RICE UNIVERSITY

The Plant Circadian Clock: Roles in Jasmonate Accumulation and Postharvest Plant Performance

by
John Liu

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

Doctor of Philosophy

APPROVED, THESIS COMMITTEE

Dr. Janet Braam
Wiess Professor of BioSciences

Dr. Bonnie Bartel
Ralph and Dorothy Looney Professor of BioSciences

Dr. Michael Gustin
Professor of BioSciences

Dr. Seiichi Matsuda
E. Dell Butcher Professor of Chemistry and Professor of BioSciences

Dr. Weiwei Zhong
Assistant Professor of Biosciences

HOUSTON, TEXAS

November 2015

ABSTRACT

The Plant Circadian Clock: Roles in Jasmonate Accumulation and Postharvest Plant Performance

by

John Liu

Nearly all organisms have evolved circadian clocks that allow them to anticipate and prepare for cyclical changes in their environment, such as those associated with the transitions from night to day and between seasons. The plant circadian clock functions nearly cell autonomously, regulating rhythmic behaviors in a variety of processes that range from the transcriptional level up to organismal level. Although great progress has been made in determining how the clock functions in plants, not much is known about the clock's physiological relevance on particular plant processes. Specifically, the clock influences plant defense against *Trichoplusia ni* (*T. ni*), as well as basal rhythmic accumulation of jasmonic acid (JA), a plant hormone involved in the regulation of resistance against insect herbivores such as *T. ni* and necrotrophic fungi such as *Botrytis cinerea* (*B. cinerea*). Additionally, the clock affects the accumulation of glucosinolates, secondary metabolites involved in plant insect resistance, in both Arabidopsis and post-harvest cabbage (*Brassica oleracea*). Here I show that rhythmic accumulation of basal JA may be transcriptionally regulated through clock controlled JA-biosynthesis gene transcript accumulation. Additionally, the clock may influence rhythmic accumulation of gene transcripts involved in JA biosynthesis independently of JA-positive feedback. Furthermore, while SA and JA act antagonistically, we note that decreased SA does not influence differential accumulation of JA-biosynthesis related gene transcripts. I also

show that phase-entrainment dependent plant resistance to *T. ni* is influenced in part by aliphatic glucosinolates. Finally, I demonstrate that postharvest vegetables kept in light/dark cycles maintain appearance, tissue integrity, chlorophyll levels, and glucosinolate levels for longer periods of time when compared to postharvest vegetables stored in constant light or constant dark. Moreover, postharvest plants exhibit improved resistance to *B. cinerea* when kept under light/dark cycles when compared to constant light storage.

Acknowledgments

I would like to thank all the members of the Braam lab, particularly Dr. Wassim Chehab, Dr. Danielle Goodspeed, Dr. Se Kim, Dr. Yeonjong Koo, and Dr. Wenbo Li for all the advice, informative discussions and most of all valued friendship that they have given me during my time with the Braam lab. I would also like to thank my extremely helpful undergraduate students: Jim Sheng and Isabella Yang, who helped me survive the long nights of circadian experiments.

Thank you to my wife who understood when I had to stay overnight to conduct experiments and for all the support she's given to me during my research.

I would like to give my greatest thanks and appreciation to my mentor, Dr. Janet Braam, who took Danielle and me into her lab and with great enthusiasm incorporated our research interests into her lab. She has been the greatest mentor I could have hoped for.

Contents

List of Figures

List of Tables

List of Equations

Abbreviations

13-HPOT	13(S)-hydroperoxy-octadecatrienoic acid
13-LOX	13-lipoxygenase
3MSO	3-methylsulfinylpropyl
4CDD	Refrigeration at 4oC in constant dark
4MO-I3M	4-methoxy-indolyl-3-methyl
4MSO	4-methylsulfinylbutyl
5MSO	5-methylsulfinylpentyl
6MSO	6-methylsulfinylhexyl
7MSO	7-methylsulfinylheptyl
8MSO	8-methylsulfinyloctyl
ACD6	*ACCELERATED CELL DEATH6*
ANOVA	Analysis of variance
AOC	ALLENE OXIDE CYCLASE
AOS	ALLENE OXIDE SYNTHASE
ATR1	ALTERED TRYTOPHAN REGULATION 1
BAT5	BILE ACID TRANSPORTER5
BCAT3	BRANCHED CHAIN AMINO ACID AMINOTRANSFERASE3
BCAT4	BRANCHED CHAIN AMINO ACID AMINOTRANSFERASE4
B. cinerea	*Botrytis cinerea*

CCA1	*CIRCADIAN CLOCK ASSOCIATED 1*
CCA1-OX	*CCA1-OVER EXPRESSOR*
CYP	Cytochrome P450
DD	Constant dark
EE	Evening element
gl-1	*glabra1-1*
GLS	Glucosinolates
HPLC-DAD	High-Performance Liquid Chromatography with Diode-Array Detection
I3M	Indoyl-3-methyl
IAA	Indole-3-acetic acid
IPMI	isopropylmalate
IPM-DH	Isopropylmalate dehyrogenase
JA	Jasmonic acid
JA-Ile	Jasmonoyl-Isoleucine
JAR1	JASMONATE RESISTANT 1
JAZ	JASMONATE ZIM-DOMAIN
LD	12-hour light/12-hour dark cycles
LHY	*LATE ELONGATED HYPOCOTYL*
LL	Constant light
MAM	METHYLTHIOALKYLMALATE SYNTHASE
MeJA	Methyl-Jasmonic acid
MS	Murashige and Skoog

NMO-I3M	1-methoxy-indolyl-3-methyl
OPC-8:0	3-oxo-2-(2'-pentenyl) cyclopentane-1-octanoic acid
OPDA	12-oxo-phytodienoic acid
OPR3	*OPDA REDUCTASE3*
OPR3-OE	*OPR3-Overexpressor*
PDF1.2	*PLANT DEFENSIN1.2*
PR1	*PATHOGENESIS-RELATED1*
PRR1	*PSEUDO-RESPONSE REGULATOR 1*
RFU	Relative fluorescence units
SA	Salicylic acid
SCF^{COI1}	Skp1, Cullin, F-box protein$^{CORONATINE\text{-}INSENSITIVE1}$
SCN	Suprachiasmatic nucleus
SE	Standard error
SOT	Sulfotransferase
SUR1	SUPERROOT1
T. ni	*Trichoplusia ni*
T-DNA	Transfer DNA
TFs	Transcription factors
TOC1	*TIMING OF CAB EXPRESSION1*
UGT	Uridine diphosphate glycosyltransferase
UNG	Uracil N-glycosylase
ZT	Zeitgeber time

Chapter 1: Introduction

1.1. Circadian clocks

The earth rotates on its axis every 24 hours resulting in organisms experiencing diurnal changes in both light and temperatures. As a result nearly all organisms have evolved an endogenous oscillator known as the circadian clock that regulates rhythmic behaviors and biological processes and enables organisms to anticipate and prepare for changes associated with the day and night (McClung, 2006).

A circadian system is composed of three components: inputs, the circadian clock and outputs (Figure 1). Inputs are external cues that synchronize the circadian clock to the outside environment. The circadian clock or oscillator is the core of the system found endogenously within the organism. Circadian clocks can be centralized in particular organs of an organism such as in the case of the suprachiasmatic nucleus (SCN) in mammals (Bell-Pedersen et al., 2005) or clocks can be found in each individual cell in the case of unicellular organisms (Bell-Pedersen et al., 2005) or plants (McClung, 2006). The circadian clock integrates external input cues to regulate outputs of the circadian clock.

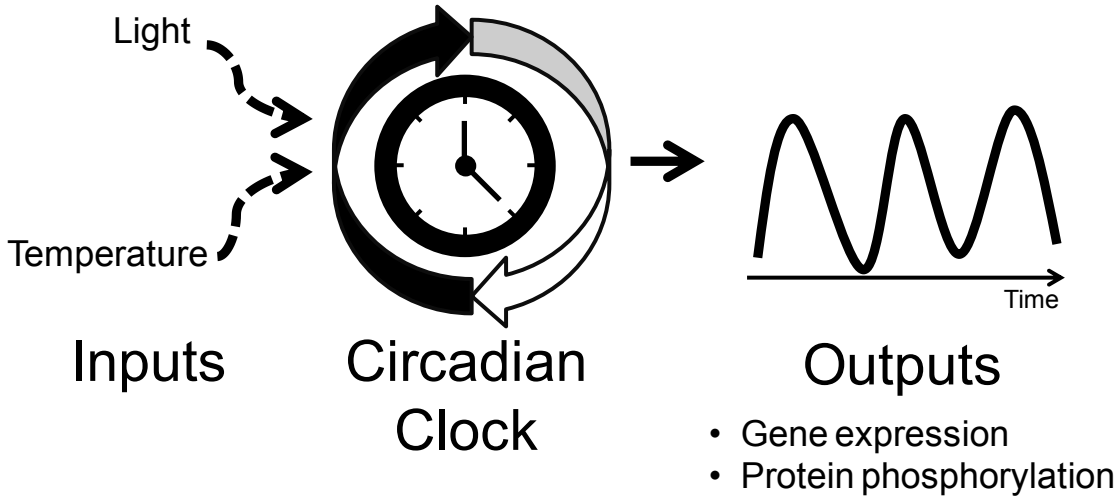

Figure 1: Schematic of circadian systems

Circadian systems are composed of three components: inputs, the circadian clock, and outputs of the circadian clock. Inputs are external cues such as changes in light and/or temperature associated with the transition from night to day that helps synchronizes the circadian clock to the outside environment. The circadian clock or oscillator is the system of regulatory feedback loops that generates rhythmic outputs. Outputs of the circadian system are biological processes that exhibit rhythmic behavior such as rhythmic changes observed in gene expression and protein phosphorylation.

Outputs of the circadian system include biological processes that exhibit rhythmic behavior that has an approximate 24-hour period, that is, the time between two successive occurrences. Circadian behaviors also exhibit specific phasing, that is, the time of day an output occurrence takes place; and amplitude, a measure of magnitude between the peak and trough of an output (Figure 2). In addition, for a rhythmic biological process to be considered as a circadian output it must satisfy three criteria. First, the output must exhibit rhythmic behavior with a period between two peaks or troughs of approximately ~24 hours (Figure 2; McClung, 2006). Second, output rhythmicity must persist even in the absence of external cues, for example under constant, or free running, environmental conditions (Figure 2; McClung, 2006). Lastly, the period of an output should exhibit temperature compensation and remain relatively unchanged over a range of external temperatures (McClung, 2006). Outputs of the oscillator can be found at both the cellular level, for example rhythmic changes in gene transcription and protein phosphorylation, to the whole-organism level, affecting changes in behavior such as sleep/wake cycles in animals (Harmer, 2009).

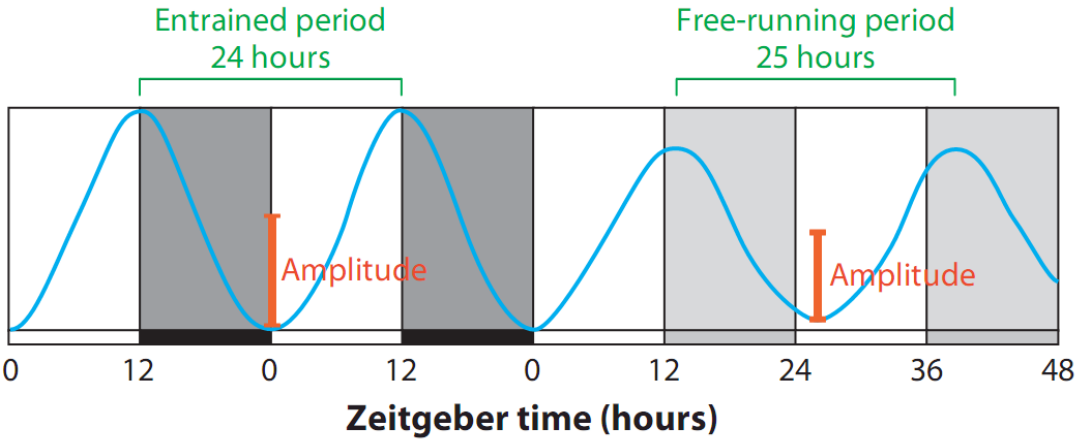

Figure 2: Traits of circadian outputs

An output regulated by the circadian clock displays rhythmic behavior under light/dark cycles (entraining conditions) and constant light (free-running conditions). Under light/dark cycles the output adopts the period of the entrainment. However, under constant light, the period of the endogenous clock is revealed. Zeitgeber time refers to the time of onset of a signal or cue that resets the circadian clock, for example ZT0 refers to the transition from dark to light. In the figure, during the entrainment period, the white bars indicate lights on or day and black bars indicate lights off or night. After transition to free-running conditions, the white bars indicates subjective day and the gray bars indicate subjective night (adapted from Harmer, 2009).

1.1.1. Adaptive advantage of the circadian system in plants

Plants are sensitive to the environment. Once germinated, plants remain fixed at a given location and must sense and react to the environment to optimize growth and reproduction. Therefore, the circadian clock provides an adaptive advantage by allowing plants to measure time and anticipate and prepare for daily and seasonal changes before they occur (Harmer, 2009; McClung, 2006; Yerushalmi and Green, 2009). Arabidopsis grown in day lengths that match the endogenous circadian period display improved performance compared to plants with endogenous periods that did not match the day length (Dodd et al., 2005). Additionally, mutants with defective circadian clocks that are unable to anticipate changes associated with day/night transitions perform worse than plants with functional oscillators; mutants plant flowered later under long day conditions and were less viable under very short day conditions (Green et al., 2002).

1.1.2. The circadian system in plants

Unlike the suprachiasmatic nucleus (SCN) found in mammals that contains a centralized circadian clock, the circadian clock in plants are relatively cell autonomous and found in each individual cell of the plant (Harmer, 2009; Yakir et al., 2011). The phase of the oscillator is synchronized with the outside environment by light/dark cycles (Millar et al., 1995a) and/or changes in temperature (Salomé and McClung, 2005) to generate robust rhythmic behavior in the organism. These behaviors persist even when plants are placed in artificial constant conditions, however over time the phases of

individual cells lose synchrony with respect to each other resulting in a dampening of overall rhythmic behavior (Yakir et al., 2011).

Most of our understanding of the circadian clock in plants comes from the plant model *Arabidopsis thaliana* (McClung, 2006). Initially, examining Arabidopsis mutants with altered periods determined that the core of the circadian clock consisted of three circadian clock-regulated genes: *LATE ELONGATED HYPOCOTYL* (*LHY*)*, CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *TIMING OF CAB EXPRESSION 1* (*TOC1*), also known as *PSEUDO-RESPONSE REGULATOR 1* (*PRR1*) (Harmer, 2009). *LHY* and *CCA1* are partially redundant morning-phased MYB-related transcription factors that form a reciprocal transcriptional feed-back loop with the evening-phased *TOC1* (Alabadí et al., 2001; Harmer and Kay, 2005). Loss of function of any of these core clock genes resulted in short period mutants (Millar et al., 1995b; Schaffer et al., 1998; Strayer et al., 2000). Overexpression of core clock components such as *CCA1* in *CCA1-OX* transgenic plants results in arrhythmicity of multiple outputs (Wang and Tobin, 1998).

As research into the circadian clock in plants progressed, the complexity of the circadian clock was revealed with the identification of additional components that form an intricate network of transcription-based interconnected feedback loops (Nagel and Kay, 2012; Pruneda-Paz and Kay, 2010). In addition the circadian clock integrates external cues to control rhythmic outputs using additional levels of regulation (Nagel and Kay, 2012), including RNA-based regulation through alternative splicing (Filichkin et al., 2010; James et al., 2012), post-translational modifications (Daniel et al., 2004), and chromatin regulation (Nagel and Kay, 2012).

1.1.3. Outputs of the circadian clock in plants

Outputs of the circadian clock in plants can be observed at the cellular level, for example in the rhythmic accumulation of transcript, up to the organismal level, for example in the daily rhythmic movement of leaves (Figure 3; Barak et al., 2000). The circadian clock can also affect the seasonal behavior of plants such as the timing of flowering (Corbesier and Coupland, 2005; Reeves and Coupland, 2000). Approximately 90% of the Arabidopsis genome exhibits rhythmic transcript accumulation under various combinations of light and temperature cycles (Michael et al., 2008). Additionally, estimates predict that the circadian clock regulates approximately a third of expressed Arabidopsis genes (Covington et al., 2008).

Clock-regulated timing of outputs is thought to provide biological benefits. For example, chloroplasts move within the cells over the course of the day; in preparation for morning chloroplasts move close to the cell surface thereby maximizing exposure to light, and away from the cell surface during night (Britz and Briggs, 1976; Koop et al., 1978). The circadian clock also influences plant response to other abiotic stress, such as cold (Fowler et al., 2005; Rikin et al., 1993) and hot temperatures (Rikin, 1992; Rikin et al., 1993). Recent studies highlight a role for the circadian clock in the response of plants to biotic stress. The biotic stress resistance promoted by the plant circadian clock suggests that plants can anticipate and thus prepare for cyclical pest and pathogen

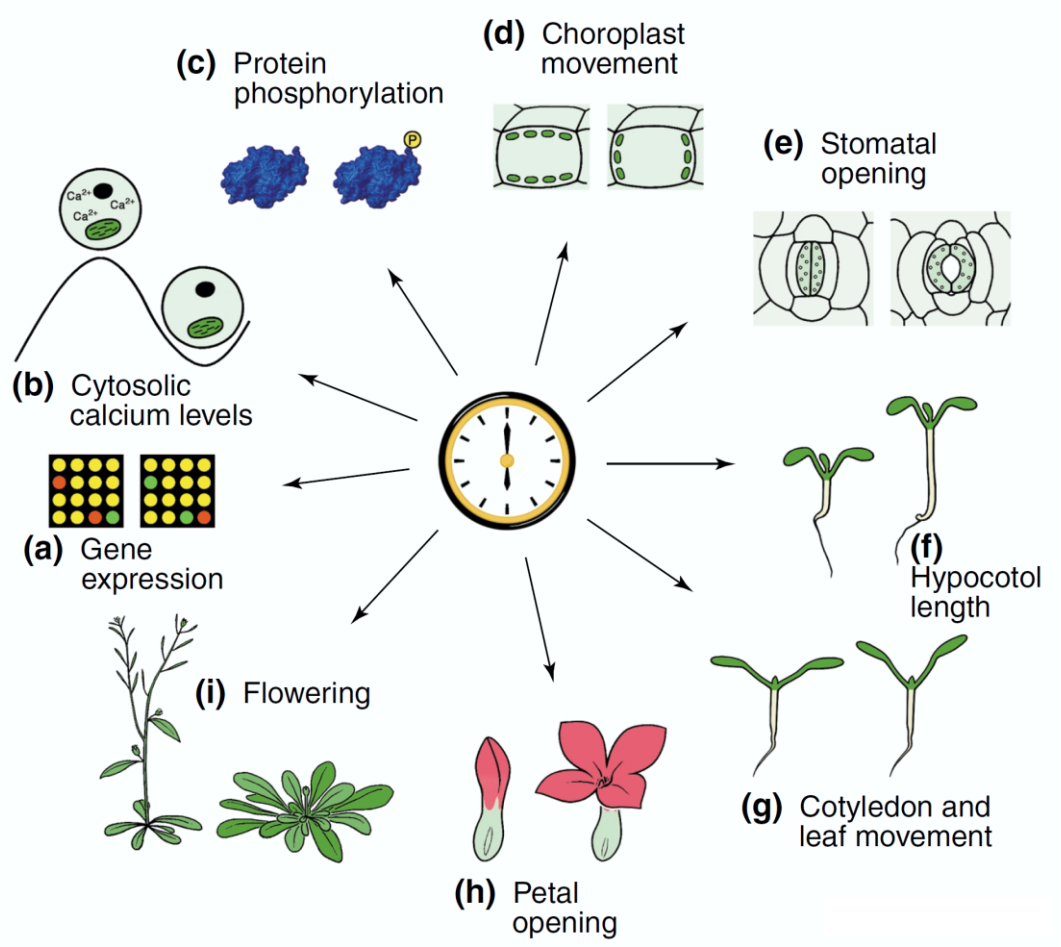

Figure 3: Examples of outputs regulated by the circadian clock in plants

Plant clocks control a plethora of biological processes. (a) Expression of approximately one-third of expressed genes in Arabidopsis shows circadian rhythms (Covington et al., 2008). (b) Cytosolic calcium levels oscillate with a circadian rhythm in Arabidopsis (Johnson et al., 1995). (c) The clock regulates the phosphorylation of some proteins (Nimmo, 2000). At an organismal level, (d) chloroplast movement (Koop et al., 1978), (e) stomatal opening, (f) hypocotyl elongation (Dowson-Day and Millar, 1999) and (g) cotyledon and leaf movements (McClung, 2006) in Arabidopsis all show circadian rhythms. (h) In Kalanchoe, petal opening shows a circadian rhythm. (i) The clock is also vital for synchronizing developmental processes such as flowering time (Reeves and Coupland, 2000) (adapted from Barak et al., 2000).

behavior, including that of biotrophic pathogens (Bhardwaj et al., 2011; Shin et al., 2012; Wang et al., 2011a, 2011b) and insect herbivores (Goodspeed et al., 2012, 2013a, 2013b).

1.2. Phytohormones

Plants use phytohormones as chemical signals to regulate diverse processes, often through regulated changes in gene transcription rates. For example auxin (indole-3-acetic acid, IAA) regulates plant growth and root development (Woodward and Bartel, 2005; Zhao, 2010). Salicylic acid (SA; Figure 4A), another phytohormone, regulates plant defense against biotrophic pathogens (Glazebrook, 2005). Jasmonic acid (JA; Figure 4B), the phytohormone of particular relevance to the research described here, has a prominent role in insect and fungal defense (Acosta and Farmer, 2010; Glazebrook, 2005).

1.2.1. Role of jasmonates in plant defense

JA along with other compounds derived from JA that are collectively referred to as jasmonates, regulate plant pollen and stamen development (Feys et al., 1994; McConn and Browse, 1996; Stintzi and Browse, 2000), wound response (León et al., 2001), and defenses to stressors such as insect herbivory (Erb et al., 2012) and necrotrophic fungi (Glazebrook, 2005). Arabidopsis reacts to fungal and insect attack by increasing JA levels, which activates hormone-specific response pathways that lead to the expression of stress response genes and initiation of defense pathways (Figure 5; Glazebrook, 2005). Examples of defense compounds include secondary metabolites, such as glucosinolates, and protease inhibitors (Kliebenstein, 2004).

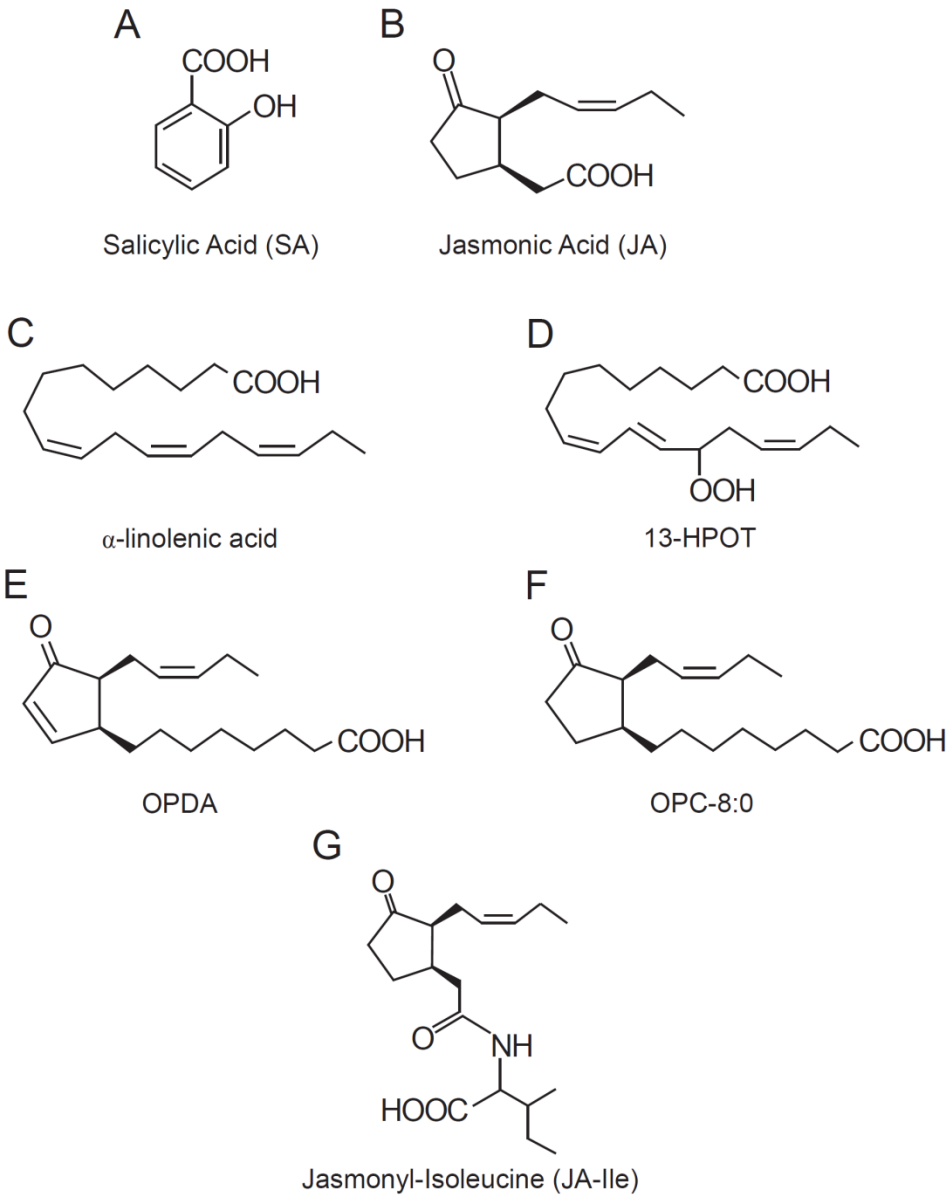

Figure 4: Structural formulas of plant hormones and JA precursors

(A) Salicylic acid, SA. (B) Jasmonic acid, JA. (C) α-linolenic acid (18:3). (D) 13-HPOT, 13(S)-hydroperoxy-octadecatrienoic acid. (E) OPDA, 12-oxo-phytodienoic acid (F) OPC-8:0, 3-oxo-2-(2-(Z)-pentenyl-cyclopentane-1-octanoic. (G) Jasmonyl-Isoleucine (JA-Ile) (adapted from Acosta and Farmer, 2010).

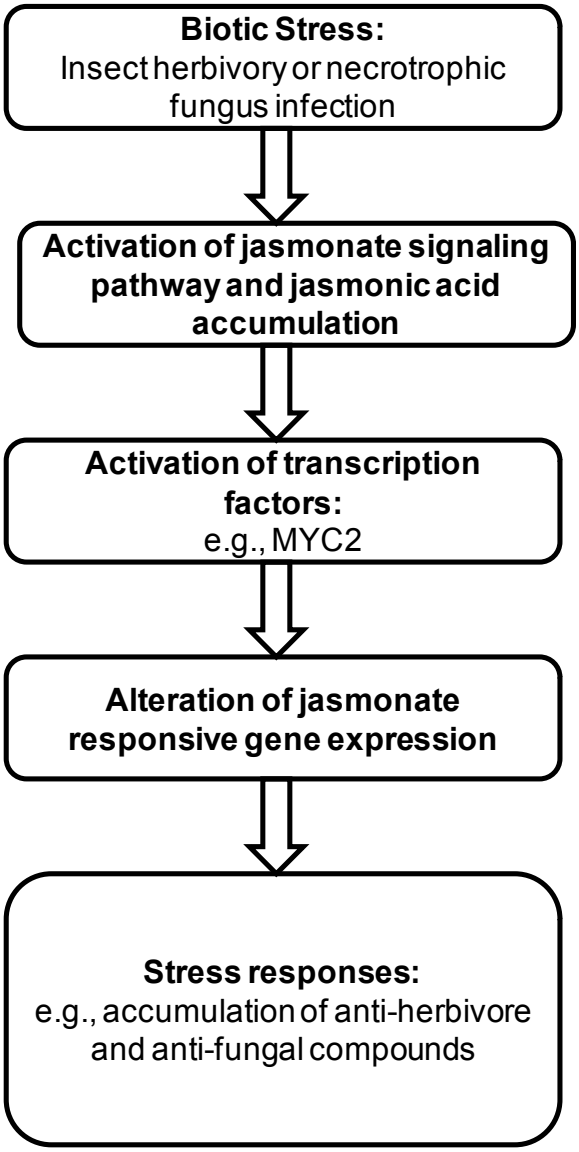

Figure 5: JA signaling pathway for stress responses

Biotic stress such as insect herbivory or necrotrophic fungal infection triggers JA-signaling activation and induces JA accumulation. Activated JA signaling leads to transcription factor mediated changes in JA-responsive gene expression, resulting in the induction of stress responses (adapted from Fujita et al., 2006).

1.2.2. Jasmonic acid biosynthesis, signaling and positive feedback

JA biosynthesis (Figure 6; Acosta and Farmer, 2010) is initiated in the plastids where α-linolenic acid (Figure 4C) is converted to 13-hydroperoxyl-linolenic acid (13-HPOT; Figure 4D) through the action of 13-lipoxygenase (13-LOX). 13-HPOT is converted to 12-oxo-phytodienoic acid (OPDA; Figure 4E) through the successive action of two enzymes, allene oxide synthase (AOS) and allene oxide cyclase (AOC). OPDA is transported out of the plastid and into the peroxisome where OPDA reductase 3 (OPR3) catalyzes the reduction of OPDA to 3-oxo-2-(2'-pentenyl) cyclopentane-1-octanoic acid (OPC-8:0; Figure 4F). OPC-8:0 undergoes three rounds of β-oxidation to form jasmonic acid (JA; Figure 4B). JA is transported to the cytosol where it can be conjugated to amino acids. Production of JA-Ile (Jasmonoyl-Isoleucine; Figure 4G), the active form of JA, leads to changes in JA-responsive-gene expression. In the absence of JA-Ile, the expression of some JA-responsive genes is repressed by the interaction of JAZ (JASMONATE ZIM-DOMAIN) proteins with transcription factors, such as MYC2. JA-Ile is recognized and bound by the SCF^{COI1} (Skp1, Cullin, F-box $protein^{CORONATINE-INSENSITIVE1}$) ubiquitin ligase complex. The binding of JA-Ile to the SCF^{COI1} complex leads to ubiquitination and degradation of JAZ proteins, thus freeing MYC2, and likely other transcription factors, that regulate expression (Figure 6). JA-responsive genes include those that encode the enzymes required for JA biosynthesis. This JA-induced expression of JA-biosynthesis genes forms a JA-positive feedback loop that amplifies JA production (Figure 6; Sasaki et al., 2001).

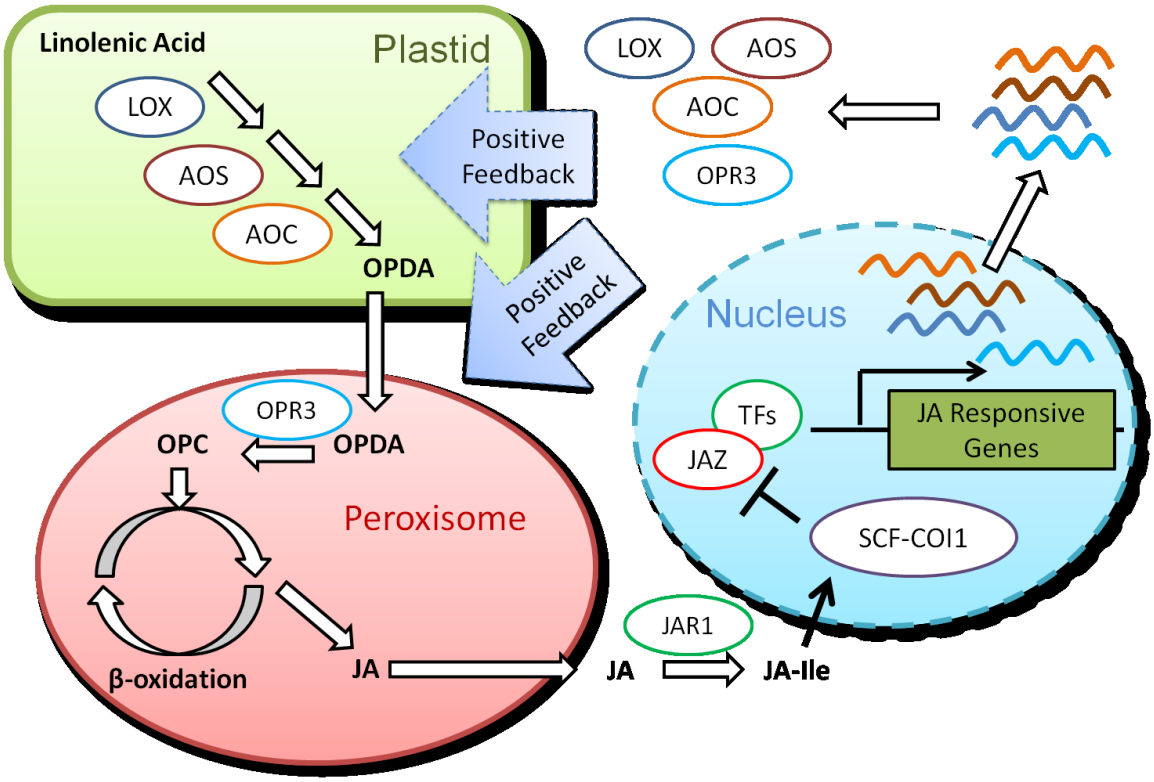

Figure 6: Overview of jasmonic acid biosynthesis, signaling and JA positive feedback

The major steps of JA biosynthesis and signaling occur in several cellular compartments of the plant cell as described in the text. Also depicted is the positive feedback loop in which JA activates JA-biosynthesis gene expression leading to amplification of JA levels. AOS, allene oxide synthase; AOC, allene oxide cyclase; JA, jasmonic acid; JA-Ile, jasmonyl isoleucine; JAR1, JASMONATE RESISTANT 1; JAZ, JASMONATE ZIM-DOMAIN; LOX, lipoxygenase; OPC, 3-oxo-2-(2'-pentenyl)cyclopentane-1-octanoic acid; OPDA, 12-oxo-phytodienoic acid; OPR3, OPDA reductase 3; TFs, transcription factors; SCF-COI1, Skp1, Cullin, F-box protein-CORONATINE-INSENSITIVE1 (adapted from Acosta and Farmer, 2010).

1.2.3. Antagonistic relationship between jasmonic acid and salicylate acid

Arabidopsis reacts to specific stressors by increasing JA and salicylic acid (SA) levels, which subsequently activate hormone-specific response pathways leading to the expression of stress response genes (Glazebrook, 2005). SA and JA signaling can act antagonistically (Glazebrook, 2005; Koornneef and Pieterse, 2008, 2008; Thaler et al., 2012). This antagonism may ensure that plants are not wasting resources in applying the wrong response to a particular stress (Ballaré, 2011; Heil and Baldwin, 2002). For example, in response to biotrophic pathogens, which derive nutrients from living tissues, plants utilize the SA response pathway (Glazebrook, 2005). SA induces rapid plant cell death around the site of infection, which deprives the biotrophic pathogens of nutrients. In contrast, a defense using such a cell death strategy would be an inappropriate response to necrotrophic pathogens, which derive nutrients from dead cells. Instead, in the case of necrotrophic pathogens, plants employ JA-dependent stress response pathways (Glazebrook, 2005). Plants regulate the JA and SA pathways to ensure a suitable response. The activation of either JA or SA pathways can lead to the suppression of the other pathway (Koornneef and Pieterse, 2008; Kunkel and Brooks, 2002; Thaler et al., 2012; Verhage et al., 2010). Mutants with impaired signaling in either the SA or JA pathway show increased expression of reporter genes responsive to the other hormonal pathway. For example, decreased SA signaling leads to an increase in stress-induced transcript accumulation of *PDF1.2* transcripts, a JA-responsive gene, and decreased JA signaling leads to an increase in stress-induced accumulation of *PR1* transcripts, a SA-responsive gene (Brooks et al., 2005; Chung et al., 2008; Dombrecht et al., 2007;

Glazebrook et al., 2003; Kloek et al., 2001). In addition, changes in SA levels can lead to opposite changes in JA levels. For example, in *NahG* transgenic plants that express bacterial salicylate hydroxylase (Katagiri et al., 1965; Yamamoto et al., 1965) leading to decreased SA levels, show increased JA levels and *PDF1.2* transcript accumulation upon pathogen infection (Spoel et al., 2003). Additionally, treating plants with SA or aspirin, a salicylate drug, decreases the levels of wound-induced JA and *AOS* transcripts (Harms et al., 1998; Pena-Cortés et al., 1993).

1.2.4. Clock-regulated basal jasmonate and salicylate hormone accumulation

JA and SA levels cycle with circadian rhythmicity under free running conditions in unstressed plants (Figure 7; Goodspeed et al., 2012). Interestingly, peak levels of SA and JA are antiphasic to each other. JA levels peak during the middle of subjective day while SA levels peak during the middle of subjective night (Figure 7).

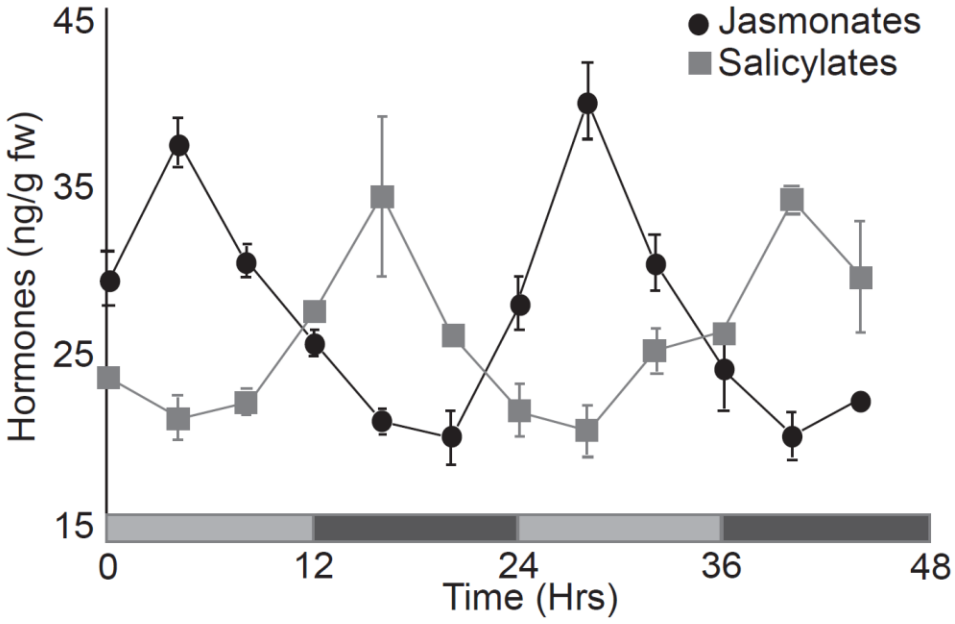

Figure 7: Jasmonate and salicylate levels accumulate with circadian rhythmicity with opposite phase of peak accumulation

Arabidopsis JA and MeJA (methyl-JA) (black circles) peak in the middle of subjective day and salicylates (grey squares) peak in the middle of subjective night under free-running conditions. Mean ± SE; n = 3. fw, fresh weight. Light grey bars indicate subjective day and dark grey bars indicate subjective night (adapted from Goodspeed et al., 2012).

1.3. Glucosinolates

1.3.1. Glucosinolate function

Glucosinolates are a class of sulfur containing organic compounds found in members of the *Brassicaceae* plant family, which includes Arabidopsis. Glucosinolates are secondary metabolites that play a major role in *Brassicaceae* plant herbivore defense (Hopkins et al., 2009). Additionally, glucosinolates provide the human health benefits attributed to *Brassicaceae* (cruciferous) vegetable consumption (Hayes et al., 2008; Higdon et al., 2007).

1.3.2. Glucosinolate classes and structure

Over 120 different chemical structures of glucosinolates have been discovered in plants (Fahey et al., 2001). All glucosinolates contain a core structure (Figure 8A) composed of a β-thioglucose, an N-hydroxyiminosulphate group and a structurally diverse side-chain. Most glucosinolates in Arabidopsis contain either aliphatic or indolic side chains (Figure 8B; Brown et al., 2003). Aliphatic glucosinolates are derived from methionine while indolic glucosinolates are synthesized from tryptophan precursors (Sønderby et al., 2010).

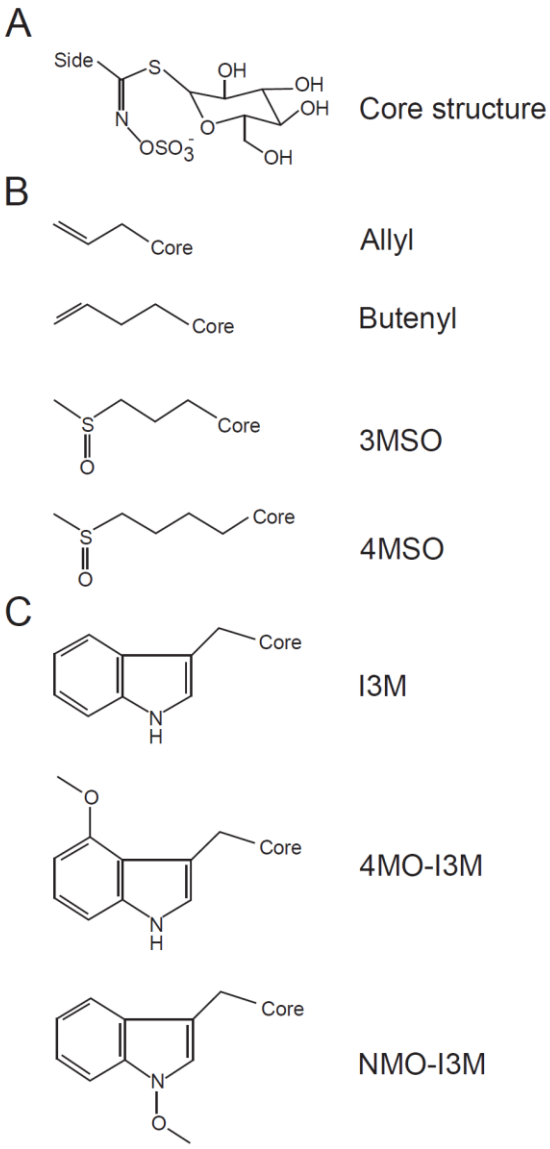

Figure 8: Structural formulas of several glucosinolates

(A) Glucoinolate core structure consisting of a β-thioglucose, an N-hydroxyiminosulphate group and a structurally diverse side-chain. (B) Examples of glucosinolate side chains. (B) Aliphatic glucosinolates: Allyl, allyl glucosinolate; Butenyl, 3-butenyl glucosinolate; 3MSO, 3-methylsulfinylpropyl glucosinolate; and 4MSO, 4-methylsulfinylbutyl glucosinolate. (C) Indolic glucosinolates: I3M, indoyl-3-methyl glucosinolate; 4MO-I3M, 4-methoxy-indolyl-3-methyl glucosinolate; and NMO-I3M, 1-methoxy-indolyl-3-methyl glucosinolate (adapted from Beekwilder et al., 2008).

1.3.3. Regulation of glucosinolate metabolism

Multiple hormones may play a role in the regulation of glucosinolate metabolism including JA, SA and ethylene, which control induction of glucosinolate accumulation (Yan and Chen, 2007). Jasmonates, in particular, strongly influence levels of aliphatic and indolic glucosinolates in Arabidopsis (Brader et al., 2001; Mewis et al., 2005; Mikkelsen et al., 2003). Aliphatic glucosinolate accumulation requires MYB28 and MYB29 since *myb28myb29* double mutants have no detectable levels of aliphatic glucosinolates (Beekwilder et al., 2008; Hirai et al., 2007). Loss of aliphatic glucosinolates results in plants with high susceptibility to insects (Beekwilder et al., 2008). Indolic glucosinolates are regulated by *ATR1* (*ALTERED TRYTOPHAN REGULATION 1;* Celenza et al., 2005; Grubb and Abel, 2006), a MYB transcription factor.

1.3.4. Glucosinolate metabolism

Glucosinolates are enzymatically cleaved by myrosinases (Figure 9A) to form the active compounds used in plant defense. Under unstressed conditions glucosinolates and myrosinases are stored in physically separate compartments. Glucosinolates can normally be found in the vacuoles of most plant tissue cells (Brown et al., 2003; Grubb and Abel, 2006; Kelly et al., 1998), while myronsinases are sequestered in the vacuoles of specialized idioblast cells, called myrosin cells (Andréasson et al., 2001; Rask et al., 2000). Separation of glucosinolates and myrosinases ensures that the break-down products of glucosinolates, which produce reactive oxygen species, are not produced unnecessarily (Halkier and Gershenzon, 2006). When tissue damage occurs due to pest or

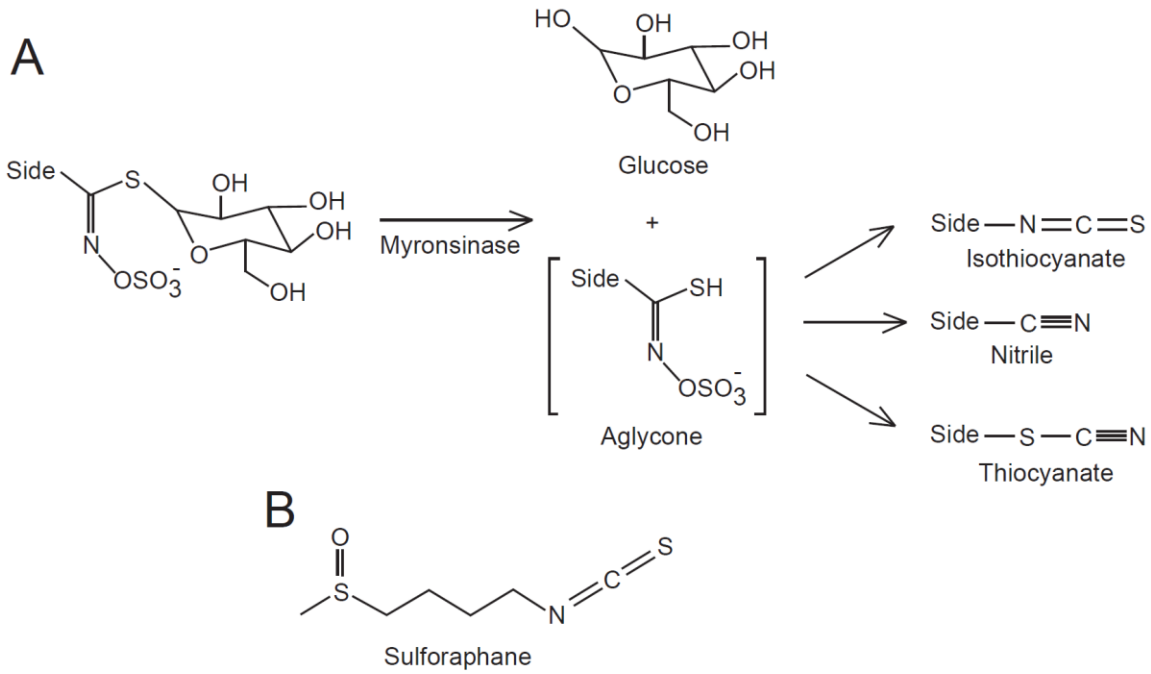

Figure 9: Glucosinolate metabolism and structural formula of sulforaphane

(A) When plants are wounded, glucosinolates are enzymatically cleaved by myrosinases, releasing glucose and an aglycone intermediate. Rearrangement of aglycone leads to products such as isothiocyanates, nitriles and thiocyanates. (B) Structural formula of the isothiocyanate sulforaphane which is derived from 4MSO (Figure 8B) (adapted from Halkier and Gershenzon, 2006).

pathogen attack, glucosinolates and myrosinases are released from their compartments and interact, resulting in the hydrolysis of thioglucoside linkages (Figure 9A; Halkier and Gershenzon, 2006; Mumm et al., 2008). Hydrolysis of glucosinolates releases two byproducts, glucose and an unstable aglycone, which undergoes rearrangement to form a variety of different products depending on the side chain present (Figure 9A; Halkier and Gershenzon, 2006; Mumm et al., 2008). Products of glucosinolates hydrolysis include isothiocynates, nitriles, and thiocynates (Figure 9A). Isothiocyanates, (Figure 9A) the most abundant product of glucosinolate hydrolysis, are important for plant defense and are toxic to pests (Mumm et al., 2008). Additionally, isothiocyanates are responsible for the anti-cancer benefits associated with consumption of *Brassicaceae* vegetables (Björkman et al., 2011).

1.3.5. Glucosinolate anti-cancer activities

Glucosinolates contribute to the human health benefits attributed to cruciferous vegetable consumption (Hayes et al., 2008; Higdon et al., 2007). Several isothiocynates such as sulforaphane, phenethyl isothiocyange, allyl isothiocynate and indole-3-carbinol have been demonstrated to have anti-cancer properties (Finley, 2005). For example, isothiocyanates may function as indirect antioxidants, inducing phase II detoxifying enzymes (Fahey et al., 2001, 2002; Finley, 2005). Furthermore, isothiocynates can modulate cell signaling, induce apoptosis and regulate cell cycles (Fahey et al., 2002; Finley, 2005).

In particular, glucoraphanin (4-methylsulfinylbutyl; 4MSO; Figure 8) is a glucosinolate that has been shown to have anticancer and antimicrobial activity when

broken down to form the isothiocynate, sulforaphane (Figure 9B; Fahey et al., 2002; Zhang et al., 1992). Sulforaphane has an inhibitory effect on tumor growth in studies of prostate (Bhamre et al., 2009) and breast (Azarenko et al., 2008) cancers.

1.3.6. Clock-regulated rhythmic accumulation of glucosinolates

Individual and total glucosinolates in Arabidopsis cycle with circadian rhythmicity under free running conditions (Figure 10A and Figure 11; Goodspeed et al., 2013b). In addition, glucosinolates cycle under free-running conditions in postharvest *Brassica oleracea* (cabbage) a close relative to Arabidopsis, when entrained in light/dark cycles (Figure 10B; Goodspeed et al., 2013b).

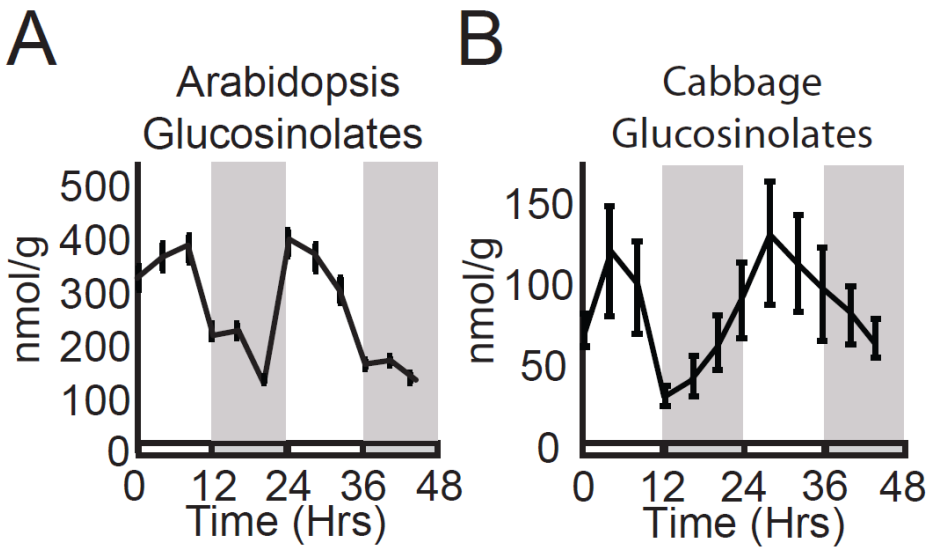

Figure 10: Circadian accumulation of glucosinolates in Arabidopsis and cabbage

Total glucosinolates measured from Arabidopsis (A) and postharvest re-entrained cabbage (B). At 4-h intervals, Arabidopsis and cabbage leaves were collected and glucosinolates were identified and quantified in comparison to reference standards using HPLC-DAD as previously described (Kliebenstein et al., 2001). Mean ± SE, n =6. White bar indicates subjective day and grey bars indicate subjective night (adapted from Goodspeed et al., 2013b.)

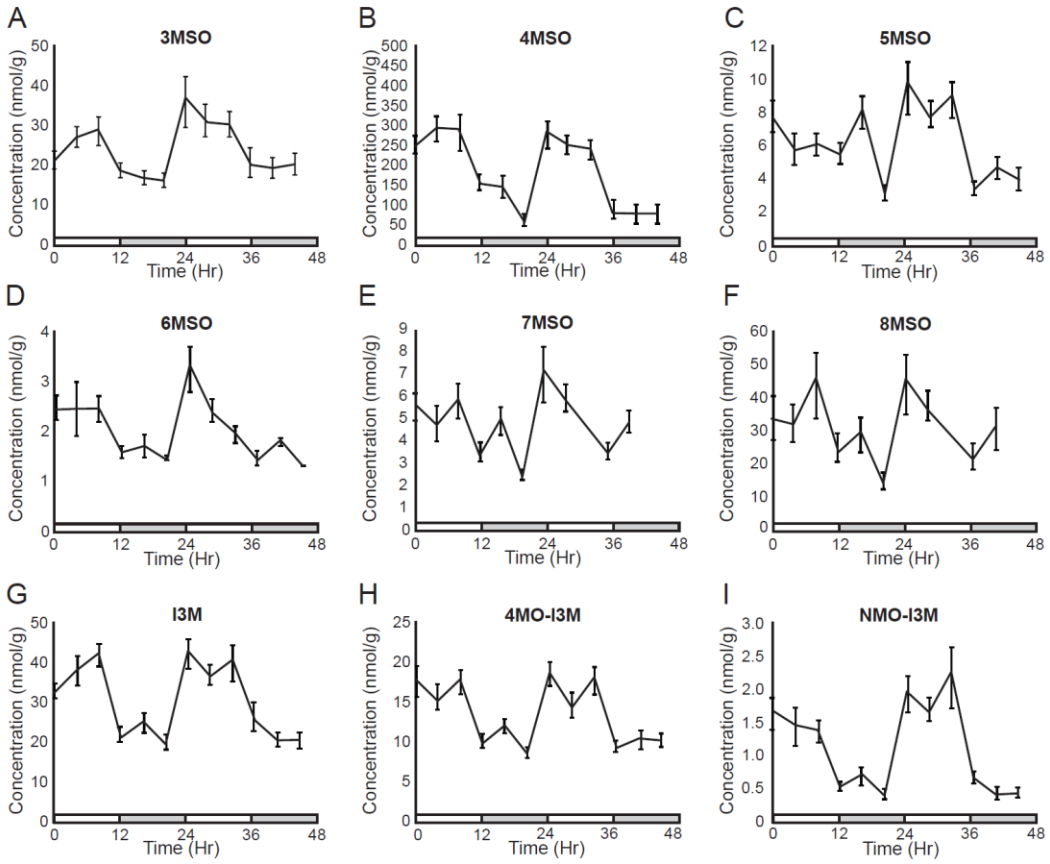

Figure 11: Circadian accumulation of individual glucosinolate species in Arabidopsis

Concentration of glucosinolate species collected from Arabidopsis under free-running conditions. At 4-h intervals, Arabidopsis leaves were collected and glucosinolates were identified and quantified in comparison to reference standards using HPLC-DAD as previously described (Kliebenstein et al., 2001). 3MSO, 3-methylsulfinylpropyl glucosinolate; 4MSO, 4-methylsulfinylbutyl glucosinolate; 5MSO, 5-methylsulfinylpentyl glucosinolate; 6MSO, 6-methylsulfinylhexyl glucosinolate; 7MSO, 7-methylsulfinylheptyl glucosinolate; 8MSO, 8-methylsulfinyloctyl glucosinolate; I3M, indolyl-3-methyl glucosinolate; 4MO-I3M, 4-methoxy-indolyl-3-methyl glucosinolate; NMO-I3M, 1-methoxy-indolyl-3-methyl glucosinolate. Mean ± SE, n =6. White bar indicates subjective day and grey bars indicate subjective night (adapted from Goodspeed et al., 2013b).

1.4. Clock-regulated plant defense against insect herbivory

1.4.1. Proper entrainment increases plant resistance against insect herbivory

Arabidopsis have enhanced herbivore resistance when exposed to the generalist insect, *Trichoplusia ni* (Cabbage loopers) that are entrained in-phase with respect to the plants, demonstrating that proper entrainment is critical for plant resistance. Plants entrained out-of-phase with the loopers had much lower resistance, losing more tissue than plants entrained in-phase with the *T. ni* (Figure 12A and B; Goodspeed et al., 2012). Conversely, *T. ni* are larger when feeding on plants entrained out-of-phase when compared to those feeding on in-phase plants (Figure 12C and D; Goodspeed et al., 2012).

1.4.2. Arabidopsis phase-dependent resistance to *Trichoplusia ni* is clock dependent

CCA1-OX (*CIRCADIAN CLOCK ASSOCIATED 1-OVER EXPRESSOR*) plants with constitutive expression of the component *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*), are arrhythmic under free-running conditions (Wang and Tobin, 1998). In contrast to wildtype plants (Figure 12), plants with defective clocks do not show phase-entrainment-dependent susceptibility to *T. ni* (Figure 13 ; Goodspeed et al., 2012). *CCA1-OX* plants have similar aerial tissue area and *T. ni* have similar mass regardless of whether the plants and insects are entrained in-phase or out-of-phase with respect to each other (Figure 13 ; Goodspeed et al., 2012), indicating that Arabidopsis phase-entrainment-dependent resistance to *T. ni* is clock dependent.

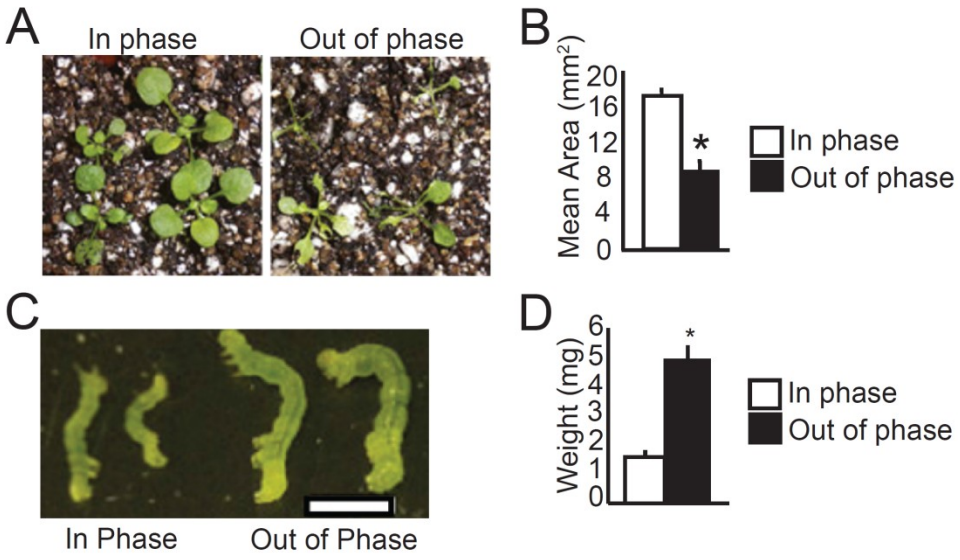

Figure 12: Arabidopsis is more resistant to insect herbivory when entrained in-phase with *T. ni*

(A) Photographs of representative plant tissue remaining from plants entrained in-phase and out-of-phase with *T. ni* entrainment after 72 hours of coincubation. (B) Area of plant tissue remaining from plants entrained in phase (white bars) and out of phase (black bars) with respect *T. ni* entrainment after 72 hours of coiincubation with *T. ni*. Mean area ± SE; n = 6; *P<0.05; two-tailed paired t test. (C) Photographs of representative of T. ni after 72-hours of coincubation with plants entrained in phase and out of phase with respect to the *T. ni*. (D) *T. ni* net weights after 72 hours of coincubation with plants. Mean area ± SE; n = 15; *P<0.05; two-tailed paired t test. Scale bar, 0.5 mm. (Adapted from Goodspeed et al., 2012.)

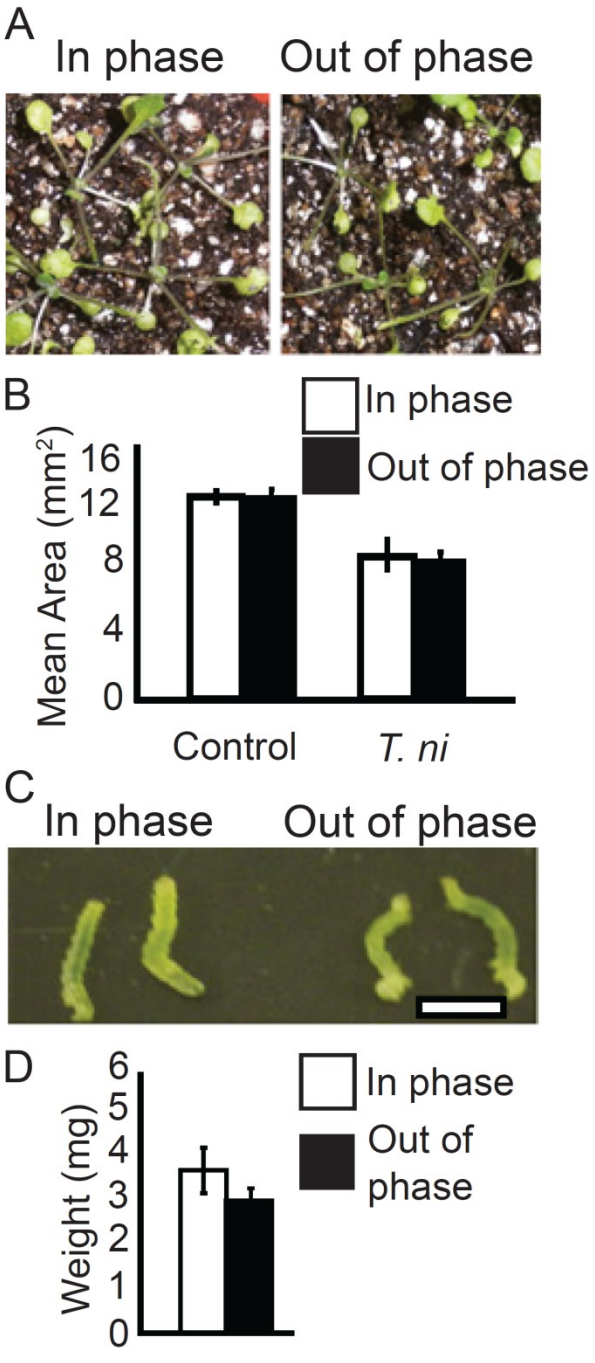

Figure 13: Arrhythmic Arabidopsis plants lack enhanced herbivory resistance when entrained in-phase with *T. ni*

(A) Photographs of representative plant tissue remaining from *CCA1-OX* plants entrained in phase and out of phase with *T. ni* entrainment after 72 hours of coincubation. (B) Area of plant tissue remaining from plants entrained in phase (white bars) and out of phase (black bars) with respect to *T. ni* entrainment after 72 hours of incubation without (control) or with *T. ni.* Mean area ± SE; n = 6; P<0.8; two-tailed paired t- test. (C) Photographs of representative of *T. ni* after 72 hours of coincubation with plants entrained in phase and out of phase with respect to the *T. ni.* Scale bar, 0.5 mm. (D) *T. ni* net weights after 72 hours of coincubation with plants. Mean area ± SE; n = 15; P<0.05; two-tailed paired t test. (Adapted from Goodspeed et al., 2012.)

1.4.3. Arabidopsis phase-dependent resistance to *Trichoplusia ni* is jasmonate dependent

A T-DNA insertion into the *AOS* (*ALLENE OXIDE SYNTHASE*) gene creates *aos* plants that are defective in JA production (Park et al., 2002). *gl-1* plants, the genetic background of *aos*, display phase-entrainment-dependent susceptibility to *T. ni* (Figure 14; Goodspeed et al., 2012) similar to that demonstrated by Col-0 plants (Figure 12). In contrast to *gl-1*, *aos* does not show phase-entrainment-dependent susceptibility to *T. ni* (Figure 14; Goodspeed et al., 2012), indicating that phase-dependent resistance to *T. ni* is JA dependent.

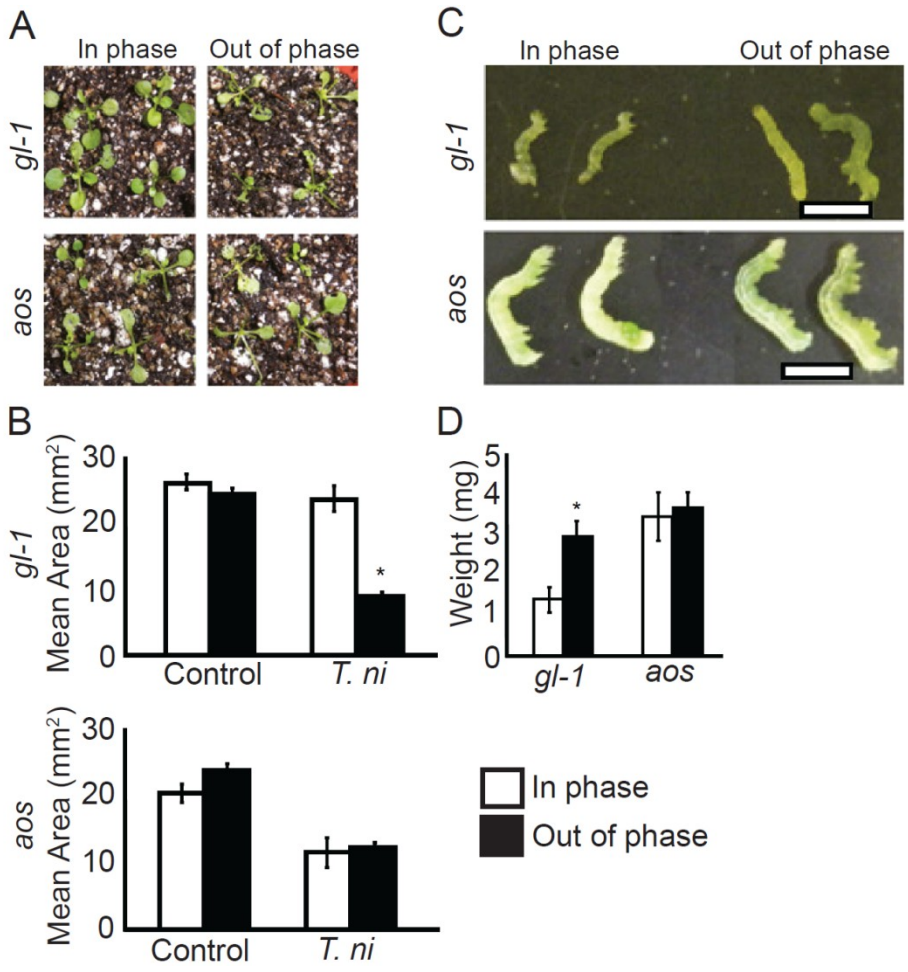

Figure 14: Jasmonates are required for enhanced herbivory resistance when entrained in-phase with *T. ni*

(A) Photographs of representative plant tissue remaining from *gl-1* and *aos* plants entrained in phase and out of phase with *T. ni* entrainment after 72 hours of coincubation. (B) Area of plant tissue remaining from plants entrained in phase (white bars) and out of phase (black bars) with respect *T. ni* entrainment after 72-hours of incubation without (control) or with *T. ni.* Mean area ± SE; n = 6; P<0.0002; two-tailed paired t test. (C) Photographs of representative of *T. ni* after 72 hours of coincubation with plants entrained in phase and out of phase with respect to the *T. ni*. Scale bar, 0.5 mm (D) *T. ni* net weights after 72 hours of coincubation with plants. Mean area ± SE; n = 15; *P<0.05; two-tailed paired t test (adapted from Goodspeed et al., 2012).

1.5. Postharvest vegetables

Approximately one-third of food produced globally is lost or wasted (FAO, 2011), yet fewer resources are devoted to postharvest research and development than to efforts for improving productivity (Kader, 2003). The modular design of plants (Watkinson and White, 1986) allows plant tissues and organs to remain biologically active even after harvest and therefore responsive to environmental conditions (Burton, 1982; Goodspeed et al., 2013b).

1.5.1. Light effects on postharvest vegetables

Plants exhibit exquisite sensitivity to light stimuli, and isolated plant leaves maintain responsiveness to light after harvest and can continue light-dependent biological processes, such as photosynthesis (Costa et al., 2013). Additionally, the clocks of postharvest fruit and vegetable tissues can be entrained with 12-hour light/12-hour darkness cycles producing rhythmic behaviors not observed in tissues stored in constant light or constant dark (Goodspeed et al., 2013b). A few studies have examined the effects of light on performance and longevity during postharvest storage (Nilsson, 2000). For example, light exposure delays broccoli senescence and yellowing (Büchert et al., 2011; Costa et al., 2013) but accelerates browning in cauliflower, a close relative of broccoli (Olarte et al., 2009; Sanz Cervera et al., 2007). Other studies report that light exposure to broccoli during postharvest storage either provides no additional benefits (Olarte et al., 2009) or decreases performance (Kasim and Kasim, 2007). Postharvest light exposure improves chlorophyll content in cabbage (Perrin, 1982), but leads to increased browning

of romaine lettuce leaves (Martínez-Sánchez et al., 2011). Although exposure of spinach to light during postharvest storage can improve nutritional value (Lester et al., 2010; Toledo et al., 2003), light can also accelerate spinach water loss, leading to wilting (Lester et al., 2010). Together, these findings are inconclusive as to whether light exposure during postharvest storage can be generally beneficial, and the variation of the results may be attributable to differences in the plant species examined and the specific conditions used during postharvest storage, such as lighting intensities, temperature, humidity or packaging. It is unknown whether light may be advantageous when present in its natural context of 24-hour light/dark periodicity perhaps through maintenance of clock function.

Chapter 2: Methods

2.1. Plant materials and growth conditions

2.1.1. Arabidopsis growth and sample preparation

2.1.1.1. Plant growth, entrainment and sample collection for transcript accumulation measurements

Col-0 (Lehle, Round Rock, TX), *aos* (CS6149; Arabidopsis Biological Resource Center, backcrossed to Col-0 three times), *NahG* (Frederic M. Ausubel, Harvard Medical School, Boston, MA), *acd6-1* (Hua Lu, University of Maryland, Baltimore County, Baltimore, MD), and *OPR3-OE* (E. Wassim Chehab, Rice University, Houston, TX) seeds (Table 1) were surface-sterilized with 70% ethanol for 5 minutes followed by 95% ethanol for 15 minutes. After two washes with sterilized water, the seeds were stratified in the dark for four days at 4°C and then sown on growth media containing half-strength Murashige and Skoog (MS) medium (PhytoTechnology Laboratories, Shawnee Mission, KS), 10 g/L sucrose (Sigma, St. Louis, MO) and 8 g/L agar (EMD, Darmstadt, Germany) (pH 5.7) and grown at 120 μE•m-2•s-1 (E = Einstein, defined as one mole of photons) at 22°C. After one week, seedlings were transferred to soil (Sunshine MVP soil; Sun Gro Horticulture) with 9 plants per pot and grown in 12-hour light/12-hour dark cycles under

140 μE•m-2•s-1 at 22°C for two weeks. The three-week-old plants were then moved into constant light, 140 μE•m-2•s-1 at 22^{o}C. After 24 hours in constant light, aerial-plant-tissue samples were collected as described in text. Treatment of samples during collection involved flash freezing the aerial leaf tissue collected from one pot of plants in liquid nitrogen.

Genotype	Mutant/Transgene	Seed Source
Col-0		Lehle seeds
aos (in *gl-1* background)	Mutant	ABRC
aos (in Col-0 background)	Mutant	Rice University – Dr. Kim
gl-1	Mutant	ABRC
CCA1-OX	Transgene	UC Davis: Dr. Harmer
OPR3-OE	Transgene	Rice University: Dr. Chehab
acd6-1	Mutant	U of Maryland, Baltimore County – Dr. Lu
NahG	Transgene	Harvard Medical School – Dr. Ausubel
myb28myb29	Mutant	UC Davis: Dr. Kliebenstein

Table 1: Arabidopsis seed genotype, mutant/transgene and seed source

2.1.1.2. Plant growth and entrainment for *Trichoplusia ni* defense assays

All Arabidopsis genotypes have the Col-0 genetic background except for *aos*, which is in the *gl-1* genetic background. Col-0 (Lehle Seeds) and *myb28myb29* (Daniel J. Kliebenstein, University of California, Davis, CA) (Table 1) seeds were surface-sterilized and grown on media as described above. After 1 week on MS media, seedlings were transferred to soil (SunshineMVP soil; Sun Gro Horticulture), with 16 plants per pot, and grown for 2 weeks at 22 °C under 12-hour light/12-hour dark cycles (140 μE·m−2·s−1). At 3 weeks of age, plants were moved into constant light, 30 μE•m-2•s-1 at 22^{o}C.

2.1.1.3. Plant growth and entrainment for *Botrytis cinerea* infection assays

Col-0 (Lehle, Round Rock, TX), *gl-1* (CS8155; Arabidopsis Biological Resource Center), *aos* (CS6149; Arabidopsis Biological Resource Center) and *CCA1-OX* (Stacey Harmer, University of California, Davis, CA) seeds were surface-sterilized and grown as described above. After one week, seedlings were transferred to soil (Sunshine MVP soil; Sun Gro Horticulture) and grown in 12-hour light/12-hour dark cycles under 140 μE•m-2•s-1 at 22°C for four weeks. At 5 weeks of age, leaves were cut at the petiole using scissors during midday, four hours after dawn and placed on 0.8% agar media.

2.1.2. Crop treatments

2.1.2.1. Cabbage treatments for *Trichoplusia ni* defense assays

Cabbage (*Brassica oleracea*) was purchased from a local organic grocer. For experiments to determine how long the ability of cabbage to be re-entrained persists after

storage at room temperature, cabbage was stored at 22°C under constant 24 hour light (120 ± 10 μE • m−2 • s−1) for 3, 6 or 9 days. After constant light storage, leaf tissue was cut into 3 cm disks and placed on 0.8% agar medium. Leaf disks were then entrained under 12-hour light/12-hour dark cycles (120 ± 10 μE • m−2 • s−1) at 22°C for 3 days.

For *T. ni* choice experiments, cabbage (*Brassica oleracea*) was purchased from a local organic grocer and entrained under 12-hour light/12-hour dark cycles (120 ± 10 μE • m−2 • s−1) or 24-hour constant light (120 ± 10 μE • m−2 • s−1) at 22°C for 3 days. After light entrainment, leaf tissue was cut into 3 cm disks and placed on 0.8% agar medium.

2.1.2.2. Crop treatments for postharvest longevity assays

Kale (*Brassica oleracea* cv. acephala group), cabbage (*Brassica oleracea*), green leaf lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*) were purchased from a local organic grocer. Leaf tissue was cut into 2 cm disks and placed on 0.5% agar. Light-treated leaf disks were stored under 120 ± 10 μE • m−2 • s−1 at 22°C for either cycles of 12 hours of light followed by 12 hours of darkness or constant 24-hour light. Leaf disks stored in constant darkness were either maintained at 22°C or refrigerated at 4°C. Samples for analysis were collected 6 hours after initiation of the light period for samples stored under 12-hour light/12-hour dark cycles or at comparable times for samples stored in constant light, in constant darkness, or under refrigeration to avoid time-of-day dependent differences in measured values.

2.1.2.3. Crop treatments for *Botrytis cinerea* infection assays

Cabbage (*Brassica oleracea*) and green leaf lettuce (*Lactuca sativa*) leaf tissue was cut into 5-cm disks and placed on 0.8% agar media. Leaf disks were then entrained for 3 days in 12-hour light/12-hour dark cycles (120 ± 10 μE • m−2 • s−1) at 22^{o}C.

2.2. *Botrytis cinerea* growth, maintenance and plant inoculation

Botrytis cinerea spores were germinated on 1.5% agar in 50% grape juice (Welch's, Concord, MA): 50% sterile water 25°C for 1 to 3 weeks until sporulation. Mature spores were collected as a sterilized water suspension after scraping spores from agar surface with a spatula. The resulting spore suspension was filtered through sterilized glass wool and centrifuged for 10 minutes at 5,000xg. The liquid was removed by aspiration and the spore pellet was washed by vortexing vigorously in 20 mL of sterile water followed by centrifugation at 5,000xg for 10 minutes. The liquid was removed by aspiration and the pellet was washed a second time by vigorous vortexing in 20 mL of sterile water followed by centrifugation at 5,000xg for 10 minutes. After the liquid was removed by aspiration, the washed spore pellet was resuspended in 1 ml of water. Spore concentrations were determined by hemocytometer counting under a light microscope and spores were diluted with 50% sterile glycerol to a final concentration of 500 spores/μL. Plant leaves were exposed to 100 *B. cinerea* spores suspended in 4 μL of 50%: organic grape juice: 50% water solution.

2.3. *Trichoplusia ni* handling and treatments

Trichoplusia ni eggs (Benzon Research, Carlisle, PA) were hatched at 25°C, incubated with pinto bean diet as described (Goodspeed et al., 2012), under 12-hour light/12-hour dark cycles for 3 days, and then the larvae were moved to constant conditions for 24 h. *T. ni* were gently moved onto plates or plants using a fine brush. Visibly similar-size *T. ni* loopers were placed with plants for co-incubation experiments. The experiments were stopped after 72 hours of co-incubation, and *T. ni* weights were determined.

2.4. Transcript accumulation measurements

Frozen Arabidopsis tissues were ground using a mortar and pestle, and the RNA from approximately 100 mg of the resulting powder was extracted using an ISOLATE II RNA plant kit (Bioline USA Inc., Taunton, MA). Reverse transcription of 1 μg of total RNA was conducted using SuperScript® III (Invitrogen, Grand Island, NY) using oligo dT primers (18 repeats).

Quantitative PCR was performed using forward and reverse primers listed in Table 2. Measurements were made using a BioRad CFX Real-Time PCR Detection System (BioRad, Hercules, CA) employing SYBR Green qPCR MasterMix Plus (Eurogentec, San Diego, CA). qPCR was performed using a protocol as shown in Table 3, consisting of Taq activation at 95^{o}C for 10 minutes. Following Taq activation, a denaturing step was carried out at 95^{o}C for 15 seconds followed by an

annealing/extension step for 1 minute at 60^{o}C. The denaturing and annealing/extension steps were repeated another 39 times for a total of 40 cycles. C_T values were obtained by manually setting a 100 RFU threshold value. Transcript accumulation of the gene of interest was normalized to *PP2A* transcript accumulation using the formula:

Equation 1: Normalization of transcript accumulation to *PP2A*

$$2^{-(CtGene - CtPP2A)}$$

Gene	Forward Primer	Reverse Primer
AOS	ACTACGGTTTACCAATCGTAG	TCTGTACACCGTGGAGTTGTA
AOC1	CCAGACCAAGCAAAGTTCAA	AGATCGCCGGTGTAGAGTT
OPR3	ACGGACCACTCCCGGCGGTTTTC	CGTGAACTGCTTCCACAACTT

Table 2: Primers used for qPCR measurement of transcript levels

Step	Function	Temperature	Time
1	UNG step	50 °C	2’
2	Taq Activation	95 °C	10’
3	Denature	95 °C	15”
4	Anneal/Extension	60 °C	1’
5	Back to step 2 for 39 times (total 35-40 cycles)		
6	Meltcurve	60-95 °C	0.5 increments
7	Hold at 4 °C		

Table 3: Protocol for qPCR reaction

2.5. Chlorophyll measurements

Frozen leaf disks were ground using a mortar and pestle, and approximately 50 mg of the resulting powder was mixed with 0.5 mL of 80% (v/v) acetone and incubated overnight at 4°C. Samples were centrifuged at 14,000 x g for 10 minutes at 4°C. The absorbance of the supernatant was measured spectrophotometrically at 645 and 663 nm using a Tecan Infinite M200 PRO (Tecan, Morrisville, NC). Chlorophyll concentrations relative to fresh weight were determined using the formula (Porra, 2002):

Equation 2: Calculation of chlorophyll concentration

$$\text{Total chlorophyll } (\mu g/g \text{ fresh weight}) = (17.76A_{645nm} + 7.34A_{663nm})/g \text{ plant tissue}$$

Chlorophyll concentrations per gram dry weight were determined after plant materials were freeze dried at −80°C at 0.04 mBar overnight using a FreeZone 4.5 liter benchtop freeze dry system (Labconco, Kansas City, MO).

2.6. Electrolyte leakage measurements

For electrolyte leakage measurements, 2 cm leaf disks were placed into 50 ml of deionized room temperature water. After 30 minutes, electrical conductivity was measured using a Horiba B-173 Twin Cond Conductivity Meter (Horiba Instruments Inc., Kyoto, Japan). To avoid complications associated with electrolyte leakage due to initially cutting out the leaf disks, the first electrolyte leakage measurements were delayed as specified in the text.

2.7. Glucosinolate measurements

Tissue frozen with liquid nitrogen was ground using a mortar and pestle. Ground tissue was then submerged in 90% methanol. Samples were placed into the -80^{o}C freezer for storage until samples were shipped. Samples were then sent to the Kliebenstein lab (UC Davis) on dry ice, where they were extracted. Glucosinolates were identified and quantified in comparison to reference standards using HPLC-DAD, as previously described (Kliebenstein et al., 2001). Total glucosinolate levels were determined by totaling the concentrations of individually quantified glucosinolate species.

Chapter 3: Rhythmic accumulation of jasmonic-acid-biosynthesis gene transcripts is independent of jasmonic acid

3.1. Introduction

The circadian clock plays a role in plant response to biotic stresses. For example, the clock helps Arabidopsis prepare for and defend against insect herbivory. Plants grown in light/dark cycles that are synchronized with *T. ni* are more resistant to herbivory when compared to plants that are entrained out-of-phase relative to *T. ni* (Figure 12; Goodspeed et al., 2012). Defense against insect herbivores involves the plant hormone jasmonic acid. Jasmonic acid (JA) and compounds derived from jasmonic acid, collectively known as the jasmonates, are important plant hormones that regulate many plant processes including those involved in fertility and plant defense against insect herbivores and necrotrophic pathogens (Acosta and Farmer, 2010).

Interestingly, JA levels are circadian clock regulated and cycle rhythmically with peak accumulation occurring during subjective day even in unstressed plants (Figure 7). However, how the circadian clock regulates cycling JA levels is not understood. Additionally, because of the JA positive feedback regulation on JA biosynthesis, it has

been difficult to determine how JA synthesis is initially activated in response to diverse stimuli. Elucidating how the clock regulates JA accumulation may shed light on JA biosynthesis initiation. One possibility is that the clock regulates the time-of-day accumulation of gene transcripts involved in jasmonate biosynthesis such as *AOS, AOC1* and *OPR3* (Figure 6), which would then lead to cyclical production of JA. Alternatively, the clock could control basal JA accumulation downstream of JA-biosynthesis-gene expression regulation. I hypothesized that the clock controls JA accumulation by regulating *AOS*, *AOC1* and/or *OPR3* transcription. I favored this hypothesis because the circadian clock affects expression of one third of expressed Arabidopsis genes (Covington et al., 2008), with significant impact on genes involved in JA response (Covington et al., 2008; Walley et al., 2007).

3.2. Jasmonic-acid-biosynthesis-gene transcript accumulation under circadian control

To examine if the clock can regulate JA biosynthesis through regulation of JA-biosynthesis gene expression, *AOS, AOC1,* and *OPR3* transcript levels were quantified in wild-type Col-0 plants under free-running unstressed conditions. Plants were grown for three weeks under 12-hour light/12-hour dark cycles and then transferred to free-running constant light conditions. After 24 hours of constant light, aerial plant tissue were collected every 4 hours beginning at subjective dawn (Zeitgeber 24, ZT24) for 48 hours. Zeitgeber refers to any external cue that entrains the circadian clock, and zeitgeber 0

(ZT0) is a reference point for the last instance of a night to day transition that synchronizes the clock in the plants.

AOS transcripts were lowest at the first two time points (ZT24 and ZT28), and increased at ZT32. The pattern of reduced transcript accumulation continued into the second subjective day (Figure 15A). Similarly, *AOC1* and *OPR3* transcripts also displayed differential transcript accumulation over a 48-hour period. Transcript accumulation was at a minimum during the first few time points of subjective day beginning at ZT24 and ZT48. *AOC1* and *OPR3* transcripts then increased during the subjective day hours to peak during subjective dusk at both ZT36 and ZT60 (Figure 15B and C). *AOC1* and *OPR3* transcript accumulation decreased over subjective night hours (Figure 15B and C). These results indicate that the clock regulates transcript levels of *AOS, AOC1* and *OPR3* with peak expression occurring at subjective dusk and/or during the first few hours of subjective night.

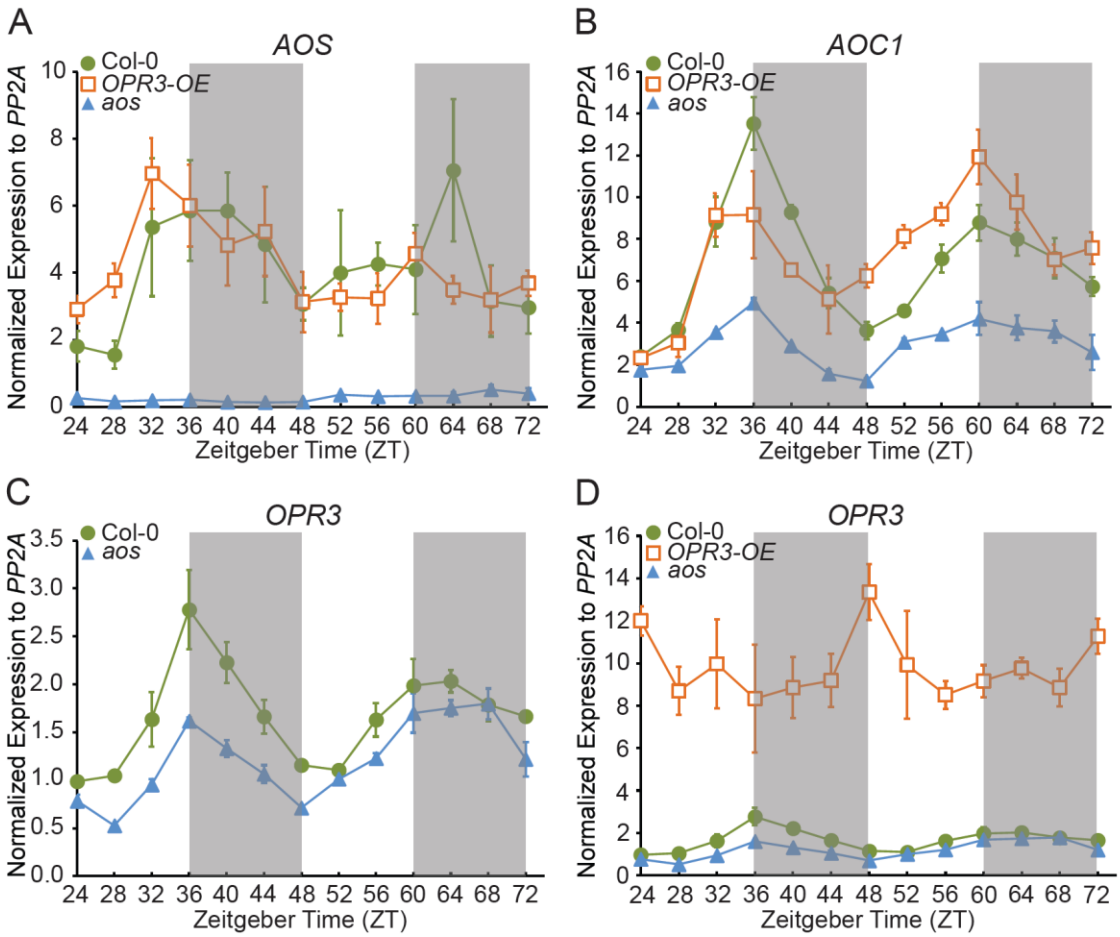

Figure 15: Differential accumulation of *AOS, AOC1* and *OPR3* under free-running conditions

AOS (A), *AOC1* (B), and *OPR3* (C and D) transcript levels in Col-0 (green circles), *aos* (blue triangles) and *OPR3-OE* (orange squares). Shoot tissue was collected every 4 hours under free-running conditions, beginning at subjective dawn, 24 hours after transfer to constant light. Quantification for each time point was normalized to *PP2A* transcript amplification. Mean ± SE; n = 3. White bars indicate subjective day, and grey bars indicate subjective night.

3.3. *AOC1* and *OPR3* transcripts show circadian periodicity in the absence of JA positive feedback

To begin to understand how the clock may regulate accumulation of transcripts encoding the JA biosynthetic enzymes, I addressed the question of whether the clock acts through JA positive feedback regulation. If transcript cycling is independent of JA, then the JA-biosynthetic gene transcripts should show rhythmic accumulation in *aos* plants, which are unable to accumulate JA (Park et al., 2002). *AOS* transcript levels in *aos* plants are considerably lower than those observed in wildtype (Figure 15A), possibly due to the T-DNA insertion into the exon of *AOS* affecting transcript abundance. Alternatively, the long DNA insertion may reduce efficiency of full length cDNA synthesis when using Oligo(dT) to prime reverse transcription starting at the 3' poly(A) tail. *AOC1* transcripts in *aos* plants showed accumulation peaks at subjective dusk (ZT36 and ZT60), corresponding to transcript peaks observed in Col-0 (Figure 15B). However, *AOC1* transcript levels in *aos* were lower in both peak and trough accumulation points than in Col-0. *OPR3* transcripts in *aos* also displayed 24-hour periodicity in accumulation largely mirroring the *OPR3* transcription pattern seen in Col-0 (Figure 15C). Together, these data indicate the *AOS* function and therefore JA positive feedback are not required for the apparent circadian periodicity of *AOC1* and *OPR3* transcript accumulation. However, my results also support that JA positive feedback may impact *AOC1* and *OPR3* transcript accumulation levels.

Since the absence JA positive feedback in *aos* affects the overall magnitude of JA-biosynthetic gene transcript levels, I next examined whether high, possibly

constitutive, JA levels would affect cycling of *AOS, AOC1* and *OPR3* transcripts. *AOS* and *AOC1* transcripts levels were measured in *OPR3-OE* transgenic plants that constitutively express *OPR3* and have higher levels of JA compared to wild-type (Col-0; Chehab et al., 2011). As expected, *OPR3* transcripts in *OPR3-OE* were higher than those observed in Col-0, and there was no evidence that *OPR3* transcripts cycled with 24-hour periodicity (Figure 15D). *AOS* transcript levels in *OPR3-OE* followed a similar accumulation pattern to Col-0, at least for the first free-running period, with an increase in *AOS* transcript levels during subjective day and a moderate decreasing trend during subjective night (Figure 15A). However, *AOS* transcripts showed no significant accumulation fluctuation during the second free-running period. Overall, *AOS* transcript levels in *OPR3-OE* are comparable to those in Col-0, indicating that increased JA levels did not significantly affect overall free-running *AOS* transcript levels. *AOC1* transcript fluctuation in *OPR3-OE* also maintained the pattern of increasing accumulation during subjective day and decreasing levels during subjective night. Overall, constitutive expression of *OPR3* does not cause dramatic effects on cycling or magnitude of *AOS* and *AOC1* transcripts under free-running conditions compared to wild type.

3.4. Conclusion

Here I examined whether cycling of basal JA levels under free-running conditions (Figure 7) may be due to clock-regulated cycling of JA-biosynthetic gene transcript levels. *AOS, AOC1* and *OPR3* transcript accumulation levels were generally higher during subjective night when compared to levels measured during subjective day in Col-0

during both consecutive 24-hour periods examined (Figure 15). The repeated pattern of peaks and troughs under free-running conditions with an approximately 24-hour period indicates that the clock may regulate transcript accumulation of *AOS, AOC1* and *OPR3*. To further verify this, additional experiments should examine transcript levels in plants with altered clock function. Additionally, while transcripts for *AOS, AOC1* and *OPR3* increased during the early hours of subjective day and peaked at roughly at ZT36 and ZT60 (Figure 15), peak basal JA accumulation occurs at ZT28 and ZT52 (Figure 7; Goodspeed et al., 2012), meaning there is an approximately 12-14 hour delay from peak transcript accumulation to peak JA accumulation. This delay could be due to additional levels of regulation at the translational, enzyme activation and/or transport levels.

AOC1 and *OPR3* transcripts in *aos* plants show differential accumulation over the course of the 48-hour time course. Similar to what was observed in Col-0, *AOC1* and *OPR3* transcript levels are higher during subjective dusk when compared to subjective dawn (Figure 15B and C). However, overall levels of *AOC1* and *OPR3* transcripts in *aos* were lower than that observed in Col-0. Together, these data indicate that JA-positive feedback is not required for cycling of the transcripts, but basal JA is required for wild-type levels of *AOS*, *AOC1* and *OPR3* transcripts. Thus far it has been difficult to elucidate how initial synthesis of JA is controlled due to the complications resulting from the positive JA feedback loop. These results indicate that cycling of JA-biosynthesis transcripts may be independent of JA feedback and provide the first example of JA-independent JA synthesis activation.

Constitutive expression of *OPR3* in *OPR3-OE* did not affect differential accumulation of *AOS* and *AOC1* transcripts when compared to Col-0 (Figure 15A and B). *AOS* and *AOC1* transcripts both accumulated at higher levels during subjective dusk and had lower levels during subjective dawn. Interestingly, while lower JA levels in *aos* decreased overall magnitude of JA-biosynthesis transcript accumulation compared to wild type (Figure 14B and C), *OPR3-OE*, which has approximately 35% higher basal JA than wild type (Chehab et al., 2011), did not have noticeably higher *AOS* or *AOC1* transcript levels compared to wild type over the 48-hour time course. However, it is unknown how basal JA levels in *OPR3-OE* behave under free-running conditions. Future experiments should examine if JA levels in *OPR3-OE* plants cycle and whether exogenous JA treatments given at times when endogenous basal JA levels are normally low would affect cycling of JA-biosynthesis transcripts.

Chapter 4: Accumulation of jasmonic acid biosynthesis gene transcripts is affected by salicylate levels

4.1. Introduction

Salicylic (SA) levels, like JA levels, display rhythmic accumulation under free-running conditions in unstressed plants (Figure 7; Goodspeed et al., 2012). However, SA levels accumulate anti-phasic to JA levels; JA levels peak during subjective dawn and SA levels peak during subjective night (Figure 7; Goodspeed et al., 2012). Because there is an antagonistic relationship between the JA and SA signaling pathways (Glazebrook, 2005; Koornneef and Pieterse, 2008, 2008; Thaler et al., 2012), one possible mechanism via which the clock could be controlling JA cycling is through control of SA levels, which in turn could act through negative regulation to the accumulation the of JA-biosynthesis-related gene transcripts or JA accumulation. Therefore, I hypothesized that if the circadian clock acts through SA accumulation or signaling to control JA biosynthesis, then plants with altered SA levels will also see an effect in rhythmic JA accumulation and/or levels of transcripts encoding JA biosynthetic enzymes.

4.2. Free-running accumulation of jasmonic acid biosynthesis gene transcripts are altered in plants with low or high SA

To examine if altered SA levels affect daily basal accumulation of JA-biosynthetic gene transcripts, free-running *AOS, AOC1* and *OPR3* transcript levels in 3-week old *NahG* and *acd6-1* tissue were compared to those collected from Col-0 plants over a 48-hour period. *NahG* plants produce bacterial salicylate hydroxylase, which breaks down SA (Katagiri et al., 1965; Yamamoto et al., 1965), leading to decreased levels of SA (Delaney et al., 1994; Gaffney et al., 1993). In contrast, *acd6-1* (*accelerated cell death6-1*) mutants produce higher levels of SA compared to wild type (Lu et al., 2003, 2009; Rate et al., 1999), although the underlying defect in *acd6-1* mutants is as yet undefined (Lu et al., 2009).

I first examined transcript levels in Col-0. Similar to the previously described Col-0 results (Figure 15A), *AOS* transcripts during the first 24-hour period (ZT24-ZT48) were relatively lower during subjective day (ZT24-ZT36) compared to subjective night (ZT40 and ZT44; Figure 16A). However, the apparent peak or phase of *AOS* transcript accumulation was observed at ZT40 (Figure 16A), which is delayed approximately 8 hours from that observed previously (Figure 15A). Yet, both sets of *AOS* transcript measurements showed a trough at ZT48 (Figures 14A and 15A). Additionally, both sets of Col-0 data showed weak cycling during the second 24-hour period (ZT48 to ZT72); that is, slightly elevated *AOS* transcript levels during the middle of subjective night followed by a decrease before subjective dawn (ZT72; Figures 14A and 15A). Similar to

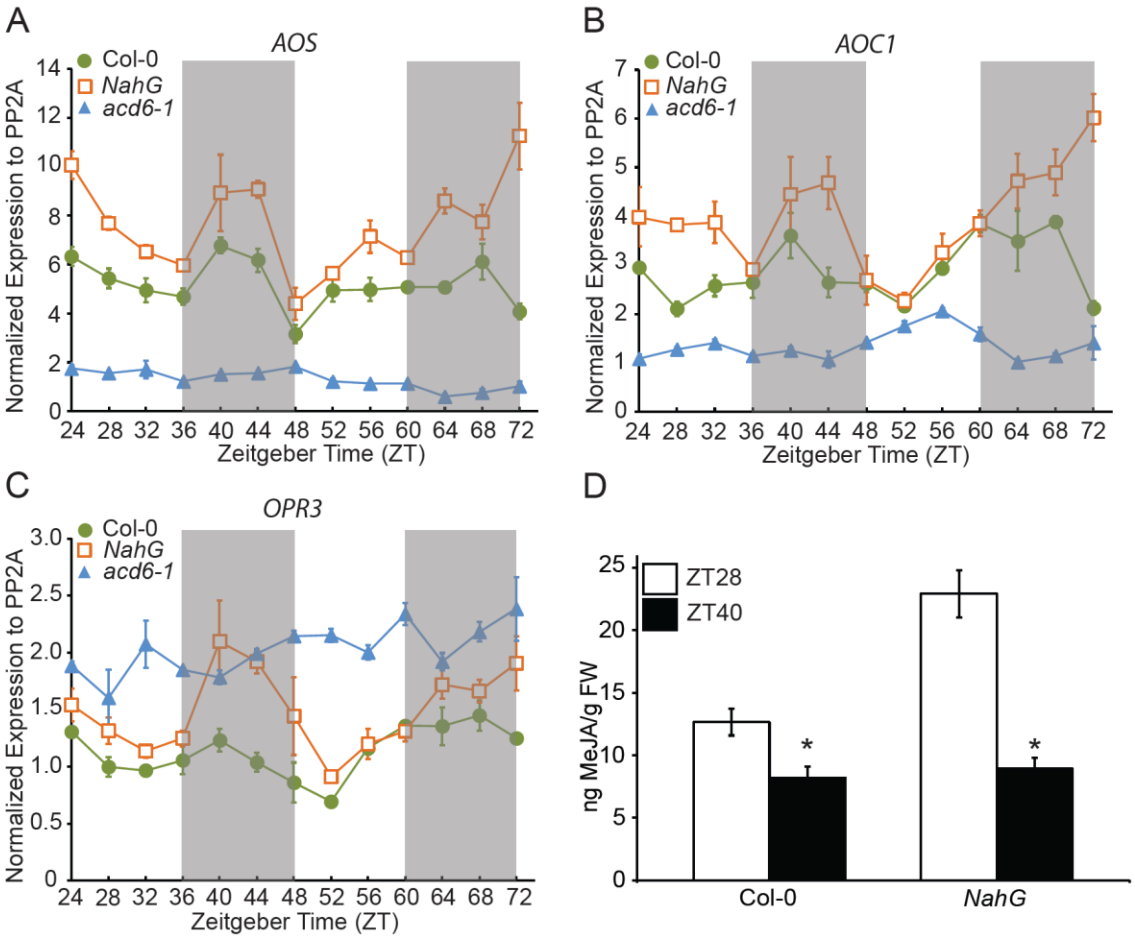

Figure 16:Accumulation of *AOS, AOC1* and *OPR3* in plants with altered SA levels

AOS (A), *AOC1* (B), and *OPR3* (C) transcript levels in Col-0 (green circles), *acd6-1* (blue triangles) and *NahG* (orange squares). Aerial tissue was collected every 4 hours under free-running conditions, beginning at subjective dawn, 24 hours after transfer to constant light. Quantification for each time point was normalized to PP2A transcript amplification. Mean ± SE; n = 3. White bars indicate subjective day, and grey bars indicate subjective night. (D) Jasmonate (JA and MeJA) levels in Col-0 and *NahG* plants from aerial tissues collected under free-running conditions at ZT28 and ZT40.

Col-0, *AOS* transcripts levels in *NahG* decreased starting at ZT24 over the course of the day but show a sharp increase from ZT36 to ZT40. This increase in *AOS* transcript abundance is followed by a sharp decrease at ZT48, mirroring the pattern observed in Col-0 (Figure 16A). During the second 24-hour period, *AOS* transcripts in *NahG* continued to increase from ZT48 to ZT72 (Figure 16A), in contrast to Col-0 that showed a slight dip in *AOS* transcript accumulation at ZT72. Overall, *AOS* transcript levels at all time points were higher in *NahG* when compared to Col-0, particularly during the first 24-hour period and at ZT72. In contrast to both Col-0 and *NahG*, *acd6-1* did not show differential accumulation of transcripts over the 48-hour free running period (Figure 16A). Additionally, *AOS* transcripts in *acd6-1* were consistently lower than those observed in Col-0 and *acd6-1* over the 48-hour period.

In comparison to the Col-0 data described previously, *AOC1* transcript accumulation in the Col-0 that was used for *NahG* and *acd6-1* comparisons also showed lower levels during subjective day, with a slight, though less dramatic increase in *AOC1* transcript accumulation during subjective night (Figures 14B and 15B). However, a more distinct pattern was observed during the second 24-hour period; *AOC1* transcript levels were lower during subjective day (ZT48-ZT52; Figure 16B), with increasing levels that peaked during subjective night (ZT60-68) followed by a decrease before subjective dawn (ZT72), similar to what was described previously (Figure 15B). Comparable to *AOS* transcripts in *NahG, AOC1* transcript levels in *NahG* also started with a decrease in transcript abundance at ZT36 from initial levels detected at ZT24 (Figure 15A and B). This decrease in *AOC1* transcript levels was followed by a sharp increase during the middle of subjective night (ZT40 and ZT44), and then a subsequent decrease at ZT48

(Figure 16B). During the second 24-hour period, *NahG AOC1* transcript levels continued to rise beginning at ZT52, in contrast to Col-0 (Figure 16B). *AOC1* transcript levels in *acd6-1* are lower than those observed in Col-0 over the 48-hour free running time course (Figure 16B). During the first 24-hour period, *AOC1* transcripts in *acd6-1* did not demonstrate any difference in transcript levels during subjective day and subjective night. In contrast, there did appear to be an increase in *AOC1* transcripts during the second subjective day period (ZT52-ZT56) when compared to the prior (ZT40-ZT44) and subsequent subjective night (ZT64-ZT68) periods (Figure 16B).

OPR3 transcript levels in the Col-0 used for *NahG* and *acd6-1* comparisons showed a similar pattern of transcript accumulation to that observed previously in Figure 15C. *OPR3* transcripts were lower during subjective day hours when compared to subjective night hours during both the first and second 24-hour periods (Figure 16C). Similar to *AOS* data described in Figure 16A, peak *OPR3* transcript accumulation appeared delayed by approximately 4 hours compared to what was observed previously (Figure 15A). *OPR3* transcripts in *NahG* displayed a distinct difference between transcript accumulation during subjective day and subjective night during the first 24-hour period (ZT24 to ZT48; Figure 16C). Again, similar to *AOS* and *AOC1* transcripts, *OPR3* transcripts in *NahG* began increasing over the remaining time course during the second 24-hour period (Figure 16C). In contrast to Col-0, *OPR3* transcripts in *acd6-1* did not show any evidence of rhythmic cycling within a 24-hour period over the 48-hour time course (Figure 16C). Additionally, *OPR3* transcripts were consistently higher in *acd6-1* as compared to Col-0 (Figure 16C).

To determine if the increased accumulation of JA-biosynthetic enzymes *AOS, AOC1* and *OPR3* in measured *NahG* compared to wild type would result in increased JA accumulation, I measured JA in *NahG* and Col-0 under free-running conditions at two time points during subjective day (ZT28) and subjective night (ZT40). Consistent with Figure 7, jasmonate levels are higher during subjective day (ZT28) than during subject night (ZT40) for both genotypes. The results from *NahG* indicate that reduced SA may not affect time-of-day differential accumulation of jasmonates but does affect the overall magnitude of JA accumulation, specifically during subjective day periods.

4.3. Conclusions

The results indicate that the accumulation pattern of transcripts in Col-0 (Figure 15) was altered in this set of measurements as compared to the previously described set both in terms of accumulation pattern and phase of peak accumulation (Figure 16). This is most likely due to differences in growth conditions that were unobserved and will require the experiments described in Figure 15 and Figure 16 be conducted again. Nevertheless, a few interesting observations can be made. SA signaling can inhibit JA responses as described above (Glazebrook, 2005; Koornneef and Pieterse, 2008, 2008; Thaler et al., 2012); here I have shown that SA suppresses accumulation of JA-biosynthetic gene transcripts, with *NahG* plants having higher *AOS, AOC1* and *OPR3* transcript levels compared to wild type, particularly during subjective night periods (ZT40-ZT44 and ZT64-ZT68; Figure 16A-C). Additionally, at least during the first 24-hour period, both Col-0 and *NahG* showed differential accumulation of *AOS, AOC1* and

OPR3 transcript levels when measured during subjective dusk (ZT36) , subjective night (ZT40) and subjective dawn (ZT48) (Figure 16A-C), indicating that the lack of SA may not affect cycling of JA-biosynthetic genes. However, while one would expect the pattern to repeat during the second 24-hour period, indicating that the clock controls cycling of transcript accumulation, *AOS, AOC1* and *OPR3* transcripts in *NahG* continued to increase during the second 24-hour period. To determine if clock controlled cycling of transcripts is lost over time or if period length is affected in plants lacking SA, subsequent experiments should include longer time courses to examine what occurs during subsequent subjective day/night cycles. The results from *acd6-1* produced two intriguing observations. First, *acd6-1* plants showed drastic changes in the accumulation pattern of *AOS, AOC1* and *OPR3* transcripts over the 48-hour time course when compared to Col-0. Whereas Col-0 showed evidence of differential transcript accumulation at least during the first 24-hour period, *acd6-1* did not (Figure 16A-C). Second, while *AOS* and *AOC1* transcript levels in *acd6-1* appeared lower than Col-0 (Figure 16A and B), *OPR3* transcripts in *acd6-1* were higher than those in Col-0 (Figure 16C). Whether these two observations are due to increased SA levels (Lu et al., 2009) or the *acd6-1* mutation itself acting through an unknown mechanism will require further investigation.

Chapter 5: Glucosinolates play a role in phase-dependent resistance of Arabidopsis plants to insect herbivory

5.1. Introduction

Arabidopsis displays phase-dependent increased resistance to *T. ni* when plants are exposed to *T. ni* that are entrained in-phase with the plants and decreased resistance to *T. ni* when plants are exposed to *T. ni* that are entrained out-of-phase with the plants (Figure 12; Goodspeed et al., 2012). Plant phase-dependent resistance to *T. ni* is JA dependent since *aos* plants that are JA-deficient do not show phase-dependent susceptibility to *T. ni.* Defense against insect herbivores also involves glucosinolates, whose levels are regulated by jasmonates (Grubb and Abel, 2006). Since JA (Figure 7; Goodspeed et al., 2012) and glucosinolates (Figure 10; Goodspeed et al., 2013b) cycle with circadian rhythmicity under free running conditions in unstressed plants, we hypothesized that phase-dependent plant resistance to *T. ni* might require glucosinolates.

5.2. Loss of aliphatic glucosinolates reduce benefits attributed to proper entrainment in Arabidopsis resistance against *Trichoplusia ni*

To examine if glucosinolates are required for phase-dependent plant defense against *T. ni*, Col-0 and *myb28myb29* plants were exposed to *T. ni* that were entrained in-phase and out-of-phase with respect to the plants. *myb28myb29* mutants lack the ability to generate aliphatic glucosinolates (Sønderby et al., 2007, 2010). Col-0 and *myb28myb29* mutants were grown in either 12-hour-light/ 12-hour-dark cycles (LD) or 12-hour-dark/ 12-hour-light cycles (DL) at 22^{o}C for 3 weeks. After 3 weeks of growth, plants were moved to free-running constant light conditions for 24 hours at 22^{o}C. Subsequently, 4-day-old *T. ni* (entrained with 3 days of LD, 1 day of LL) were placed on the plants. Following 3 days of insect/plant co-incubation under constant light, relative tissue area loss of the plants was quantified.

Both Col-0 and *myb28myb29* plants showed increased resistance to *T. ni* when the plants were entrained in-phase with the *T. ni* when compared to plants entrained out-of-phase with respect to the *T. ni* (Figure 17). However, the resistance enhancement based on synchronized entrainment of plants to *T. ni* was significantly reduced in *myb28mby29* plants when compared to the resistance benefit seen in Col-0 plants (Figure 17; $p < 0.05$, two-way interaction ANOVA).

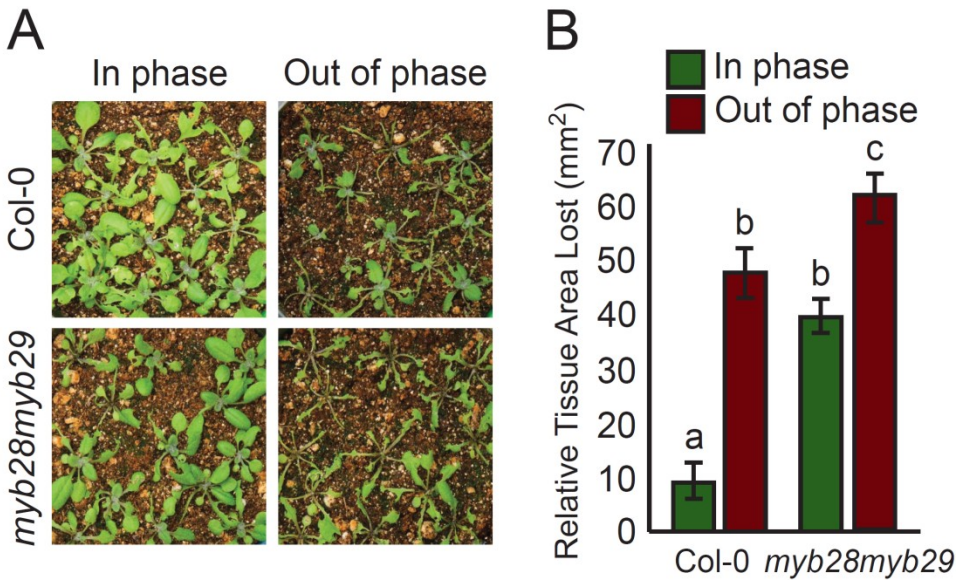

Figure 17: Enhanced phase-dependent Arabidopsis resistance to *T. ni* is reduced in plants lacking aliphatic glucosinolates

(A) Photographs of representative Col-0 and *myb28myb29* plant tissue remaining from plants entrained in phase and out of phase with *T. ni* entrainment after 72 hours of coincubation. (B) Area of plant tissue remaining from plants entrained in phase (green bars) and out of phase (red bars) with respect *T. ni* entrainment after 72 hours of coincubation with *T. ni*. Mean area ± SE; n = 6;. Phenotypes with different letters were statistically different at $p < 0.05$ using a one-way ANOVA with Tukey's honest significance multiple comparison test; those with the same letter designation were statistically nonsignificant for a difference (adapted from Goodspeed et al., 2013b.)

5.3. Conclusion

The decrease in phase-dependent-enhanced resistance to *T. ni* found in *myb28myb29* plants when compared to Col-0 indicates that aliphatic glucosinolates may play a role in clock enhanced resistance of plants to cabbage loopers. However, there was still a significant increase in resistance of *myb28myb29* plants entrained in-phase with the loopers compared to *myb28myb29* plants entrained out-of-phase. This indicates that while aliphatic glucosinolates have a role in clock-mediated enhanced resistance of Arabidopsis to insects, other factors also contribute to this phenomenon.

Chapter 6: Storing vegetables in light/dark cycles improves postharvest performance compared to storage in constant light or constant dark[1]

6.1. Introduction

Fruits and vegetables after harvest can respond to repeated cycles of 12-hour light/12-hour dark, resulting in circadian clock function and rhythmic behaviors (Goodspeed et al., 2013b). Postharvest cabbage that has been re-entrained using light/dark cycles displays rhythmic accumulation of glucosinolates under free-running conditions (Figure 10) and exhibits phase-entrainment-dependent resistance to *T. ni* (Figure 18; Goodspeed et al., 2013a) similar to Arabidopsis (Figure 12; Goodspeed et al., 2012). When postharvest cabbage is exposed to *T. ni* that are entrained with the same light/dark cycles, the cabbage display less tissue loss and *T. ni* gain less weight than when the postharvest cabbage exposed to *T. ni* is entrained out-of-phase (Figure 18).

[1] Parts of this chapter have been published (Liu et al., 2015)

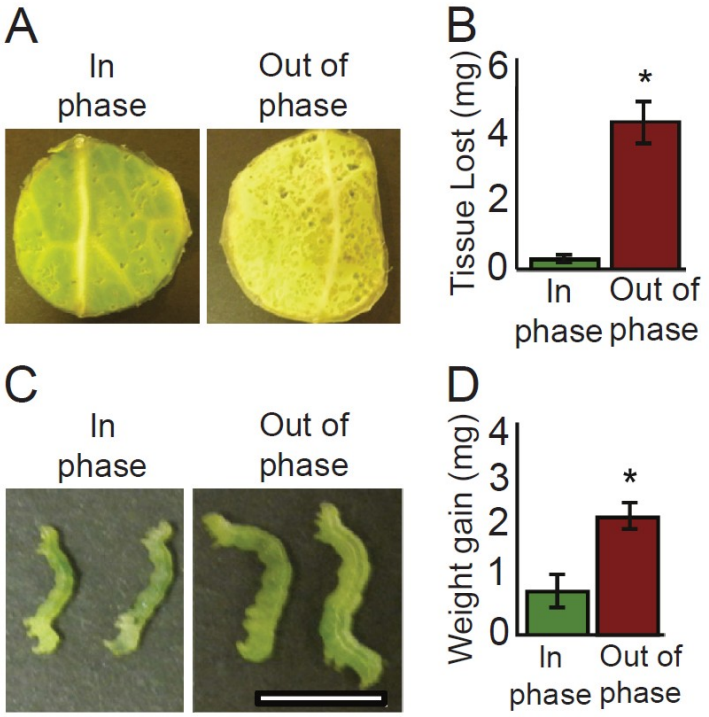

Figure 18: Postharvest cabbage can be re-entrained by light/dark cycles to demonstrate phase-dependent herbivore resistance

(A) Photographs of representative cabbage disks entrained in phase and out of phase with *T. ni* entrainment after 72 hours of coincubation. Dark spots on disks are sites of herbivory damage. (B) Tissue weight loss remaining from cabbage disks entrained in phase (green bars) and out of phase (red bars) with respect T. ni entrainment after 72 hours of coincubation with *T. ni*. Mean area ± SE; n = 6. *p < 0.005, unpaired t test. (C) Photographs of representative of *T. ni* after 72 hours of coincubation with cabbage disks entrained in phase and out of phase with respect to the *T. ni*. Scale bar, 0.5 mm (D) *T. ni* net weights after 72 hours of coincubation with plants. Mean area ± SE; n = 18; *P<0.005; two-tailed paired t test. (Adapted from Goodspeed et al., 2013b.)

Because a functional plant circadian clock is physiologically advantageous (Dodd et al., 2005; Goodspeed et al., 2012, 2013a), we hypothesized that postharvest storage under conditions that simulate day/night cycles, thereby potentially maintaining biological rhythms, would improve postharvest longevity.

6.2. Vegetables stored in light/dark cycles are visibly healthier than those stored in constant light or constant dark

To address the question of whether storing postharvest vegetables in light/dark cycles would increase longevity, I examined the effect of light treatment on green leafy vegetables, including commonly consumed kale (*Brassica oleracea* cv. acephala group), cabbage (*Brassica oleracea*), green leaf lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*), because I anticipated that the leaf organ would likely maintain light sensitivity and responsiveness even after harvest. To determine whether daily light/dark cycles during postharvest storage can affect leaf longevity, I compared the overall appearance of leaf disks that were stored at 22°C under cycles of 12-hour light/12-hour darkness (LD) versus leaf disks stored under constant light (LL) or constant darkness (DD) for various lengths of time (Figure 19).

Under cycles of 12-hour light/12-hour darkness, kale leaf disks were dark green after 3 days of storage (Figure 19A; LD). After 6 days and 15 days of storage, the kale disks showed lighter green coloration than the kale disks stored for 3 days (Figure 19A; LD). However, the kale leaf disks stored under constant light were lighter green than the kale disks stored under light/dark cycles and showed some brown or yellow discoloration

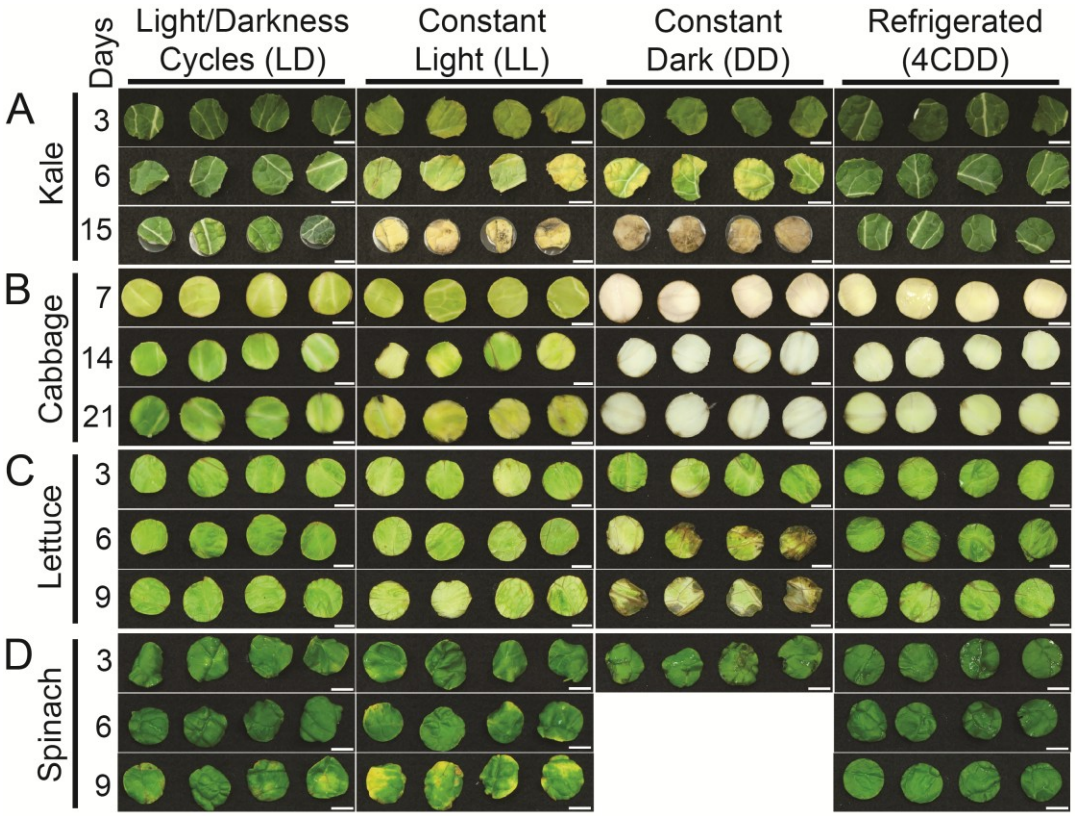

Figure 19: Leaf disk appearance depends upon light exposure during post harvest storage

Representative photographic images of kale (A), cabbage (B), lettuce (C), and spinach (D) leaf disks after varying number of days (number of days indicated at left) of storage at 22°C under 12-hours light/12-hours darkness (Light/Dark Cycles, LD), constant light (LL), or constant dark (DD), or at 4°C under constant dark (Refrigerated, 4CDD). Spinach disks disintegrated after 3 days when stored under constant darkness and therefore images were not available (Scale bars, 1 cm) (Liu et al., 2015).

after 3 and 6 days (Figure 19A; LL). By 15 days, the kale leaf disks stored under constant light lost nearly all green coloration and showed light and dark shades of browning with shape changes resulting from leaf folding and shrinkage (Figure 19A; LL). The kale leaf disks stored under constant darkness resembled those stored under constant light, except that the 3-day kale samples were darker green than the 3-day constant light-stored kale leaf disks (Figure 19A; DD), suggesting that the constant light may have constituted a greater stress on the kale leaves than constant darkness. These results indicate that postharvest storage with daily cycling of light and darkness improved the appearance of the kale leaf tissue compared to storage under either constant light or constant darkness. However, the preservation benefit obtained from postharvest storage under light/dark cycles at 22°C appeared to be less than that provided by refrigeration; kale leaf disks stored at 4°C with constant darkness, were comparable in their dark green coloration whether stored for 3, 6 or 15 days (Figure 19A; 4CDD).

Cabbage leaf disks stored under cycles of 12-hour light/12-hour darkness showed brown spots along the disk edges that increased in intensity over the storage period of 7, 14, and 21 days (Figure 19B; LD). However, although the 7-day cabbage leaf samples were light green in coloration, the 14- and 21-day cabbage leaf disks stored under light/dark cycles had darker green coloration (Figure 19B; LD), suggesting increased photosynthetic activity over storage time. In contrast, although the cabbage leaf disks stored under constant light were also light green after 3 days of storage, the 14- and 21-day cabbage leaf disks were more yellow and included more brown discolorations (Figure 19B; LL). Remarkably, the absence of light exposure during post-harvest storage had a dramatic effect on the cabbage leaf disk coloration. Cabbage leaf disks stored under

constant darkness at either 22°C or 4°C were pale tan or yellow after 3 days of storage (Figure 19B; DD and 4CDD). The constant darkness-exposed cabbage leaf disks stored at 22°C appeared nearly white in color by 14 and 21 days; those at 4°C had a yellowish appearance after 2 or 3 weeks of storage (Figure 19B; DD and 4CDD).

Lettuce and spinach leaf disks tissue were nearly uniformly green, with little difference in color intensity between 3 and 6 days of storage under cycles of 12-hour light/12-hour darkness (Figure 19C and D; LD). By 9 days of storage under light/dark cycles, however, both lettuce and spinach leaf disks looked slightly less green, and most of the spinach leaf disks had distinct patches of yellow (Figure 19C and D; LD). In contrast, the loss of green coloration and increased yellowing over time was much more apparent in the lettuce and spinach leaf disks stored under constant light; the lettuce leaf disks were pale green by 9 days (Figure 19C; LL), and all the spinach disks had large yellow patches (Figure 19D; LL). Lettuce and spinach leaf disks stored under constant darkness displayed small brown patches by 3 days (Figure 19C and D; DD). After 6 and 9 days of storage under constant darkness, the lettuce disks had large wet patches of darkened tissue (Figure 19C; DD). However, the spinach leaf disks stored under constant darkness at 22°C for 6 days completely disintegrated and therefore could not be moved for photographic imaging. Lettuce and spinach leaf disks stored in constant darkness at 4°C largely maintained dark green coloration at 6 days and were lighter green at 9 days, similar to that of the disks stored under light/dark cycles at 22°C (Figure 19C and D; 4CDD). However, after 6 days of storage at 4°C, the lettuce leaf disks also displayed browning around the vascular tissues (Figure 19C; 4CDD).

Overall, the image analysis shown in Figure 19 suggests that postharvest storage under cycles of 12-hour light and 12-hour darkness may enable kale, cabbage, lettuce, and spinach leaf tissues to maintain physiological functioning for longer durations after harvest. The reduction in green color and appearance of brown discoloration suggests that postharvest storage in constant light or constant darkness may accelerate loss of tissue viability.

6.3. Vegetables stored in light/dark cycles have higher chlorophyll content compared to those stored in constant light or constant dark

To further characterize kale, cabbage, lettuce and spinach leaf health and viability during postharvest storage, I compared chlorophyll content in leaf samples after storage under cycles of 12-hour light/12-hour darkness to that of leaf tissues stored under constant light or constant darkness. Three sets of comparative data are shown (Figures 19-21). Because my primary focus was to determine whether light/dark cycles were advantageous relative to constant light or constant dark storage conditions, I first conducted two-way comparative statistical analyses between data derived from the samples stored under light/dark cycles relative to comparable samples stored under the alternative condition (that is, constant light, constant dark or refrigerated/dark). Figure 20 presents statistical analysis of storage-dependent differences in chlorophyll levels relative to dry weight at each time point. Figure 21 shows similar analyses but of storage-dependent differences in chlorophyll levels relative to fresh weight. Finally, to evaluate

whether there were significant changes in chlorophyll levels of each plant type over time, statistical analyses of differences in chlorophyll content at the beginning and end of the experiments for kale, cabbage, lettuce, and spinach are shown in Figure 22. Consistent with the loss of green coloration in the representative leaf disk samples shown in Figure 19A, postharvest storage of kale leaf disks in either constant light or constant dark led to significantly greater losses in kale chlorophyll content within 3 or 6 days of postharvest storage compared to storage under 12-hour light/12-hour dark cycles (Figures 19A and 20A). Kale leaf disks stored for 15 days under constant light lost 97% and 93% of their original chlorophyll content relative to dry and fresh weight, respectively (Figure 22A and E); kale leaf disks stored under constant darkness lost 88% and 89% of their chlorophyll content relative to total dry and fresh weight, respectively (Figure 22A and E). In contrast, 15 days of storage under cycles of 12-hour light/12-hour darkness led to loss of only 36% and 9% of the kale leaf disk chlorophyll relative to dry and fresh weight, respectively (Figure 22A and E). Kale leaf disks stored at 4°C under constant darkness, however, performed statistically better than those stored under cycles of light/dark (Figures 19A and 20A), with no significant decreases in chlorophyll content relative to total dry or fresh weight over the full 15 days of the experiment (Figure 22A and E).

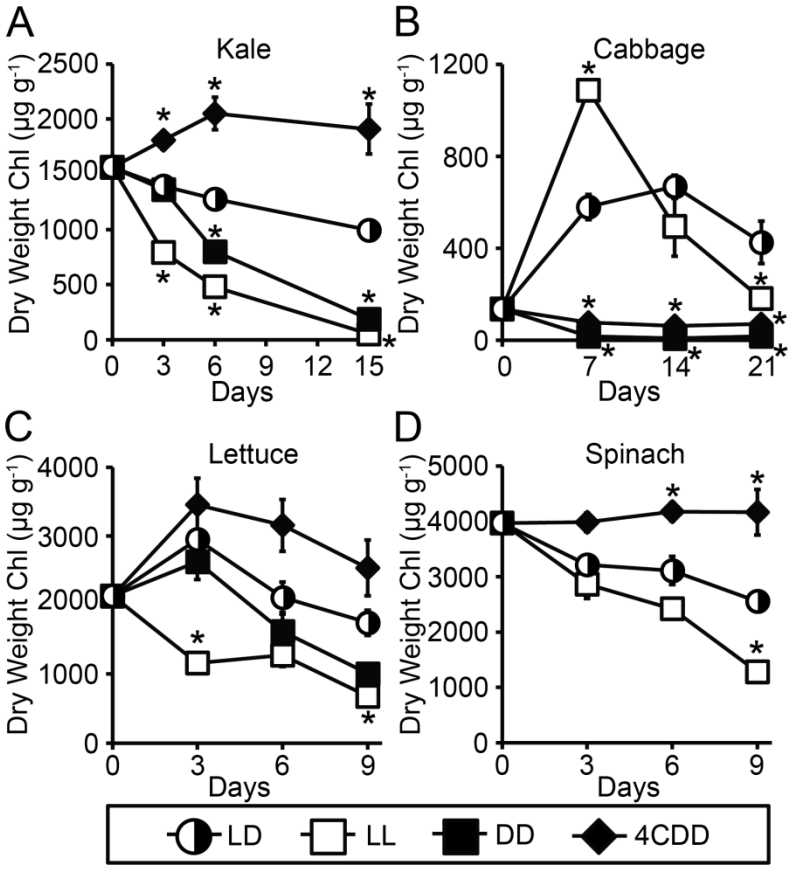

Figure 20: Chlorophyll content retention was higher in kale, cabbage, lettuce and spinach leaves stored in light/dark cycles compared to constant light or constant darkness at 22°C

Chlorophyll content relative to dry weight was quantified from leaf tissue disks of kale (A), cabbage (B), lettuce (C) and spinach (D) stored under cycles of 12-hours light/12-hours darkness (LD, half-filled circles), constant light (LL, open squares), or constant darkness (DD, filled squares) at 22°C, or under constant darkness at 4°C (4CDD, filled diamonds). Mean ± SE; n = 4. Asterisks indicate significant differences ($p < 0.05$. ANOVA Test with Bonferroni Post Hoc analysis) between data derived from leaf disks stored under light/dark cycles (22°C) and that derived from leaf disks stored under other conditions at each time point (Liu et al., 2015).

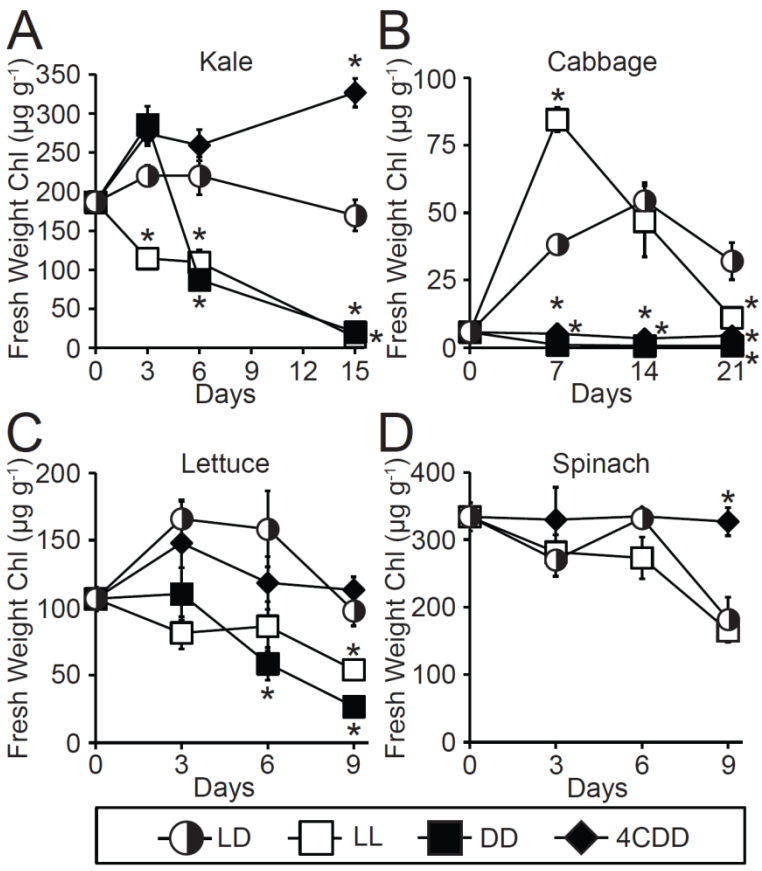

Figure 21: Chlorophyll content in fresh weight leaf tissue was maintained at higher levels in light/darkness stored vegetables compared to constant light or constant darkness at 22°C

Chlorophyll content was quantified from leaf disk tissues of kale (A), cabbage (B), lettuce (C) or spinach (D) stored over time under cycles of 12-hours light/12-hours darkness (LD, half-filled circles), constant light (LL, open squares), or constant darkness (DD, filled squares) at 22°C, or under constant darkness at 4°C (4CDD, filled diamonds). Mean ± SE; n = 4. Asterisks represent significant differences ($p<0.05$. ANOVA Test with Bonferroni Post Hoc analysis) between data derived from leaf disks stored under light/dark cycles (22oC) and that derived from leaf disks stored under other conditions at each time point (Liu et al., 2015).

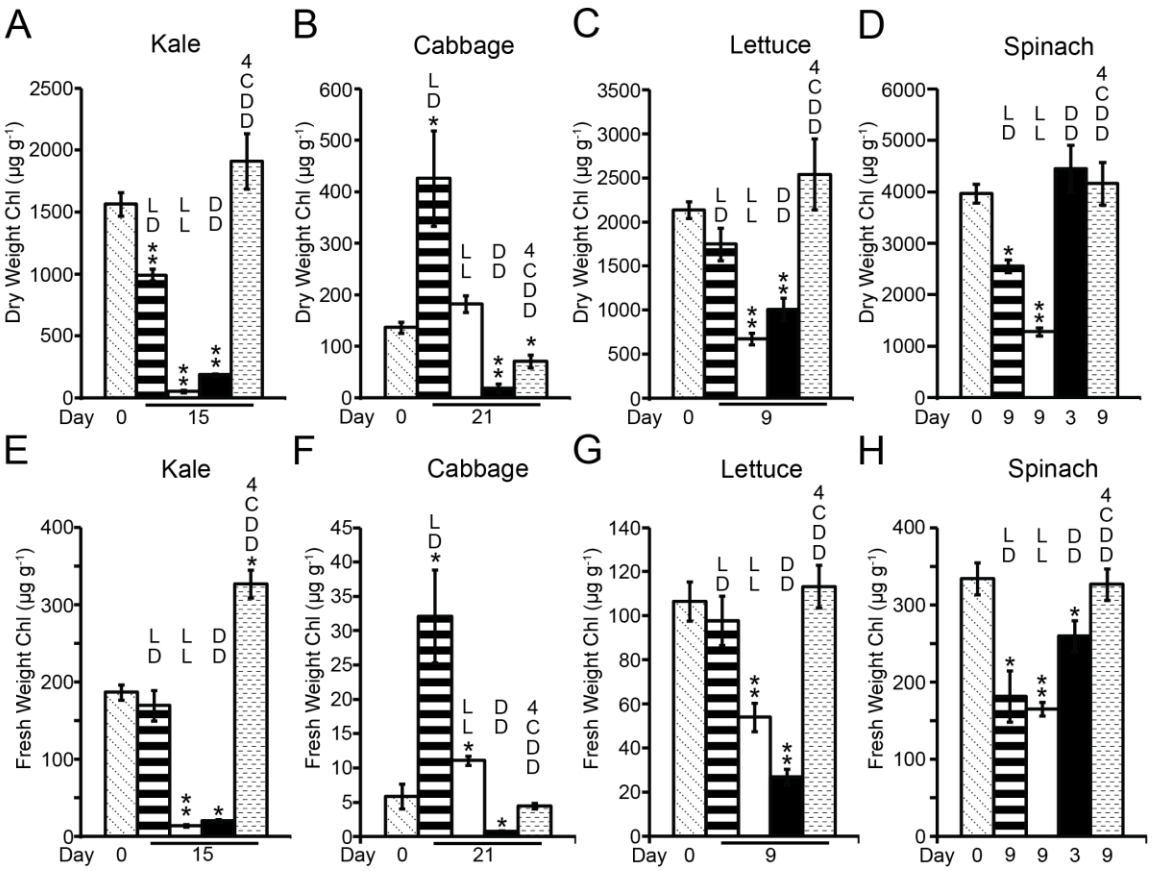

Figure 22: Chlorophyll content in fresh and dry weight leaf tissue was maintained at higher levels over time when stored in light/darkness cycles or under refrigeration

Chlorophyll content was quantified from dry weight leaf (A-D) and fresh weight (E-H) leaf disk tissues of kale (A, E), cabbage (B, F), lettuce (C, G) or spinach (D, H) stored over time under cycles of 12 hours light/12 hours darkness (LD), constant light (LL), or constant darkness (DD) at 22°C, or under constant darkness at 4°C (4CDD). Mean ± SE; n = 4. *p<0.05. **p<0.005. Unpaired student t-test analysis between day 0 concentrations and concentrations at the specified final time-point. (Liu et al., 2015)

Postharvest storage of cabbage leaf disks under light/dark cycles resulted in significantly higher chlorophyll levels than storage under constant dark either at 22°C or 4°C at all time points examined (Figures 19B and 20B). Indeed, cabbage leaf disks began with only modest chlorophyll levels (Figures 19B and 20B). However, when the cabbage leaf disks were stored under either constant light or light/dark cycles, chlorophyll content increased over time (Figures 19B and 20B) with significantly higher levels remaining even after three weeks of storage (Figure 22B and F). Light-induced synthesis is likely responsible for the elevated chlorophyll levels (Figures 19B, 20B and 21B and F) and the enhanced green coloration observed in the cabbage leaf images (Figure 19B) of the samples stored under light/dark cycles or constant light but absent in the cabbage leaf disks stored under constant darkness either at 22°C or 4°C (Figures 19B, 20B, and 21B and F). Storage under light/dark cycles was also more successful than constant light exposure in maintaining higher chlorophyll levels after long-term storage of 3 weeks (Figures 19B and 20B). Over time, storage under constant light may be counterproductive; whereas cabbage leaf disks stored for 7 days under constant light had significantly higher chlorophyll content than leaf disks stored under light/dark cycles (Figures 19B and 20B), by three weeks of storage, the leaf disks stored under light/dark cycles retained at least 2-fold more chlorophyll than samples stored under constant light (Figures 19B and 20B). These results indicate that light during postharvest storage can have a profound effect on chlorophyll levels in cabbage, consistent with previous reports (Perrin, 1982), and that diurnal cycling of light and darkness prolongs this benefit during longer term storage.

Storage under cycles of 12-hour light/12-hour darkness also promoted chlorophyll retention (relative to both dry and fresh weight) in lettuce leaf disks, comparable to that of lettuce leaf disks stored under refrigeration; chlorophyll levels were statistically indistinguishable between lettuce leaf disks stored under light/dark cycles versus those refrigerated under constant darkness conditions after 3, 6 or 9 days of storage (Figures 19C and 20C). Postharvest storage of lettuce leaf disks either at 22°C under light/dark cycles or under refrigeration resulted in no significant change in chlorophyll content over the course of the 9-day experiment, whereas the lettuce leaf disks stored under either constant light or constant darkness, lost more than 50% of their starting chlorophyll content (Figure 22C and G).

Chlorophyll content of spinach leaf disks was not significantly affected by treatment conditions for the first 3 days of postharvest storage (Figures 19D, 20D and 21D and H). However, the spinach leaf disks stored at 22°C in constant darkness disintegrated by 6 days and were therefore unable to be further analyzed. Spinach leaf disks stored under light/dark cycles had similar chlorophyll content to those stored under constant light with relatively stable chlorophyll retention until day 9 when chlorophyll levels in both samples decreased significantly from initial levels (Figures 19D, 20D and 21D and H). In contrast, refrigeration led to stable chlorophyll levels in the spinach leaf disks over the course of the experiment (Figure 22D and H).

These results indicate that chlorophyll content of postharvest green leafy vegetables varies depending upon the storage conditions and suggests that storage under 12-hour cycles of light and darkness, known to maintain the plant circadian clock

(Goodspeed et al., 2013b), can improve kale, cabbage and lettuce chlorophyll content maintenance relative to storage in constant light or constant dark. Perhaps surprisingly light/dark cycles during postharvest storage may be at least as beneficial as refrigeration with respect to chlorophyll content for cabbage and lettuce.

6.4. Vegetables stored in light/dark cycles have improved tissue integrity compared to those stored in constant light or constant dark

Over time during postharvest storage, plant tissues typically show visible signs of tissue disintegration (e.g., Figure 19). To determine if maintaining light/dark cycles during storage of post harvest leafy vegetables could prolong tissue integrity, I compared electrolyte leakage from kale, cabbage, lettuce, and spinach leaf disks stored over time under cycles of 12-hour light/12-hour darkness to leaf disks stored under constant light or constant darkness at 22°C or constant darkness at 4°C. Figure 23 shows that postharvest storage under light/dark cycles and refrigeration were comparable, with respect to leaf tissue integrity maintenance of kale, cabbage and lettuce, as measured by electrolyte leakage, (Figure 23A-C; LD and 4CDD). When directly comparing light/dark storage to other conditions, a statistically significant benefit to diurnal stimuli during storage was apparent relative to constant light for kale (Figure 23A; LL), constant darkness for cabbage and lettuce (Figure 23B, C; DD), and constant darkness (at 3 days) and constant light (at 6 days) for spinach (Figure 23D; LL, and Figure 23E; DD).

Postharvest storage under constant dark was detrimental to kale, cabbage, and lettuce tissue integrity, with at least 4-fold increases in electrolyte leakage, whereas storage under light/dark cycles at 22°C or refrigeration resulted in no significant increase in electrolyte leakage over the course of the experiment (Figure 24). Constant light treatment also led to significant increases in electrolyte leakage from kale and lettuce leaf disks, but not cabbage leaf disks, over the storage periods examined (Figure 24).

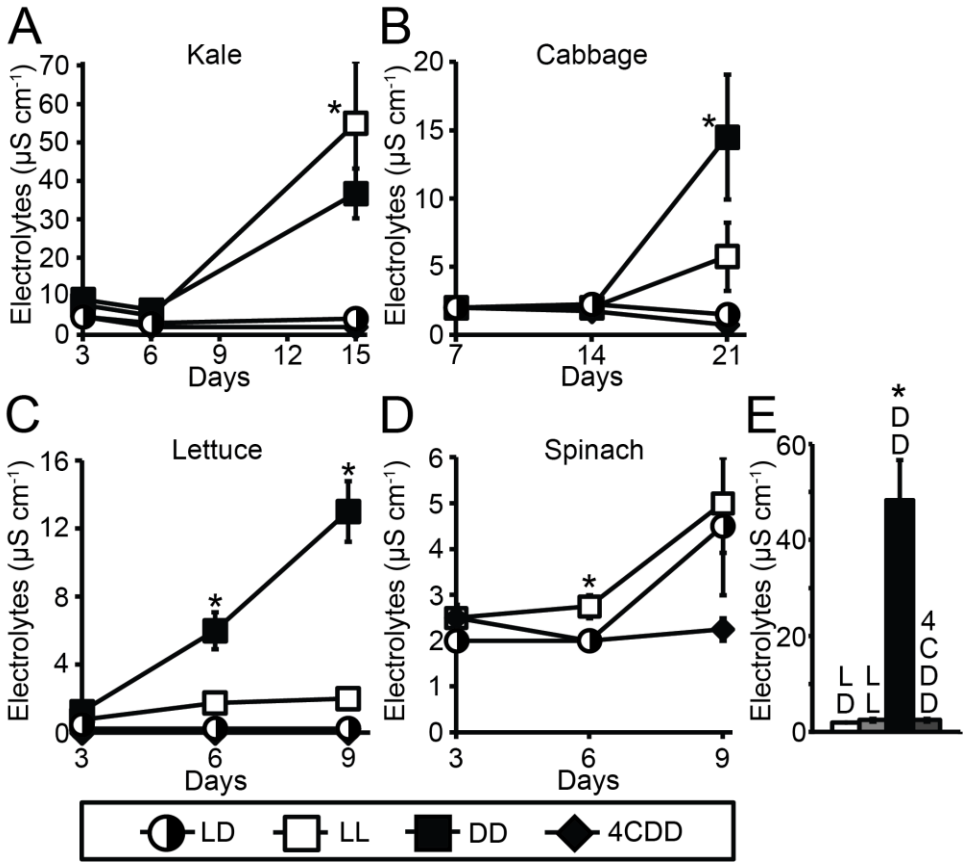

Figure 23: Electrolyte leakage from kale, cabbage, lettuce, and spinach leaf disks was affected by light exposure during storage

Electrolytes released from leaves of kale (A), cabbage (B), lettuce (C) or spinach (D & E) were measured after storage under cycles of 12-hour light/12-hour darkness (LD, half-filled circles), constant light (LL, open squares), or constant darkness (DD, filled squares) at 22°C or under constant darkness at 4°C (4CDD, filled diamonds). Mean ± SE; n = 4. Asterisks indicate significant differences ($p < 0.05$, ANOVA Test with Bonferroni Post Hoc analysis) between data derived from leaf disks stored under light/dark cycles (22°C) and that derived from leaf disks stored under other conditions at each time point (Liu et al., 2015).

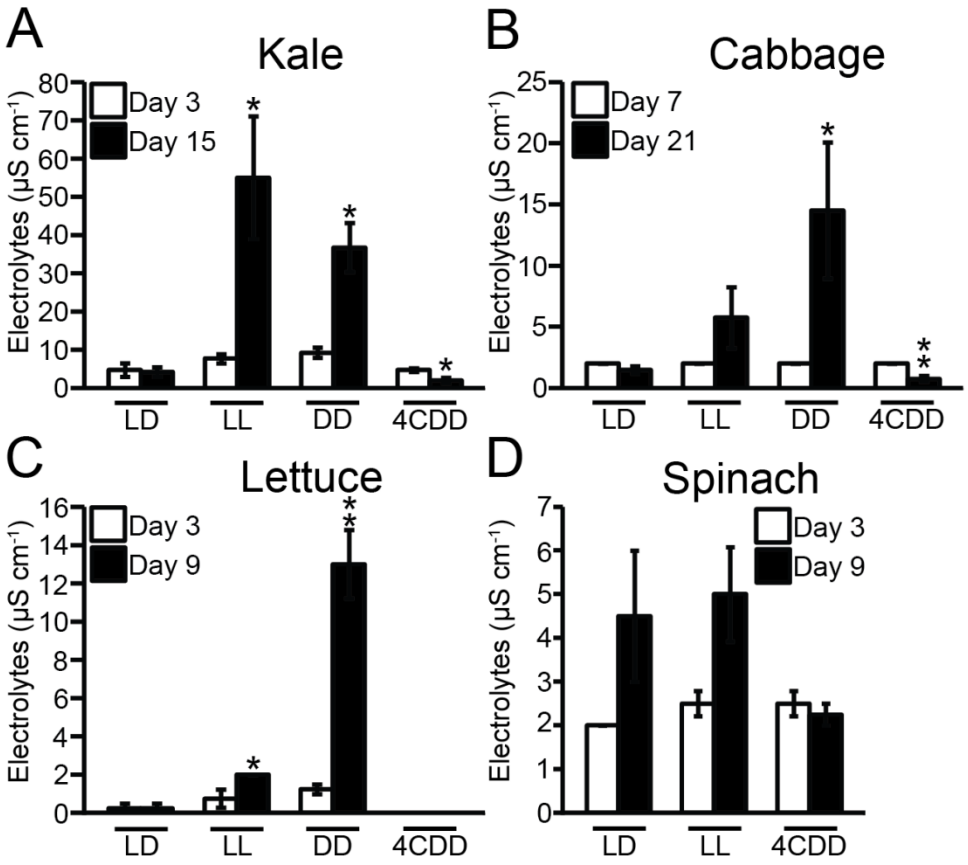

Figure 24: Electrolyte leakage from kale, cabbage, lettuce, and spinach leaf disks is increased when stored under constant light or constant dark in kale, cabbage and spinach

Electrolytes released from leaves of kale (A), cabbage (B), lettuce (C) or spinach (D) were measured after storage under cycles of 12-hour light/12-hour darkness (LD), constant light (LL), or constant darkness (DD) at 22°C or under constant darkness at 4°C (4CDD). Mean ± SE; n = 4. *p<0.05. **p<0.005. Unpaired student t-test analysis between measured initial levels and levels at the specified final time-point. (Liu et al., 2015)

Overall the results shown in Figure 23 and Figure 24 provide evidence that daily cycles of light and darkness during postharvest storage resulted in superior leaf tissue integrity maintenance largely comparable to refrigeration, whereas either constant light or constant dark storage conditions were detrimental.

6.5. Vegetables stored in light/dark cycles have increased glucosinolate retention compared to those stored in constant light or constant dark

Next I was interested in determining whether plant maintenance under daily cycles of light and darkness affects human-health relevant metabolite content. In particular, I sought to examine whether kale and cabbage stored under light/dark cycles maintain their glucosinolate content longer than when stored under constant light, constant darkness, or refrigeration.

Figure 25A shows total glucosinolate levels in kale leaf disks after 0, 3, 6 and 15 days of postharvest storage under different conditions. Individual glucosinolate levels are shown in Figure 26. Total glucosinolate levels were comparable between kale leaf disks stored at 22°C under light/dark cycles and leaf disks stored at 4°C in the dark (Figure 25A); after 15 days of postharvest storage under these conditions, total glucosinolate levels decreased by less than 35% (Figure 28A). In comparison to light/dark storage conditions, constant light or constant darkness exposure during storage resulted in significantly reduced glucosinolate content in the kale leaf disks (Figure 25A). By 15

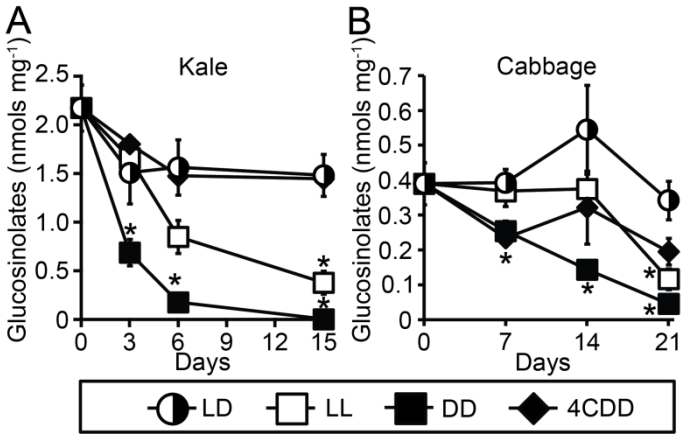

Figure 25: Maintenance of total glucosinolate levels in kale and cabbage leaves when stored under light/dark cycles

Glucosinolate species was quantified from leaf disks of kale (A) and cabbage (B) stored under cycles of 12-hour light/12-hour darkness (LD, half-filled circles), constant light (LL, open squares), or constant darkness (DD, filled squares) at 22°C or under constant darkness at 4°C (4CDD, filled diamonds). Mean ± SE; n = 4. Asterisks indicate significant differences ($p < 0.05$. ANOVA Test with Bonferroni Post Hoc analysis) between samples kept in light/darkness cycles (22°C) and those stored in constant light (22°C), constant dark (22°C) and under constant darkness (4°C) for a specified time point (Liu et al., 2015).

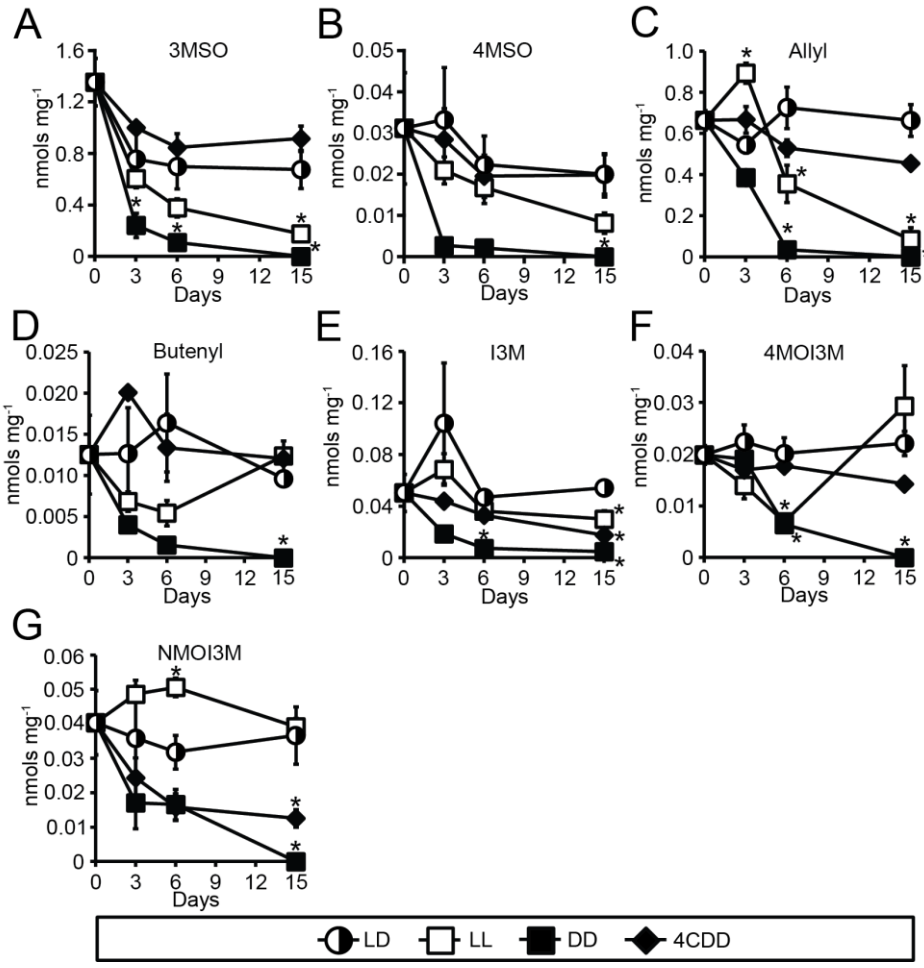

Figure 26: Accumulation of individual glucosinolate species in kale disks

Kale disks stored in 12 hour light/12 hour dark (22^{o}C) (LD, half-filled circles), constant light (22^{o}C) (LL, open squares); constant dark (22^{o}C) (DD, filled squares), or refrigerated at 4^{o}C in the dark (4CDD, filled diamonds). 3MSO, 3-methylsulfinylpropyl glucosinolate; 4MSO, 4-methylsulfinylbutyl glucosinolate; I3M, indoyl-3-methyl glucosinolate; 4MOI3M, 4-methoxy-indoyl-3-methyl glucosinolate; NMOI3M, 1-methoxy-indolyl-3-methyl glucosinolate. Mean ± SE; n = 4. Asterisks represent significant differences ($p<0.05$. ANOVA Test with Bonferroni Post Hoc analysis) between data derived from leaf disks stored under light/dark cycles (22^{o}C) and that derived from leaf disks stored under other conditions at each time point (Liu et al., 2015).

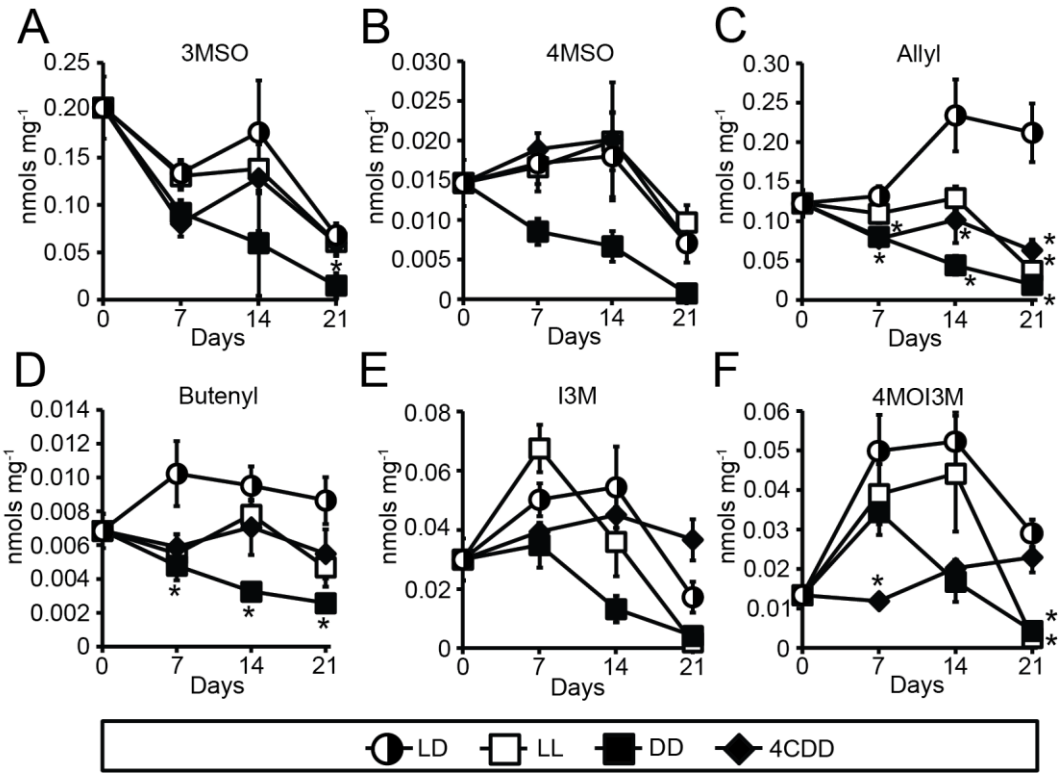

Figure 27: Accumulation of individual glucosinolate species in cabbage disks

Cabbage disks stored in 12-hour light/12-hour dark (22°C) (LD, half-filled circles), constant light (22°C) (LL, open squares); constant dark (22°C) (DD, filled squares), or refrigerated at 4°C in the dark (4CDD, filled diamonds). 3MSO, 3-methylsulfinylpropyl glucosinolate; 4MSO, 4-methylsulfinylbutyl glucosinolate; I3M, indoyl-3-methyl glucosinolate; 4MOI3M, 4-methoxy-indoyl-3-methyl glucosinolate. Mean ± SE; n = 4. Asterisks represent significant differences ($p<0.05$. ANOVA Test with Bonferroni Post Hoc analysis) between data derived from leaf disks stored under light/dark cycles (22°C) and that derived from leaf disks stored under other conditions at each time point (Liu et al., 2015).

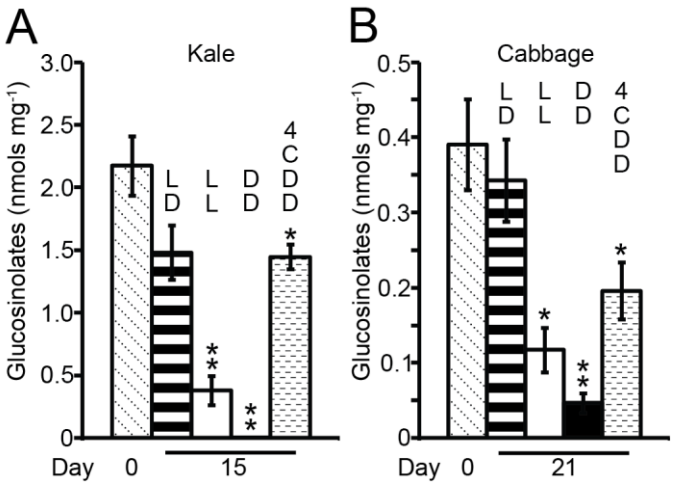

Figure 28: Maintenance of total glucosinolate levels in kale and cabbage leaves when stored under light/dark cycles

Glucosinolate species was quantified from leaf disks of kale (A) and cabbage (B) stored under cycles of 12-hour light/12-hour darkness (LD), constant light (LL), or constant darkness (DD) at 22°C or under constant darkness at 4°C (4CDD). Mean ± SE; n = 4. *p<0.05. **p<0.005. Unpaired student t-test analysis between day 0 concentrations and concentrations at the specified final time-point (Liu et al., 2015).

days of postharvest storage under constant light or constant darkness at 22°C, the kale leaf disks lost over 80% and 99% of initial levels, respectively.

Daily cycles of light and darkness also promoted maintenance of glucosinolate content during postharvest storage of cabbage (Figures 24B and 26). Total glucosinolate content in the cabbage leaf disks stored under light/dark cycles remained stable with no significant fluctuation in levels over the 21 days of analysis (Figures 24B and 27B). In comparison to total glucosinolate levels in light/dark-stored cabbage, the glucosinolate levels were significantly lower by 7 days when cabbage leaf disks were stored under constant darkness (Figure 25B; DD) and by 21 days when stored under constant light (Figure 25B; LL). Total glucosinolate levels declined by 70% and 88%, respectively, in cabbage disks stored at 22°C under constant light or constant darkness (Figure 28B). Remarkably, glucosinolate levels of the cabbage leaf disks stored at 4°C also showed a significant decrease by 21 days, with a loss of 50% of the initial glucosinolate levels (Figure 28B), indicating that storage under cycle of light/darkness led to enhanced retention of this valuable phytochemical even relative to refrigeration.

6.6. Conclusions

Detached kale, cabbage, lettuce and spinach leaves show enhanced tissue longevity through continued exposure to diurnal light/dark cycles during storage. In addition, human-health relevant metabolites, such as glucosinolates and chlorophyll, are also retained at higher levels under diurnal storage conditions, suggesting that postharvest vegetables that retain natural rhythms during storage may be of greater nutritional value.

These results provide additional evidence that postharvest plant tissues retain the ability to sense external stimuli and respond in ways that affect tissue integrity and cellular metabolite levels. However, it is currently unknown whether the benefits associated with maintaining postharvest vegetables in light/dark cycles is due to a robustly entrained circadian clock and/or an optimization of light exposure allowing for improved physiological function. Future experiments could examine whether temperature cycles, which can also entrain the circadian clock (Millar, 2004; Salomé and Mcclung, 2005), can provide improved postharvest performance when compared to storage under constant temperatures. Additionally, future studies could examine if the concentrations of other human-health relevant compounds also show improved maintenance when vegetables are stored under light/dark cycles.

Chapter 7: Light/dark cycles affect postharvest plant tissue resistance to necrotrophic pathogens

7.1. Introduction

Light/dark cycles that better mimic natural conditions can improve postharvest performance (Figures 18-27), and proper circadian entrainment can improve plant resistance to *T. ni* under free-running conditions (Figures 11 and 17). Therefore we hypothesized that employing light/dark cycles to maintain robust daily rhythms maybe advantageous for plant resistance against pathogens such as *Botrytis cinerea*.

7.2. Light/dark cycles enhanced Arabidopsis leaf fungal resistance

To determine if light/dark cycles can improve plant resistance to *B. cinerea*, a necrotrophic plant pathogen, I compared fungal resistance of detached Arabidopsis leaves maintained under diurnal conditions (12-hour light/12-hour dark cycles), known to sustain robust biological rhythms, to that of leaves under constant light, conditions under which plant circadian rhythms eventually dampen (Yakir et al., 2011). Leaves from 5-week-old Arabidopsis (Col-0) grown in 12-hour light/12-hour dark cycles were

inoculated with *B. cinerea* spores 4 hours after dawn and then incubated under either 12-hour light/12-hour dark cycles or constant light. After 72 and 96 hours, *B. cinerea* lesions on leaves incubated under constant light were significantly larger than the lesions on leaves exposed to light/dark cycles (Figure 29). These results indicate that maintenance of light/dark cycles can enhance Arabidopsis resistance to *B. cinerea*.

Next, I examined whether a functional plant circadian clock is necessary for the enhanced *B. cinerea* resistance conferred by 12-hour light/12-hour dark cycles relative to constant light. *B. cinerea* leaf lesion diameters were compared on *CCA1-OX* leaves, which, in addition to being arrhythmic under constant light conditions (Wang and Tobin, 1998), lacks the ability to anticipate night-to-day transitions and displays attenuated diurnal rhythms under light/dark cycles (Green et al., 2002). In contrast to Col-0, *CCA1-OX* leaves did not display any significant differences in lesion diameters when incubated in light/dark cycles compared to incubation under constant light (Figure 29), evidence that an accurately functioning internal oscillator is required for the fungal resistance benefit obtained by diurnal light/dark transitions. However, we also found that under constant light conditions *CCA1-OX* leaves displayed higher *B. cinerea* resistance after 72 and 96 hpi compared to Col-0 (Figure 29). In addition, even under light/dark cycles, *CCA1-OX* leaves showed increased resistance compared to Col-0 at 72 hpi (Figure 29A), but this elevated resistance compared to Col-0 was lost at 96 hpi (Figure 29B). Thus, despite the overall increased fungal resistance of wild-type Arabidopsis leaves stored under light/dark cycles relative to those stored under constant light (Figure 29), oscillator

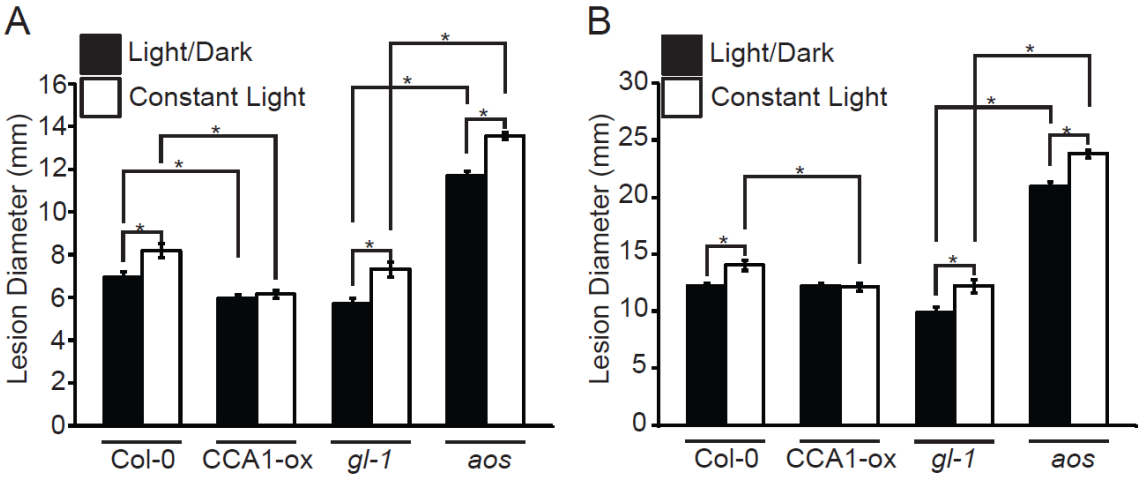

Figure 29: Arabidopsis exhibits differential sensitivity to *B. cinerea* when stored under light/dark cycles versus constant light

Col-0, *CCA1-OX*, *gl-1* and *aos* leaf lesion diameters at 72 hpi (A) and 96 hpi (B) with fungal spores. Mean lesion diameters ± SEM. n = 30. Asterisks denote significant lesion diameter differences between light/dark cycles and constant light treated leaf tissue ($P < 0.005$, Unpaired student t-test).

function, at least that conferred by proper *CCA1* expression, can be detrimental for plant fungal resistance under some environmental conditions (Figure 29).

To examine if jasmonates are required for the increased fungal resistance conferred by light/dark cycles relative to constant light, I compared lesion diameters on *aos* leaves to those on leaves of *gl-1*, the genetic background of *aos*, under the two light conditions. Similar to what was observed in Col-0 leaves, *gl-1* leaves showed significantly smaller fungal lesions at 72 and 96 hpi when the leaves were subjected to light/dark cycles than when incubated under constant light (Figure 29). Jasmonate production is required for limiting *B. cinerea* lesion growth as expected; *aos* leaf lesions were significantly larger than those found on *gl-1* leaves when compared at 72 and 96 hpi regardless of whether the leaves were incubated under light/dark cycles or constant light (Figure 29). However, *aos* fungal lesions were smaller at 72 and 96 hpi when the leaves were incubated under light/dark cycles than when the leaves were exposed to constant light (Figure 29). Whereas *gl-1* lesion diameters were 28% larger when the leaves were subjected to constant light versus light/dark cycles, *aos* leaves displayed a 16% increase in lesion diameter when incubated under constant light versus light/dark cycles; therefore jasmonate production is required for the full benefit of diurnal cycling.

7.3. Postharvest cabbage and lettuce displays enhanced resistance to *Botrytis cinerea* when stored in 12-hour light/12-hour dark cycles when compared to storage under constant light

B. cinerea is responsible for large agricultural losses due to the prolific nature of grey mold disease infecting plants from early seedling stages through the postharvest consumer retail chain, resulting in up to 20% yield loss (Dean et al., 2012). I sought to test whether diurnal storage might improve postharvest crop fungal resistance. To determine if the *B. cinerea* resistance benefit obtained by Arabidopsis leaves incubated under light/dark cycles could be obtained by postharvest green leafy vegetables, we compared *B. cinerea* lesion diameters formed on green leaf lettuce (*Lactuca sativa*) and cabbage (*Brassica oleracea*) leaf disks incubated under 12-hour light/12-hour dark cycles to those on leaf disks exposed to constant light. Fungal lesion diameters on both lettuce and cabbage leaf disks were significantly larger at 48 hpi (Figure 30A) and 72 hpi (Figure 30B and C) when leaf tissues were exposed to constant light than when placed under light/ dark cycles. Indeed, the differential fungal growth was substantial; after 72 hpi, lesion diameters were approximately 2- and 1.7-fold larger on lettuce and cabbage disks, respectively, when leaf tissue was exposed to constant light compared to lesion growth on disks incubated under light/dark cycles (Figure 30C). These results suggest that postharvest storage that maintains diurnal light/dark cycles may dramatically affect fungal susceptibility of green leafy vegetables, such as lettuce and cabbage.

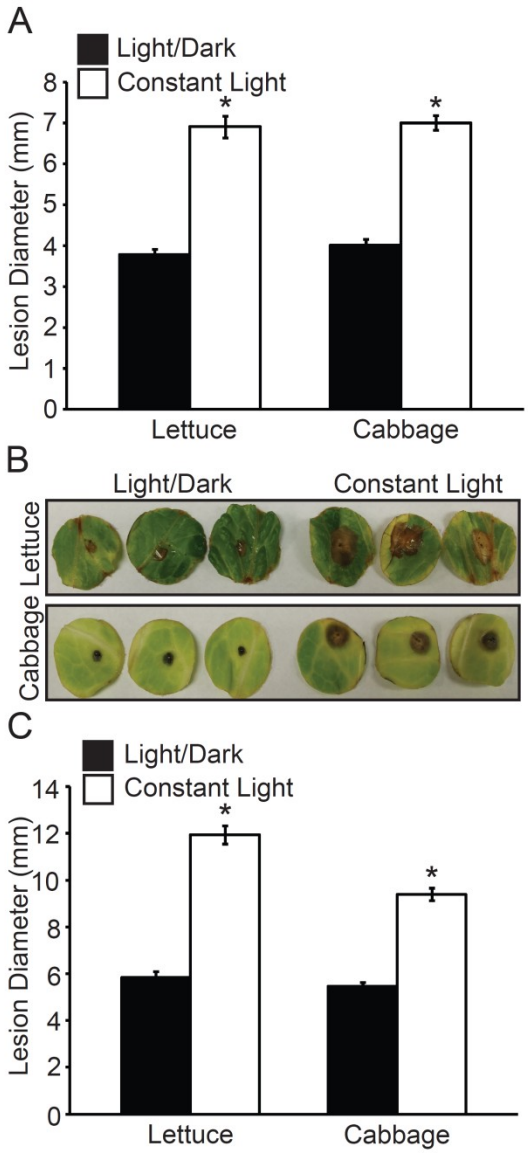

Figure 30: Cabbage and lettuce leaf tissue exhibits differential sensitivity to B. cinerea when stored under light/dark cycles versus constant light

(A) Photographs of representative lettuce and cabbage leaves 72 hpi with fungal spores. Lettuce and cabbage leaf lesion diameters at 72 hpi (B) and 96 hpi (C). Mean lesion diameters ± SEM. n = 40. Asterisks denote significant lesion diameter differences between light/dark cycles and constant light treated leaf tissue ($P < 0.005$, Unpaired student t-test).

7.4. Conclusions

The Arabidopsis leaf lesion diameter assays indicate that detached leaves have increased resistance to *B. cinerea* when kept under light/dark cycles as compared to leaves kept in constant light (Figure 29). This benefit appears to be dependent on a functional clock since *CCA1-OX* did not display a difference in fungal resistance when kept under either light/dark cycles or constant light (Figure 29). However, *CCA1-OX* plants also displayed enhanced resistance to *B. cinerea* after 72 hpi in light/dark cycles and after both 72 and 96 hpi under constant light (Figure 29). Why *CCA1-OX* plants display enhanced resistance to *B. cinerea* under certain conditions is unknown.

Increased leaf resistance to *B. cinerea* attributed to light/dark cycles involves both jasmonate-dependent and -independent mechanisms, as demonstrated by enhanced fungal resistance of *aos* when stored under light/dark cycles, similar to *gl-1*; however this benefit is reduced compared to that of wild type (Figure 29). An alternative possibility is that light/dark cycles may reduce fungal growth relative to constant light. However, this possibility is unlikely given that fungal lesion growth on *CCA1-OX* is indistinguishable between leaves incubated under constant light versus light/dark cycles (Figure 29).

The results also demonstrate a practical advantage for daily rhythms in plant fungal resistance. We find that postharvest lettuce and cabbage leaves, similar to detached leaves of Arabidopsis, harbor significantly greater *B. cinerea* resistance when maintained under light/dark cycles than when placed under constant light conditions. Therefore, the use of light/dark cycles to treat postharvest vegetables may not only

improve shelf-life and nutritional content (Figures 18-27), it may also help decrease postharvest losses due to biotic pathogens.

Chapter 8: Conclusions and Future Directions

We have shown that the clock may regulate rhythmic basal accumulation of JA through clock controlled JA-biosynthesis gene transcript accumulation independent of JA-positive feedback. Additionally, keeping postharvest vegetables in light/dark storage could have profound effects on improving postharvest longevity, nutritional content and resistance to fungal infection.

8.1. Potential mechanism for clock-regulated jasmonic acid biosynthesis

JA is critical for plant defense against insect herbivores and necrotrophic fungal infection, yet the mechanism behind initiation of JA synthesis remains unknown. Complicating the matter is JA-positive feedback (Figure 6), wherein JA accumulation acts through positive feedback to up regulate additional JA production. Basal JA levels cycle under free-running conditions (Figure 7; Goodspeed et al., 2012), indicating that the clock may regulate JA accumulation. This work provides evidence that the clock might act as a JA-independent initiator of JA-biosynthesis since both wild type and *aos* plants that lack JA show evidence of clock-regulated rhythmic accumulation of JA-biosynthesis gene transcripts (Figure 15). While JA, total glucosinolates and *T. ni*

feeding behavior show similar phase of peak accumulation or activity, there is a 12-14 hour delay from the peak of JA-biosynthesis gene transcript accumulation (Figure 15) and basal JA accumulation (Figures 7 and Figure 31; Goodspeed et al., 2012) that indicates additional levels of regulation may exist. These could include regulation of translation, enzyme activation or transport of precursors or enzymes required for JA biosynthesis, all of which may or may not be further influenced by the circadian clock.

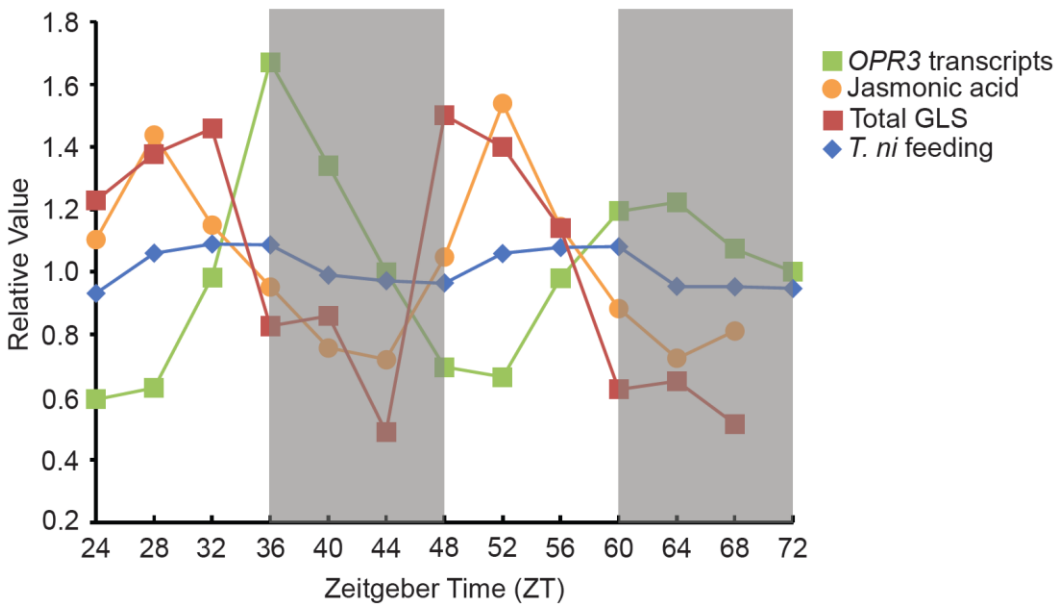

Figure 31: Timing of rhythmic JA and glucosinolate accumulation in relation to *T. ni* feeding

OPR3 transcript accumulation (green squares), JA levels (orange circles), total glucosinolate levels (red squares) and *T. ni* feeding behavior (blue diamonds). Shoot tissue was collected every 4 hours under free-running conditions, beginning at subjective dawn, 24 hours after transfer to constant light for *OPR3* transcripts, JA (adapted from Goodspeed et al., 2012), and glucosinolate measurements (adapted from Goodspeed et al., 2013b). *T. ni* feeding activity was measured as described by Goodspeed et al. (Goodspeed et al., 2012). Relative values were calculated by dividing the average value at a particular ZT to the median value of the data set.

8.2. Influence of aliphatic glucosinolates in phase-entrainment enhanced plant resistance to *T. ni*

Plants are more resistant to *T. ni* when both the insect and plant are entrained in-phase with respect to each other as opposed to when plants are entrained out of phase with the *T. ni* (Figure 12; Goodspeed et al., 2012). A functioning clock (Figure 13; Goodspeed et al., 2012) and JA are both required (Figure 14; Goodspeed et al., 2012) for phase-entrainment-dependent enhanced resistance (Figure 12; Goodspeed et al., 2012). Here we examined whether aliphatic glucosinolates, which show basal cycling under free-running conditions (Figure 10A; Goodspeed et al., 2013b), play a role in phase-entrainment-dependent enhanced plant resistance to *T. ni.* In-phase entrained *myb28myb29* mutants, which lack aliphatic glucosinolates (Sønderby et al., 2007, 2010), have decreased resistance to *T. ni* when compared to wild-type plants (Figure 17), indicating that glucosinolates play a role in plant-entrainment enhanced resistance to insects. However, other defense mechanisms or compounds may also play a role in mediating in-phase enhanced resistance to *T. ni* since *myb28myb29* mutants still retained higher resistance to *T. ni* than *myb28myb29* mutants entrained out of phase with respect to *T. ni* (Figure 17). For example, a similar phase-dependent resistance to *T. ni* assay could be conducted on plants that have altered levels of indolic glucosinolates such as *sur1*(Boerjan et al., 1995). *sur1* contains a mutation in *SUPERROOT1* (SUR1), a *C-S* lyase involved in glucosinolate biosynthesis, and lacks detectable levels of aliphatic and indolic glucosinolates (Mikkelsen et al., 2004). Alternatively, phase-dependent resistance to *T. ni* could also be examined in *atr1* (*altered tryptophan regulation1*), which contains a

mutation in a MYB transcription factor and has reduced levels of indolic glucosinolates (Celenza et al., 2005).

8.3. Light/dark cycles improve postharvest performance

Here we examined whether kale, cabbage, lettuce and spinach leaf tissue maintain the ability to respond to light/dark cycles during postharvest storage and whether tissue deterioration would be reduced if they were placed under conditions that better mimic the natural light cycles of the environment. Our goal was to expose plant tissues to diurnal conditions known to maintain the functioning of the circadian clock (Figures 10 and 17; Goodspeed et al., 2013b) and thereby capitalize on physiological enhancements conferred by robust circadian rhythms. Plants grown under light/dark cycles that match the endogenous cycling of their internal circadian clock have a growth and reproductive advantage over plants exposed to light/dark cycles that do not match their internal oscillator (Dodd et al., 2005; Green et al., 2002).

We found that storing green leafy vegetables in light/dark cycles improved several postharvest performance markers compared to those stored under constant light or constant darkness. Similarly, a modest reduction in senescence was noted for postharvest broccoli stored under natural light/dark cycles (Büchert et al., 2011). Surprisingly, we found that storage in light/dark cycles resulted in several aspects of postharvest performance being comparable to storage under refrigeration, a commonly practiced method of postharvest storage thought to slow down cellular breakdown (Kader, 2003). The longevity of kale and lettuce leaf color, chlorophyll levels, and tissue integrity, which

are important contributors to the appeal of green leafy vegetables to consumers (Hutchings, 1999), were largely indistinguishable whether the kale and lettuce leaf samples were stored at 22°C under light/dark cycles or were stored under refrigeration in constant darkness (Figures 18, 22 and 23). Spinach leaf samples also maintained green coloration and chlorophyll levels under light/dark cycles at 22°C just as well as when they were refrigerated, but refrigeration was more successful at preventing spinach leaf tissue breakdown. Significant improvement of green coloration and chlorophyll content was seen when cabbage leaves were stored under light/dark cycles at 22°C compared to refrigeration, demonstrating that light may not only be important for clock entrainment but also can provide the additional benefit of promoting continued photosynthesis during postharvest storage. Promotion of photosynthesis and/or chlorophyll levels was previously observed in post-harvest crops stored under light (Ayala et al., 2009; Büchert et al., 2011; Olarte et al., 2009). However, constant light during post-harvest storage can also cause detrimental physiological activity, such as respiration leading to browning (Ayala et al., 2009) and transpiration contributing to weight loss (Barbieri et al., 2009; Kasim and Kasim, 2007; Sanz Cervera et al., 2007). Therefore, cycling of light treatment with darkness periods may not only maintain clock function but may also prevent physiological damage that may occur in plant tissues under too much light.

In addition to improvement of green leafy vegetable appearance by postharvest storage under light/dark cycles, we found that light/dark treatment of crop plants may improve human health benefits through maintenance of phytochemical content (Figures 19-21, 24 and 27). Chlorophyll, which is responsible for the visual appeal of green leafy vegetables (Hutchings, 1999), also has beneficial impacts on human health upon

ingestion. Chlorophyll can limit efficacy of carcinogens, such aflatoxin B1 (Egner et al., 2003; Jubert et al., 2009; McQuistan et al., 2012; Simonich et al., 2007, 2008) and can activate Phase II detoxifying enzymes (Fahey et al., 2005). Additional anticancer benefits may derive from glucosinolates in kale and cabbage. Glucosinolates, sulfur-containing compounds that play a major role in Brassicaceae plant herbivore defense (Hopkins et al., 2009), also underlie the human health benefits attributed to Brassicaceae (cruciferous) vegetable consumption (Hayes et al., 2008; Higdon et al., 2007). Previous studies have shown that glucosinolate levels can be maintained by refrigeration (Rangkadilok et al., 2002) or exposure to radiation (Banerjee et al., 2014); here we find that post-harvest storage under light/dark cycles can also lead to sustained glucosinolate levels (Figures 24 and 27).

Light/dark cycles also maintain the circadian clock function of other edible crops after harvest, including zucchini, carrots, sweet potatoes, and blueberries (Goodspeed et al., 2013b). These fruits and vegetables displayed time-dependent differences in insect resistance strongly suggesting temporal fluctuations in diverse metabolites, some of which may have important human health impact. Whether continued promotion of circadian periodicity postharvest can also improve longevity of tissue integrity and phytochemical content in diverse vegetables and fruits, as we have shown with kale, cabbage, lettuce, and spinach, remains to be investigated.

8.4. Light/dark cycles improves postharvest fungal resistance

In addition to showing that light/dark cycles improve postharvest performance (Figures 18-27), we show that keeping detached leaves in light/dark cycles improves resistance to *B. cinerea* when compared to leaves kept in constant light in both Arabidopsis (Figure 29) and postharvest cabbage and lettuce (Figure 30). Enhanced resistance to *B. cinerea* when stored in light/dark cycles is at least in part due to the function of *CCA1* since plants that have constitutive *CCA1* expression do not display differences in fungal lesion diameter (Figure 29). Interestingly, *CCA1-OX* leaves displayed increased resistance to *B. cinerea* when compared to wild type, particularly when stored under constant light (Figure 29), though it is unknown why this occurs. One possibility is that under constant light, the clock in *CCA1-OX* plants, which are arrhythmic under free-running conditions (Wang and Tobin, 1998), have constitutively higher defenses under constant light conditions.

Tests with *aos* revealed that the increased resistance of Arabidopsis stored in light/dark cycles is due in part to JA levels since *aos* plants have increased lesion diameters compared to wild type (Figure 29). Interestingly, *aos* still shows enhanced resistance to *B. cinerea* when stored in light/dark cycles in comparison to constant light (Figure 29). This indicates that JA-independent pathways are also involved in defense against *B. cinerea*. One possibility is that other defense hormones, such as ethylene (Berrocal-Lobo et al., 2002; Ferrari et al., 2003; Thomma et al., 1999), play a role in plant light/dark-cycle-enhanced resistance to *B. cinerea*.

To test whether clock function in fungal defense is potentially shared by other plants, including important food crops, we compared fungal resistance of Arabidopsis, green leaf lettuce (*Lactuca sativa*), and cabbage (*Brassica oleracea*) leaves maintained under diurnal light/dark cycles to those stored under constant light. Continued exposure of the plant leaves to light/dark cycles significantly enhanced resistance to *B. cinerea*. Altogether, these results highlight an important role for maintenance of diurnal storage conditions for postharvest crops to control fungal resistance.

8.5. Future work

8.5.1. Circadian control of jasmonate accumulation

Basal jasmonate is rhythmic under free-running conditions with anti-phasic accumulation patterns (Figure 7), indicating clock control of JA accumulation. However, how the clock regulates JA accumulation is unknown. Additionally, because of the JA-positive feedback regulation on JA biosynthesis, it has been difficult to determine how JA accumulation is initially activated in response to diverse stimuli. The mechanism underlying initiation of JA biosynthesis independent from JA-positive feedback is not known. The positive feedback loop, whereby JA signaling leads to elevated expression of genes encoding JA biosynthesis enzymes, creates a "chicken-or-egg" dilemma.

One possibility is that the clock regulates the time-of-day accumulation of gene transcripts involved in jasmonate biosynthesis such as *AOS, AOC1* and *OPR3* (Figure 6), which would then lead to cyclical production of JA. Alternatively, the clock could control basal JA accumulation downstream of JA-biosynthesis-gene expression regulation. I

examined the transcript accumulation of several JA-biosynthesis genes and found evidence of differential accumulation of *AOS, AOC1* and *OPR3* under free-running conditions with an approximately 24-hour period independent of JA-positive feedback (Figure 15), indicating that clock may be influencing JA biosynthesis through regulation of JA-biosynthesis-gene expression. However, how this regulation occurs remains unknown. One possibility is that the clock regulates expression of *AOS, AOC1* and *OPR3* directly through clock-related transcription factors such as the core clock component *CCA1* (Alabadí et al., 2001). A second possibility is that the clock could influence JA levels using antagonistic relationship between JA and SA (Glazebrook, 2005; Koornneef and Pieterse, 2008, 2008; Thaler et al., 2012). Like JA, SA also cycles rhythmically under free-running conditions but with anti-phasic accumulation patterns (Figure 7; Goodspeed et al., 2012). Therefore, it is possible that the clock could be directly controlling one hormone, which then acts antagonistically on the accumulation of the second hormone.

CCA1 binds to specific *cis*-motifs known as the Evening Element (EE; Figure 32A; Harmer et al., 2000) and the CCA1-binding site (Figure 32A; Wang et al., 1997) in the promoter regions of clock regulated genes. A simple sequence based *cis*-motif search of the upstream 3kb promoter region of *AOS, AOC1* and *OPR3* genes reveals several instances of the Evening Element and CCA1-binding site sequences (Figure 32B). Therefore, examination of JA-biosynthesis gene expression in plants with altered *CCA1* transcript levels can help determine if the clock could be influencing JA accumulation through direct transcriptional regulation. If CCA1 regulates expression of JA biosynthesis genes, then JA levels may be higher in plants that constitutively express *CCA1* and lower in plants with repressed levels of *CCA1* expression.

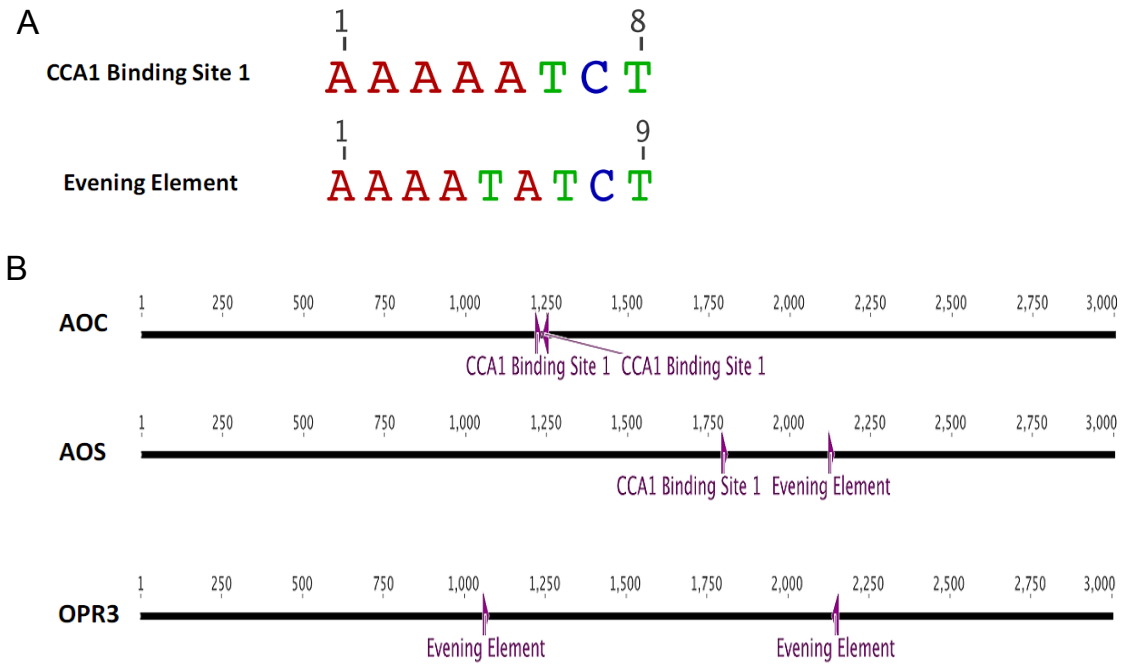

Figure 32: Potential CCA1 binding sites

(A) *cis*-motif sequences that can be bound by the CCA1 transcription factor (Harmer et al., 2000; Wang et al., 1997). (B) Instances of the CCA1 Binding site and Evening element sequences that occur in the 3kb upstream promoter region of *AOC1, AOS* and *OPR3*.

Examination of JA-biosynthesis gene expression in plants with altered SA levels revealed that low SA did not appear to affect differential accumulation of JA-biosynthesis gene transcripts compared to wild type, at least for the first 24-hour period under free-running conditions (Figure 16). However, a clear cyclical differential accumulation of JA-biosynthesis gene transcripts was not observed in *acd6-1* (Figure 16), which have high basal SA levels (Lu et al., 2003, 2009; Rate et al., 1999). However, several questions remain to be addressed: first, whether SA levels in *NahG* and *acd6-1* cycle under free-running conditions, and second, whether altered SA levels in *NahG* and *acd6-1* affect rhythmic basal JA accumulation under free-running conditions. Finally, the question of whether altered *AOS, AOC1* and *OPR3* transcript accumulation seen in *acd6-1* (Figure 16) is due to higher SA levels in general or due to the mutation in *ACD6* affecting JA-biosynthesis in a non-SA related process remains. For example, a mutation to *ACD6* could be affecting overall clock function. Measurements of JA and SA in *NahG* and *acd6-1* under free-running conditions can address the first two questions, while measurements of JA-biosynthesis gene transcripts, clock-reporter transcripts, and JA levels under free-funning conditions in a *NahG* x *acd6-1* double mutant could potentially address the third question. If SA is involved in the regulation of rhythmic basal JA accumulation, then JA accumulation should be altered in *NahG* and *acd6-1* plants under free-running conditions. Additionally, if clock-reporter gene expression such as *CCA1* cycles under free-running conditions in *acd6-1*, then the mutation to *ACD6* does not affect clock function. Furthermore, if increased SA is responsible for the alteration of JA-biosynthetic gene transcript accumulation in *acd6-1*, then a *NahG acd6-1* double

mutant with decreased SA levels would display restored rhythmic cycling of JA-biosynthesis transcript accumulation.

Additionally, while I have shown that the circadian clock may regulate JA-biosynthesis through the timing of JA-biosynthesis gene transcript accumulation (Figure 15); one question that remains is how the circadian clock can regulate the rhythmic decrease of JA levels (Figure 7). Here, we have specifically measured methyl jasmonate concentrations (Figure 7) that represent the total amount of jasmonic acid after chemically induced methylation during extraction (Schmelz et al., 2004) and naturally derived methyl jasmonate formed in plants by a jasmonic acid carboxyl methyltransferase (Seo et al., 2001). In addition to methyl jasmonate, jasmonic acid can also be converted to a variety of other compounds *in planta* which could account for the decrease in jasmonic acid levels. One possibility is that JA is conjugated to an amino acid such as isoleucine to form JA-Ile (Staswick and Tiryaki, 2004; Staswick et al., 2002). JA-Ile in turn, can be metabolized by amidohydrolases (Widemann et al., 2013). Alternatively, the circadian clock may regulate the flow of various JA precursors along different pathways. For example, in addition to forming JA (Figure 6), OPDA is found in arabidopsides, plastid-localized galactolipid-derivatives (Böttcher and Weiler, 2007; Stelmach et al., 2001), but the mechanism via which OPDA is incorporated into arabidopsides remains unclear (Acosta and Farmer, 2010).

8.5.2. Circadian accumulation of glucosinolates

Here we have shown that glucosinolate levels accumulate with circadian rhythmicity in Arabidopsis and in cabbage (Figure 10 Figure 11; Goodspeed et al.,

2013b). However, how the clock regulates rhythmic accumulation of glucosinolates is not understood. While jasmonates strongly influence the levels of aliphatic and indolic glucosinolates in Arabidopsis (Brader et al., 2001; Mewis et al., 2005; Mikkelsen et al., 2003), further research is required to determine whether this is the mechanism through which the clock regulates glucosinolate levels. To determine that a functional clock is required for rhythmic accumulation of glucosinolates, individual and total glucosinolates need be measured in plants with altered clock function such as CCA1-OX (Wang and Tobin, 1998) or *lux* (Hazen et al., 2005) under free-running conditions to examine whether this property is altered or lost.. Glucosinolate levels can also be examined under free running conditions in *aos* plants that lack JA (Park et al., 2002) to determine if the clock regulates rhythmic accumulation of glucosinolates through JA.

Further research can also address how the clock regulates biosynthesis and/or degradation of glucosinolates, which are derived from amino acids. In Arabidopsis there are 40 known glucosinolates mostly derived from Met and Trp (Sønderby et al., 2010), which form aliphatic and indolic glucosinolates, respectively. Biosynthesis of glucosinolates involves three independent steps: (1) chain elongation of Met, (2) formation of the core glucosinolate structure, and (3) secondary modification of the amino acid sign chain (Grubb and Abel, 2006).

Side chain elongation of Met (Figure 33) begins with deamination by a branched-chain amino acid aminotransferase (BCAT4, Schuster et al., 2006) to form 2-oxo acid in the cytosol. 2-oxo acid can then be transported into the chloroplast by a bile acid transporter, BAT5 (Sawada et al., 2009a), where it undergoes 3 successive reactions: (1)

condensation with acetyl-CoA by a methylthioalkylmalate synthase (MAM; Kroymann et al., 2001; Textor et al., 2007), (2) isomerization by isopropylmalate isomerase (IPMI; Knill et al., 2009; Sawada et al., 2009), and (3) oxidative decarboxylation by an isopropylmalate dehydrogenase (IPM-DH; Sawada et al., 2009b). This results in the elongation of 2-oxo acid by a methylene group, which can be transaminated by BCAT3 (Knill et al., 2008) to form homoMet. HomoMet can then be used in the construction of the core glucosinolate.

The glucosinolate core (Figure 33) is formed through 5 different biochemical steps using 13 enzymes (Sønderby et al., 2010). First, aldoximes are derived from precursor amino acids by cytochrome P450 of the CYP79 family. CYP79F1 and CYP79F2 act on Met derivatives (Chen et al., 2003; Hansen et al., 2001a) while CYP79B2 and CYP79B3 act on Trp precursors (Hull et al., 2000; Mikkelsen et al., 2000). Aldoximes are then activated through oxidation by members of the CYP83 family. CYP83B1 acts on Trp-derived aldoximes, while CYP83A1 acts on aliphatic aldoximes (Bak and Feyereisen, 2001; Hansen et al., 2001b; Naur et al., 2003). The activated aldoximes can then be non-enzymatically conjugated to a sulfur donor to form *S*-alkyl-thiohydroximates, which are then converted to thiohydroximates by the *C*-*S* lyase SUR1 (Mikkelsen et al., 2004). Thiohydroximates are subsequently *S*-glycosylated by glucosyltransferases of the UGT74 family to form desulfoglucosinolates (Sønderby et al., 2010). UGT74B1 acts on Phe-derived thiohydroximates (Douglas Grubb et al., 2004), and co-expression evidence suggests that UGT74C1 acts on Met-derived thiohydroximates (Gachon et al., 2005). The desulfoglucosinolates are then sulfated by sulfotransferases SOT16, 17 and 18 to form glucosinolates. SOT16 acts on Trp-derived

desulfoglucosinolates, while SOT17 and SOT18 act on aliphatic substrates (Piotrowski et al., 2004).

Diurnal (diurnal.mocklerlab.org) is a web-based tool that collates together various diurnal and circadian genome-wide expression results from array studies of common model plants (Mockler et al., 2007). Additionally, Diurnal uses a pattern-matching algorithm called HAYSTACK to calculate a Pearson correlation score between expression data and a set of user-defined models to determine a best-fitting model (Mockler et al., 2007). Examples of models used to represent cyclical diurnal or circadian behavior include: spike, cosine, sine, and box-like patterns (Mockler et al., 2007). Using the Diurnal tool to examine the transcript accumulation pattern of the genes associated with glucosinolate biosynthesis described above, I found that in Col-0, 15 of the 16 genes cycle under 12-hour light/12-hour dark (22^{o}C) diurnal conditions, as determined by a correlation score (>0.8) between the best fit model and the array data (Table 4). When array data was examined for samples collected under free running conditions, 6 of the 16 genes had a correlation score greater than 0.8; indicating that *BCAT4, MAM, CYP79B3, CYP83B1, CYP83A1* and *UGT74C1* may be under circadian regulation (Table 4). Future experiments could confirm that glucosinolate biosynthesis gene transcripts show clock-dependent cycling under free-running conditions, as well as determine how the clock regulates cycling of glucosinolate biosynthesis and/or accumulation.

Another question to examine is how the clock affects rhythmic glucosinolate decreases in plants. As described previously, glucosinolates are broken down enzymatically by myrosinases that are normally kept separate from glucosinolates in

intact plant tissue (Andréasson et al., 2001; Rask et al., 2000). The interaction of glucosinolates and myrosinases occurs due to tissue damage as a result of pest or pathogen attack. In addition to damage induced changes in glucosinolate content, dynamic changes in glucosinolate content have been observed in intact tissues. For example, changes in glucosinolate composition during seed germination and early plant development (Brown et al., 2003; Petersen et al., 2002), as well as changes in glucosinolate content associated with altered sulfur availability (Falk et al., 2007) have been reported, suggesting that non-defensive glucosinolate turnover in intact tissue may also exist. One proposal suggests that myrosinase-type enzymes acting in conjunction with nitrilases and nitrile-specifier proteins (NSPs), which help promote nitrile formation, could break down glucosinolates without forming toxic isothiocyanates (Burow and Wittstock, 2008; Kissen and Bones, 2009). Nitrilases are involved in the hydrolysis of nitriles to form carboxylic acid and ammonia. Arabidopsis has four nitrilases, three of which form the NIT1 group which is active on nitriles derived from glucosinolates (Janowitz et al., 2009; Piotrowski, 2008). However, a nitrilase-dependent glucosinolate turnover pathway has not been shown conclusively *in planta*, and the overall mechanism for glucosinolate breakdown in intact plant is not well understood (Wittstock and Burow, 2010).

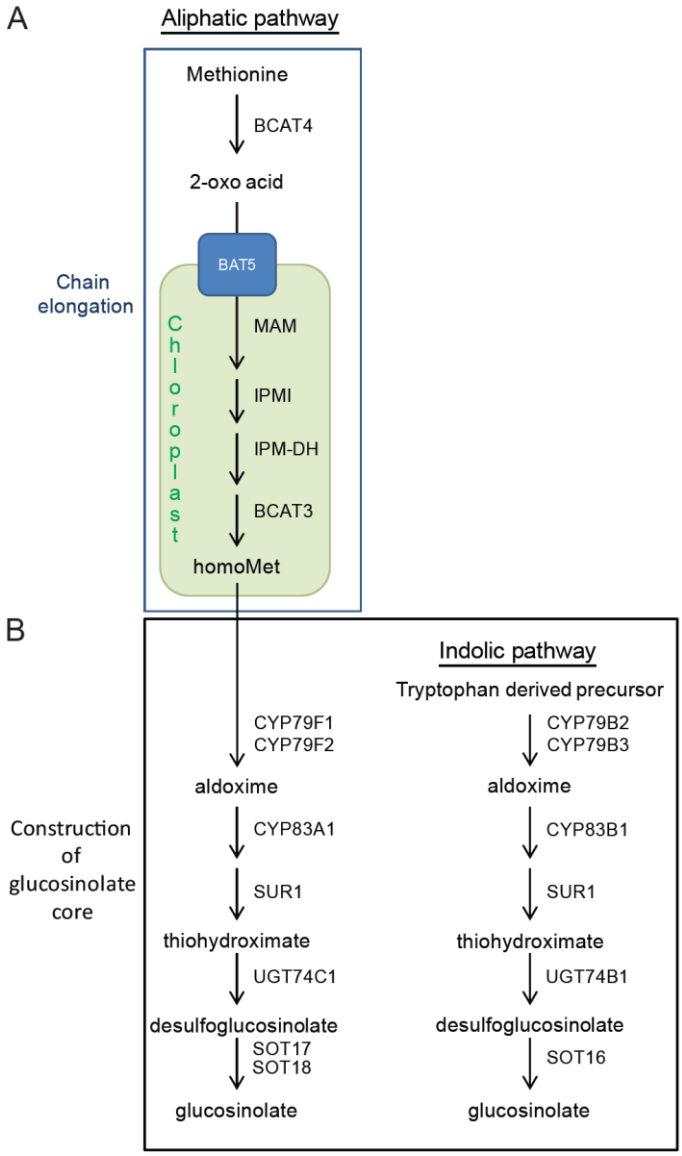

Figure 33: Overview of core glucosinolate biosynthesis

Enzymes involved in biosynthesis of the glucosinolate core amino acid precursors. (A) Chain elongation of methionine precursors. (B) Construction of core glucosinolate from methionine and tryptophan precursors. BAT5, bile acid transporter; BCAT, branched-chain amino acid aminotransferase; CYP, cytochrome P450; IPMI, isopropylmalate isomerase; IPM-DH, isopropylmalate dehydrogenase; MAM, methylthioalkylmalate synthase; SOT, sulfotransferase; SUR1, SUPERROOT1; UGT, uridine diphosphate glycosyltransferase. (Adapted from Sønderby et al., 2010).

Gene Name	Gene ID	Diurnal correlation score	Free-running correlation score
BCAT4	AT3G19710	0.9	0.8
BAT5	AT4G12030	1.0	0.6
MAM	AT5G23010	1.0	0.9
IPMI	AT4G13430	1.0	0.7
IPM-DH	AT5G14200	N/A	N/A
BCAT3	AT3G49680	0.9	0.7
CYP79F1	AT1G16410	1.0	0.7
CYP79F2	AT1G16400	N/A	N/A
CYP79B2	AT4G39950	0.7	0.7
CYP79B3	AT2G22330	1.0	0.8
CYP83B1	AT4G31500	0.9	0.8
CYP83A1	AT4G13770	1.0	0.8
SUR1	AT2G20610	1.0	0.7
UGT74B1	AT1G24100	0.9	0.4
UGT74C1	AT2G31790	1.0	0.8
SOT16	AT1G74100	0.8	0.6
SOT17	AT1G18590	1.0	0.7

Table 4: Analysis of glucosinolate biosynthesis gene transcript accumulation under diurnal and free-running conditions

Transcript levels of genes associated with glucosinolate biosynthesis were taken from plants grown under diurnal (12-hour light/12-hour dark, 22^{o}C)(Hazen et al., 2009) or under free-running conditions (24-hour light, 22^{o}C)(Harmer et al., 2000). They were measured at 12 time points and analyzed using the Diurnal web-based tool (diurnal.mocklerlab.org). Diurnal was used to compare the time-course data to user-defined models and a correlation score was generated. A score >0.8 represents a signficant correlation (Mockler et al., 2007)

8.5.3. Improving postharvest produce performance using entrainment

Here we have shown that keeping postharvest kale, cabbage, lettuce and spinach in light/dark cycles improves several postharvest performance markers when compared to storage under constant light or constant dark. Light/dark cycles improved maintenance of visual appeal (Figure 19), tissue integrity (Figures 22 and 23), as well as several chemical compounds with human health benefits including chlorophyll (Figures 19-21) and glucosinolates (Figures 24-27). Surprisingly, when measuring tissue integrity and glucosinolate levels, keeping postharvest vegetables in light/dark cycles was comparable to the performance achieved from refrigeration, a standard industry practice (Kader, 2003). Additionally, we have shown that storing postharvest vegetables in light/dark cycles improves resistance to *B. cinerea* when compared to storage under constant light (Figure 30).

One remaining question generated from this work is whether the improvement observed when storing postharvest vegetables in light/dark cycles compared to constant light or constant dark is due to the benefits of maintaining a robust circadian rhythm. Alternatively, the benefits observed with storage under light/dark cycles could be due to an optimization of the light exposure required to provide energy for sustained cellular metabolism, without inducing the stress of constant light (Ayala et al., 2009; Barbieri et al., 2009; Kasim and Kasim, 2007), which can cause tissue damage, or constant dark, which can induce plant senescence (Thimann and Satler, 1979; Velerskov, 1987). One way to address this question would be to maintain postharvest vegetables in either constant dark or constant light and employ diurnal changes in temperatures to entrain the

clock; a temperature change of just 4^{o}C can entrain the clock in plants (Millar, 2004; Salomé and Mcclung, 2005). The postharvest performance of temperature entrained vegetables could then be compared to that of those maintained under constant temperatures. This method would answer the question of whether it was an optimization of light exposure that provided the benefits associated with storage under light/dark cycles.

Several questions of practical importance also remain. First, here we have found that postharvest performance is improved in plants when kept under 12-hour light/12-hour dark cycles at 22^{o}C, specifically. However, more work can be done to determine the optimal levels of light exposure and temperature to achieve maximum benefits; these optimal conditions may vary for different types of vegetables. Optimizing storage conditions could decrease the amount of food lost or wasted every year in the world.

Secondly, we have shown that chlorophyll and glucosinolates, two classes of chemical compounds with human health benefits, are maintained for longer periods when stored under light/dark cycles. Another question that would be beneficial to address is what other human health relevant compounds are maintained for longer periods of time when postharvest vegetables are stored in light/dark cycles. Furthermore, while glucosinolate levels were comparable between light/dark stored and refrigerated kale and cabbage (Figure 25), chlorophyll content was higher in light/dark stored cabbage compared to refrigerated cabbage. Therefore, future work can examine if certain metabolite concentrations are actually improved when storage occurs under light/dark cycles.

Addressing these question could be particularly valuable in developing countries that have difficulties maintaining cold chain practices (Kader, 2010; Kitinoja et al., 2011). The use of light/dark cycles could not only improve longevity, which would decease food lost, but could also improve produce nutritional content using a storage practice that may be easier and less expensive to implement than cold chain practices.

References

Acosta, I.F., and Farmer, E.E. (2010). Jasmonates. The Arabidopsis Book *8*, e0129.

Alabadí, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Más, P., and Kay, S.A. (2001). Reciprocal Regulation Between TOC1 and LHY/CCA1 Within the Arabidopsis Circadian Clock. Science *293*, 880–883.

Andréasson, E., Jørgensen, L.B., Höglund, A.-S., Rask, L., and Meijer, J. (2001). Different Myrosinase and Idioblast Distribution in Arabidopsis and Brassica napus. Plant Physiol. *127*, 1750–1763.

Ayala, F., Echávarri, J.F., Olarte, C., and Sanz, S. (2009). Quality characteristics of minimally processed leek packaged using different films and stored in lighting conditions. International Journal of Food Science & Technology *44*, 1333–1343.

Azarenko, O., Okouneva, T., Singletary, K.W., Jordan, M.A., and Wilson, L. (2008). Suppression of microtubule dynamic instability and turnover in MCF7 breast cancer cells by sulforaphane. Carcinogenesis *29*, 2360–2368.

Bak, S., and Feyereisen, R. (2001). The Involvement of Two P450 Enzymes, CYP83B1 and CYP83A1, in Auxin Homeostasis and Glucosinolate Biosynthesis. Plant Physiol. *127*, 108–118.

Ballaré, C.L. (2011). Jasmonate-induced defenses: a tale of intelligence, collaborators and rascals. Trends in Plant Science *16*, 249–257.

Banerjee, A., Variyar, P.S., Chatterjee, S., and Sharma, A. (2014). Effect of post harvest radiation processing and storage on the volatile oil composition and glucosinolate profile of cabbage. Food Chemistry *151*, 22–30.

Barak, S., Tobin, E.M., Green, R.M., Andronis, C., and Sugano, S. (2000). All in good time: the Arabidopsis circadian clock. Trends in Plant Science *5*, 517–522.

Barbieri, G., Bottino, A., Orsini, F., and De Pascale, S. (2009). Sulfur fertilization and light exposure during storage are critical determinants of the nutritional value of ready-to-eat friariello campano (*Brassica rapa* L. subsp. *sylvestris*). Journal of the Science of Food and Agriculture *89*, 2261–2266.

Beekwilder, J., van Leeuwen, W., van Dam, N.M., Bertossi, M., Grandi, V., Mizzi, L., Soloviev, M., Szabados, L., Molthoff, J.W., Schipper, B., et al. (2008). The Impact of the Absence of Aliphatic Glucosinolates on Insect Herbivory in Arabidopsis. PLoS ONE *3*, e2068.

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L., and Zoran, M.J. (2005). Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet *6*, 544–556.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. The Plant Journal *29*, 23–32.

Bhamre, S., Sahoo, D., Tibshirani, R., Dill, D.L., and Brooks, J.D. (2009). Temporal changes in gene expression induced by sulforaphane in human prostate cancer cells. Prostate *69*, 181–190.

Bhardwaj, V., Meier, S., Petersen, L.N., Ingle, R.A., and Roden, L.C. (2011). Defence Responses of Arabidopsis thaliana to Infection by Pseudomonas syringae Are Regulated by the Circadian Clock. PLoS ONE *6*, e26968.

Björkman, M., Klingen, I., Birch, A.N.E., Bones, A.M., Bruce, T.J.A., Johansen, T.J., Meadow, R., Mølmann, J., Seljåsen, R., Smart, L.E., et al. (2011). Phytochemicals of Brassicaceae in plant protection and human health – Influences of climate, environment and agronomic practice. Phytochemistry *72*, 538–556.

Boerjan, W., Cervera, M.T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., Caboche, M., Onckelen, H.V., Montagu, M.V., and Inzé, D. (1995). Superroot, a recessive mutation in Arabidopsis, confers auxin overproduction. Plant Cell *7*, 1405–1419.

Böttcher, C., and Weiler, E.W. (2007). cyclo-Oxylipin-galactolipids in plants: occurrence and dynamics. Planta *226*, 629–637.

Brader, G., Tas, É., and Palva, E.T. (2001). Jasmonate-Dependent Induction of Indole Glucosinolates in Arabidopsis by Culture Filtrates of the Nonspecific Pathogen Erwinia carotovora. Plant Physiol *126*, 849–860.

Britz, S.J., and Briggs, W.R. (1976). Circadian Rhythms of Chloroplast Orientation and Photosynthetic Capacity in Ulva123. Plant Physiol *58*, 22–27.

Brooks, D.M., Bender, C.L., and Kunkel, B.N. (2005). The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. Molecular Plant Pathology *6*, 629–639.

Brown, P.D., Tokuhisa, J.G., Reichelt, M., and Gershenzon, J. (2003). Variation of glucosinolate accumulation among different organs and developmental stages of Arabidopsis thaliana. Phytochemistry *62*, 471–481.

Büchert, A.M., Gómez Lobato, M.E., Villarreal, N.M., Civello, P.M., and Martínez, G.A. (2011). Effect of visible light treatments on postharvest senescence of broccoli (Brassica oleracea L.). Journal of the Science of Food and Agriculture *91*, 355–361.

Burow, M., and Wittstock, U. (2008). Regulation and function of specifier proteins in plants. Phytochem Rev *8*, 87–99.

Burton, W.G. (1982). Post-harvest Physiology of Food Crops (London ; New York: Longman).

Celenza, J.L., Quiel, J.A., Smolen, G.A., Merrikh, H., Silvestro, A.R., Normanly, J., and Bender, J. (2005). The Arabidopsis ATR1 Myb Transcription Factor Controls Indolic Glucosinolate Homeostasis. Plant Physiol. *137*, 253–262.

Chehab, E.W., Kim, S., Savchenko, T., Kliebenstein, D., Dehesh, K., and Braam, J. (2011). Intronic T-DNA Insertion Renders Arabidopsis opr3 a Conditional Jasmonic Acid-Producing Mutant. Plant Physiol. *156*, 770–778.

Chen, S., Glawischnig, E., Jørgensen, K., Naur, P., Jørgensen, B., Olsen, C.-E., Hansen, C.H., Rasmussen, H., Pickett, J.A., and Halkier, B.A. (2003). CYP79F1 and CYP79F2 have distinct functions in the biosynthesis of aliphatic glucosinolates in Arabidopsis. The Plant Journal *33*, 923–937.

Chung, H.S., Koo, A.J.K., Gao, X., Jayanty, S., Thines, B., Jones, A.D., and Howe, G.A. (2008). Regulation and Function of Arabidopsis JASMONATE ZIM-Domain Genes in Response to Wounding and Herbivory. Plant Physiol. *146*, 952–964.

Corbesier, L., and Coupland, G. (2005). Photoperiodic flowering of Arabidopsis: integrating genetic and physiological approaches to characterization of the floral stimulus. Plant, Cell & Environment *28*, 54–66.

Costa, L., Millan Montano, Y., Carrión, C., Rolny, N., and Guiamet, J.J. (2013). Application of low intensity light pulses to delay postharvest senescence of Ocimum basilicum leaves. Postharvest Biology and Technology *86*, 181–191.

Covington, M.F., Maloof, J.N., Straume, M., Kay, S.A., and Harmer, S.L. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol *9*, R130.

Daniel, X., Sugano, S., and Tobin, E.M. (2004). CK2 phosphorylation of CCA1 is necessary for its circadian oscillator function in Arabidopsis. PNAS *101*, 3292–3297.

Dean, R., Van Kan, J. a. L., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J., et al. (2012). The Top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology *13*, 414–430.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., et al. (1994). A Central Role of Salicylic Acid in Plant Disease Resistance. Science *266*, 1247–1250.

Dodd, A.N., Salathia, N., Hall, A., Kévei, E., Tóth, R., Nagy, F., Hibberd, J.M., Millar, A.J., and Webb, A.A.R. (2005). Plant Circadian Clocks Increase Photosynthesis, Growth, Survival, and Competitive Advantage. Science *309*, 630–633.

Dombrecht, B., Xue, G.P., Sprague, S.J., Kirkegaard, J.A., Ross, J.J., Reid, J.B., Fitt, G.P., Sewelam, N., Schenk, P.M., Manners, J.M., et al. (2007). MYC2 Differentially Modulates Diverse Jasmonate-Dependent Functions in Arabidopsis. Plant Cell *19*, 2225–2245.

Douglas Grubb, C., Zipp, B.J., Ludwig-Müller, J., Masuno, M.N., Molinski, T.F., and Abel, S. (2004). Arabidopsis glucosyltransferase UGT74B1 functions in glucosinolate biosynthesis and auxin homeostasis. The Plant Journal *40*, 893–908.

Dowson-Day, M.J., and Millar, A.J. (1999). Circadian dysfunction causes aberrant hypocotyl elongation patterns in Arabidopsis. The Plant Journal *17*, 63–71.

Egner, P.A., Muñoz, A., and Kensler, T.W. (2003). Chemoprevention with chlorophyllin in individuals exposed to dietary aflatoxin. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis *523–524*, 209–216.

Erb, M., Meldau, S., and Howe, G.A. (2012). Role of phytohormones in insect-specific plant reactions. Trends in Plant Science *17*, 250–259.

Fahey, J.W., Zalcmann, A.T., and Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry *56*, 5–51.

Fahey, J.W., Haristoy, X., Dolan, P.M., Kensler, T.W., Scholtus, I., Stephenson, K.K., Talalay, P., and Lozniewski, A. (2002). Sulforaphane Inhibits Extracellular, Intracellular, and Antibiotic-Resistant Strains of Helicobacter pylori and Prevents Benzo[a]pyrene-Induced Stomach Tumors. Proceedings of the National Academy of Sciences of the United States of America *99*, 7610–7615.

Fahey, J.W., Stephenson, K.K., Dinkova-Kostova, A.T., Egner, P.A., Kensler, T.W., and Talalay, P. (2005). Chlorophyll, chlorophyllin and related tetrapyrroles are significant inducers of mammalian phase 2 cytoprotective genes. Carcinogenesis *26*, 1247–1255.

Falk, K.L., Tokuhisa, J.G., and Gershenzon, J. (2007). The Effect of Sulfur Nutrition on Plant Glucosinolate Content: Physiology and Molecular Mechanisms. Plant Biology *9*, 573–581.

FAO (2011). Global food losses and food waste: extent, causes and prevention (Rome: Food and Agriculture Organization of the United Nations).

Ferrari, S., Plotnikova, J.M., De Lorenzo, G., and Ausubel, F.M. (2003). Arabidopsis local resistance to Botrytis cinerea involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. The Plant Journal *35*, 193–205.

Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. Plant Cell *6*, 751–759.

Filichkin, S.A., Priest, H.D., Givan, S.A., Shen, R., Bryant, D.W., Fox, S.E., Wong, W.-K., and Mockler, T.C. (2010). Genome-wide mapping of alternative splicing in Arabidopsis thaliana. Genome Res. *20*, 45–58.

Finley, J.W. (2005). Proposed Criteria for Assessing the Efficacy of Cancer Reduction by Plant Foods Enriched in Carotenoids, Glucosinolates, Polyphenols and Selenocompounds. Ann Bot *95*, 1075–1096.

Fowler, S.G., Cook, D., and Thomashow, M.F. (2005). Low Temperature Induction of Arabidopsis CBF1, 2, and 3 Is Gated by the Circadian Clock. PLANT PHYSIOLOGY *137*, 961–968.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Current Opinion in Plant Biology *9*, 436–442.

Gachon, C.M.M., Langlois-Meurinne, M., Henry, Y., and Saindrenan, P. (2005). Transcriptional co-regulation of secondary metabolism enzymes in Arabidopsis: functional and evolutionary implications. Plant Mol Biol *58*, 229–245.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of Salicylic Acid for the Induction of Systemic Acquired Resistance. Science *261*, 754–756.

Glazebrook, J. (2005). Contrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens. Annual Review of Phytopathology *43*, 205–227.

Glazebrook, J., Chen, W., Estes, B., Chang, H.-S., Nawrath, C., Métraux, J.-P., Zhu, T., and Katagiri, F. (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. The Plant Journal *34*, 217–228.

Goodspeed, D., Chehab, E.W., Min-Venditti, A., Braam, J., and Covington, M.F. (2012). Arabidopsis synchronizes jasmonate-mediated defense with insect circadian behavior. Proceedings of the National Academy of Sciences *109*, 4674–4677.

Goodspeed, D., Chehab, E.W., Covington, M.F., and Braam, J. (2013a). Circadian control of jasmonates and salicylates: The clock role in plant defense. Plant Signaling & Behavior *8*, e23123.

Goodspeed, D., Liu, J.D., Chehab, E.W., Sheng, Z., Francisco, M., Kliebenstein, D.J., and Braam, J. (2013b). Postharvest Circadian Entrainment Enhances Crop Pest Resistance and Phytochemical Cycling. Current Biology *23*, 1235–1241.

Green, R.M., Tingay, S., Wang, Z.-Y., and Tobin, E.M. (2002). Circadian Rhythms Confer a Higher Level of Fitness to Arabidopsis Plants. Plant Physiol. *129*, 576–584.

Grubb, C.D., and Abel, S. (2006). Glucosinolate metabolism and its control. Trends in Plant Science *11*, 89–100.

Halkier, B.A., and Gershenzon, J. (2006). Biology and Biochemistry of Glucosinolates. Annual Review of Plant Biology *57*, 303–333.

Hansen, C.H., Wittstock, U., Olsen, C.E., Hick, A.J., Pickett, J.A., and Halkier, B.A. (2001a). Cytochrome P450 CYP79F1 from Arabidopsis Catalyzes the Conversion of Dihomomethionine and Trihomomethionine to the Corresponding Aldoximes in the Biosynthesis of Aliphatic Glucosinolates. J. Biol. Chem. *276*, 11078–11085.

Hansen, C.H., Du, L., Naur, P., Olsen, C.E., Axelsen, K.B., Hick, A.J., Pickett, J.A., and Halkier, B.A. (2001b). CYP83B1 Is the Oxime-metabolizing Enzyme in the Glucosinolate Pathway in Arabidopsis. J. Biol. Chem. *276*, 24790–24796.

Harmer, S.L. (2009). The Circadian System in Higher Plants. Annual Review of Plant Biology *60*, 357–377.

Harmer, S.L., and Kay, S.A. (2005). Positive and Negative Factors Confer Phase-Specific Circadian Regulation of Transcription in Arabidopsis. Plant Cell *17*, 1926–1940.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.-S., Han, B., Zhu, T., Wang, X., Kreps, J.A., and Kay, S.A. (2000). Orchestrated Transcription of Key Pathways in Arabidopsis by the Circadian Clock. Science *290*, 2110–2113.

Harms, K., Ramirez, I., and Peña-Cortés, H. (1998). Inhibition of Wound-Induced Accumulation of Allene Oxide Synthase Transcripts in Flax Leaves by Aspirin and Salicylic Acid. Plant Physiol *118*, 1057–1065.

Hayes, J.D., Kelleher, M.O., and Eggleston, I.M. (2008). The cancer chemopreventive actions of phytochemicals derived from glucosinolates. Eur J Nutr *47*, 73–88.

Hazen, S.P., Schultz, T.F., Pruneda-Paz, J.L., Borevitz, J.O., Ecker, J.R., and Kay, S.A. (2005). LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. PNAS *102*, 10387–10392.

Hazen, S.P., Naef, F., Quisel, T., Gendron, J.M., Chen, H., Ecker, J.R., Borevitz, J.O., and Kay, S.A. (2009). Exploring the transcriptional landscape of plant circadian rhythms using genome tiling arrays. Genome Biology *10*, R17.

Heil, M., and Baldwin, I.T. (2002). Fitness costs of induced resistance: emerging experimental support for a slippery concept. Trends in Plant Science *7*, 61–67.

Higdon, J.V., Delage, B., Williams, D.E., and Dashwood, R.H. (2007). Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. Pharmacological Research *55*, 224–236.

Hirai, M.Y., Sugiyama, K., Sawada, Y., Tohge, T., Obayashi, T., Suzuki, A., Araki, R., Sakurai, N., Suzuki, H., Aoki, K., et al. (2007). Omics-based identification of Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis. PNAS *104*, 6478–6483.

Hopkins, R.J., van Dam, N.M., and van Loon, J.J.A. (2009). Role of Glucosinolates in Insect-Plant Relationships and Multitrophic Interactions. Annual Review of Entomology *54*, 57–83.

Hull, A.K., Vij, R., and Celenza, J.L. (2000). Arabidopsis cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. PNAS *97*, 2379–2384.

Hutchings, J.B. (1999). Food Color and Appearance (Gaithersburg, Maryland: Aspen Publishers, Inc.).

James, A.B., Syed, N.H., Bordage, S., Marshall, J., Nimmo, G.A., Jenkins, G.I., Herzyk, P., Brown, J.W.S., and Nimmo, H.G. (2012). Alternative Splicing Mediates Responses of the Arabidopsis Circadian Clock to Temperature Changes. Plant Cell *24*, 961–981.

Janowitz, T., Trompetter, I., and Piotrowski, M. (2009). Evolution of nitrilases in glucosinolate-containing plants. Phytochemistry *70*, 1680–1686.

Johnson, C.H., Knight, M.R., Kondo, T., Masson, P., Sedbrook, J., Haley, A., and Trewavas, A. (1995). Circadian oscillations of cytosolic and chloroplastic free calcium in plants. Science *269*, 1863–1865.

Jubert, C., Mata, J., Bench, G., Dashwood, R., Pereira, C., Tracewell, W., Turteltaub, K., Williams, D., and Bailey, G. (2009). Effects of Chlorophyll and Chlorophyllin on Low-Dose Aflatoxin B1 Pharmacokinetics in Human Volunteers. Cancer Prev Res *2*, 1015–1022.

Kader, A.A. (2003). A Perspective on Postharvest Horticulture (1978-2003). HortScience *38*, 1004–1008.

Kader, A.A. (2010). Handling of Horticultural Perishables in Developing vs. Developed Countries. Acta Hort. *877*, 121–126.

Kasim, R., and Kasim, M.U. (2007). Inhibition of yellowing in Brussels sprouts (B. oleraceae var. gemmifera) and broccoli (B. oleraceae var. italica) using light during storage. Journal of Food Agriculture and Environment *5*, 126.

Katagiri, M., Maeno, H., Yamamoto, S., Hayaishi, O., Kitao, T., and Oae, S. (1965). Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide II. The mechanism of salicylate hydroxylation to catechol. Journal of Biological Chemistry *240*, 3414–3417.

Kelly, P.J., Bones, A., and Rossiter, J.T. (1998). Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of Brassica juncea. Planta *206*, 370–377.

Kissen, R., and Bones, A.M. (2009). Nitrile-specifier Proteins Involved in Glucosinolate Hydrolysis in Arabidopsis thaliana. J. Biol. Chem. *284*, 12057–12070.

Kitinoja, L., Saran, S., Roy, S.K., and Kader, A.A. (2011). Postharvest technology for developing countries: challenges and opportunities in research, outreach and advocacy. J. Sci. Food Agric. *91*, 597–603.

Kliebenstein, D.J. (2004). Secondary metabolites and plant/environment interactions: a view through Arabidopsis thaliana tinged glasses. Plant, Cell & Environment *27*, 675–684.

Kliebenstein, D.J., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J., and Mitchell-Olds, T. (2001). Genetic Control of Natural Variation in Arabidopsis Glucosinolate Accumulation. Plant Physiol. *126*, 811–825.

Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F., and Kunkel, B.N. (2001). Resistance to Pseudomonas syringae conferred by an Arabidopsis thaliana coronatine-insensitive (coi1) mutation occurs through two distinct mechanisms. The Plant Journal *26*, 509–522.

Knill, T., Schuster, J., Reichelt, M., Gershenzon, J., and Binder, S. (2008). Arabidopsis Branched-Chain Aminotransferase 3 Functions in Both Amino Acid and Glucosinolate Biosynthesis. Plant Physiol. *146*, 1028–1039.

Knill, T., Reichelt, M., Paetz, C., Gershenzon, J., and Binder, S. (2009). Arabidopsis thaliana encodes a bacterial-type heterodimeric isopropylmalate isomerase involved in both Leu biosynthesis and the Met chain elongation pathway of glucosinolate formation. Plant Mol Biol *71*, 227–239.

Koop, H.-U., Schmid, R., Heunert, H.-H., and Milthaler, B. (1978). Chloroplast migration: a new circadian rhythm inAcetabularia. Protoplasma *97*, 301–310.

Koornneef, A., and Pieterse, C.M.J. (2008). Cross Talk in Defense Signaling. Plant Physiol. *146*, 839–844.

Kroymann, J., Textor, S., Tokuhisa, J.G., Falk, K.L., Bartram, S., Gershenzon, J., and Mitchell-Olds, T. (2001). A Gene Controlling Variation in Arabidopsis Glucosinolate Composition Is Part of the Methionine Chain Elongation Pathway. Plant Physiol. *127*, 1077–1088.

Kunkel, B.N., and Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen defense. Current Opinion in Plant Biology *5*, 325–331.

León, J., Rojo, E., and Sánchez-Serrano, J.J. (2001). Wound signalling in plants. J. Exp. Bot. *52*, 1–9.

Lester, G.E., Makus, D.J., and Hodges, D.M. (2010). Relationship between Fresh-Packaged Spinach Leaves Exposed to Continuous Light or Dark and Bioactive Contents: Effects of Cultivar, Leaf Size, and Storage Duration. Journal of Agricultural and Food Chemistry *58*, 2980–2987.

Liu, J.D., Goodspeed, D., Sheng, Z., Li, B., Yang, Y., Kliebenstein, D.J., and Braam, J. (2015). Keeping the rhythm: light/dark cycles during postharvest storage preserve the tissue integrity and nutritional content of leafy plants. BMC Plant Biology *15*, 92.

Lu, H., Rate, D.N., Song, J.T., and Greenberg, J.T. (2003). ACD6, a Novel Ankyrin Protein, Is a Regulator and an Effector of Salicylic Acid Signaling in the Arabidopsis Defense Response. Plant Cell *15*, 2408–2420.

Lu, H., Salimian, S., Gamelin, E., Wang, G., Fedorowski, J., LaCourse, W., and Greenberg, J.T. (2009). Genetic analysis of acd6-1 reveals complex defense networks and leads to identification of novel defense genes in Arabidopsis. The Plant Journal *58*, 401–412.

Martínez-Sánchez, A., Tudela, J.A., Luna, C., Allende, A., and Gil, M.I. (2011). Low oxygen levels and light exposure affect quality of fresh-cut Romaine lettuce. Postharvest Biology and Technology *59*, 34–42.

McClung, C.R. (2006). Plant circadian rhythms. The Plant Cell Online *18*, 792–803.

McConn, M., and Browse, J. (1996). The Critical Requirement for Linolenic Acid Is Pollen Development, Not Photosynthesis, in an Arabidopsis Mutant. Plant Cell *8*, 403–416.

McQuistan, T.J., Simonich, M.T., Pratt, M.M., Pereira, C.B., Hendricks, J.D., Dashwood, R.H., Williams, D.E., and Bailey, G.S. (2012). Cancer chemoprevention by dietary chlorophylls: A 12,000-animal dose–dose matrix biomarker and tumor study. Food and Chemical Toxicology *50*, 341–352.

Mewis, I., Appel, H.M., Hom, A., Raina, R., and Schultz, J.C. (2005). Major Signaling Pathways Modulate Arabidopsis Glucosinolate Accumulation and Response to Both Phloem-Feeding and Chewing Insects. Plant Physiol. *138*, 1149–1162.

Michael, T.P., Mockler, T.C., Breton, G., McEntee, C., Byer, A., Trout, J.D., Hazen, S.P., Shen, R., Priest, H.D., Sullivan, C.M., et al. (2008). Network Discovery Pipeline Elucidates Conserved Time-of-Day–Specific cis-Regulatory Modules. PLoS Genet *4*, e14.

Mikkelsen, M.D., Hansen, C.H., Wittstock, U., and Halkier, B.A. (2000). Cytochrome P450 CYP79B2 from Arabidopsis Catalyzes the Conversion of Tryptophan to Indole-3-acetaldoxime, a Precursor of Indole Glucosinolates and Indole-3-acetic Acid. J. Biol. Chem. *275*, 33712–33717.

Mikkelsen, M.D., Petersen, B.L., Glawischnig, E., Jensen, A.B., Andreasson, E., and Halkier, B.A. (2003). Modulation of CYP79 genes and glucosinolate profiles in Arabidopsis by defense signaling pathways. Plant Physiol. *131*, 298–308.

Mikkelsen, M.D., Naur, P., and Halkier, B.A. (2004). Arabidopsis mutants in the C–S lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. The Plant Journal *37*, 770–777.

Millar, A.J. (2004). Input signals to the plant circadian clock. J. Exp. Bot. *55*, 277–283.

Millar, A.J., Straume, M., Chory, J., Chua, N.H., and Kay, S.A. (1995a). The regulation of circadian period by phototransduction pathways in Arabidopsis. Science *267*, 1163–1166.

Millar, A.J., Carre, I.A., Strayer, C.A., Chua, N.-H., and Kay, S.A. (1995b). Circadian clock mutants in Arabidopsis identified by luciferase imaging. Science *267*, 1161–1163.

Mockler, T.C., Michael, T.P., Priest, H.D., Shen, R., Sullivan, C.M., Givan, S.A., McEntee, C., Kay, S.A., and Chory, J. (2007). The Diurnal Project: Diurnal and Circadian Expression Profiling, Model-based Pattern Matching, and Promoter Analysis. Cold Spring Harb Symp Quant Biol *72*, 353–363.

Mumm, R., Burow, M., Bukovinszkine'Kiss, G., Kazantzidou, E., Wittstock, U., Dicke, M., and Gershenzon, J. (2008). Formation of Simple Nitriles upon Glucosinolate Hydrolysis Affects Direct and Indirect Defense Against the Specialist Herbivore, Pieris rapae. J Chem Ecol *34*, 1311–1321.

Nagel, D.H., and Kay, S.A. (2012). Complexity in the Wiring and Regulation of Plant Circadian Networks. Current Biology *22*, R648–R657.

Naur, P., Petersen, B.L., Mikkelsen, M.D., Bak, S., Rasmussen, H., Olsen, C.E., and Halkier, B.A. (2003). CYP83A1 and CYP83B1, Two Nonredundant Cytochrome P450 Enzymes Metabolizing Oximes in the Biosynthesis of Glucosinolates in Arabidopsis. Plant Physiol. *133*, 63–72.

Nilsson, T. (2000). Postharvest handling and storage of vegetables. In Fruit and Vegetable Quality: An Integrated View, R.L. Shewfelt, and B. Bruckner, eds. (Pennsylvania: CRC Press), pp. 96–121.

Nimmo, H.G. (2000). The regulation of phosphoenolpyruvate carboxylase in CAM plants. Trends in Plant Science *5*.

Olarte, C., Sanz, S., Federico Echávarri, J., and Ayala, F. (2009). Effect of plastic permeability and exposure to light during storage on the quality of minimally processed broccoli and cauliflower. LWT - Food Science and Technology *42*, 402–411.

Park, J.-H., Halitschke, R., Kim, H.B., Baldwin, I.T., Feldmann, K.A., and Feyereisen, R. (2002). A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. The Plant Journal *31*, 1–12.

Pena-Cortés, H., Albrecht, T., Prat, S., Weiler, E.W., and Willmitzer, L. (1993). Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta *191*, 123–128.

Perrin, P.W. (1982). Poststorage effect of light, temperature and nutrient spray treatments on chlorophyll development in cabbage. Can. J. Plant Sci. *62*, 1023–1026.

Petersen, B.L., Chen, S., Hansen, C.H., Olsen, C.E., and Halkier, B.A. (2002). Composition and content of glucosinolates in developing Arabidopsis thaliana. Planta *214*, 562–571.

Piotrowski, M. (2008). Primary or secondary? Versatile nitrilases in plant metabolism. Phytochemistry *69*, 2655–2667.

Piotrowski, M., Schemenewitz, A., Lopukhina, A., Müller, A., Janowitz, T., Weiler, E.W., and Oecking, C. (2004). Desulfoglucosinolate Sulfotransferases from Arabidopsis thaliana Catalyze the Final Step in the Biosynthesis of the Glucosinolate Core Structure. J. Biol. Chem. *279*, 50717–50725.

Porra, R.J. (2002). The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynthesis Research *73*, 149–156.

Pruneda-Paz, J.L., and Kay, S.A. (2010). An expanding universe of circadian networks in higher plants. Trends in Plant Science *15*, 259–265.

Rangkadilok, N., Tomkins, B., Nicolas, M.E., Premier, R.R., Bennett, R.N., Eagling, D.R., and Taylor, P.W.J. (2002). The Effect of Post-Harvest and Packaging Treatments on Glucoraphanin Concentration in Broccoli (Brassica oleracea var. italica). J. Agric. Food Chem. *50*, 7386–7391.

Rask, L., Andréasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B., and Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol *42*, 93–114.

Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S., and Greenberg, J.T. (1999). The gain-of-function Arabidopsis acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. The Plant Cell Online *11*, 1695–1708.

Reeves, P.H., and Coupland, G. (2000). Response of plant development to environment: control of flowering by daylength and temperature. Current Opinion in Plant Biology *3*, 37–42.

Rikin, A. (1992). Circadian rhythm of heat resistance in cotton seedlings: synthesis of heat-shock proteins. European Journal of Cell Biology *59*, 160–165.

Rikin, A., Dillwith, J.W., and Bergman, D.K. (1993). Correlation between the circadian rhythm of resistance to extreme temperatures and changes in fatty acid composition in cotton seedlings. Plant Physiology *101*, 31–36.

Salomé, P.A., and McClung, C.R. (2005). PSEUDO-RESPONSE REGULATOR 7 and 9 Are Partially Redundant Genes Essential for the Temperature Responsiveness of the Arabidopsis Circadian Clock. Plant Cell *17*, 791–803.

Salomé, P.A., and Mcclung, C.R. (2005). What makes the Arabidopsis clock tick on time? A review on entrainment. Plant, Cell & Environment *28*, 21–38.

Sanz Cervera, S., Olarte, C., Echávarri, J.F., and Ayala, F. (2007). Influence of Exposure to Light on the Sensorial Quality of Minimally Processed Cauliflower. Journal of Food Science *72*, S012–S018.

Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., et al. (2001). Monitoring of Methyl Jasmonate-responsive Genes in Arabidopsis by cDNA Macroarray: Self-activation of Jasmonic Acid Biosynthesis and Crosstalk with Other Phytohormone Signaling Pathways. DNA Res *8*, 153–161.

Sawada, Y., Toyooka, K., Kuwahara, A., Sakata, A., Nagano, M., Saito, K., and Hirai, M.Y. (2009a). Arabidopsis Bile Acid:Sodium Symporter Family Protein 5 is Involved in Methionine-Derived Glucosinolate Biosynthesis. Plant Cell Physiol *50*, 1579–1586.

Sawada, Y., Kuwahara, A., Nagano, M., Narisawa, T., Sakata, A., Saito, K., and Yokota Hirai, M. (2009b). Omics-Based Approaches to Methionine Side Chain Elongation in Arabidopsis: Characterization of the Genes Encoding Methylthioalkylmalate Isomerase and Methylthioalkylmalate Dehydrogenase. Plant Cell Physiol *50*, 1181–1190.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carré, I.A., and Coupland, G. (1998). The late elongated hypocotyl Mutation of Arabidopsis Disrupts Circadian Rhythms and the Photoperiodic Control of Flowering. Cell *93*, 1219–1229.

Schmelz, E.A., Engelberth, J., Tumlinson, J.H., Block, A., and Alborn, H.T. (2004). The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. The Plant Journal *39*, 790–808.

Schuster, J., Knill, T., Reichelt, M., Gershenzon, J., and Binder, S. (2006). BRANCHED-CHAIN AMINOTRANSFERASE4 Is Part of the Chain Elongation Pathway in the Biosynthesis of Methionine-Derived Glucosinolates in Arabidopsis. Plant Cell *18*, 2664–2679.

Seo, H.S., Song, J.T., Cheong, J.-J., Lee, Y.-H., Lee, Y.-W., Hwang, I., Lee, J.S., and Choi, Y.D. (2001). Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses. PNAS *98*, 4788–4793.

Shin, J., Heidrich, K., Sanchez-Villarreal, A., Parker, J.E., and Davis, S.J. (2012). TIME FOR COFFEE Represses Accumulation of the MYC2 Transcription Factor to Provide Time-of-Day Regulation of Jasmonate Signaling in Arabidopsis. Plant Cell *24*, 2470–2482.

Simonich, M.T., Egner, P.A., Roebuck, B.D., Orner, G.A., Jubert, C., Pereira, C., Groopman, J.D., Kensler, T.W., Dashwood, R.H., Williams, D.E., et al. (2007). Natural chlorophyll inhibits aflatoxin B1-induced multi-organ carcinogenesis in the rat. Carcinogenesis *28*, 1294–1302.

Simonich, M.T., McQuistan, T., Jubert, C., Pereira, C., Hendricks, J.D., Schimerlik, M., Zhu, B., Dashwood, R.H., Williams, D.E., and Bailey, G.S. (2008). Low-dose dietary chlorophyll inhibits multi-organ carcinogenesis in the rainbow trout. Food Chem Toxicol *46*, 1014–1024.

Sønderby, I.E., Hansen, B.G., Bjarnholt, N., Ticconi, C., Halkier, B.A., and Kliebenstein, D.J. (2007). A Systems Biology Approach Identifies a R2R3 MYB Gene Subfamily with Distinct and Overlapping Functions in Regulation of Aliphatic Glucosinolates. PLoS ONE *2*.

Sønderby, I.E., Geu-Flores, F., and Halkier, B.A. (2010). Biosynthesis of glucosinolates – gene discovery and beyond. Trends in Plant Science *15*, 283–290.

Spoel, S.H., Koornneef, A., Claessens, S.M.C., Korzelius, J.P., Pelt, J.A.V., Mueller, M.J., Buchala, A.J., Métraux, J.-P., Brown, R., Kazan, K., et al. (2003). NPR1 Modulates Cross-Talk between Salicylate- and Jasmonate-Dependent Defense Pathways through a Novel Function in the Cytosol. Plant Cell *15*, 760–770.

Staswick, P.E., and Tiryaki, I. (2004). The Oxylipin Signal Jasmonic Acid Is Activated by an Enzyme That Conjugates It to Isoleucine in Arabidopsis. THE PLANT CELL ONLINE *16*, 2117–2127.

Staswick, P.E., Tiryaki, I., and Rowe, M.L. (2002). Jasmonate Response Locus JAR1 and Several Related Arabidopsis Genes Encode Enzymes of the Firefly Luciferase Superfamily That Show Activity on Jasmonic, Salicylic, and Indole-3-Acetic Acids in an Assay for Adenylation. Plant Cell *14*, 1405–1415.

Stelmach, B.A., Müller, A., Hennig, P., Gebhardt, S., Schubert-Zsilavecz, M., and Weiler, E.W. (2001). A Novel Class of Oxylipins,sn1-O-(12-Oxophytodienoyl)-sn2-O-(hexadecatrienoyl)-monogalactosyl Diglyceride, from Arabidopsis thaliana. J. Biol. Chem. *276*, 12832–12838.

Stintzi, A., and Browse, J. (2000). The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci U S A *97*, 10625–10630.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R., Somers, D.E., Más, P., Panda, S., Kreps, J.A., and Kay, S.A. (2000). Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog. Science *289*, 768–771.

Textor, S., Kraker, J.-W. de, Hause, B., Gershenzon, J., and Tokuhisa, J.G. (2007). MAM3 Catalyzes the Formation of All Aliphatic Glucosinolate Chain Lengths in Arabidopsis. Plant Physiol. *144*, 60–71.

Thaler, J.S., Humphrey, P.T., and Whiteman, N.K. (2012). Evolution of jasmonate and salicylate signal crosstalk. Trends in Plant Science *17*, 260–270.

Thimann, K.V., and Satler, S. (1979). Relation between senescence and stomatal opening: Senescence in darkness*. Proc Natl Acad Sci U S A *76*, 2770–2773.

Thomma, B.P.H.J., Eggermont, K., Tierens, K.F.M.-J., and Broekaert, W.F. (1999). Requirement of Functional Ethylene-Insensitive 2Gene for Efficient Resistance of Arabidopsis to Infection by Botrytis cinerea. Plant Physiol. *121*, 1093–1101.

Toledo, M.E.A., Ueda, Y., Imahori, Y., and Ayaki, M. (2003). L-ascorbic acid metabolism in spinach (Spinacia oleracea L.) during postharvest storage in light and dark. Postharvest Biology and Technology *28*, 47–57.

Velerskov, B. (1987). Irradiance-dependent senescence of isolated leaves. Physiologia Plantarum *71*, 316–320.

Verhage, A., van Wees, S.C.M., and Pieterse, C.M.J. (2010). Plant Immunity: It's the Hormones Talking, But What Do They Say? Plant Physiol. *154*, 536–540.

Walley, J.W., Coughlan, S., Hudson, M.E., Covington, M.F., Kaspi, R., Banu, G., Harmer, S.L., and Dehesh, K. (2007). Mechanical Stress Induces Biotic and Abiotic Stress Responses via a Novel cis-Element. PLoS Genetics *3*, e172.

Wang, Z.-Y., and Tobin, E.M. (1998). Constitutive Expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) Gene Disrupts Circadian Rhythms and Suppresses Its Own Expression. Cell *93*, 1207–1217.

Wang, G.-Y., Shi, J.-L., Ng, G., Battle, S.L., Zhang, C., and Lu, H. (2011a). Circadian Clock-Regulated Phosphate Transporter PHT4;1 Plays an Important Role in Arabidopsis Defense. Mol Plant *4*, 516–526.

Wang, W., Barnaby, J.Y., Tada, Y., Li, H., Tör, M., Caldelari, D., Lee, D., Fu, X.-D., and Dong, X. (2011b). Timing of plant immune responses by a central circadian regulator. Nature *470*, 110–114.

Wang, Z.Y., Kenigsbuch, D., Sun, L., Harel, E., Ong, M.S., and Tobin, E.M. (1997). A Myb-related transcription factor is involved in the phytochrome regulation of an Arabidopsis Lhcb gene. Plant Cell *9*, 491–507.

Watkinson, A.R., and White, J. (1986). Some Life-History Consequences of Modular Construction in Plants. Phil. Trans. R. Soc. Lond. B *313*, 31–51.

Widemann, E., Miesch, L., Lugan, R., Holder, E., Heinrich, C., Aubert, Y., Miesch, M., Pinot, F., and Heitz, T. (2013). The Amidohydrolases IAR3 and ILL6 Contribute to Jasmonoyl-Isoleucine Hormone Turnover and Generate 12-Hydroxyjasmonic Acid Upon Wounding in Arabidopsis Leaves. Journal of Biological Chemistry *288*, 31701–31714.

Wittstock, U., and Burow, M. (2010). Glucosinolate Breakdown in Arabidopsis: Mechanism, Regulation and Biological Significance. Arabidopsis Book *8*.

Woodward, A.W., and Bartel, B. (2005). Auxin: Regulation, Action, and Interaction. Ann Bot *95*, 707–735.

Yakir, E., Hassidim, M., Melamed-Book, N., Hilman, D., Kron, I., and Green, R.M. (2011). Cell autonomous and cell-type specific circadian rhythms in Arabidopsis: Circadian rhythms in individual plant cells. The Plant Journal *68*, 520–531.

Yamamoto, S., Katagiri, M., Maeno, H., and Hayaishi, O. (1965). Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide I. Purification and general properties. Journal of Biological Chemistry *240*, 3408–3413.

Yan, X., and Chen, S. (2007). Regulation of plant glucosinolate metabolism. Planta *226*, 1343–1352.

Yerushalmi, S., and Green, R.M. (2009). Evidence for the adaptive significance of circadian rhythms. Ecology Letters *12*, 970–981.

Zhang, Y., Talalay, P., Cho, C.G., and Posner, G.H. (1992). A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. PNAS *89*, 2399–2403.

Zhao, Y. (2010). Auxin Biosynthesis and Its Role in Plant Development. Annual Review of Plant Biology *61*, 49–64.

Appendix A: Postharvest cabbage loses the ability to be re-entrained after three days in constant light[2]

Postharvest cabbage can be re-entrained using light/dark cycles and displays phase-dependent-enhanced resistance to *T. ni* (Figure 18; Goodspeed et al., 2013b). To examine if the ability to be re-entrained is lost over time, I analyzed the phase-dependent enhancement of resistance on cabbage disks entrained at different time points after purchase. Based on grocer information, cabbage was harvested between 24 and 72 hours before purchase. After purchase, cabbage was kept in constant light at 22^{o}C for either 0, 3, 6, or 9 days before entraining the cabbage in 12 hour light/12 hour dark cycles (LD) at 22^{o}C for 3 days. Entrained cabbage was then moved to free-running conditions consisting of constant light (LL) for 24 hours at 22^{o}C. After 24 hours, 4 day old *T. ni* (3 days of LD, 1 day of LL) were placed on the plants. Following 3 days of insect/cabbage co-incubation under LL, *T. ni* weight gain was measured.

[2] These data have been published (Goodspeed et al., 2013b)

A significant difference in looper performance in the cabbage that was entrained in LD cycles was observed either immediately or up to 3 days post-purchase (Figure 34). *T. ni* entrained in-phase with the cabbage were significantly smaller than the *T. ni* entrained out-of-phase (Figure 34). However, cabbage loses the ability to demonstrate phase-dependent herbivory resistance if more than 3 days passed before initiating entrainment (Figure 34). This indicates that the ability of the cabbage to be re-entrained is lost after 1 week postharvest.

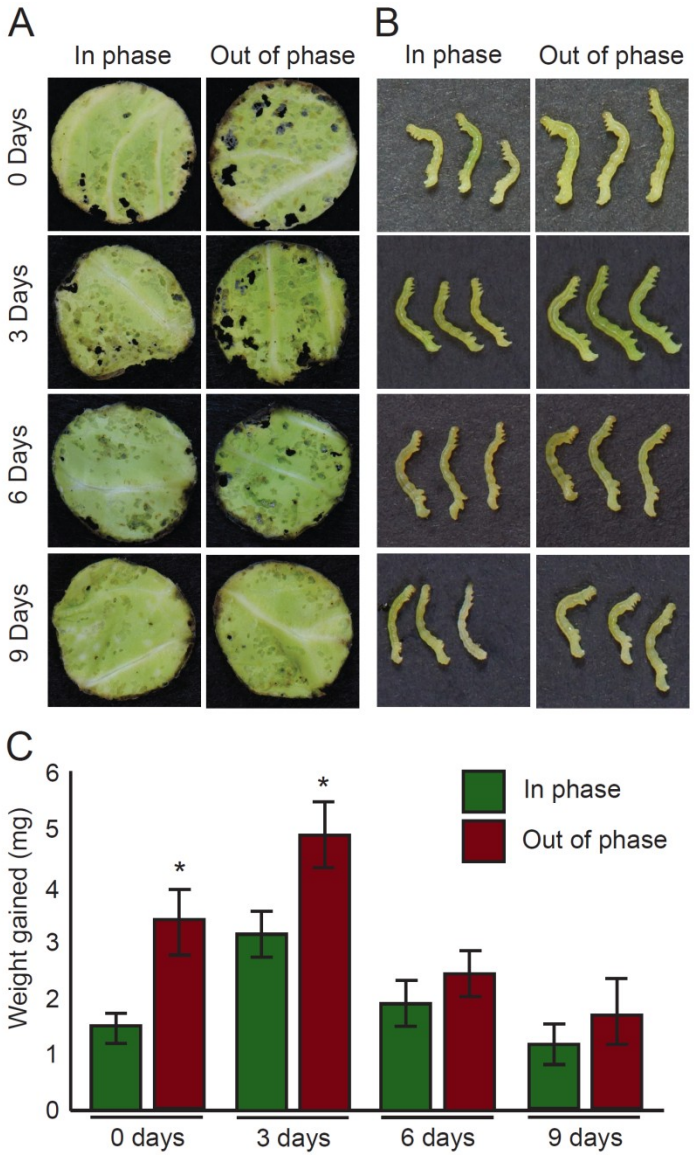

Figure 34: The ability of postharvest cabbage to demonstrate enhanced herbivory resistance after in-phase entrainment is lost over time

(A) Photographs of representative cabbage disks entrained in phase and out of phase with *T. ni* entrainment after 72 hours of coincubation. Entrainment was initiated the day of purchase (0 days) or 3, 6, or 9 days, as indicated, after purchase. Dark spots and holes on disks are sites of herbivory damage. (B) Photographs of representative of *T. ni* after 72 hours of coincubation with cabbage disks entrained in phase and out of phase with respect to the *T. ni*. Scale bar, 0.5 mm (C) *T. ni* weight gained after 72 hours of coincubation with plants. Mean area ± SE; n = 18; *P<0.05; two-tailed paired t test (Goodspeed et al., 2013b).

Appendix B: *T. ni* prefer to feed on postharvest cabbage entrained in constant light over cabbage entrained in 12-hour light/12-hour dark cycles[3]

Previous non-choice experiments with postharvest cabbage and *T. ni* show that cabbage entrained in light/dark cycles are more resistant to *T. ni* when compared to cabbage entrained in constant light or constant dark (Goodspeed et al., 2013b). Cabbage entrained in light/dark cycles lose less mass and *T. ni* gain less mass when compared to cabbage that are entrained in constant light or constant dark (Figure 35; Goodspeed et al., 2013b). To examine which tissues *T. ni* prefers to feed on if given a choice between cabbage entrained in light/dark cycles or constant light, I conducted a choice experiment. Postharvest cabbage was entrained in either 12-hour light/12-hour dark (LD) cycles or constant light (LL) cycles at 22^{o}C for 3 days. Three-day-old *T. ni* entrained with LD

[3] These data have been published (Goodspeed et al., 2013b)

cycles were placed in an enclosure containing samples of both LD and LL entrained cabbage. Cabbage mass loss was then scored following 3 days of insect/cabbage co-incubation under LL. As shown in Figure 36, the LD entrained cabbage lost significantly less mass compared to the LL entrained cabbage, indicating that entrainment of cabbage is advantageous to its defense against herbivory and that *T. ni* prefer to feed on leaf tissue that is not entrained under light/dark cycles.

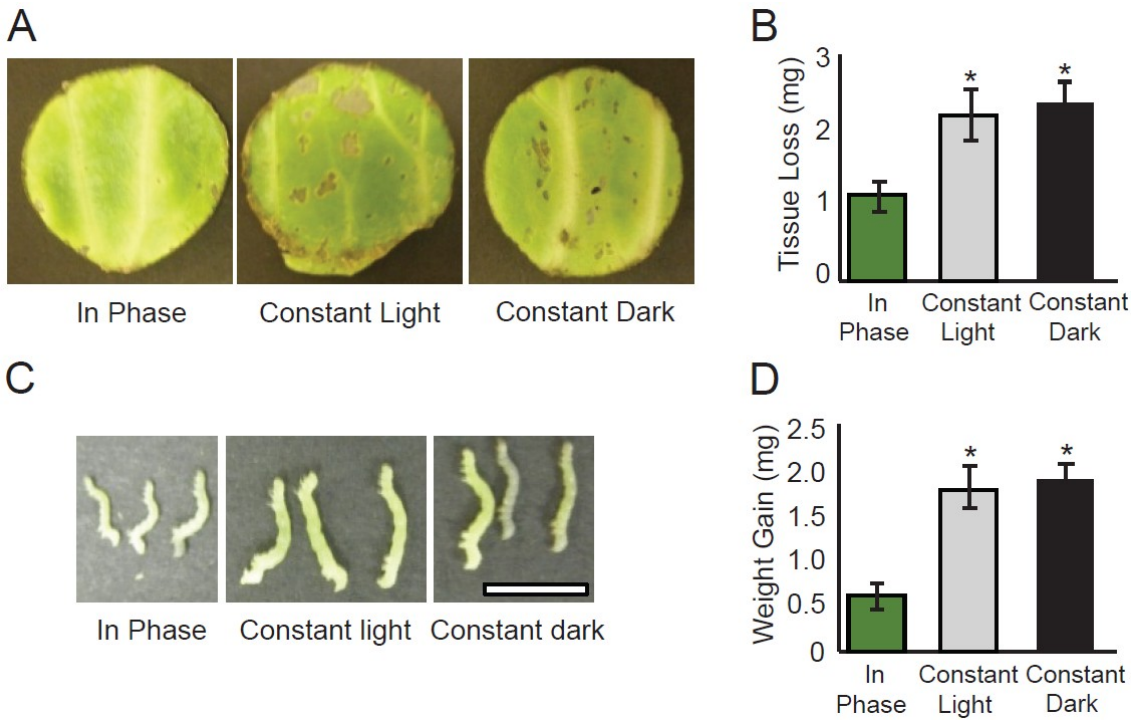

Figure 35: Maintenance of light/dark cycles enhances *T. ni* resistance

(A) Photographs of representative cabbage disks maintained in light/dark cycles in phase with *T. ni* or maintained in constant light or darkness 72 hours after incubation with *T. ni*. Dark spots and holes on disks are sites of herbivory damage. (C) Tissue weight loss from cabbage described in (A). Mean area ± SE; n = 6. *P<0.005, unpaired t test.(C) Photographs of representative of *T. ni* described in (A). Scale bar, 0.5 mm (D) *T. ni* weight gained after 72 hours of coincubation with plants. Mean area ± SE; n = 6; *P<0.005; two-tailed paired t test (Adapted from Goodspeed et al., 2013b.)

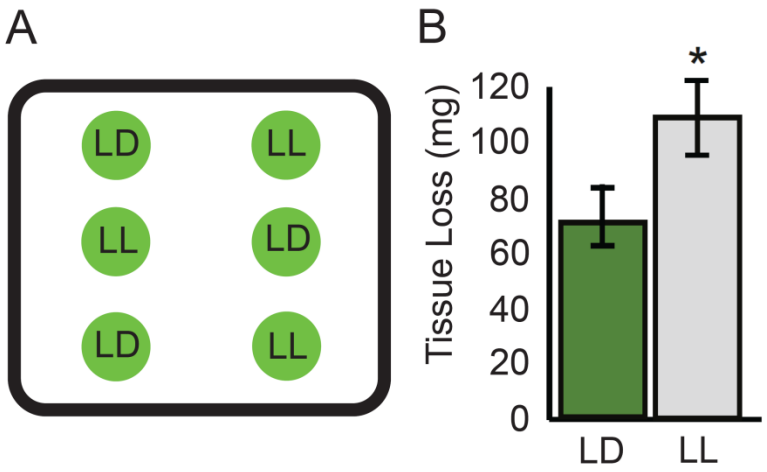

Figure 36: T. ni prefer to feed on cabbage entrained under constant light

(A) Schematic depicting set up for choice experiment. LD, denotes cabbage disk entrained under light/dark cycles. LL, denotes cabbage disk entrained under constant light. Cabbage disks were entrained for 3 days in LD or LL. (B) Tissue weight loss from cabbage disks that were entrained in light/dark cycles in phase with T. ni or maintained in constant light and then provided together to the T. ni as shown in (A) for 72 hours of coincubation, enabling the insects to choose among the different disks. Mean area ± SE; n = 15; *P<0.05; two-tailed paired t test (Adapted from Goodspeed et al., 2013b.)