering)

and *lux-2* (LUX Arrhythmo) has lost function of a MYB domain transcription factor (Doyle et al., 2002; Hazen et al., 2005). Both *ELF4* and *LUX* are required for sustained circadian rhythms in constant conditions.

#### 3.3.1.1. Arrhythmic clock mutants show no entrainment phase difference in *T. ni* resistance

Wild type Col-0, *elf4-1*, and *lux-2* plants were entrained in either 12-hour LD or 12-hour DL conditions and then transferred to LL for 24 hours. *T. ni*, entrained in 12-hour LD conditions, were released onto plants and allowed to eat freely for 72 hours. The *T. ni* were collected and weighed, and the plants were photographed to record tissue loss. *T. ni* weights were not significantly different whether they fed on in-phase entrained *elf4-1* or out-of-phase entrained *elf4-1*, with average *T. ni* weights of 2.19 mg and 2.45 mg, respectively (Figure 5E). Similarly, *T. ni* weights did not significantly differ whether they fed on in-phase or out-of-phase entrained *lux-2* plants, with weight averages of 1.26 mg and 1.62 mg, respectively (Figure 5F). These data show that without a functional circadian clock, plant entrainment does not help against herbivore attack.

#### 3.3.1.2. Arrhythmic clock mutants are preferred over wild type plants by *T. ni*

An additional test for a role of circadian clock functions in herbivory defense was conducted giving *T. ni* a choice to feed on clock mutants versus wild type plants. Our hypothesis predicts that a functional circadian clock would regulate plant defense responses making wild type plants less attractive or palatable to the *T. ni*, and instead *T.*

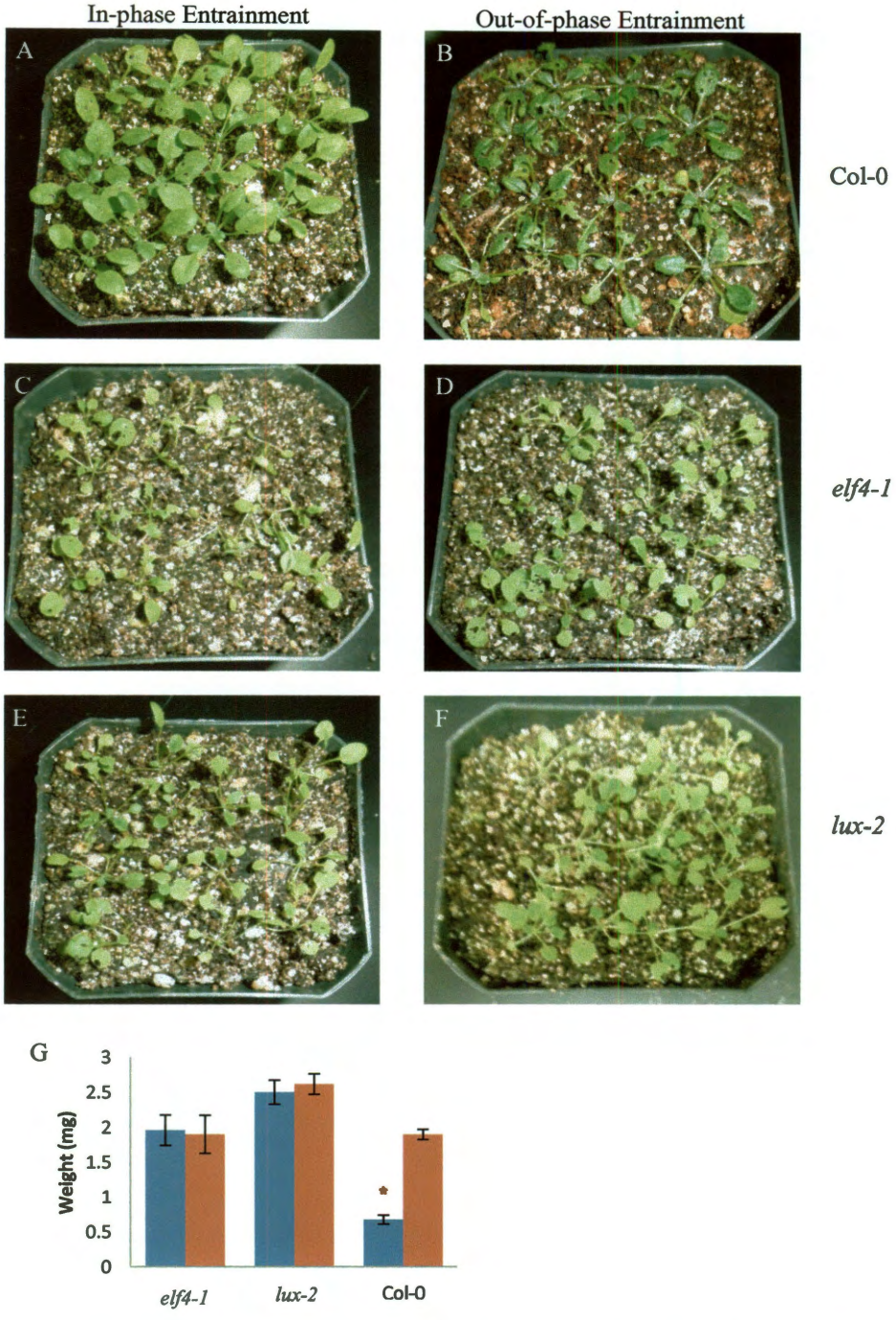


**Figure 5. Arrhythmic clock mutants lack entrainment-dependent variance in *T. ni* resistance**
Plants were grown for 3 weeks in either12-hour LD or 12-hour DL cycles at 22C. *T. ni* were entrained for 3 days in 12-hour LD cycle at 22C. Plants and *T. ni* were moved to LL conditions 24 hours to uncouple diurnal rhythms then *T. ni* were added to plants and allowed to feed freely for 72 hours in constant light. Six pots were used for each condition, 5 *T. ni* were added to each pot. (A-B) Col-0 plants after experiment. (A) In-phase entrained Col-0 (B) Out-of-phase entrained Col-0 (C-D) *elf4-1* plants after experiment. (C) In-phase entrained *elf4-1* (D) Out-of-phase entrained *elf4-1* (E-F) *lux2* plants after experiment (E) In-phase entrained *lux2* (F) Out-of-phase entrained *lux2* (G) Mean *T. ni* weight after 72 hour feeding on either in-phase entrained (blue) or out-of-phase entrained (red) (error bars=standard error, Asterisk= significant difference $P$= 0.0018) Experiment was repeated 3 times with similar results.

*ni* would preferentially eat the "less fit" plant. Three- week-old potted wild type, *elf4-1*, and *lux-2* plants were entrained in 12-hour LD and then placed together into a small flat. The flats were moved to constant light for 24 hours and then *T. ni* were released onto the soil in between the mutant and the wild type plants and allowed to feed freely for 72 hours under constant light. To assess *T. ni* feeding preference, wild type, *elf4-1* and *lux-2* shoots were harvested and weighed. Control plants were treated similarly except were not subjected to *T. ni*. The shoot weight difference between control *elf4-1* to *T. ni*-treated *elf4-1* was significantly greater than the weight difference between untreated and *T. ni*-treated wild type (Figures 6A, 6C, 6E, 6G and 6H). Similarly, *T. ni*-treated *lux-2* plants lost more shoot mass relative to untreated *lux-2* that *T. ni*-treated wild type (Figures 6B, 6D, 6F, 6I and 6J). Indeed, wild type shoot weight was not significantly different whether or not exposed to *T. ni* (Figure 6B). These results indicate that when *T. ni* are given a choice between plants with a functional circadian clock and those without, the *T. ni* prefer to feed on the clock mutant plants. These data further strengthen our hypothesis that the circadian clock confers advantage to plants, likely enabling them to link herbivore behavior to times of day and thus anticipate and defend against herbivore attack.

### 3.4. Jasmonate synthesis deficient mutants show no entrainment phase difference in *T. ni* resistance

The plant JA signaling pathway is critical for orchestrating defense against herbivorous attack (Howe 2004, Kessler and Baldwin 2002). Although some genes

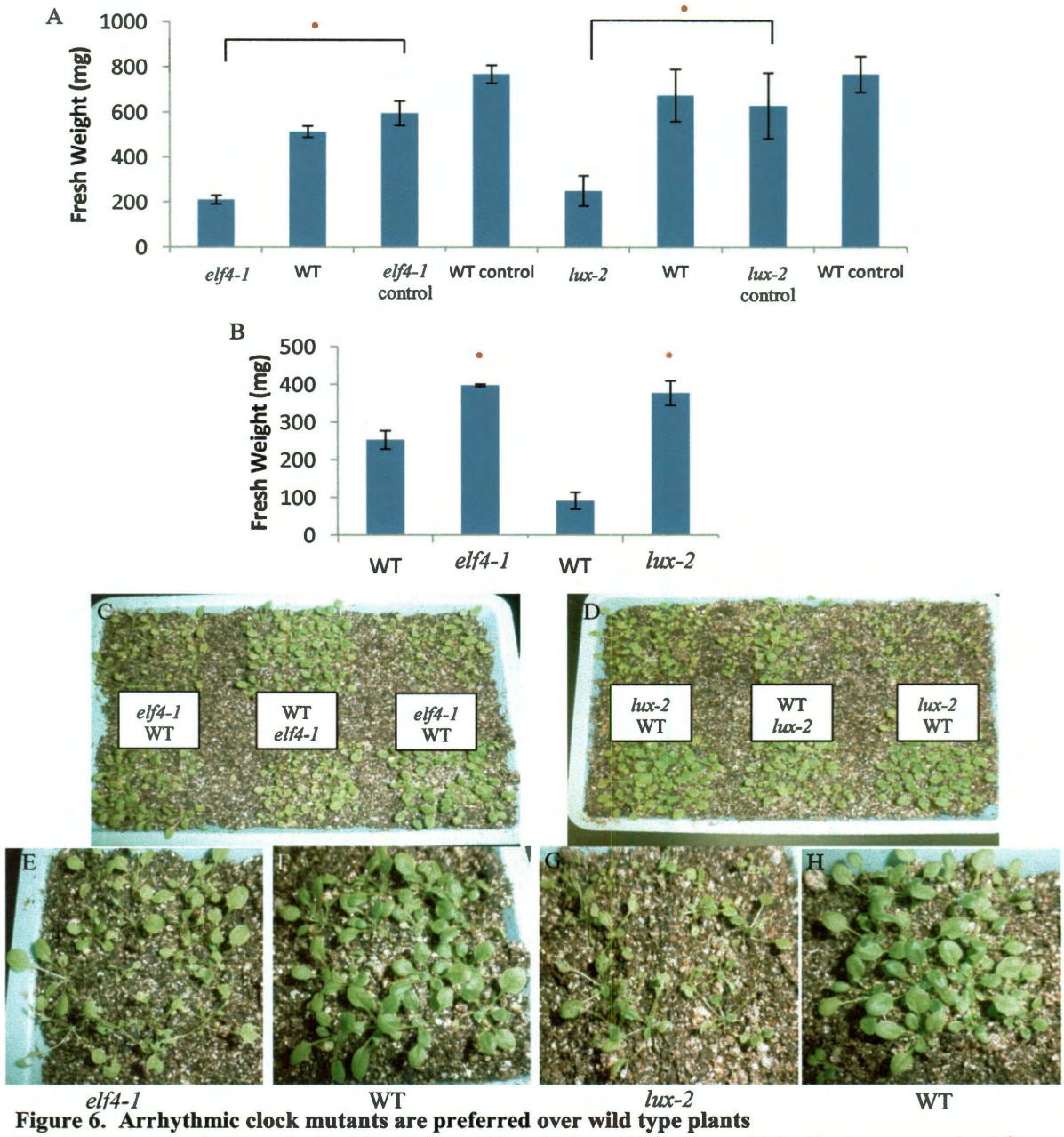


**Figure 6. Arrhythmic clock mutants are preferred over wild type plants**

Plants were grown for 3 weeks in either12-hour LD or 12-hour DL cycles at 22C. *T. ni* were entrained for 3 days in 12-hour LD cycle at 22C. Plants and *T. ni* were moved to LL conditions 24 hours to uncouple diurnal rhythms then *T. ni* were added to plants and allowed to feed freely for 72 hours in constant light. Three flats were used for each mutant, 15 *T. ni* were added to each flat. Controls were plants subjected to the same conditions with no *T. ni* added (A) *elf4-1* and *lux2* shoot weights after experiment, (error bars=standard error; asterisk=significant difference $P$= 0.0024, 0.0088 respectively) (B) *elf4-1, lux2* and WT mean shoot weight difference after experiment (error bars=standard error; asterisk=significant difference $P$=0.034, 0.0089 respectively) (C-D) Alternating arrangement of mutant and wild type plants after 72 hours of *T. ni* feeding (C) *elf4-1* (D) *lux-2* (E-H) Close up images of plant tissue loss (E) *elf4-1* (F) WT (G) *lux-2* (H) WT. Experiment was repeated 3 times with similar results.

encoding components of the JA pathway, such as *AOS, JAR1, OPR3,* and *JAZ,* are under circadian control (Covington et al. 2008), the circadian clock has not been shown to be important for JA-regulated herbivory defense. To determine whether JAs are required for circadian clock-dependent enhancement of *T. ni* defense, I tested whether the loss of a functional JA synthesis pathway eliminated the enhancement of plant defense conferred by in-phase entrainment of plants with the herbivore. Two JA-biosynthesis mutants were chosen for analysis. *aos* lacks allene oxide synthase required early in the jasmonate biosynthesis pathway (Park et al., 2002). The *jar1* mutant lacks the ability to convert jasmonic acid into the biologically active jasmonyl-isoleucine (Suza and Staswick, 2008). Plants were entrained in either 12-hour LD or 12-hour DL cycles, and *T. ni* were entrained in 12-hour LD cycles. Five *T. ni* were released to 16 plants and allowed to eat freely for 72 hours. *T. ni* were collected and weighed, and plants were photographed to record overall tissue damage. Unlike with wild type (Figure 2), entrainment of *aos* and *jar1* had no significant effect on *T. ni* weight (Figure 7). These results suggest that the JA pathway is required for plants to demonstrate an advantage in *T. ni* defense through circadian entrainment.

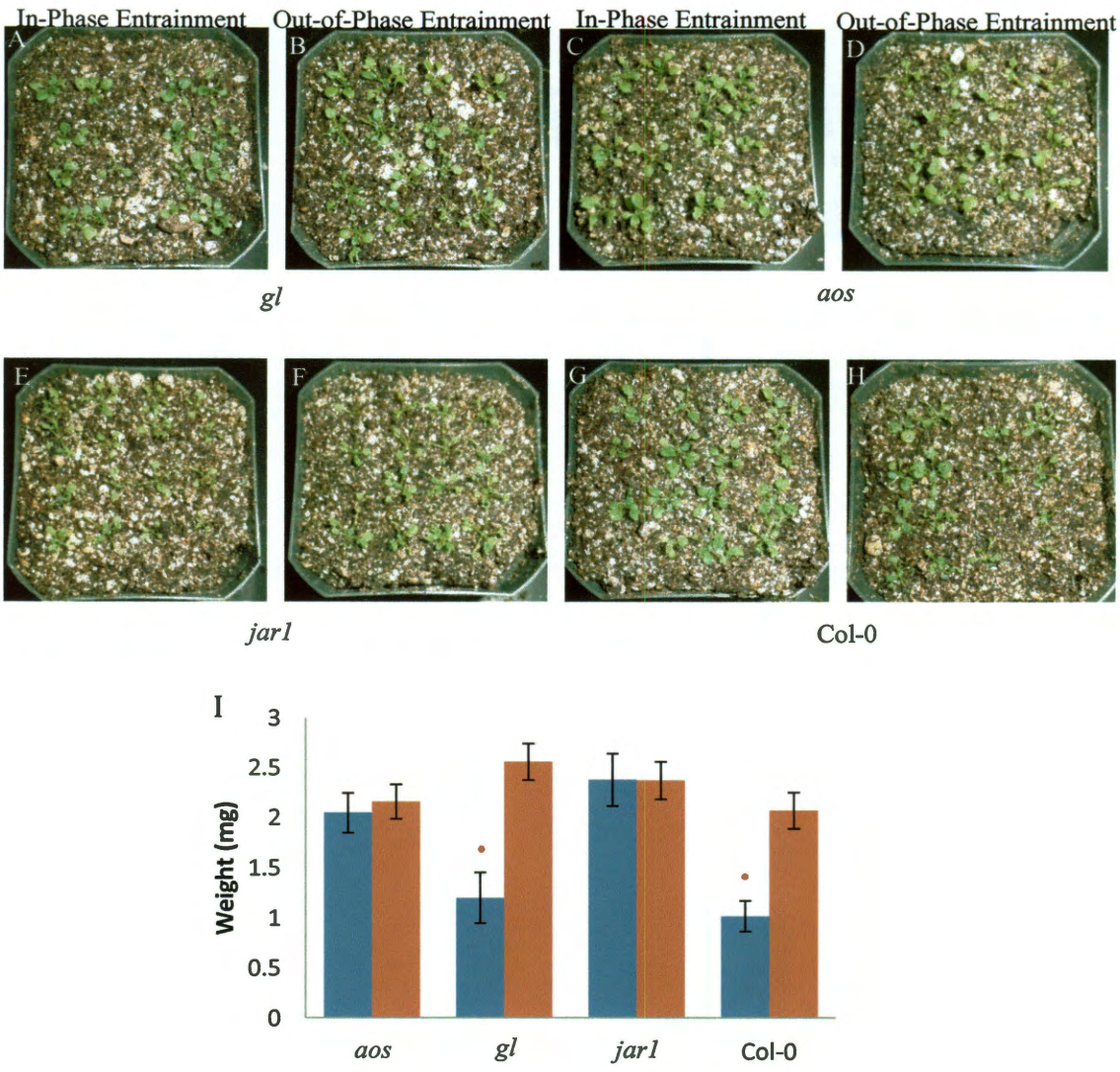


**Figure 7. JA synthesis deficient mutants lack entrainment phase dependence in *T. ni* resistance**

Plants were grown for 3 weeks in either12-hour LD or 12-hour DL cycles at 22C. *T. ni* were entrained for 3 days in 12-hour LD cycle at 22C. Plants and *T. ni* were moved to LL conditions 24 hours to uncouple diurnal rhythms then *T. ni* were added to plants and allowed to feed freely for 72 hours in constant light. Six pots were used for each condition, 5 *T. ni* were added to each pot. (A-B) *gl* plants after 72 hours of *T. ni* feeding. (A) In- phase entrained plants (B) Out-of-Phase entrained plants (C-D) *aos* plants after 72 hours*T. ni* feeding. (C) In- phase entrained plants (D) Out-of-Phase entrained plants (E-F) *jar1* plants after 72 hours of feeding. (E) In- phase entrained plants (F) Out-of-Phase entrained plants (G-H) Col-0 plants after 72 hours of feeding. (G) In- phase entrained plants (H) Out-of-Phase entrained plants (I) Mean *T. ni* weight after 72 hour feeding on either in-phase entrained (blue) or out-of-phase entrained (red) plants (error bars=standard error, Asterisk= significant difference *P*= 0.00093 *gl,* 0.05 Col-0) Experiment was repeated 3 times with similar results.

# Chapter 4

# Discussion and Future Work

## 4.1. Discussion

Although the components of the circadian clock have been largely defined (reviewed in Harmer 2009), the physiological relevance of circadian rhythms has not been extensively studied. Recently, links between the circadian clock and plant productivity and immunity have been revealed. Plants properly entrained to their environment show increased photosynthesis, enhanced growth, and increased survival, resulting in a competitive advantage (Dodd et al., 2005). CCA1, a core clock component, enhances resistance against biotrophic pathogens through the (R)-gene-mediated defense mechanism (Wang et al., 2011). The finding that many genes that are wound-responsive are also circadian regulated (Walley et al., 2007) led to the hypothesis that plants might use circadian clock information to upregulate wound-response gene expression and other defense behaviors in anticipation of potential herbivore attack. In this study, I demonstrate that clock entrainment of Arabidopsis plants matched to entrainment of their herbivore, *T. ni* results in increased plant resistance, as measured by *T. ni* weight gain and

plant tissue destruction. Plants entrained to be out of phase relative to *T. ni* suffer more herbivory.

A functional circadian clock is required for this defense advantage because arrhythmic clock mutants fail to show enhanced resistance after in-phase entrainment. Furthermore, when *T. ni* are given the choice between arrhythmic clock mutants and wild type, they choose to feed preferentially on the clock mutants.

This research also determined that *T. ni* feeding is cyclic with a 24-hour period even under constant darkness, thus demonstrating a role for the circadian clock in insect feeding behavior throughout the day. Our results also suggest that under constant light conditions the circadian regulation of their feeding habits is dampened. These results are consistent with other insect studies showing that circadian regulation is dampened in insects under constant light conditions (Tomioka and Matsumoto, 2010). These results indicate that the circadian clock influences *T. ni* feeding behavior with increased feeding at dusk.

Taken together, these results suggest that the circadian clock enables plants to predict when *T. ni* are more likely to attack and garner defenses in anticipation. The idea that the circadian clock improves plant defense is also consistent with a recent report that Arabidopsis defense against using *Hyaloperonospora arabidopsis* (*Hpa*), a biotrophic pathogen, is enhanced by the circadian clock with greater resistance at dawn (Wang et al., 2011).

Genes involved in JA biosynthesis are circadian regulated (Covington et al., 2008) and JAs are required for plant defense against insect herbivory (Kessler and Baldwin 2002) but a direct link between the circadian clock and JAs for insect defense has not been identified. I found that the inability to synthesize biologically active JAs of *aos* and *jar1* results in reduced *T. ni* resistance with coincident circadian entrainment of the plants and herbivore.

Three possibilities arise as to why both arrhythmic clock mutants and JA synthesis deficient mutants lose their ability to resist infestation under different entrainment conditions. First, the circadian clock and JA could be acting independently of each other in insect defense. If this is the case, losing either active JA or a functional clock results in plants being susceptible to insect herbivory. Second, JA could be required for the clock to function, therefore if JA is not synthesized loss of clock function arises and the plant loses resistance to infestation. A third possibility is that the circadian clock acts upstream of JA synthesis. Since some JA biosynthetic genes and responses are circadian regulated, the third possibility may be most likely.

Our results indicate that the circadian clock not only modulates the response to herbivory, possibly through the JA pathway, but anticipates when herbivores are going to feed. This allows the plants to prepare a defense against herbivores, conferring a higher level of fitness to the plant. This is consistent with our hypothesis that plants use circadian rhythms to anticipate and defend against stresses the plant encounters throughout the course of the day.

### 4.2. Future work

Further testing with the *T. ni* feeding study may need to be done to determine if under LL conditions the circadian clock's control on *T. ni* feeding behavior is dampened. Since *T. ni* survival is reduced in constant light, repeating the experiment in shaded conditions may increase survival and give us a better understanding of whether the clock control on *T. ni* feeding behavior is dampened in constant light. We propose two possible methods. One method would be to put a mesh over the plates. This will filter the light causing "shaded" conditions. Second, a fake plant, for example, a plastic plant, could be used to produce shade over the petri dishes causing a shade response. All other experimental conditions would remain the same.

The entrainment studies support a possible link between the circadian clock and the JA pathway. Some genes encoding components of the JA biosynthesis pathway are circadian regulated (Covington et al. 2008). Whether levels of JA accumulate with a circadian pattern has not yet been tested. To examine levels of JA, JA can be extracted from unwounded plants, collected every 4 hours over 48 hours and run on a GC-MS. Plants will be entrained in 12-hour LD cycles for 3 weeks then moved to LL for 24 hours before harvesting shoots. Another possibility is that JA sensitivity may change with a rhythmic pattern. A circadian-regulated change in sensitivity is known as gating. To test circadian regulation of JA-sensitivity *aos* mutants entrained in LD cycles can be supplied a constant level of exogenous methyl-JA and JA-responsive gene expression can be monitored. If expression changes throughout the day, one could conclude that JA

sensitivity changes with a circadian pattern. These experiments will determine if JA accumulates in a circadian pattern or if JA sensitivity is circadian regulated.

Alternatively, JA could act independently of the circadian clock, but both could be needed to make plants resistant to insect herbivory. To test this hypothesis, double mutants can be made with both a JA mutant (*jar1*) and a clock mutant (*lux-2*). This double mutant would be entrained and insect herbivory susceptibility can be assessed. If these two pathways act independently of each other, then an additive effect, that is an even greater susceptibility to insect herbivory, should occur. If JA and the clock work in the same pathway then no difference in susceptibility is predicted.

Finally, JA could be needed for clock function. If this is the case, then core clock components would be arrhythmic in JA mutants. To test this hypothesis, semi-quantitative RT-PCR can be performed on the JA mutants using primers for the core clock components, *CCA1* and *TOC1*. If the circadian clock is abolished in JA deficient mutants, then these core clock components will show no rhythmic expression, but if the circadian clock is still present, then these core clock components will still have a rhythmic expression.

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