APPROVAL
SHEET

Title of Thesis: Investigating Jasmonic Acid Reciprocal Regulation of the Circadian Clock in *Arabidopsis thaliana*

Name of Candidate: James Doffermyre

Master of Science, 2020

Thesis and Abstract Approved: 

Hua Lu

Professor

Department of Biological Sciences

Date Approved: 6/24/2020

ABSTRACT

Title: INVESTIGATING JASMONIC ACID RECIPROCAL REGULATION OF THE CIRCADIAN CLOCK IN ARABIDOPSIS THALIANA

James M Doffermyre, Master of Science, 2020

Directed by: Dr. Hua Lu, Professor, Biological Sciences

Recent studies have established that jasmonic acid (JA)-mediated plant defense is regulated by the circadian clock. However, reciprocation of this regulation is unclear. The Lu lab previously revealed that dosages of methyl-jasmonate, a JA agonist, affects clock activity depending on the JA receptor COI1. The question thus becomes; what part of the JA pathway, after JA perception, affects the circadian clock?

JAZ proteins are co-receptors of JA that bind COI1 to negatively regulate JA signaling. MYC2 is a transcription factor which positively regulates JA signaling. Loss-of-function mutations of MYC2 and JAZ genes confer altered JA sensitivity. The goal of this thesis is to use clock-regulated luciferase reporters to assess which step in the JA signaling pathway is involved in clock regulation. Towards this goal, we have two specific aims. Aim 1: Create luciferase reporter constructs as tools for assessing clock activity. Aim 2: Elucidate which stage(s) of the JA pathway participate in a reciprocal signal to the circadian clock. We have successfully created three reporter constructs; one of which shows solid rhythmicity in both transient assays with tobacco and in stable *Arabidopsis* transgenic plants. This construct can be used as an additional tool to investigate clock regulation by the JA pathway. Additionally, JA pathway mutants, jin1-7 (a MYC2

mutant) and jaz10-1 were crossed with plants expressing known clock reporters: the luciferase driven by the CCA1 and the GRP7 promoters respectively. Future experiments will be carried out to test if these mutants show altered clock activity in the presence of JA.

INVESTIGATING JASMONIC ACID RECIPROCAL REGULATION OF THE CIRCADIAN CLOCK IN *ARABIDOPSIS THALIANA*

By

James M. Doffermyre

Thesis submitted to the Faculty of the Graduate School of the University of Maryland, Baltimore County, in partial fulfillment of the requirements for the degree of

Master of Science

2020

ACKNOWLEDGEMENTS

I would like to thank everyone who supported me during this endeavor. I could not have done this without you.

To my god-parents, Marcella and Mark Lasslett, thank you for providing me the stable and safe home I needed for this journey.

To all of my lab mates,
Min Gao, Yubing Yang, Jessica Allison, Matt Fabian, Riki Egoshi, and Jebrail Dempsey, thank you for teaching me everything you know and welcoming me as part of the group.

To my mentor, Dr. Hua Lu, thank you for the opportunity to work in your lab. Thank you for having the patience with me to work through my mistakes. It was a learning experience that you cannot get from a book, and I will cherish it forever.

TABLE OF CONTENTS

LIST OF FIGURES & TABLES

BACKGROUND

Arabidopsis thaliana, the circadian clock, and clock-regulated defenses

Arabidopsis thaliana is the model organism utilized across myriad labs that study plant molecular biology. It is a useful model plant organism for several reasons. It has a short life cycle of approximately 7-8 weeks to plant seeds and harvest new seeds from those plants. Its genome is similar to a lot of common crops including tobacco and tomato. As well, *Arabidopsis* has a relatively small, fully sequenced genome which is easily manipulated for testing, and the full genome sequence is available for free to the public thanks to The *Arabidopsis* Information Resource (TAIR). (Woodward and Bartel 2018, Berardini et al. 2015). The similarity of *Arabidopsis* to other plant species has proven valuable in the efforts to study pathogens which afflict various crops. Indeed the spread of disease across entire crops poses a serious potential threat to the U.S. and world food supplies (Collinge et al. 2010, Schaad et al. 2006). Thus, it is imperative to study the mechanisms by which plants defend themselves and how we can possibly assist them.

One well-characterized control mechanism of plants' abilities to ward off pathogens is their circadian clock. Circadian rhythm is described as an organism's physiological and behavioral responses to cycles of light, temperature and other environmental inputs and such responses often exhibit rhythmic oscillation. Circadian clocks are the actual mechanisms by which an organism responds to environmental cues through the expression of different proteins which are often transcription factors (NIH, 2017).

The circadian clock of *Arabidopsis thaliana* consists of a set of core proteins (e.g. CCA1, LHY and TOC1) that are well characterized and mainly behave as transcriptional activators and/or repressors. These expression-oscillating, core clock proteins maintain regulation of one another through negative feedback loops. One such loop involves CCA1 and LHY which are expressed in the morning and TOC1 which is expressed at night (Hernando, Romanowski and Yanovsky 2017). CCA1 and LHY negatively regulate TOC1 expression, and TOC1 in turn positively regulates expression of CCA1 and LHY. The circadian clock of *Arabidopsis,* of course, is not limited to these three genes, rather it involves many other core clock genes. The circadian clock of *Arabidopsis* is responsible for regulating myriad physiological processes including timing of growth and flowering, hormone signaling, and, most pertinently, biotic and abiotic stress responses (Hernando et al. 2017, Lu, McClung and Zhang 2017, Campos, Kang and Howe 2014).

It is well documented that the circadian clock of *Arabidopsis* controls the ambient expression of defense genes. In order to defend itself against pathogens (such as *Pseudomonas syringae*) and herbivores (such as *Trichoplusia ni*) at different times of day, the plant has evolved a system of preemptively expressing defense genes in a circadian fashion. Expression of the defense hormone jasmonic acid (JA) and related defense genes, such as COI1 and MYC2, peaks in the midday so the plant may mitigate damage caused by those stressors (Lu, McClung and Zhang 2017).

Interestingly, some of the output pathways regulated by the circadian clock, like hormone signaling regulated by JA, can also reciprocally regulate clock activity. However, the mechanism of such reciprocal regulation is not well understood. Therefore, the goal of

this research is to elucidate how JA-mediated defense signaling reciprocates regulation of the circadian clock in the model plant, *Arabidopsis thaliana*.

Jasmonate-mediated pathway for defense

JA is a fatty acid-derived chemical that gives rise to various bioactive jasmonates to serve as phytohormones for signaling many biological processes including growth, flowering time, senescence, freeze tolerance, and defense (Figures 1 & 2) (Campos et al. 2014, Chini et al. 2016, Turner, et al., 2002. Howe et al. 2018). Among the JA derivatives, (+)-7-Iso-jasmonyl-L-isoleucine (JA-Ile) has the most biological activities (Campos et al. 2014, Howe et al. 2018).When a molecular pattern from an herbivore, microbe, or physical damage is recognized by the plant cell, JA synthesis is induced. JA is then converted into JA-Ile in the cytosol and then travels to the nucleus where it binds the receptor COI1 in the SCF^{COI1} protein complex (Figures 1 & 2) (Campos et al. 2014, Howe et al. 2018). Activated SCF^{COI} binds JAZ repressor proteins to catalyze their degradation. Degradation of JAZ proteins frees up transcription factors (e.g. MYC2)

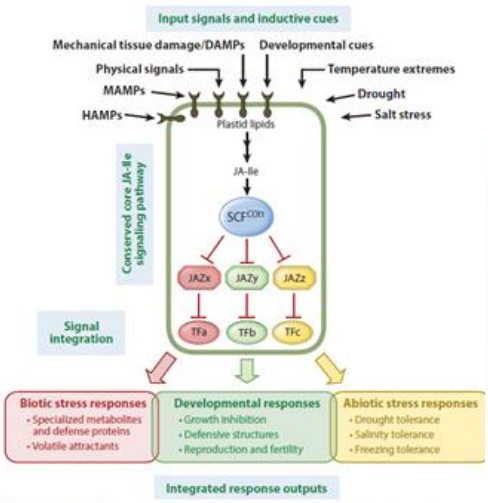

Figure 1 – Schematic of generic conserved JA-Ile signaling pathway (Howe et al., 2018). TFs in the diagram are often the MYC proteins which are of interest in this proposed study.

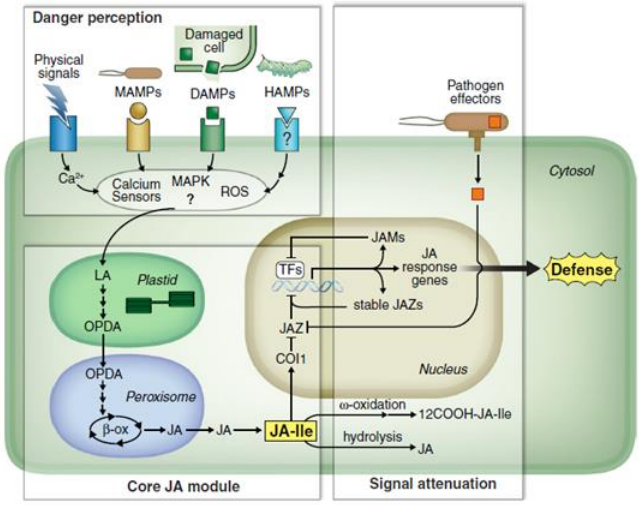

Figure 2 – Schematic of JA biosynthesis pathway (Campos et al., 2014). TFs in the diagram are often the MYC proteins which are of interest in this proposed study.

leading to transcriptional reprogramming of many related genes downstream. (Figures 1 & 2) (Campos et al. 2014). Below, the respective roles of several key parts of the JA signaling pathway are more specifically elaborated.

COI1 is an F-Box protein that recruits other necessary proteins in order to form an SCF^{COI} ubiquitin-ligase complex (Xu et al. 2002, Xie et al. 1998). This SCF^{COI1} complex acts as a co-receptor that binds to JA-Ile, and subsequently binds to the JAZ repressor proteins at the Jas domain (Lorenzo et al. 2004, Pauwels and Goossens 2011). Once these elements all come together, the SCF^{COI} complex catalyzes the interaction between JAZ and ubiquitin-conjugating enzymes. Poly-ubiquitinated JAZ proteins are then targeted for degradation by the 26S proteasome (Kipreos and Pagano 2000) leading to the release of the previously bound MYC protein to initiate transcription and activate the JA response pathway (Figures 1 & 2). Loss-of-function mutant *coi1* confers various JA insensitive phenotypes (Lorenzo et al. 2004). As well, the Lu laboratory has shown that COI1 is required for JA reciprocal regulation of the circadian clock (Zhang et al. 2019). But which downstream step(s) are involved in this reciprocal regulation is still unknown.

MYC family proteins are transcription factors (TFs) that have a JAZ-INTERACTING DOMAIN (JID) which binds the Jas domain of JAZ proteins. MYC2 plays a major role in JA signaling. It is specifically responsible for the positive regulation of genes involved in responding to physical wounds to the plant and negative regulation of genes involved in responding to a bacterial attack (Lorenzo et al. 2004). MYC2 is sometimes referred to in the literature as JIN1 for its mutant phenotype of JA insensitivity. The mutant *jin1* shows significant insensitivity to JAs and appears to grow larger roots than wildtype in the presence of JA chemicals. When subjected to chemical stressors known not to

activate the JA pathway, relative root growth was the same in *jin1* plants as it was in WT plants. *jin1* plants also show down-regulation of genes known to be associated with physical wound response and enhanced resistance to bacterial infection (Lorenzo et al. 2004). Therefore, MYC2/JIN1 has been specifically identified as a regulator of JA-mediated stress and defense responses. *jin1-7* plants were used as one of the mutant background strains for testing possible reciprocal signaling to the clock.

JASMONATE ZIM-DOMAIN (JAZ) family proteins are repressor proteins. There are at least 13 different JAZ proteins that respectively bind TFs or other JAZ proteins (Figure 3) (Howe et al. 2018, Pauwels and Goossens 2011). By binding co-repressors NINJA and TOPLESS, or even making JAZ dimers, JAZ repressors bind MYC proteins and prevent them from binding the mediator complex for transcription initiation. De-repression of MYC TFs happens by the catalyzed polyubiquitination and degradation of JAZ proteins (Lorenzo et al. 2004, An et al. 2017, Campos et al. 2014, Howe et al. 2018, Thines et al. 2007, Pauwels and Goossens 2011).

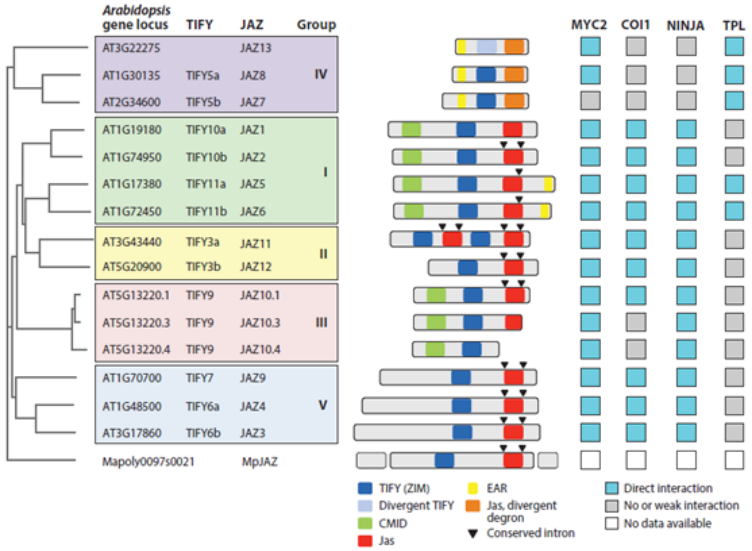

Figure 3 – Phylogenetic tree of JAZ protein family. Diagram depicts conserved functional domains throughout the protein family, and a well-characterized list of proteins that JAZ interact with (Howe et al., 2018).

JA pathway may control some aspect of the circadian clock

Innate immune responses are important for plants, and the antagonistic relationship between plant defense and plant growth/reproduction is well documented (Howe et al. 2018). Thus, in order for the plant to prioritize defense over growth, other genes need to be temporarily silenced or else the plant will not be able to properly allocate resources. Perhaps some of the genes that are down-regulated are circadian clock genes. To establish precedence for reciprocal signaling from the JA pathway to the circadian clock,

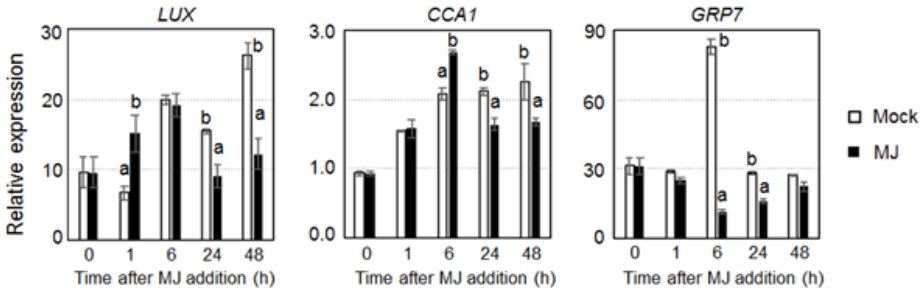

Figure 4 – RNA qRT-PCR analysis of *Arabidopsis* clock gene expression after treatment with methyl jasmonate. Figure shows that LUX and CCA1 are up-regulated and then down-regulated, while GRP7 is outright down-regulated. This indicates that the JA signal pathway likely has some control over the expression of clock genes (Zhang et al, 2019).

Zhang et al. (2019) showed, through transient assay analysis (Figure 4), that when wildtype plants are treated with methyl-jasmonate (MJ), expression of clock genes LUX, CCA1, and GRP7 was suppressed. Zhang et al. (2019) further monitored the luciferase reporter expression that was under the control of the CCA1 gene promoter (CCA1:LUC) with MJ treatment. They showed that period, phase and overall rhythmicity were not affected, but the amplitude of CCA1 expression was significantly reduced. Furthermore, Zhang et al. (2019) showed that in the *coi1-17* background, the period and amplitude of expression of clock gene GRP7 is not altered. Thus, it was established that JA signaling reciprocally regulates the circadian clock, depending on the JA receptor COI1. However, it is unknown what specific downstream component(s) of the JA signaling pathway are

involved in this reciprocal regulation. One specific aim of this thesis is to elucidate the molecular components in the JA signaling pathway that is involved in clock regulation.

Genes of interest for study of reciprocal regulation

CCA1 is a core clock oscillator gene that transcriptionally regulates the expression of many genes in *Arabidopsis*, including some defense genes (Hernando et al. 2017, Lu et al. 2017). Previous studies in the Lu lab have demonstrated that CCA1 has its expression altered when JA-Ile is present. When MJ is added, the rhythmicity of CCA1 expression is not significantly altered, but the amplitude is significantly changed (Figure 4) (Zhang et al., 2019). Thus, CCA1 one is a prime candidate for study as a target for reciprocal regulation by the JA signaling network.

We were also interested in three other gene promoters, LUX ARRHYTHMO (LUX), PHT4;1, and CPR5 to test if their expression was circadian clock and JA-regulated. The reasons for choosing these gene promoters are outlined below.

LUX ARRHYTHMO (LUX) is a Myb transcription factor (similar to CCA1) that is part of the evening cycle of the core circadian clock in *Arabidopsis*. It is a transcription factor that directly regulates expression of several other members of the core clock, including GI, LNK1, PRR7, PRR9, CCA1, LHY, and LUX itself (Hazen et al. 2005). Importantly, LUX binds the promoter region of JAZ5 (Figure 5) (Zhang et al., 2018), likely as a transcription inhibitor. This makes LUX a key contributor to the JA-mediated defense pathway and a possible target of regulatory reciprocation.

PHT4;1 is a phosphate transporter important for salicylic acid-mediated defense (Wang et al. 2011, Wang et al. 2014). Wang et al. (2014a) demonstrated that expression of PHT4;1 was positively regulated by CCA1. EMSA assays show that CCA1 directly binds to the promoter region of PHT4;1. As well, PHT4;1 is expressed in a circadian manner (Figure 6) (Wang et al. 2014, Wang et al. 2011). Thus, any change in CCA1 expression should have affected PHT4;1 expression.

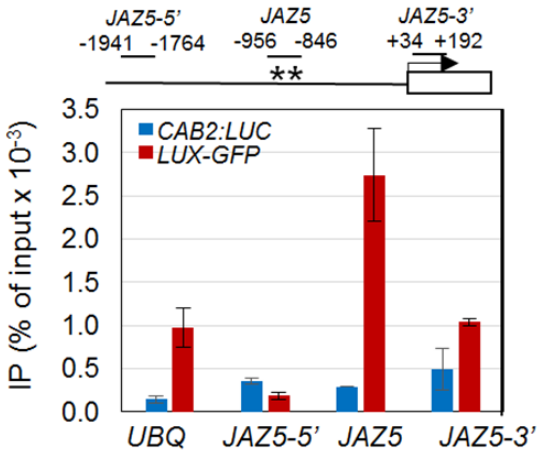

Figure 5 – Chromatin immunoprecipitation of LUX and JAZ5 promoter. LUX binds to the promoter region of JAZ5, and likely acts as a transcription inhibitor (Zhang et al., 2019).

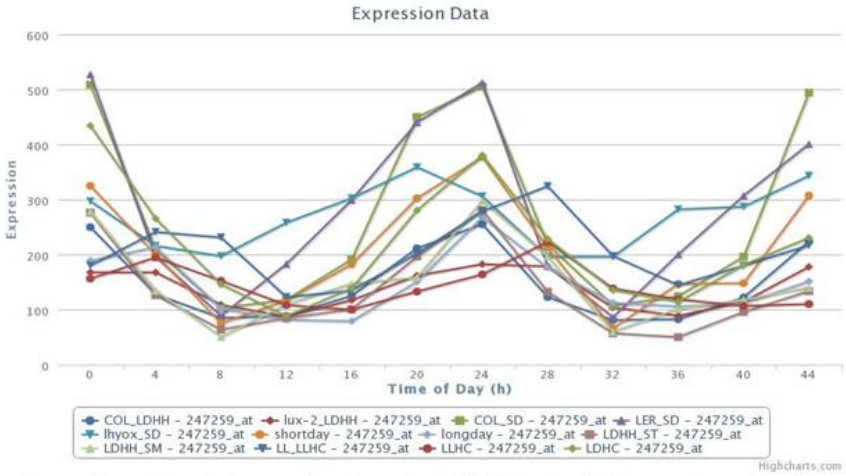

Figure 6 – *in silico* clock assay data show that PHT4;1 has rhythmic expression when the plant is under normal conditions (Mocklerlab.org).

CPR5 (***c****onstitutively* expressed member of ***p****athogenesis* ***r****elated* (PR) *protein*) (Jing and Dijkwel 2008), is a regulator of programmed cell death and cell reproduction (Kirik et al. 2001). CPR5 also plays a role in cell wall biogenesis (Brininstool et al. 2008). As

rebuilding the cell wall is a response to herbivory, CPR5 is likely controlled by the JA pathway. Preliminary data from computer simulations show CPR5 is expressed in a circadian rhythmic manner when the plant is not under attack (Figure 7) (Mocklerlab.org). Thus, if CPR5 has its expression altered, this would indicate that the circadian basis for its normal expression has been interrupted.

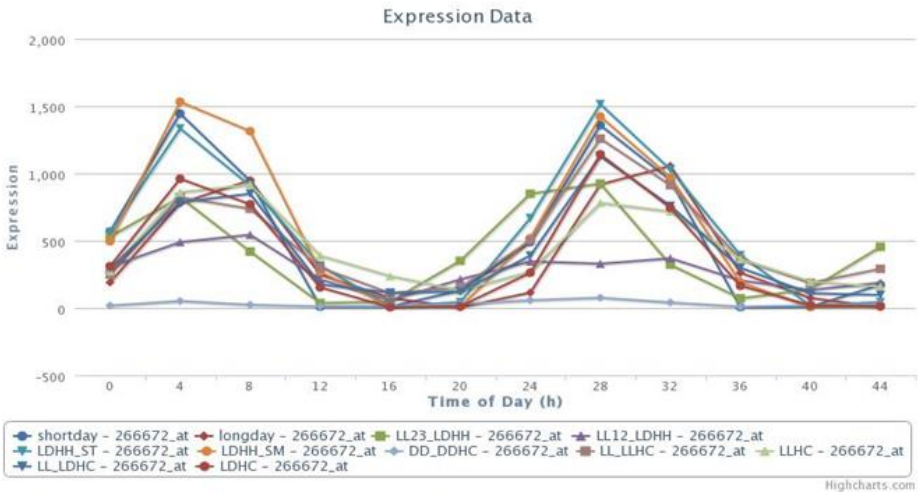

Figure 7 – *in silico* Clock assay data show that CPR5 has rhythmic expression when the plant is under normal conditions (Mocklerlab.org).

Figures 6 and 7 were obtained from using an online database and simulation software at Mocklerlab.org. Refer to the following explanation of the legends. COL: Col-0, wildtype plants, LER: Ler-0, wildtype plants, lhyox: Overexpression of LHY gene, lux-2: Non-functioning mutant of LUX gene ,shortday: 6 hours light and 18 hours dark, longday: 18 hours light and 6 hours dark, LDHH: 12/12 hour, light/dark, 24 hour warm (28°C), LLHC: 24 hour light, 12/12 hour warm (28°C) and cold (12°C), LDHC: 12/12 hour light/dark and 12/12/ hour warm/cold, DDHC: 24 hour dark, 12/12 hour warm and cold.

Use of luciferase reporters to assess reciprocal regulation by JA

In order to detect whether reciprocal regulation of the circadian clock happens by the JA pathway, we used luciferase reporters under the control of clock gene promoters in a luciferase assay. Our lab uses firefly luciferase because one enzyme yields only one reaction with luciferin (Gould and Subramani 1988). Whenever the promoter of a gene of interest is being read by transcription machinery in the plant, so too should our reporter construct, allowing simultaneous expression of our gene of interest and luciferase enzyme. Assuming there is no lag between transcription and translation of luciferase, the fact that one enzyme causes one light-producing reaction enables us to detect expression of a gene in question in real time.

During this research, we created three luciferase (LUC) reporters including PHT4;1:LUC, LUX:LUC, and CPR5:LUC. One of these constructs, PHT4;1:LUC, showed robust rhythmicity in a transient assay with tobacco leaves. We further made stable transgenic plants expressing PHT4;1:LUC and a LUC assay revealed strong rhythmicity of the reporter in *Arabidopsis*. In addition, JA mutants with impaired MYC2 gene (*jin1-7*) and JAZ10 (*jaz10-1*) were crossed with two known clock reporters, CCA1:LUC and

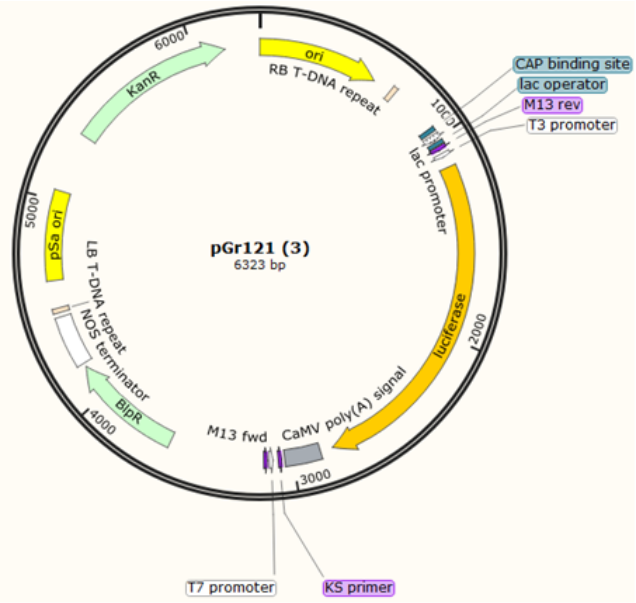

Figure 8 – Vector map of pGr121. pGr121 confers resistance to the antibiotic kanamycin, the herbicide glufosinate, and contains the gene for firefly luciferase with a multiple restriction cloning site immediately upstream.

GRP7:LUC. We have screened for homozygous mutant and transgene in the F2 populations. Future experiments will be conducted with these JA mutants expressing clock reporters to assess the clock activity under normal and JA-stressed conditions in order to elucidate which signal component(s) of JA signaling are involved in clock regulation.

METHODS

Creation of luciferase reporter constructs

To fuse *Arabidopsis* clock gene promoters and the gene for firefly luciferase, we used a derivative of the pGreen plasmid named pGr121, which contains the gene for luciferase and a multiple restriction cloning site immediately upstream of luciferase. pGr121 confers resistance to the herbicide glufosinate in plants and confers resistance to the antibiotic kanamycin in bacteria (Hellens et al. 2000) (Figure 8). We used PCR to amplify the promoter regions for genes of interest, and inserted the promoter fragments into the pGr1212 vector immediately upstream of the luciferase gene.

The promoter regions were defined as the section of an *Arabidopsis* chromosome 1500 base pairs (bp) upstream from the start codon of a gene of interest. The complementary sequence of approximately the first 20 bases immediately upstream from the start codon were used to create the reverse primer for PCR, and the forward primer was created approximately 1500 bases upstream from the start codon with an emphasis on matching annealing temperatures and a C-G anchor. In order to facilitate the ligation process, primers were given restriction enzyme cut sites on their 5' ends so that the amplicon would be created with restriction sites on both ends. PCR primers were designed accordingly for the genes PHT4;1, LUX and CPR5. The restriction enzymes we chose were NotI and PstI. The sequences for all the primer pairs can be found in the Table of Primers.

The promoter amplicons and plasmid vector were digested with restriction enzymes NotI and PstI in separate reactions. The digested products were purified from electrophoresis gel and ligated together. This method was successful in assembling the first construct we worked on, the LUX:LUC construct, however, it yielded almost prohibitively low concentrations of DNA to perform ligation. We switched to an "in-gel" ligation technique to assemble the PHT4;1:LUC and CPR5:LUC constructs which was more successful.

A sample of each ligation product was transformed by heat-shock to *E.coli*, and then plated on nutrient agar plates with kanamycin for selection. The bacteria incubated at 37°C overnight, and multiple colonies were picked and streaked on a new plate to preserve them. The re-streaked bacteria were genotyped by PCR to make sure they contained the desired reporter construct. For bacteria with a positive genotyping result, we cultured and miniprepped them to obtain the plasmids. Samples of those plasmids were digested with NotI and PstI to confirm the insertion (Figure 9). Then they were sent to Macrogen Inc. for sequencing to confirm successful assembly. Once a

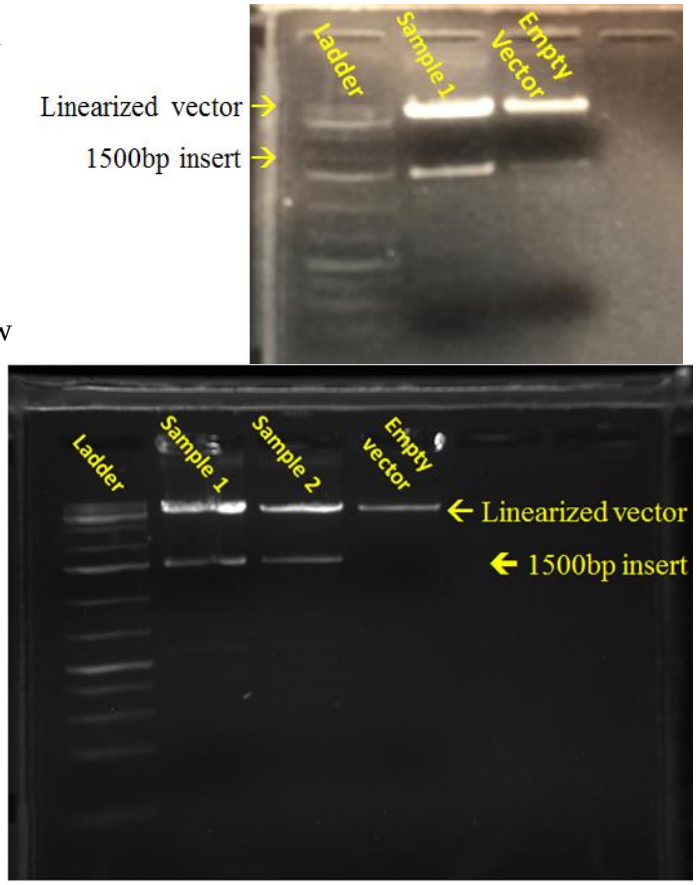

Figure 9 – Completed reporter constructs were digested with restriction enzymes NotI and PstI to confirm that the promoter regions of respective genes were inserted into the multiple cloning site on pGr121. Image of gel shows the release of the 1500bp insert and linearized pGr121, 6300bp. Images from restriction digest results confirm the successful creation of PHT4;1 (top) and LUX (bottom) reporter constructs

reporter construct assembly was confirmed, it was transformed into *Agrobacteria tumefaciens* for further analyses.

Agrobacteria infiltration of tobacco leaves for luciferase assay

Agrobacteria cultures (OD600 of 0.8-1.0) containing different reporters were grown overnight. 1 ml culture was collected for centrifugation at 8,000g for 3 min, re-suspended in 4 ml of inducing media, and grown at 30°C for 3-4 hours. The culture was collected for centrifugation followed by resuspension in infiltration medium to an OD of 0.1-0.4. The bacterial suspension was injected into expanding tobacco leaves by gentle pressure infiltration through the abaxial epidermis, using a 1-ml plastic syringe without a needle (1-2 ml per leaf). The infusion process can be monitored visually by observing a spread of opacity in the leaf as the bacterial suspension fills airspaces. Infused areas were subsequently outlined with a marker. Plants were transferred back to the growth chamber with 180 µmol m^{-2} s^{-1} photo flux density, 60% humidity, 22°C, and a 12 h light/12 h dark (LD) cycle. After 24 hours leaf discs were excised from leaves from the infiltrated areas using a puncher. 12 discs were taken for each construct and floated on 200µl water containing 0.25mM D-luciferin in a 96-well plate. Luciferase activity was measured at 1-h intervals for three days with an Omega Luminescence Reader (BMG LABTECH, Inc.) in LL with 90 µmol m^{-2} s^{-1} photon flux density.

Creating stable transgenic lines of Arabidopsis carrying luciferase reporter constructs

In order to create stable transgenic *Arabidopsis* plants, ecotype Columbia-0 (Col-0) plants were grown in a chamber that maintains 22°C with a 12/12 hour light/dark cycle. When flowers began to blossom, designated plants' flowers were dipped in a culture of

Agrobacteria carrying one the reporter constructs for transfection. Transfected plants' seeds were collected two weeks after *Agrobacteria* infection and deemed generation T_0. T_0 seeds were planted and grown for about 3 weeks and sprayed with the herbicide BASTA. Plasmid pGr121 confers resistance to BASTA. Surviving plants' seeds were collected, and deemed the T_1 generation with an expected genotype ratio of 1:2:1 (homo trans-hetero-homo WT). Young T_1 plants were again sprayed with BASTA and genotyped by PCR of leaf tissue extract. Homozygotes for the transgene were selected and all other plants discarded. Seeds were collected from these homozygotes and these were deemed the T_2 generation. So far, we have obtained two independent *Arabidopsis* transgenic lines expressing the PHT4;1:LUC reporter.

Assessment of clock activity in stable transgenic Arabidopsis plants

Luciferase assays were used to assess the clock activity in these transgenic plants. Seeds of T_2 plants were grown in ½ MS media with 1% sucrose in (LD) light conditions at 22°C for five days. Seedlings were transferred to a 96-well plate, each seedling in their own well, with 200µL of a solution of ½ MS media, 0.5% sucrose, 0.4% agar and 0.25mM D-luciferin for one day in LD followed by one day in long light (LL). The plants were measured for luminescence with an Omega Luminescence Reader (BMG LABTECH, Inc.) in LL with 90 µmol m^{-2} s^{-1} photon flux density. Luciferase activity was measured at 1-hour intervals for five days, and analyzed for amplitude, period and phase with the R package MetaCycle (Wu et al. 2016).

RESULTS

Luciferase assay results in tobacco and Arabidopsis

Tobacco is a heterologous plant system that has been commonly used to provide quick assessment of gene expression in an *Agrobacterium*-mediated transient assay. We set out to use this system to test the three constructs. After infiltrating the *Agrobacterium* strain into tobacco leaves, we collected leaf discs for luciferase assay. Our data show that only PHT4;1:LUC gave luminescence in tobacco. The LUX:LUC and CPR5:LUC constructs have not shown any luminescence (Figure 10). The lack of detection of luminescence of the LUX:LUC and CPR5:LUC constructs suggests that the constructs do not function in tobacco. It is also possible that the promoters we used require additional cis-elements to be functional.

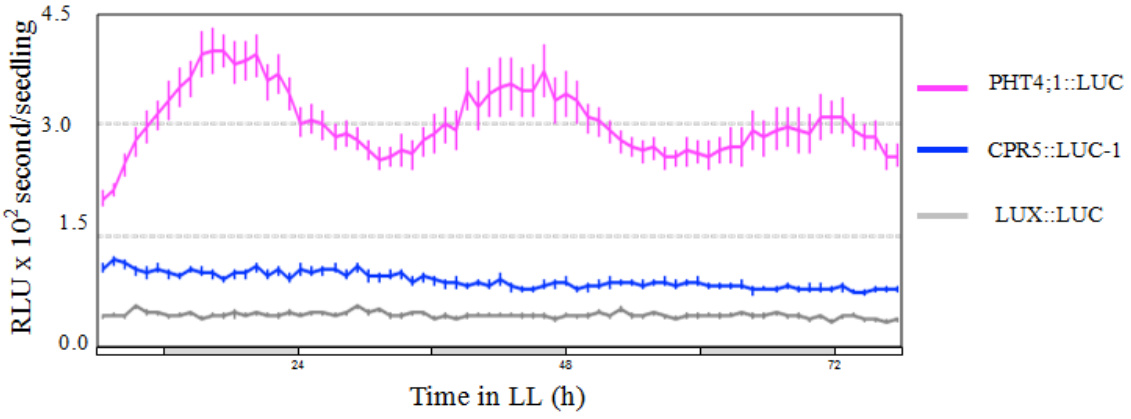

Figure 10 – Transient tobacco assay shows rhythmic expression of PHT4;1 over 72 hour program which indicates the reporter construct works as desired. Lack of luciferase detection for LUX and CPR5 may indicate that the constructs do not work in tobacco. Graphs show average of 12 samples (one row on 96-well plate) for each genotype gathered from the luciferase assay data.

To address these problems, we further made stable transgenic Arabidopsis plants via *Agrobacterium*-mediated transformation. We obtained two independent lines expressing the PHT4;1:LUC reporter, from which we further obtained homozygous seeds after two generations. Our luciferase assays with these two homozygous transgenic seedlings showed robust rhythmic expression of PHT4;1:LUC reporter (Figure 11). Compared with a known reporter CCA1:LUC, the PHT4;1:LUC reporter had lower amplitude, a similar period of about 24 hours and a lagging phase at 7.5 hours after the light is on. These data suggest that different clock reporters may be functioning simultaneously with different rhythms in the same tissue or even the same cell.

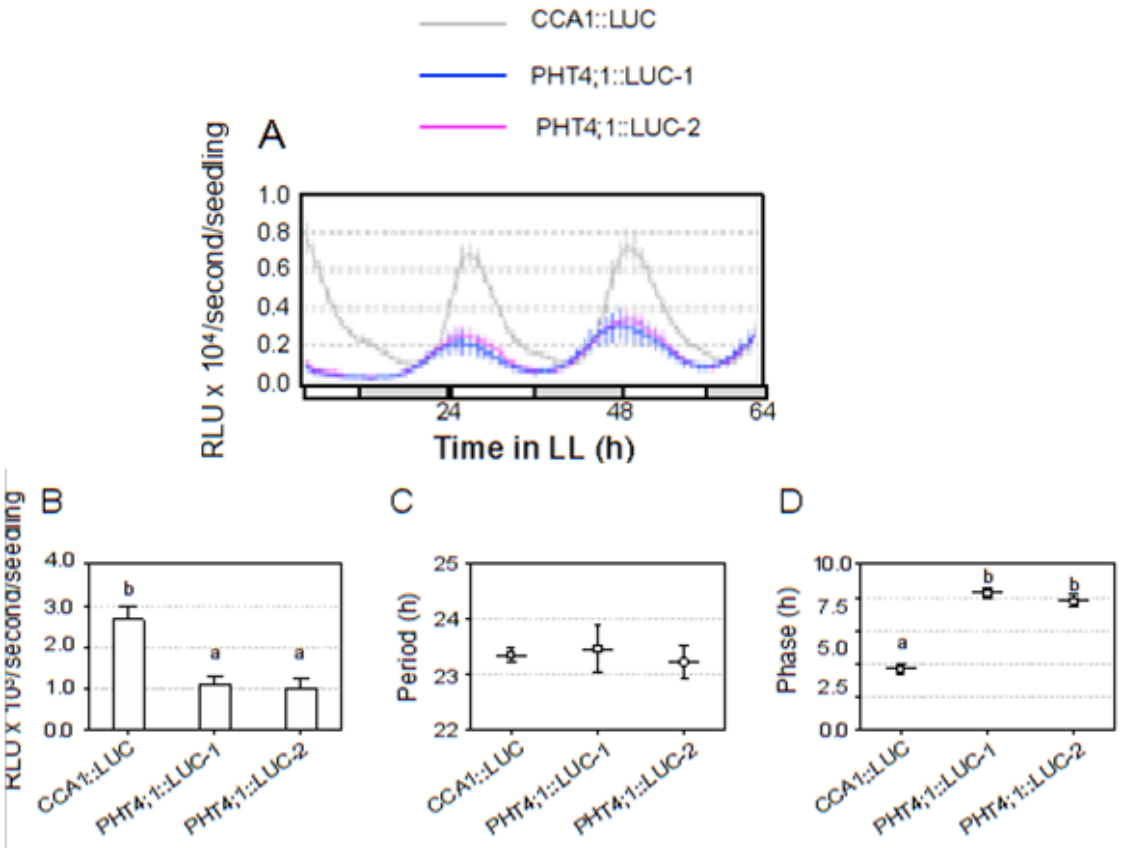

Figure 11 – Luciferase assay shows the rhythmic expression of PHT4;1:LUC reporter in two homozygous transgenic plants. CCA1 is used as control. Compared with a known reporter CCA1:LUC, the PHT4;1:LUC reporter had lower amplitude, a similar period of about 24 hours and a lagging phase at 7.5 hours after the light is on.

Elucidating which stage(s) of the JA pathway participate in a reciprocal signal to the circadian clock

To test which stage(s) of the JA pathway participate in a reciprocal signal to the circadian clock, we crossed *jin1-7* and *jaz10-1*, mutations disrupting *MYC2* and *JAZ10* genes respectively, with known reporters CCA1:LUC or GRP7:LUC. The F1 plants were allowed to make F2 seeds. We grew the F2 seeds and screened for homozygous mutants that also carried one of the reporters using the mutant-specific primers (Table of Primers). We have successfully obtained homozygous *jaz10-1* mutants expressing CCA1:LUC or GRP7:LUC reporter, and homozygous *jin1-7* mutants expressing CCA1:LUC or GRP7:LUC reporter. These plants will be used together with wildtype plants expressing CCA1:LUC or GRP7:LUC reporter in clock assays to assess how JA signaling affects the circadian clock.

DISCUSSION

With the progress of this project, we were able to show successful assembly of luciferase reporter constructs for genes LUX, PHT4;1 and CPR5. We were only able to show that the PHT4;1:LUC construct is functional in tobacco and *Arabidopsis*. However, we were not able to show the functionality of the LUX:LUC and CPR5:LUC reporters in both systems. We suspect that the lack of function of these two constructs is possibly due to the missing of some key promoter elements on the constructs. With the PHT4;1:LUC reporter successfully created, we will be able to assess when and how much of this gene is expressed, and how its rhythm is affected by JA signaling and pathogen invasion. We also successfully created JA signaling mutants expressing clock reporters CCA1:LUC or GRP7:LUC. These materials will be used for clock assays to assess the function of JA in regulating clock activity.

One potential reason for the CPR5:LUC construct not functioning in tobacco is that the cloned promoter has one point mutation approximately 1000 bases upstream of the start codon. We will attempt the process again to produce a clone with 100% fidelity. The LUX reporter construct did not have any mutations, according to our sequencing data. It is also possible that the promoter for LUX and/or CPR5 from *Arabidopsis* cannot be read by tobacco transcription machinery. If this is indeed the case, then stable transgenic lines of *Arabidopsis* carrying CPR5:LUC and LUX:LUC respectively should indicate the functionality of the reporter constructs. The failed attempts to generate stable Arabidopsis transgenic plants expressing these two constructs could be due to some technical issues

involved in transformation. Thus, we will continue attempting to transfect flowering Col-0 plants with *Agrobacteria* carrying these respective constructs, in order to create said stable transgenic lines.

FUTURE EXPERIMENTS

One of the original aims of this project, but which could not be finished due to time constraints was to test some of the reporter systems with exogenous JA to see how defense signal activation could affect the rhythm of the circadian clock. The materials to be used in the future clock assay include Col-0 expressing PHT4;1:LUC, *jaz10-1* expressing CCA1:LUC or GRP7:LUC, and *jin1-7* expressing CCA1:LUC or GRP7:LUC. We will perform standard clock assays involving these materials, using JA and mock treatments. In addition, we can challenge these plants with pathogen infection to further elucidate the role of defense activation in reciprocal clock regulation.

We will create stable transgenic lines of *jin1*-7 and *jaz10*-1 mutant plants carrying each respective reporter; CPR5:LUC, PHT4;1:LUC, and LUX:LUC, as well as Col-0 wildtype plants expressing those same reporters. We will examine the expression patterns of the clock gene LUX and defense genes PHT4;1 and CPR5 under normal conditions and stress conditions simulated by exogenous application of JA.

Our expected results from these experiments are as follows. In Col-0 plants with no exogenous JA, we would expect to see normal, circadian rhythmic expression of all genes in question. In Col-0 plants that have been given exogenous JA we expect to see a disruption of overall expression and the rhythmicity of expression of clock genes and clock-regulated defense genes. This disruption to gene expression in Col-0 plants would serve as the reference point for when mutant genes of the JA pathway are tested. If we apply exogenous JA to *jin1-7* and/or *jaz10-1* plants and expression of clock genes

increases significantly higher than what is seen in Col-0 plants treated with JA, then that would indicate that either MYC2 and/or JAZ10 are involved in reciprocating the signal to the clock along with COI1 as demonstrated by Zhang et. al (2019). But if we see no significant difference in luciferase activity between JA mutant plants and Col-0 plants upon exogenous JA application, then it would indicate that the JA mutants in question are not part of the reciprocation signal.

If it turns out that JAZ10 and/or MYC2 are involved in the reciprocal signal from the JA pathway to the circadian clock, then the clock gene targets of the JA genes would need to be identified. This could be accomplished with chromatin immunoprecipitation by MYC2 antibodies followed by high throughput sequencing (ChIP-Seq). Additionally, growth and survivability assays can be performed on JA mutant plants to assess the importance of the role of reciprocal regulation between the JA pathway and circadian clock.

APPENDIX

Table of Primers for PCR processes

Name	Sequence	Purpose
CRP5Pro_Not1_F	CAAAGCGGCCGCAAGAATTTAGACAAAATGAG	Forward primer for cloning CPR5 promoter
CPR5Pro_Pst1_R	GTTTCTGCAGAGCCTTAAAATATAACTTATGGGG	Reverse primer for cloning CPR5 promoter
LUXPro_Not1_F	AAAAGCGGCCGCGTTCAAGTAAACTGAAGATATATAC	Forward primer for cloning LUX promoter
LUXPro_Pst1_R	AAAACTGCAGTTCAAACTCTCTAATTTCTCG	Reverse primer for cloning LUX promoter
PHT4;1Pro_Not1_F	CAAAGCGGCCGCAAATAAAATAATAGAGAAAGG	Forward primer for cloning PHT4;1 promoter
PHT4;1Pro_Pst1_R	GTTTCTGCAGGGCGGAGATAGATTTTTTCTTC	Reverse primer for cloning PHT4;1 promoter
LUC Reverse	GCTTCTGCCAACCGAACGGACA	Reverse primer to genotype presence of luciferase gene
LUX-ChIP-f2	GGGGAAATCTCAGAGAATCAG	Forward primer to genotype presence of LUX promoter
SUP3-EMSA-f1	TTGTTATTGGTATTGCCGTATTATTGTA	Forward primer to genotype presence of PHT4;1 promoter
CPR5pro-493	CGGAGCCACAAACATTGTGCG	Forward primer to genotype presence of CPR5 promoter
LBa1	TGGTTCACGTAGTGGGCCATCG	Forward primer genotype *jin1-7* mutant plant
AT1G32640g2539R	CTTTACCAGCTAATCCCGCAC	Reverse primer genotype *jin1-7* mutant plant
SAIL LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC	Reverse primer genotype *jaz10-1* mutant plant
AT5G13220g2200F	GTAGTTTCCGAGGTTCGTTTGG	Forward primer genotype *jaz10-1* mutant plant

REFERENCES

An, C. P., L. Li, Q. Z. Zhai, Y. R. You, L. Deng, F. M. Wu, R. Chen, H. L. Jiang, H. Wang, Q. Chen & C. Y. Li (2017) Mediator subunit MED25 links the jasmonate receptor to transcriptionally active chromatin. *Proceedings of the National Academy of Sciences of the United States of America,* 114**,** E8930-E8939.

Berardini, T. Z., L. Reiser, D. H. Li, Y. Mezheritsky, R. Muller, E. Strait & E. Huala (2015) The arabidopsis information resource: Making and mining the "gold standard" annotated reference plant genome. *Genesis,* 53**,** 474-485.

Bernhardt, K., S. K. Vigelius, J. Wiese, N. Linka & A. P. M. Weber (2012) Agrobacterium-mediated Arabidopsis thaliana transformation: an overview of T-DNA binary vectors, floral dip and screening for homozygous lines. *Endocytobiosis & Cell Research,* 22**,** 19-28.

Brininstool, G., R. Kasili, L. A. Simmons, V. Kirik, M. Hulskamp & J. C. Larkin (2008) Constitutive Expressor of Pathogenesis-Related Genes5 affects cell wall biogenesis and trichome development. *Bmc Plant Biology,* 8**,** 11.

Campos, M. L., J. H. Kang & G. A. Howe (2014) Jasmonate-Triggered Plant Immunity. *Journal of Chemical Ecology,* 40**,** 657-675.

Chini, A., S. Gimenez-Ibanez, A. Goossens & R. Solano (2016) Redundancy and specificity in jasmonate signalling. *Current Opinion in Plant Biology,* 33**,** 147-156.

Collinge, D. B., H. J. L. Jorgensen, O. S. Lund & M. F. Lyngkjaer. 2010. Engineering Pathogen Resistance in Crop Plants: Current Trends and Future Prospects. In *Annual Review of Phytopathology, Vol 48,* eds. N. K. VanAlfen, G. Bruening & J. E. Leach, 269-291. Palo Alto: Annual Reviews.

Guo, Q., Y. Yoshida, I. T. Major, K. Wang, K. Sugimoto, G. Kapali, N. E. Havko, C. Benning & G. A. Howe (2018) JAZ repressors of metabolic defense promote growth and reproductive fitness in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America,* 115**,** E10768-E10777.

Hazen, S. P., T. F. Schultz, J. L. Pruneda-Paz, J. O. Borevitz, J. R. Ecker & S. A. Kay (2005) LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 10387-10392.

Hellens, R. P., E. A. Edwards, N. R. Leyland, S. Bean & P. M. Mullineaux (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant Molecular Biology,* 42**,** 819-832.

Hernando, C. E., A. Romanowski & M. J. Yanovsky (2017) Transcriptional and post-transcriptional control of the plant circadian gene regulatory network. *Biochimica et Biophysica Acta-Gene Regulatory Mechanisms,* 1860**,** 84-94.

Hevia, M. A., P. Canessa & L. F. Larrondo (2016) Circadian clocks and the regulation of virulence in fungi: Getting up to speed. *Seminars in Cell & Developmental Biology,* 57**,** 147-155.

Howe, G. A., I. T. Major & A. J. Koo. 2018. Modularity in Jasmonate Signaling for Multistress Resilience. In *Annual Review of Plant Biology, Vol 69,* ed. S. S. Merchant, 387-415. Palo Alto: Annual Reviews.

Jing, H. C. & P. P. Dijkwel (2008) CPR5: A Jack of all trades in plants. *Plant Signal Behav,* 3**,** 562-3.

Kipreos, E. T. & M. Pagano (2000) The F-box protein family. *Genome Biology,* 1**,** 7.

Kirik, V., D. Bouyer, U. Schobinger, N. Bechtold, M. Herzog, J. M. Bonneville & M. Hulskamp (2001) CPR5 is involved in cell proliferation and cell death control and encodes a novel trans membrane protein. *Current Biology,* 11**,** 1891-1895.

Lorenzo, O., J. M. Chico, J. J. Sanchez-Serrano & R. Solano (2004) Jasmonate-insensitive1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell,* 16**,** 1938-1950.

Lu, H., C. R. McClung & C. Zhang. 2017. Tick Tock: Circadian Regulation of Plant Innate Immunity. In *Annual Review of Phytopathology, Vol 55,* eds. J. E. Leach & S. E. Lindow, 287-311. Palo Alto: Annual Reviews.

NIH (August 2017). Circadian Rhythms. https://www.nigms.nih.gov/education/pages/Factsheet_CircadianRhythms.aspx

Pauwels, L. & A. Goossens (2011) The JAZ Proteins: A Crucial Interface in the Jasmonate Signaling Cascade. *Plant Cell,* 23**,** 3089-3100.

Schaad, N. W., J. Abrams, L. V. Madden, R. D. Frederick, D. G. Luster, V. D. Damsteegt & A. K. Vidaver (2006) An assessment model for rating high-threat crop pathogens. *Phytopathology,* 96**,** 616-621.

Thines, B., L. Katsir, M. Melotto, Y. Niu, A. Mandaokar, G. H. Liu, K. Nomura, S. Y. He, G. A. Howe & J. Browse (2007) JAZ repressor proteins are targets of the SCFCO11 complex during jasmonate signalling. *Nature,* 448**,** 661-U2.

Turner, J. G., C. Ellis & A. Devoto (2002) The jasmonate signal pathway. *Plant Cell,* 14**,** S153-S164.

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

Wang, G. Y., C. Zhang, S. Battle & H. Lu (2014) The phosphate transporter PHT4;1 is a salicylic acid regulator likely controlled by the circadian clock protein CCA1. *Frontiers in Plant Science,* 5**,** 10.

Woodward, A. W. & B. Bartel (2018) Biology in Bloom: A Primer on the Arabidopsis thaliana Model System. *Genetics,* 208**,** 1337-1349.

Wu, G., R. C. Anafi, M. E. Hughes, K. Kornacker & J. B. Hogenesch (2016) MetaCycle: an integrated R package to evaluate periodicity in large scale data. *Bioinformatics,* 32**,** 3351-3353.

Xie, D. X., B. F. Feys, S. James, M. Nieto-Rostro & J. G. Turner (1998) COI1: An Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science,* 280**,** 1091-1094.

Xu, L. H., F. Q. Liu, E. Lechner, P. Genschik, W. L. Crosby, H. Ma, W. Peng, D. F. Huang & D. X. Xie (2002) The SCFCOl1 ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. *Plant Cell,* 14**,** 1919-1935.

Zhang, C., M. Gao, N. C. Seitz, W. Angel, A. Hallworth, L. Wiratan, O. Darwish, N. Alkharouf, T. Dawit, D. Lin, R. Egoshi, X. P. Wang, C. R. McClung & H. Lu (2019) LUX ARRHYTHMO mediates crosstalk between the circadian clock and defense in Arabidopsis. *Nature Communications,* 10**,** 14.