

ABSTRACT

DUAN, JIAQI. Time of day Heat Stress Response in *Arabidopsis thaliana*. (Under the Guidance of Dr. Colleen Doherty.)

Abiotic stress presents a great threat to plant and crop health, and is one of the contributors to reduction in agricultural production. Heat stress is one of the major abiotic stresses that can inhibit normal plant growth and function. The circadian clock is an essential mechanism that regulates many major biochemical processes in higher order organisms including plants. There has been evidence to suggest that the circadian clock regulates abiotic stresses such as drought, cold and UVB in plants. Therefore, I examined how time of day modulates heat stress by comparing at the transcriptional profile in response to heat stress at two times of day in *Arabidopsis thaliana* using RNA sequencing analysis.

RNA sequencing analysis revealed that many genes were activated by moderate heat stress in a time of day dependent manner, and generated different transcriptional expression patterns. Many genes known to respond to heat shock showed a response to heat both in the morning and at night, such as MBF1c. Those genes also included the majority of the heat shock proteins (HSPs) and other stress related proteins. Many of them had higher response in the morning to a different degree. There were also HSP genes that responded only to heat in the morning, such as the small HSP genes HSP21, HSP20, HSP18.2 and HSP17.6. Some of the heat shock proteins also had higher response at night to heat, such as HsfA7B and HSP40.

Based on the results from RNA sequencing, the time of day did have an effect on how plants responded to heat stress. Plants responded to heat stress generated three

major distinct transcriptional profiles. Some heat stress genes, particular the small HSP genes were gated in the morning. Some genes responded to heat stress at both times, with variation of levels at either dawn or dusk. Other genes seemed to be more gated towards evening. This experiment provided insight into how time of day affects plant responses to heat stress, and generated some preliminary evidences showing heat stress in *Arabidopsis* plants was time of day sensitive.

Time of day Heat Stress Response in *Arabidopsis thaliana*

by
Jiaqi Duan

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APPROVED BY:

Dr. Cynthia Hemenway

Dr. Guozhou Xu

Dr. Colleen Doherty
Chair of Advisory Committee

Biography

Jiaqi Duan was born in China, on January 25, 1989. She moved to the United State with her parents in 2003. She lived in Buffalo, New York and attended college as Biochemistry major at Case Western Reserve University in Cleveland, Ohio. Upon graduation in 2011, she worked in the Bay Area, California before moving to Raleigh, North Carolina to attend the graduate Biochemistry Program at NCSU with specialization in studying the relationship between abiotic stresses in plants and the circadian clock with Dr. Colleen J. Doherty. Plan after graduation is to pursue a career in Biotechnology industry.

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Chapter 1: Introduction

1.1 Plant stresses

1.1.1 Abiotic and biotic stresses

Energy, water and nutrients are necessary for plants to grow, and agriculture is generally directly related to the climate. The unavoidable presence of outside stresses impairs the normal growth and development of plants; thus, reducing the yield and production of crop worldwide. Studies have shown that in order to cope with the projective demands of growing population and biofuel consumption, the global crop production needs to be doubled by the year 2050 (Ray, Mueller, West, & Foley, 2013).

Stresses on plants can impose a series of threats to crop production and yield worldwide. Plant health and production are affected by two principal stresses: biotic and abiotic. Biotic stresses are any external stresses that imposed on plants by other organisms. Abiotic stresses are stresses that arise from an excess or deficiency in any physical or chemical environment. Some biotic stresses such as fungi, bacteria, viruses, weeds, and pests can be controlled by available crop protection tools, such as herbicides and pesticides. Abiotic stresses, such as changing in temperature and water content, soil salinity and other environmental factors, however, have limited tools to alleviate these stresses. Abiotic stresses can present a major challenge in our expedition for sustainable food production and can result in up to 80% yield gap between record yield and average yield in many major crops (Buchanan, Gruijssem, & Jones, 2015). High temperature, drought, cold, excess water and salinity are the major abiotic stresses that induced severe cellular damage in plant species. A variety of abiotic stresses cause a serious crop loss thus limiting the agricultural productivity worldwide. An example of

the causes of crop loss is shown in figure 1 (“Guide to Agricultural Risk Management,” 2013).

Causes of average crop loss 1989-2010

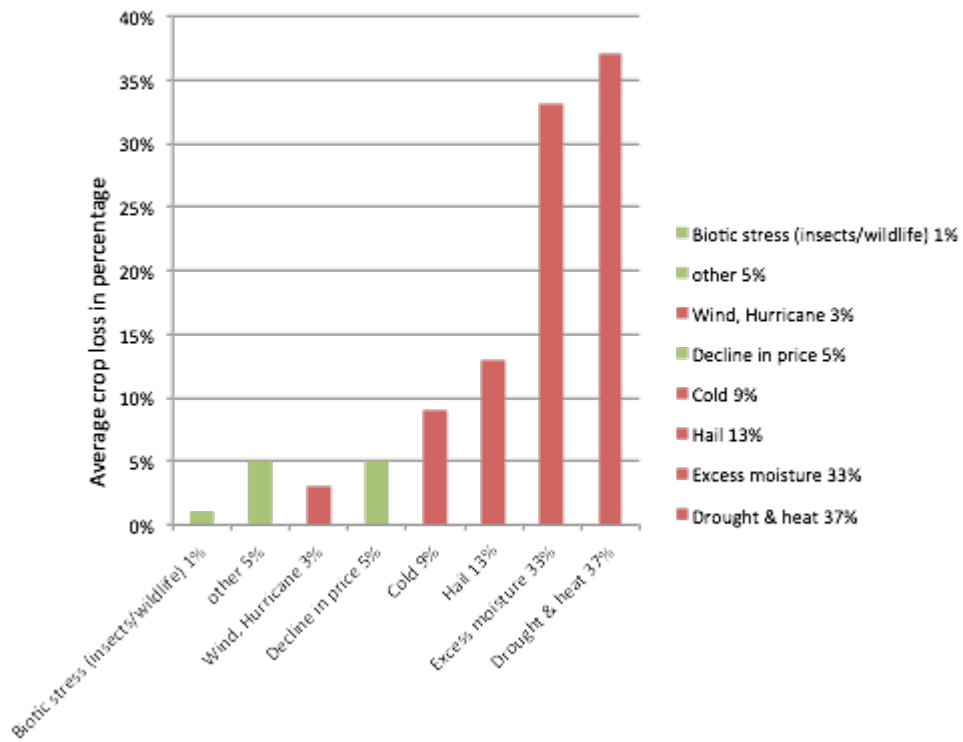


Figure 1. Causes of average crops loss from 1989-2010.

1.1.2 Heat stress

1.1.2.1 Heat stress significance

Heat stress is one of the major abiotic stresses that affects plant life on earth. Increase in temperature has a general adverse effect on plant growth due to the damaging effect on plant development, which in turn impact the aspects of plant reproduction and yield. The increasing threats of climate change including heat may lead to catastrophic loss of crop productivity. Heat waves cause massive yield losses with high risks for future global food security. During the three decades between 1988-2008, heat stress caused a reduction of 5.5% and 3.8% in the world yields of wheat and maize, respectively (Lobell, Schlenker, & Costa-Roberts, 2011). Temperature increase of 3-4°C can cause crop yield to fall by 15-35% in Africa and Asia and by 25-35% in the Middle East (Ortiz et al., 2008). In many crop species, the effect of heat stress are more notable on reproductive development and on vegetative growth, and the sudden drop in yield with high temperature is mainly associated with pollen infertility (Young, Wilen, & Bonham-Smith, 2004). In addition, the increasing demand for food from the developing countries such as China and India presents an even more challenges for agriculture to such an extent that by year 2050, the projected decline in calorie availability will worsen the malnutrition in children by nearly 20% (Nelson et al., 2009). Higher temperature poses a serious threat to crop yield, shown in figure 2, which shows the projected changes in yield as a function of potential average global temperature increases by 1°C interval for some major crops (Committee on Stabilization Targets for Atmospheric Greenhouse Gas Concentrations, Board on Atmospheric Sciences and Climate, Division on Earth and Life Studies, & National

Research Council, 2011). Thus, the growing food demand and the issue with huge crop loss due to global temperature increase impose an immediate attention to develop strategies to improve food availability.

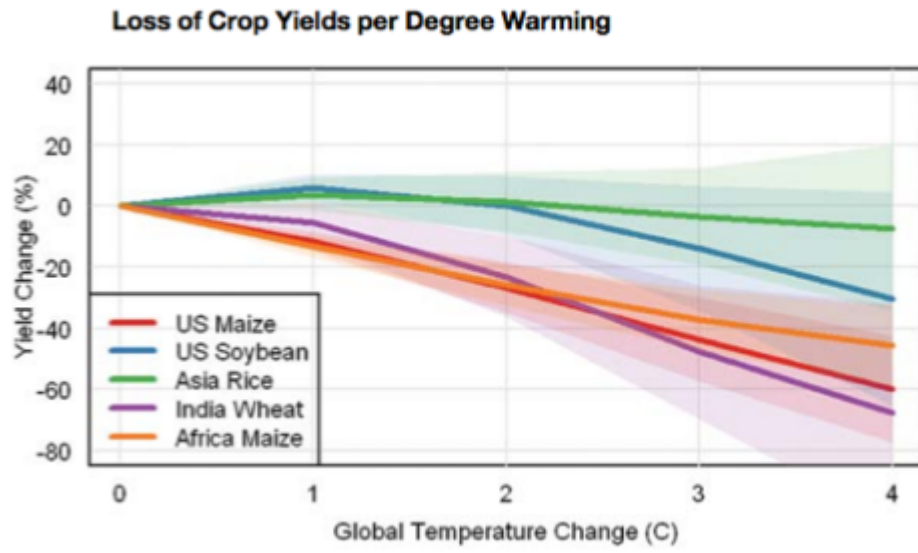


Figure 2. Loss of crop yields per degree warming.

Many physiological injuries have been observed under high temperature, such as scorching of leaves and stems, leaf abscission and senescence, shoot and root growth inhibition and fruit damage, which ultimately lead to a decreasing in plant productivity (Vollenweider & Günthardt-Goerg, 2005). In addition, heat stress can cause decreased photosynthesis, increased respiration, a major decrease in transpiration (a process of evaporation of water from plant leaves), and plant starvation. Even though all plant tissues are susceptible to exposure of heat stress, the reproductive tissues are particularly sensitive to heat. A few degrees elevation in temperature during the flowering period can result in the loss of entire grain crop cycles (Wheeler, Craufurd, Ellis, Porter, & Prasad, 2000). Heat stress can also cause plant to be more susceptible to biotic stresses like certain pests and other environmental problems. A number of pathogenic fungi are more effective in attacking trees when the host is under water and heat stress (Coder, 1999).

There are different aspects of heat stress response in plants. If a plant is in ambient temperature and suddenly experiences a dramatic increase in temperature, the ability of plant to respond and acclimate to such change in temperature is called basal thermotolerance. The second type of response involves exposing the plant to moderate level of heat stress or priming, then recovery for a period of time, and then exposing the plant to severe heat stress. The plant's ability to acclimate this treatment is called acquired thermotolerance.

1.1.2.2 Heat stress response

Heat stress not only can cause physiological changes in plants, but it also can result in biochemical changes inside the plants. Heat stress affects the stability of cellular membranes, proteins, RNA species and cellular structures. Heat stress also impacts the enzymatic reaction efficiencies and leads to a metabolic imbalance (Ruelland, Eric, & Alain, 2010; Suzuki, Nobuhiro, Shai, Ron, & Gad, 2011). Heat stress can result in the accumulation of toxic by-products, such as reactive oxygen species (ROS), and leads to oxidative stress response. In order to counter the effect of heat stress on cellular damage, plants have learned to adapt and respond to the changes in their ambient temperature by altering their transcripts, proteins and lipids. Those changes will establish a new state of balance of metabolic process and enable the plants to survive and function at a higher temperature. A general model for temperature sensing in plants is shown in figure 3 (Mittler, Ron, Andrija, & Pierre, 2012).

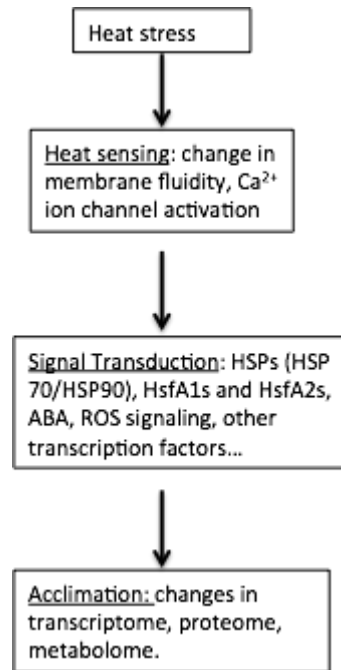


Figure 3. General model for temperature sensing in plants.

Increase in temperature can alter membrane fluidity and leads to activation of calcium channel. Influx of calcium leads to activation of signal transduction events such as activating HSPs, HsfAs and plant hormones, and cause changes in plant metabolism. This process is call acclimation. This pathway may serve as the primary sensing mechanism in plants. There is also an alternative to acclimation, which will be the activation of programmed cell death. When higher plants are exposed to heat stress, that is at least 5°C above their normal growing conditions a set of cellular and metabolic responses required for plant survival under higher temperature are initiated. These responses include triggering of the accelerated transcription and translation of heat shock proteins (HSPs) or chaperons and the production of plant hormone such as abscisic acid (ABA) and other protective molecules (Maestri et al., 2002). Under standard growth conditions, the HSPs are crucial for cellular signaling, protein folding, translocation and degradation. When under heat stress, HSPs will function to prevent protein misfolding and aggregation, which is why they are also called molecular chaperones. Increase in the production of HSPs happens when plants are experiencing either sudden or gradual increase in temperature (Nover et al., 2001). Heat shock factors (HSFs or HsfAs) are transcriptional activator of HSPs. Plants have developed the ability to acclimatize to higher temperatures, and the most important characteristics of this thermotolerance is the increased production of HSPs (Vierling, 1991). Many studies have shown that some HSFs are essential for thermotolerance, but others played a less crucial role. HSPs are categorized based on their molecular weight, ranging from HSP20s or small HSPs, HSP40s, HSP60s, HSP70s, HSP90s to HSP100s, each plays a

crucial role in preventing protein misfold, translocation, facilitate protein folding and protein stabilization.

In a general model of heat stress activation, the presence of higher temperature induces proteins to unfold, this causes HSPs to be released from their constitutive inhibitory association with HSF monomers (Voellmy, Richard, & Frank, n.d.). In many plant species, thermotolerance after experiencing a heat stress is mostly dependent upon the induction of HSP70s, which means that HSP70s are necessary for both basal and acquired thermotolerance. The induced HSP70 expression was shown to be important in assisting proteolysis, translocation, protein folding, prevent aggregation and refold denatured proteins (J.-X. Zhang et al., 2010). Another HSP, HSP101 is involved in resolubilizing protein aggregations, and seems to be not required for normal growth conditions, but plays a major role in tolerance to severe heat stresses, and also seems to be required for both basal and acquired thermotolerance (Queitsch, 2000). One major family of HSPs is the small HSPs, or sHSPs. Those sHSPs generally have low molecular weight of around 20 kDa. Induction of sHSPs gene expression indicates that these proteins also play an important role in heat stress tolerance. sHSPs family is by far the most complex group of HSPs in plants. For instance, some of the sHSPs become associated with membrane and form heat shock lipids that can stabilize membrane at early stages during the heat stress. One study showed that by introducing sHSP17.7 from carrot to potato conferred enhanced thermotolerance by affecting cellular membrane stability (Ahn & Zimmerman, 2006).

Heat shock transcription factors (HSFs or HsfAs), are the terminal components of signal transduction, they mediate HSPs expression and other heat stress induced

transcripts. Plants contain multiple HsFAs. There are 21 members of HsFAs defined in *Arabidopsis*. HSFs are categorized in three conserved classes, A, B and C, based on their structural features of their oligomerization domain (Nover et al., 2001). So far, our knowledge of plant HSF molecular mechanisms and functions is primarily based on the studying of HSFs in tomato and *Arabidopsis*. In tomato, HsfA1a is defined as the master regulator of heat stress response, and it is constitutively expressed and regulates the heat stress induced HsfA2 and HsfB1 expression (Mishra, 2002). However, in *Arabidopsis*, the heat stressed expression of HsfA2 is not regulated by HsfA1a. HsfA2 was also demonstrated to be induced by high light and H₂O₂ (Nishizawa et al., 2006). Although HsfA2 has similar functions to HsfA1a, it is exclusively found after heat stress induction and the dominant Hsf or “the working horse” of the heat stress response in tomatoes subjected to repeated cycles of heat stress and recovery (Baniwal et al., 2004). The general mechanism of HsfAs and HSPs in response to heat can be summarized in figure 4. The figure shows signaling components involved in heat stress response leading to thermotolerance. The network involves HsfAs regulate HSP genes. Arrows indicates connection with experimental evidence. The arrow with a question mark represents unidentified factors in the corresponding signaling transduction pathway.

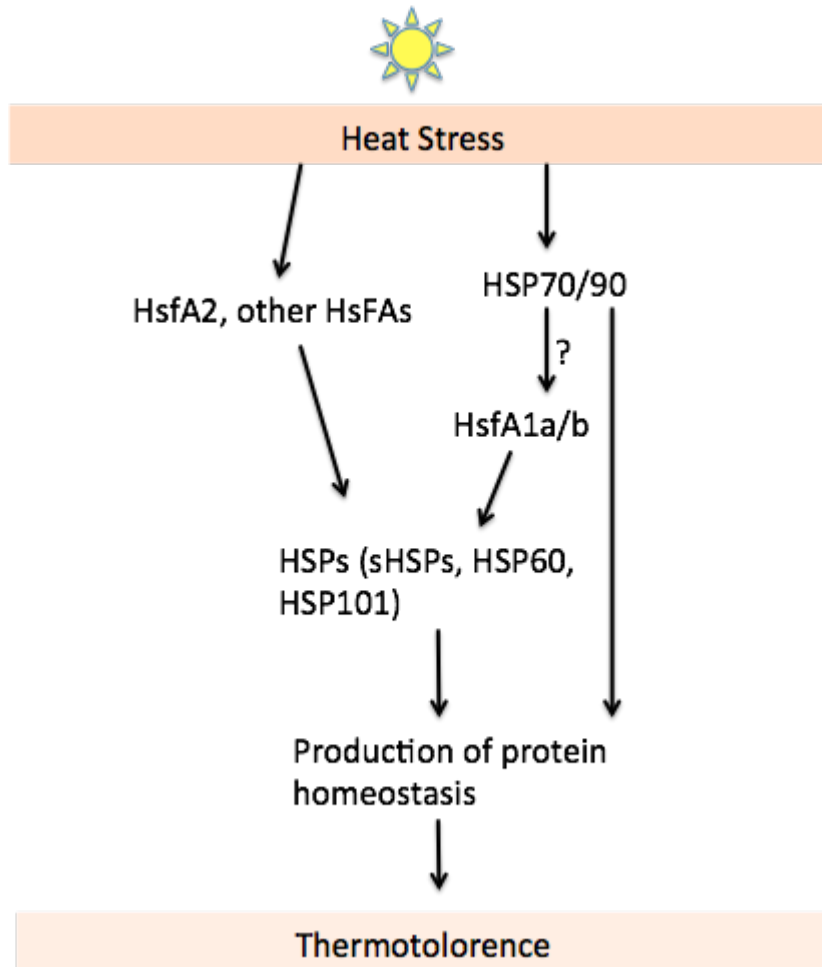


Figure 4. General signaling pathways of HsfAs and HSPs involved in thermotolerance.

1.2 Circadian clock

Plants, as sessile organism, have developed numerous of ways to rapidly sense and adapt to environmental changes. One of these most studied ways that involved in those adjustments is the mechanism of the circadian clock . Circadian rhythms are the periodicity in physiological, behavioral and biochemical changes that respond to primarily light and darkness in an organism's environment ("Circadian Rhythms Fact Sheet - National Institute of General Medical Sciences," n.d.) . Circadian clock is a ubiquitous endogenous time keeping mechanism that plays essential roles in the temporal organization of biochemical activities and in coordination of these activities with daily environmental cycles (C. Robertson McClung & Gutiérrez, 2010). In order for a biological mechanism to be referred to as the circadian clock, that mechanism needs to satisfy all four characteristics of the circadian clock. One of the most define features of the circadian clock would be the endogenous time have a period of close to, but not exactly, 24 hours (C. R. McClung, 2006; Vitaterna, Takahashi, & Turek, 2001) . The second feature would be that the clock is endogenously generated and the persistence of the rhythmicity even in the absence of periodic input signals, such as light and temperature or other external time cues. The third characteristic property of circadian clock is the ability to be entrained, or synchronized, by external environmental time cues, such as the light-dark cycle and temperatures. This means that circadian rhythms can persist without external cues, however, when such cues are present the clock is aligned to them. However, if an alteration in external cues occurs (e.g. travel through time zones), the clock will be reset and aligned to the new cues, such as light temperature and or/nutrient availability. This new alignment is referred to as

entrainment, in order for the internal time of an organism to match the local time. The last characteristic of circadian clocks is the ability to compensate for changes in temperature. It is interesting that the influence of temperature can affect the phase of a cycle without significantly changing the rate of cycling, thus the period stays the same (C. R. McClung, 2006; Vitaterna et al., 2001).

Two main input signals for entraining the plant circadian clock are light and temperature. Among all external cues, light is the one of the major factors regulating plant development and physiology by providing energy for photosynthesis. There are photoreceptors exist in plants for optimal function in perceiving both light intensities and qualities.

1.2.1 Circadian clock in other species

In different species, many rhythmic outputs and processes are regulated by the clock. In mammals, processes like digestion, maintaining body temperature, metabolism, time of sleep and hormone regulation are all controlled by the circadian clock (Eckel-Mahan & Sassone-Corsi, 2013). Circadian clocks not only exist and regulate processes in higher order organisms, but also exists in single cell organism like cyanobacterium, and can regulate processes like photosynthesis, nitrogen fixation and cell division (Golden & Canales, 2003).

1.2.2 Plant circadian clock

Higher organisms, such as plants, have developed a time-keeping mechanism that allows them to anticipate daily changes of environmental parameters, such as light and temperature. This circadian clock efficiently coordinates plant growth and development. A number of physiological and biochemical processes have been shown to have circadian rhythms, such as leaf movements, germination, photosynthesis, the fragrance emission, the stomatal opening, hypocotyl length and flowering time, gene expression and protein phosphorylation (C. Robertson McClung & Robertson McClung, 2011). The plant circadian clock regulates most of these and other biological processes (Gehan, Greenham, Mockler, & McClung, 2015).

The clock regulation of gene expression is extensive. Microarray data showed that nearly 30% of the *Arabidopsis* transcriptome cycles during free run in constant light/dark and temperature conditions following entrainment conditions with photocycles (Covington, Maloof, Straume, Kay, & Harmer, 2008). Moreover, *Arabidopsis* grown in various combinations of photocycles, thermocycles and free run conditions, which covered 6 diurnal and 5 circadian conditions for a total of 11 conditions, nearly 89% of the *Arabidopsis* transcriptome cycles in at least one condition (Michael et al., 2008).

The *Arabidopsis* circadian clock consists of three main interconnected transcription-translation feedback loops (TTFLs): a morning, a central, and an evening loop (Harmer, 2009; C. Robertson McClung & Gutiérrez, 2010; Michael et al., 2008). Each of these loops has multiple components and the overall *Arabidopsis* circadian clock feedback regulation network is very complex. The central feedback mechanism is based on the

inverse regulation of the two morning expressed MYB transcription factors, CCA1 and LHY, and an evening expressed pseudoresponse regulator TOC1 in the central loop. The central loop also includes other components like CHE and JMJD5 (Alabadí et al., 2001; Strayer et al., 2000; Wang & Tobin, 1998). The morning loop is composed of PRR5, PRR7 and PRR9, and the evening loop consists of GI, ZTL, LUX, ELF3 and PRR3. CCA1 and LHY directly repress the expression of TOC1 by binding to the EVENING ELEMENT in its promoter and activate PRR7 and PRR9, which in turn, represses CCA1 ((Alabadí et al., 2001; Strayer et al., 2000; Wang & Tobin, 1998)). TOC1 not only represses the morning-expressed components like CCA1, PRR5, PRR7 and PRR9, but also represses the evening-expressed components like PRR3 and GI, which in turn represses the expression of TOC1 through interaction with ZTL (Nagel & Kay, 2012). JMJD5 is a gene that encodes histone demethylase that activates CCA1 and LHY, however, CCA1 and LHY repress the expression of JMJD5 (Lu et al., 2011). A summary of the general circadian clock regulatory network is shown in figure 5. Figure shows the major components of the circadian clock regulatory network in the morning, central (core) and evening loop.

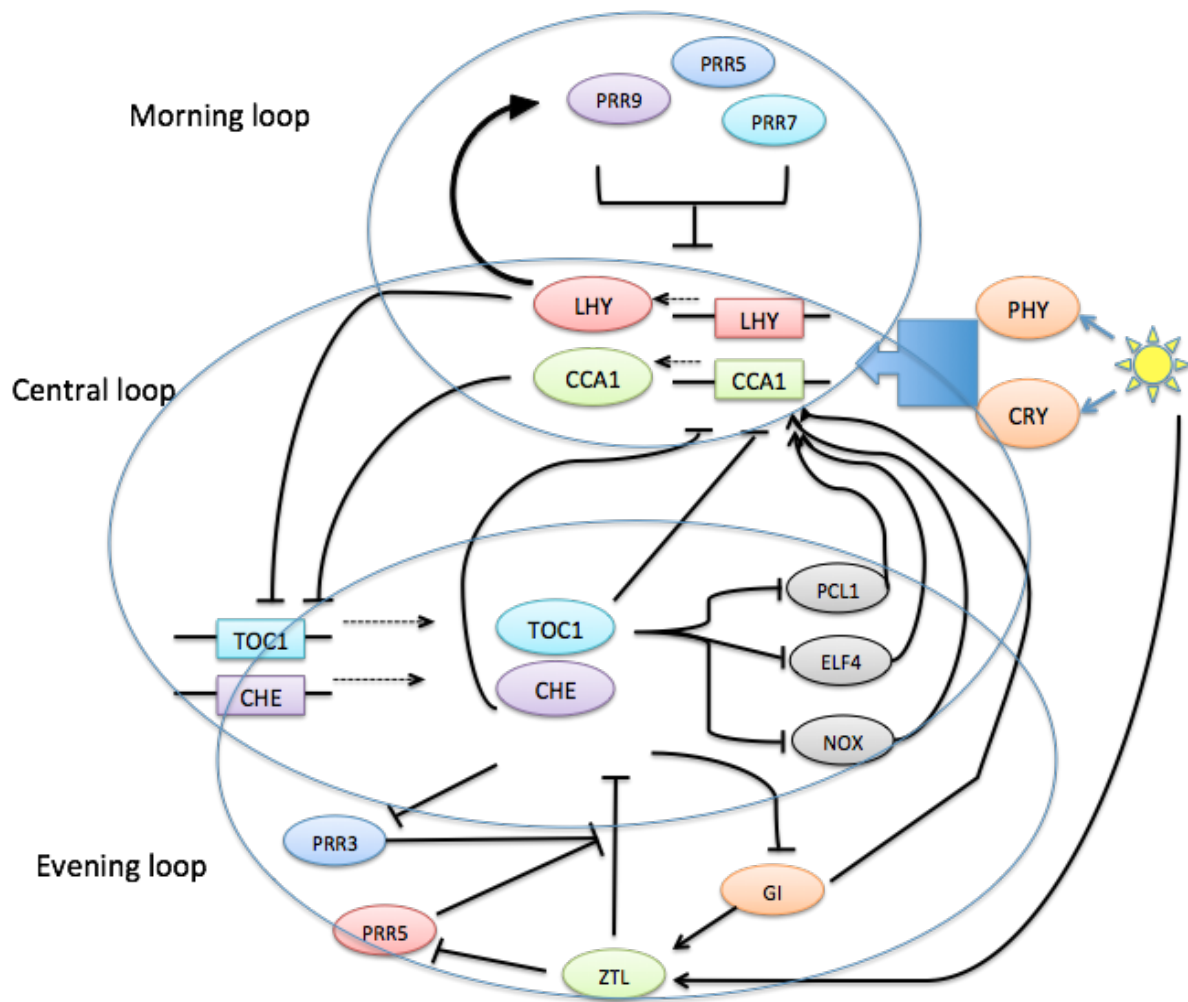


Figure 5. A regulatory network of the circadian clock in *Arabidopsis thaliana*.

1.3 Circadian clock and abiotic stresses

1.3.1 Circadian clock and cold, hormone, drought, UVB and salt stresses

More and more evidence has suggested that not only does the plant circadian clock play a role in regulating the response to environmental signals, such as light and temperature, but it also plays a role in modulating responses to various abiotic stresses. The time of day has an effect on plant abiotic stress response, and many abiotic stress responses are regulated or even gated by the circadian clock. Transcriptomic analysis has shown that circadian clock controls many abiotic stress responsive genes. In *Arabidopsis*, nearly 50% of the genes that were response to heat, osmotic stress, salinity or dehydration, had rhythmic expression under constant light condition (Covington et al., 2008).

Not only does the clock drive the basal cycling expression of stress related genes, but the clock also controls the how stress genes response to environmental signals through a mechanism called gating. For example, exposing *Arabidopsis* plants to cold temperature (4°C) will induce CBF1, 2 and 3 (also known as DREB 1B, C and A, respectively), which encode transcriptional activators that induce expression of a series of genes that can increase plant freezing and chilling tolerance (Fowler et al.) have explored the regulation of CBFs by the circadian clock in *Arabidopsis*. Their results have indicated that the level to which CBFs transcripts accumulated in response to cold temperature depend on the time of the that the time were exposed to cold temperature and that this was regulated or gated by the circadian clock(Fowler, Cook, & Thomashow, 2005). In addition, scientists have further studied how the circadian clock regulates cold response pathway, and the results from the genetic analysis and ChIP

experiments indicated that this regulation involved direct positive action of two transcription factors that are core component of the clock, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY). Their results have shown that the circadian clock provided positive regulation of the CBF cold-response pathway and enhanced freezing and chilling tolerance through the action of the core clock components CCA1 and LHY (Dong, Farré, & Thomashow, 2011). In addition to *Arabidopsis*, the CBF expression in peach leaves and bark tissue was also gated by the circadian clock (Artlip, Wisniewski, Bassett, & Norelli, 2013).

One of the major players in specifying spatial pattern of plant growth and development is auxin, a hormone essential for almost all stages of plant development. It helps the plant to coordinate itself in response to environmental cues like water, temperature, light and gravity. A study has found that the circadian clock regulates the expression of components from almost every step in the auxin signaling transduction pathway, from synthesis of compounds to response to external cues. The researchers demonstrated this observation by showing that plants had differential sensitivity to auxin at different times of day. The clock regulated plant sensitivity to auxin at both the level of transcription and stem growth (Covington & Harmer, 2007).

UVB light can also harm plant normal growth and function. In *Arabidopsis*, the circadian clock regulates UV-B mediated changes in gene expression. The circadian clock components were able to inhibit UVB induced gene expression in a gene specific manner and acted downstream of the initial UVB sensing by COP1 and UVR8. Additionally, time of day changes in plant sensitivity to UVB damage were observed. In wild type *Arabidopsis* plants, it was observed that plants were more sensitive to UVB

treatment during the night period than during the light periods under diel cycles, but this was not observed in circadian clock mutant plants (Takeuchi, Newton, Burkhardt, Mason, & Farré, 2014).

Drought stress is another major problem causing a decrease in crop yield and production. One study have shown that *Arabidopsis* plants responded to drought stress were gated by the circadian clock, with the highest dehydration induced changes in gene expression are detected at dusk by transcriptome studies (Wilkins, Bräutigam, & Campbell, 2010). Abscisic acid, or ABA, an important plant hormone, responses to abiotic stresses such as drought by inducing a signal transduction pathway. In one study, an evidence was provided of the feedback mechanism linking the circadian clock with plant response to drought. A essential clock component, TOC1 (time of CAB expression 1) bound to the promoter of the ABA related gene and controlled its circadian expression. TOC1 was induced by ABA, and this induction enhanced the phase of TOC1 binding and regulated ABA related gene circadian expression (Legnaioli, Cuevas, & Mas, 2009).

Furthermore, from our laboratory, we have observed that in *Arabidopsis* plants, salt stress response genes expressed differently depending on the time of day salt treatment, with the highest salinity inductions in the morning.

1.3.2 Circadian clock and abiotic stresses in other plant species

Although the circadian clock is an important time-keeping system that controls nearly all of the physiological responses to abiotic stresses in plants, little information is available on the impacts of the clock on stress responses in major crops. Rhythmic

expression of abiotic stress genes was also found in other plant species, such as barley and soybean in recently studies ((Habte, Müller, Shtaya, Davis, & von Korff, 2014), (Marcolino-Gomes et al., 2014)). In barley, osmotic stress up-regulated the expression of clock and stress responsive genes and enhanced their expression peak (Habte et al., 2014). In another study with soybeans, drought stress induced changes in gene expression of several circadian clock like components, which all had a decrease of expression in stressed plants. Also, the rhythmic expression pattern of the soybean drought responsive genes has changed significantly after exposure to drought. In addition, several stress responsive genes in soybean displayed diurnal oscillations (Marcolino-Gomes et al., 2014). A second study in soybean observed that multiple paralogues of clock genes were present in soybean and these genes mediated flooding and drought responses. Differential expression of many clock genes under flooding and drought conditions was observed, and it is proposed that those core clock genes along with other genes mediated flooding and drought responses through alternative splicing to fine tune photosynthesis and energy utilization under stress conditions (Syed et al., 2015).

1.3.3 Circadian clock gates abiotic stresses

From the above evidences, it is clear that there exists a connection between the circadian clock and the abiotic stresses, and all the evidences have suggested that circadian clock plays an essential role in modulating plant abiotic stress response pathways. This clear linking and gating mechanism between the clock and abiotic stress responses is summarized in figure 6.

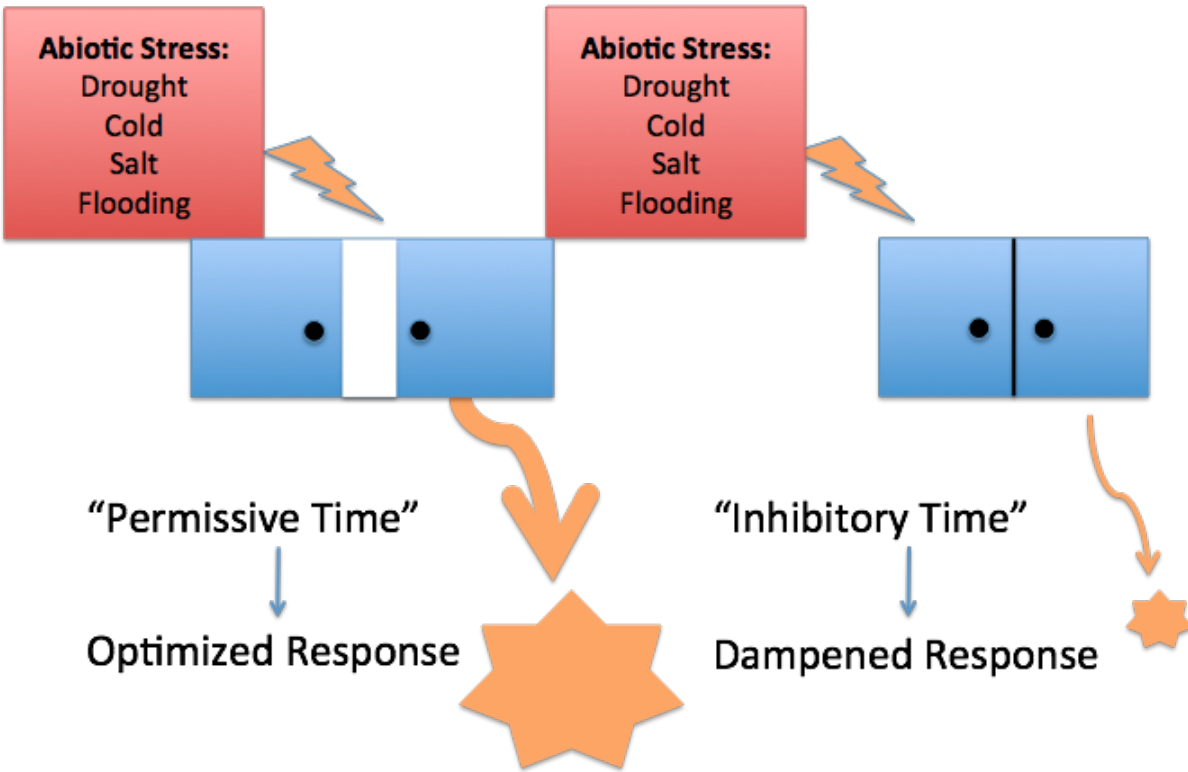


Figure 6. General proposed mechanism of circadian clock gating of abiotic stresses.

Figure 6 outlines that at a “Permissive Time”, the clock gate is open to abiotic stresses like drought, cold, salt and flooding, this will allow the plant to produce an optimized response pathway to deal with these stresses and to establish a tolerance. However, during a “Inhibitory Time”, the clock gate is not open to abiotic stresses will result a dampened or no response in the plant.

1.3.4 Circadian clock and heat stress

Circadian clock plays a role in heat stress. Circadian clock associated protein ZEITLUPE (ZTL) was a client or target for HSP90. HSP90 can effectively bind to ZTL and prevent aggregation. The maturation of a clock component, ZTL, by Hsp90 was essential for proper function of the *Arabidopsis* circadian clock. In addition, Hsp90 depletion by either inhibitor or RNAi lengthens circadian period by 0.6 hour, and ZTL was diminished (Kim et al., 2011), representative results shown in figure 7. Figure on the left shows the reduction of HSP90 by GDA lengthens the period. Plants expressing luciferase driven by two promoters of the morning core clock genes CCA1::LUC and LHY::LUC were treated with GDA (HSP90 inhibitor) or DMSO and monitored under red light. Figure on the right shows the reduction of HSP90 by GDA diminishes ZTL levels. Seedlings were grown in normal conditions and treated with GDA or DMSO, and sampled every 4 hours, and the levels of ZTL were found to be diminished.

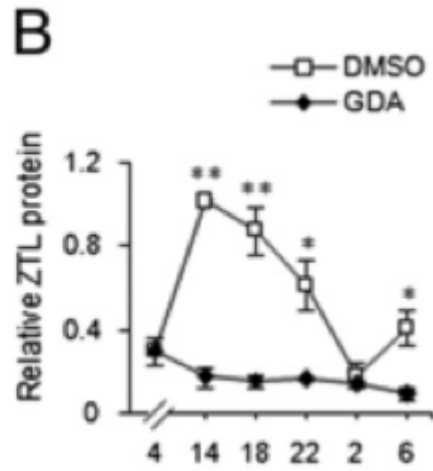
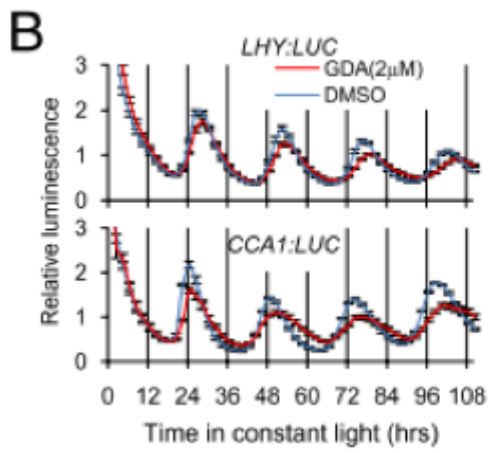


Figure 7. Figure adapted from Kim et al. 2011.

The circadian clock can perceive environmental cues to reset the time. In one study, the authors have shown that a member of HSF family, HsfB2b, was involved in the input pathway to the plant circadian clock. They have found using yeast one-hybrid approach, that HsfB2b bound to the promoter of a clock component PRR7. Constitutive expression of HsfB2b lead to dramatic reduction in PRR7 transcript, and resulted late flowering and elongated hypocotyls. HsfB2b overexpression sustained circadian rhythm under heat and salt stress, but the HsfB2b mutant shortened the period under same conditions. The authors also found that HsfB2b is involved in the regulation of hypocotyl growth under warm and short days condition (Kolmos, Chow, Pruneda-Paz, & Kay, 2014).

In addition to the two above evidences demonstrating the links between circadian clock and heat stress response, some of the well known HSPs and HSFs also show cycling pattern under different circadian conditions, shown in figure 8 (Mockler et al., 2007). Time of day hours is on the bottom with indication of either morning or night. LDHC is the real world condition of Light (12h) Dark (12h) / Hot (12h) Cold (12h). LL_LDHC is entrained on LDHC and subjected to constant light.

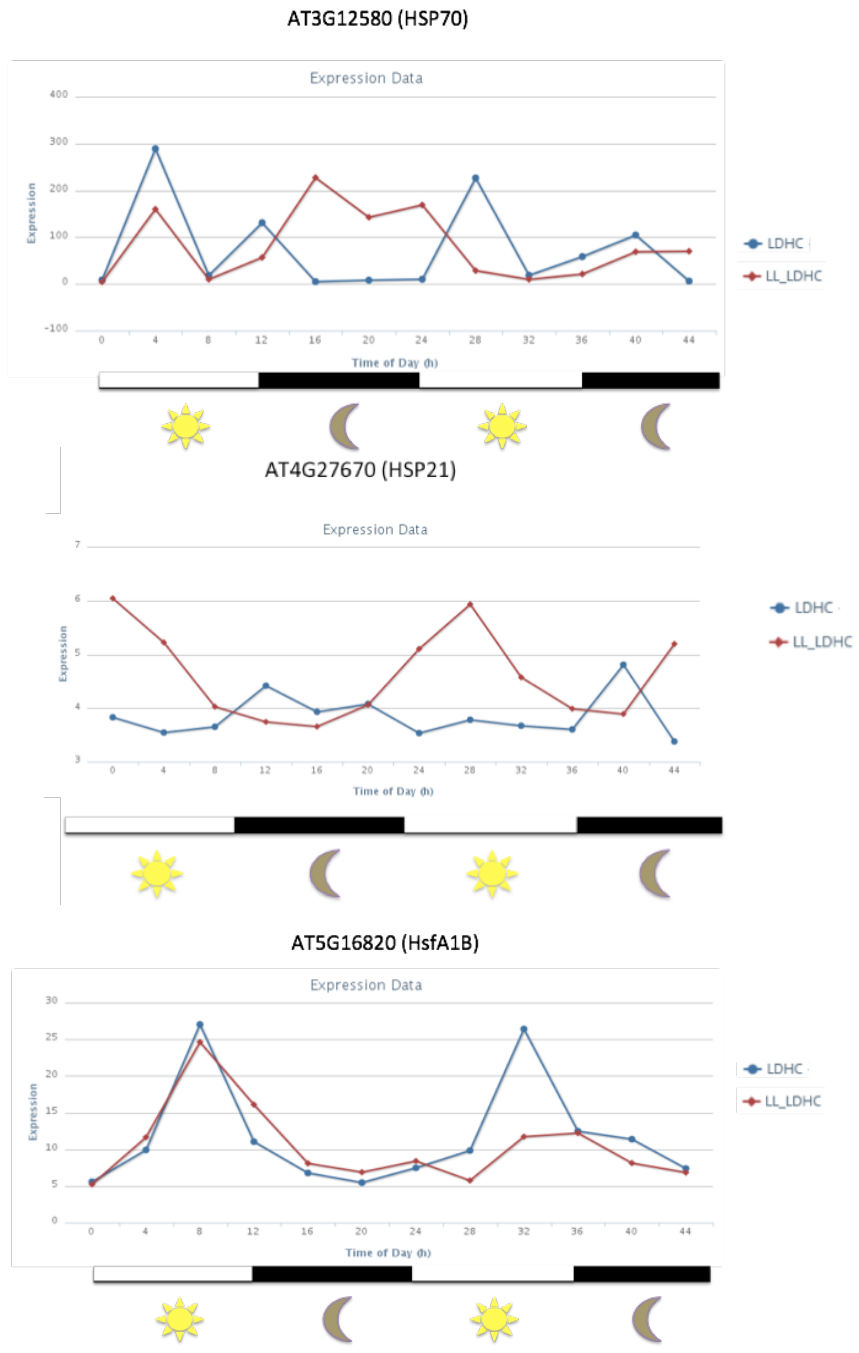


Figure 8. The cycling pattern of HSP70, HSP21, HsfA1B under different circadian conditions.

Heat stress is highly conserved response involving multiple regulatory networks and pathways. HSPs and HSFs are well known key components coordinating heat stress response. There is another recently identified component that also play an important role in heat stress tolerance, which is multiprotein bridging factor 1c (MBF1c) protein (Suzuki, Bajad, Shuman, Shulaev, & Mittler, 2008). MBF1c is a transcription co-activator, first discovered in yeast, and it is a highly conserved protein involved in different developmental and metabolic pathways in different organisms, ranging from yeast to humans (Mishima et al., 1999; Takemaru, Harashima, Ueda, & Hirose, 1998). In *Arabidopsis thaliana*, MBF1 has three different homologues and are encodes by three genes, MBF1a, b, and c (Tsuda & Yamazaki, 2004), and only MBF1c is required for thermotolerance. MBF1c increase transcription by bridging the complex between transcription factors and TATA box binding protein (TBP). Without the binding of MBF1c, there is some basal level of transcription, but with the binding the MBF1c, the level of transcription increase dramatically.

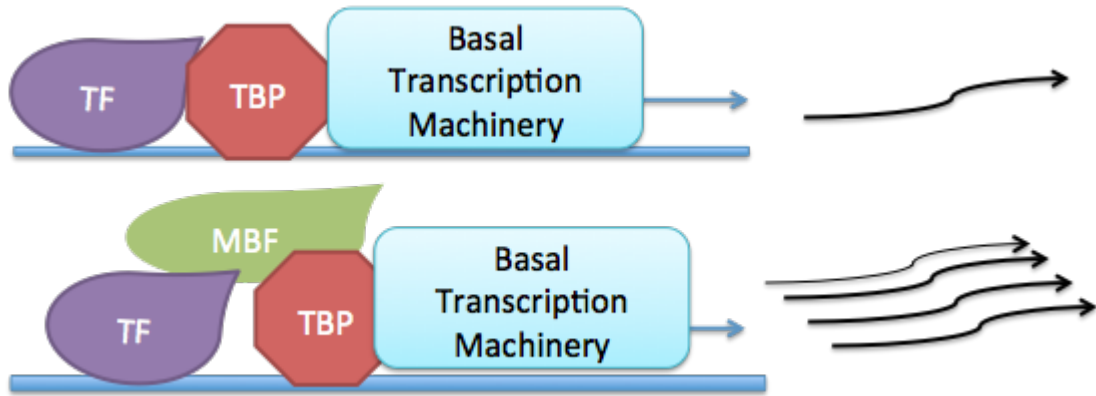


Figure 9. General mechanism of MBF1c as transcription co-factor.

Chapter 2: Results and Discussion

2.1 General experiment setup

Based on the data from previous studies of relationships between heat stress and clock components, there seems to be a correlation and a link s between the circadian clock and the regulation of heat stress response (Cha, Joon-Yung, Kim, Woe-yeon, & Kim, 2015; Kolmos et al., 2014; Mockler et al., 2007). Therefore, my hypothesis is: heat stress response in plant is gated or regulated by the circadian clock.

In order to test this hypothesis, experiments were performed to test the transcriptional response to heat stress at different times during a day. The model plant that I used for all of my experiments was *Arabidopsis thaliana*, ecotype Columbia-0. The following terms are used to describe the conditions for the following experiments:

L=light, D=dark

L/light indicated light was on in the growth chamber, which illustrated daytime. D/dark indicated light was off in the growth chamber, which illustrated nighttime.

LD- light,dark, LL-light, light,

A diagram of the representative experimental condition is shown in figure 10 below.

The temperature in the growth chamber was 23°C(LD), which was the regular temperature for *Arabidopsis* plants. This was the growth condition I used for all of my plants in all of my experiment. For high temperature treatment, the plants were transferred to a 30°Cchamber for heat shock.

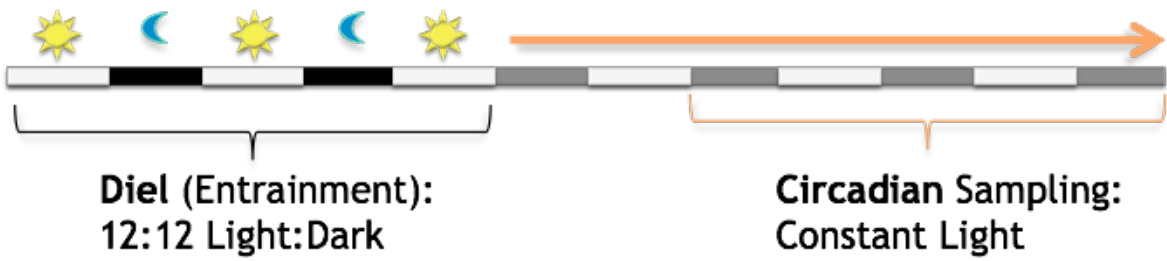


Figure 10. Representative growth and experimental conditions.

Plants were first entrained under the condition of 12 hours of light and 12 hours of dark in a 23°C regular growth chamber (or diel condition, means light and dark). Circadian sampling was when the light in the chamber turned on constantly after the plants were entrained under diel condition, this would be the constant light condition. White bars indicating the daytime, black bars indicating the nighttime, gray bars indicating “subjective night” when plants are in constant light, but the plants expect it to be dark. To test if the time of day gates heat stress, I looked at the transcriptional response for alterations of gene expression upon heat stress at different times during the day. In this case, it would be either in the morning or at night. Four biological replicates of *Arabidopsis* plants were grown on different plates in the control chamber at 23°C with the light on and off for 12 hours each day for 10-14 days or until they’ve reached 4-leaf stage. The experimental set of plants were transferred to a 30°C chamber at either morning or at night, that was 1 hour after dawn and 1 hour after dusk. Plants were treated with heat of 30°C for 1 hour, and seedlings were harvested and flash frozen in liquid nitrogen. The control plants were moved along with the experimental set but returned to the control chamber for 1 hour. The control plants were also harvested at either morning or night along with the experimental plants at the same time. Figure 11 below illustrates the representative experimental setup and sampling time points. The first figure shows the sampling or harvesting time indicated by the arrows. After the light was on or off for about 0.5 hour indicated by the white or black bars in the top figure, the plants were either treated or not treated with 30°C heat stress indicated by the below figure.

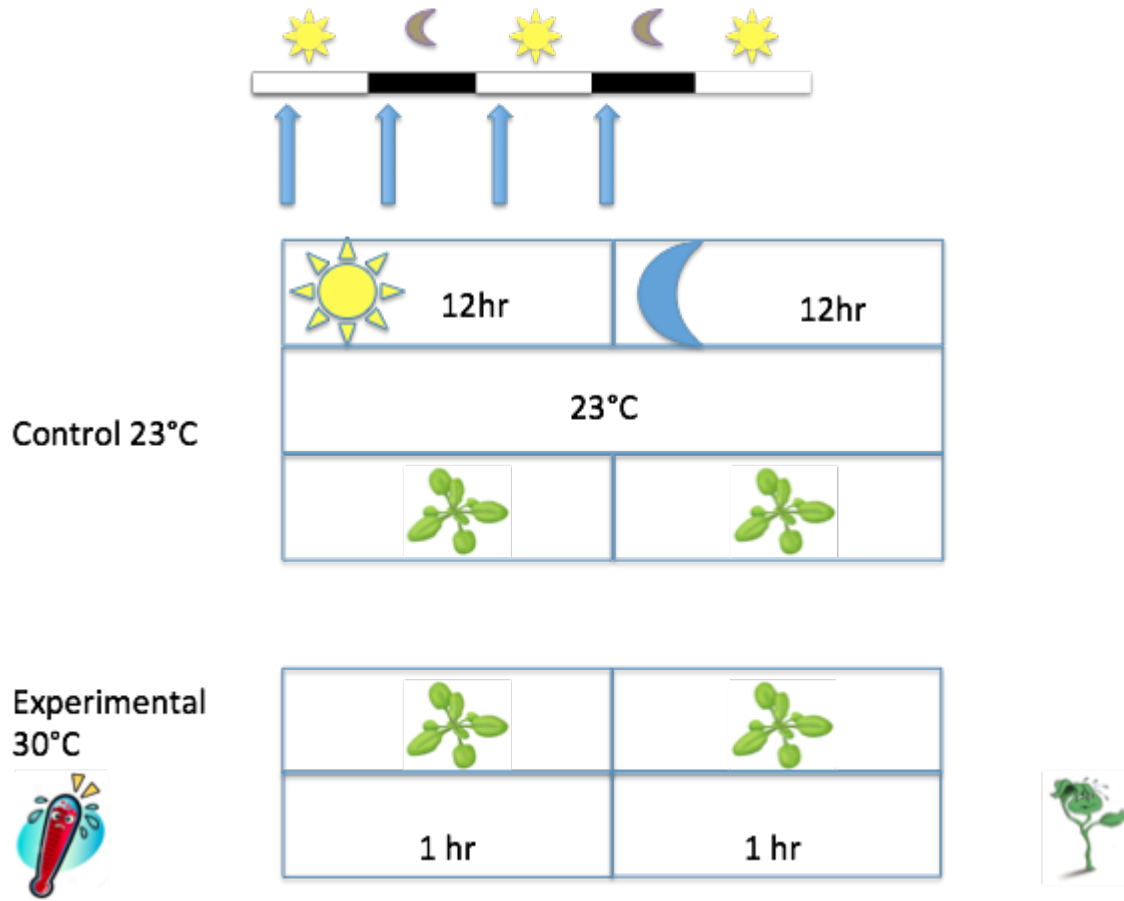


Figure 11. A simple diagram illustrates experimental setup and sampling time points.

After the plants were harvested, RNA extractions were performed on all samples, then mRNA isolation and cDNA library preparation were done for all the samples using NEB next generation sequencing library preparation kit. After library preparation, all samples were sent to Genomic Sciences Laboratory (GSL) at NC State for RNA sequencing.

2.2 RNA sequencing results showed differentially expressed genes

RNA sequencing was performed in order to determine how time of day affected the transcriptional response to heat stress. From the RNA-seq results, there are many genes that were up-regulated due to heat stress. Many of those genes were HSPs and HsfAs. Since heat is a form of abiotic stresses, thus heat can induce many signaling pathways and trigger many stress related genes for plant to establish thermotolerance. Since the plants were treated with heat shock at 30°C directly from the regular growth chamber of 23°C without pre-treatment of heat priming, the plants would respond to such sudden heat treatment with basal thermotolerance. Once experiencing heat, plants commonly respond to such stress by activating the transcription of many HSP and HsfA genes. I also observed such response in my experimental results. In addition to heat stress, my experiment also took into the account for the time of day. The plants were treated with heat in either morning or at night. There were many genes that were up-regulated in the morning upon heat treatment. Example genes that are up-regulated specifically in the morning are shown in figure 12.

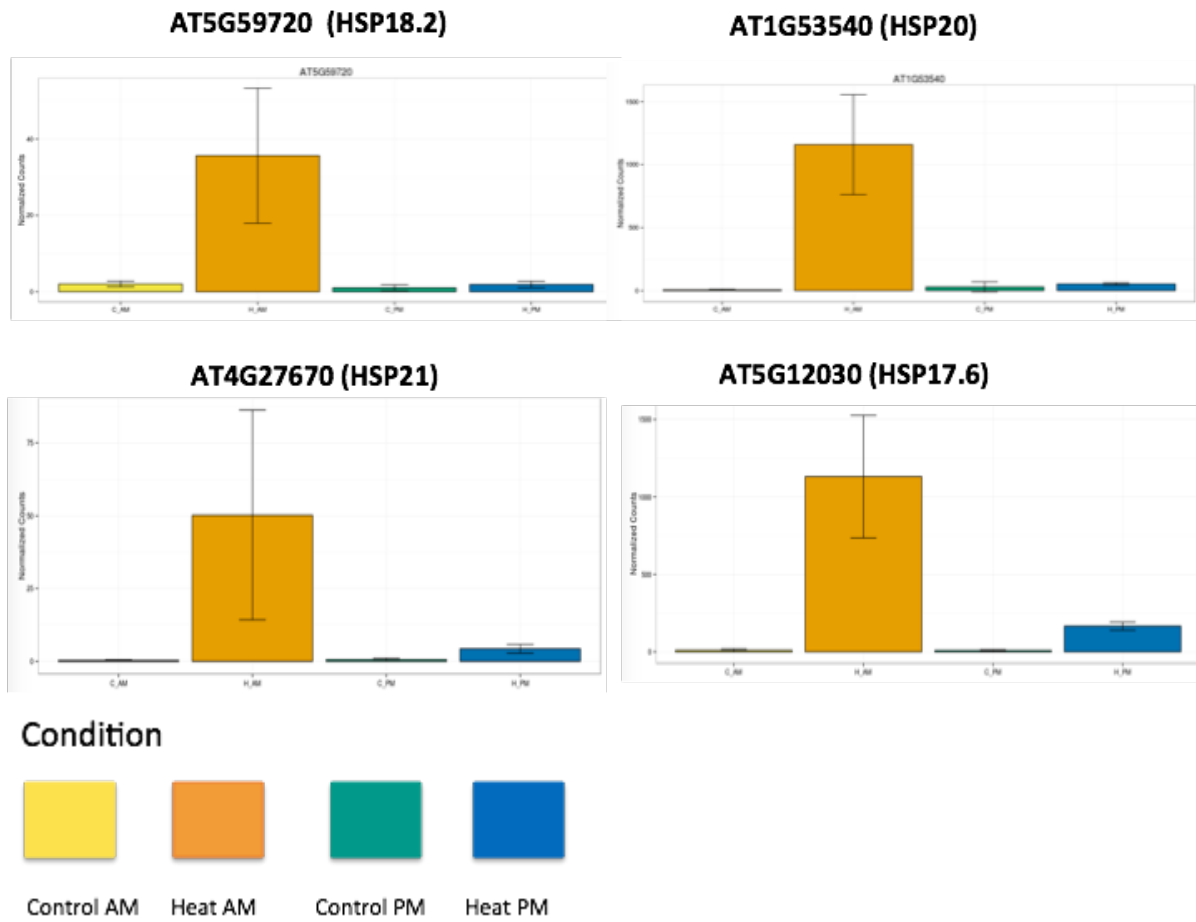


Figure 12. RNA sequencing data of transcription of genes up-regulated in the morning.

Figure showing the graphed normalized counts for each gene with error bars. HSP18.2, HSP20, HSP21 and HSP17.6 gene expressions are shown in the figure. Legend on the bottom shows the condition of each colored bar. Conditions: yellow-control harvested 1.5hr after dawn; orange-heat treated at 30°C for 1hr and harvested 1.5hr after dawn; green-control harvested 1.5hr after dusk, blue-heat treated at 30°C for 1hr and harvested 1.5hr after dusk. As shown in the above figure, HSP18.2, HSP20, HSP21 and HSP17.6 have higher counts or higher expression in plants with heat stress than the control plants. Those genes also had some degree of up-regulation at night upon heat treatment, but much less than in the morning. Interestingly, these four HSP genes with higher expression in the morning are small HSP genes. This indicated that these small HSPs were involved in heat stress response particularly in the morning.

In addition to the RNA sequencing analysis, qRT-PCR analysis with many of the HSPs and HsfAs to see how time of day would affect heat stress response was also performed. The experiment condition and treatment for qRT-PCR was the same as for the RNA sequencing analysis. Four biological replicates were used for qRT-PCR analysis. From the qRT-PCR results, many of the HSP genes and other stress genes also showed higher gene expression in the morning than at night with heat treatment, similar to what was observed in RNA sequencing data. Among the genes that showed up-regulation in the morning upon heat stress, two of those genes also showed up in RNA sequencing results, HSP21 and HSP17.6, shown in below graphs.

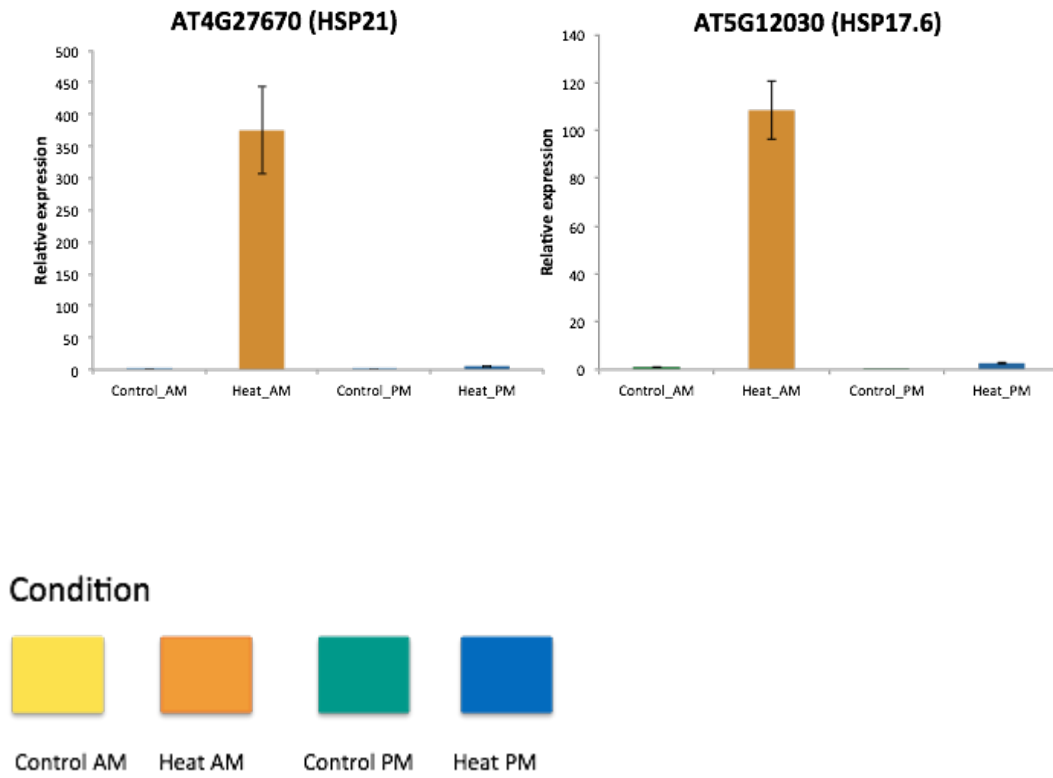


Figure 13. qRT-PCR analysis results for expression response of HSP21 and HSP17.6 with time of day heat stress treatment.

The qRT-PCR analysis (figure 13) of HSP21 and HSP17.6 showed the same transcriptional response to heat treatment, with higher expression in the morning than at night by both qRT-PCR and RNA-seq analysis, confirming the results from RNA sequencing analysis showing higher HSP21 and HSP17.6. The RNA sequencing data showed that HSP18.2, HSP20, HSP21 and HSP17.6 seemed to be only responded to heat in the morning, with a very minimal response at night. These sets of data suggested that HSP18.2, HSP20, HSP21 and HSP17.6 responded to heat stress were time of day sensitive, with higher expression to heat in the morning.

In addition to genes with higher expression in the morning with heat treatment, RNA sequencing analysis also showed some genes were also expressed higher at both morning and evening upon heat treatment. Genes like MBF1c, HsfA7B, HSP70 and HSP40, had similar transcriptional response in the morning and at night. The graphs of normalized counts for those genes are shown in the below figure.

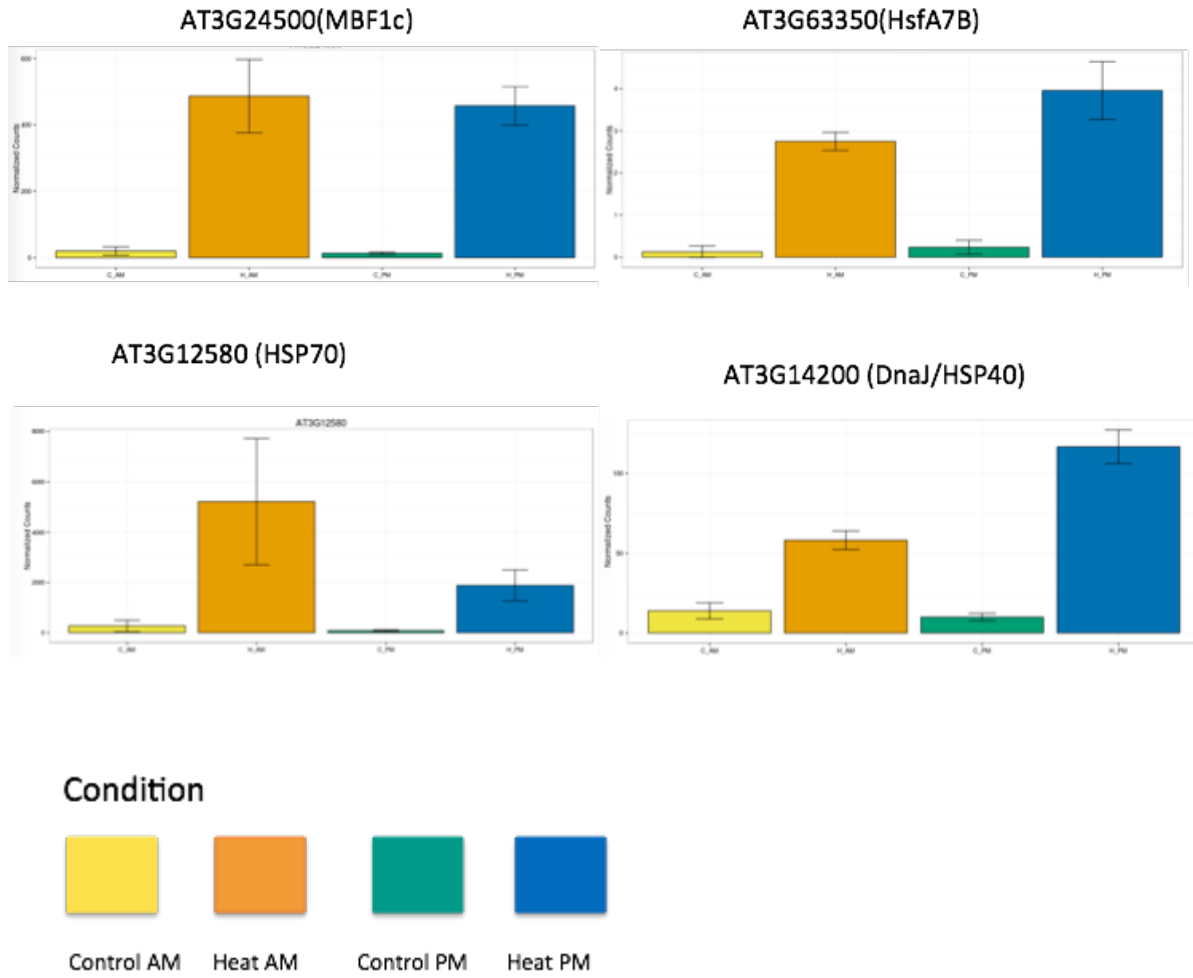


Figure 14. RNA sequencing data of transcription of genes up-regulated in the morning and evening.

RNA sequencing results showed MBF1c, HsfA7B, HSP70 and HSP40 not only responded to heat stress in the morning, but also showed a transcriptional response to heat stress in the evening compared with the control plants. From this data, we observed stress related genes with responses to heat shock that were not time of day sensitive at the timepoints we examined. MBF1c is a known stress gene and a transcriptional co-activator that is involved in number of abiotic stresses, especially in heat stress tolerance, and MBF1c is a key regulator of thermotolerance in *Arabidopsis* (Suzuki et al., 2008). MBF1c response to heat shock did not depend on the time of day of heat treatment. Likewise, neither did HsfA7B, a heat shock transcription factor involved in heat stress response pathway, also responds to heat shock at both dawn and dusk. Those genes did response to heat in the morning as well in the evening, but their responses to heat were slightly different depending on the time of day heat treatment. MBF1c had similar up-regulation in the morning and evening due to heat stress. HSP70 had higher expression in the morning than at night. HsfA7B and HSP40 had slightly higher expressions in the evening upon heat stress. Overall, based on our RNA sequencing data, although those four genes responded to heat at both dawn and dusk, MBF1c and HsfA7B did not show a clear dependence on the time of day. HSP70 and HSP40 had a time of day dependent effect with HSP70 more responsive to heat stress in the morning and HSP40 more responsive to heat stress in the evening.

Transcripts which responded to heat only in the evening were also observed in the RNA-seq data. (Figure 15). Interestingly, some of those genes were the actual components of the circadian clock network in *Arabidopsis*.

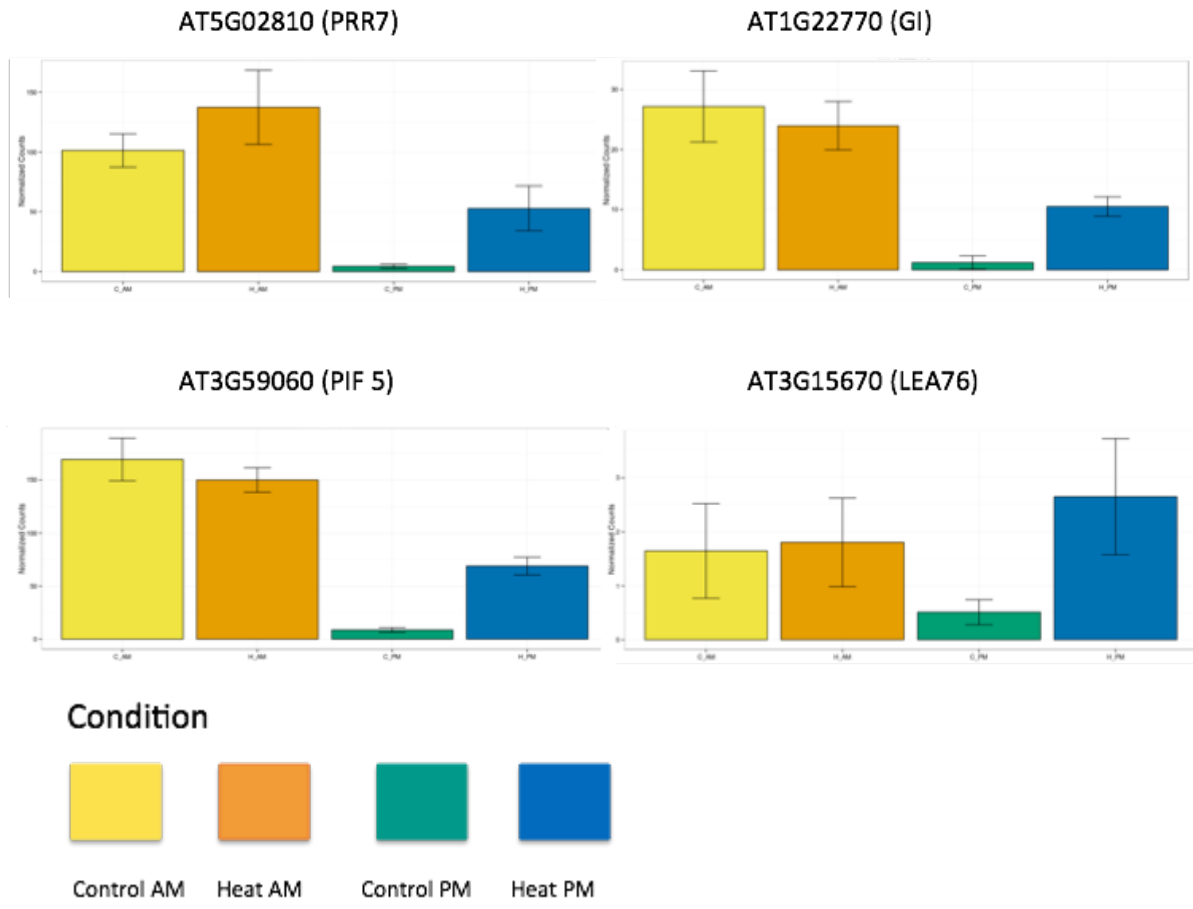
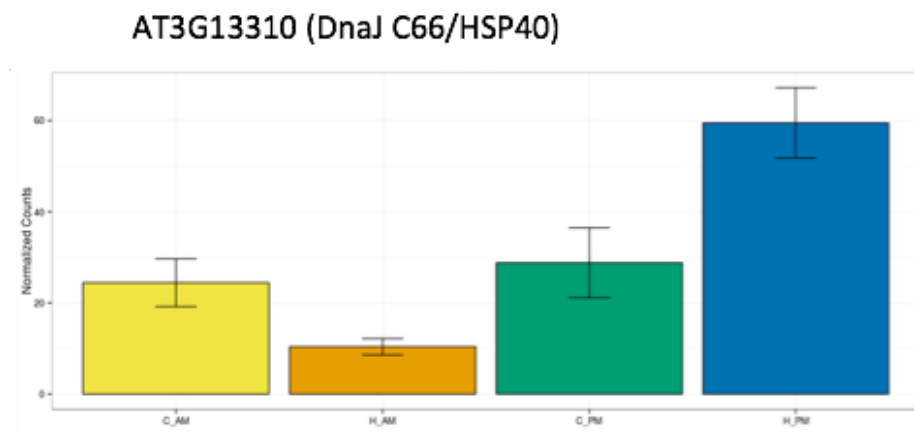
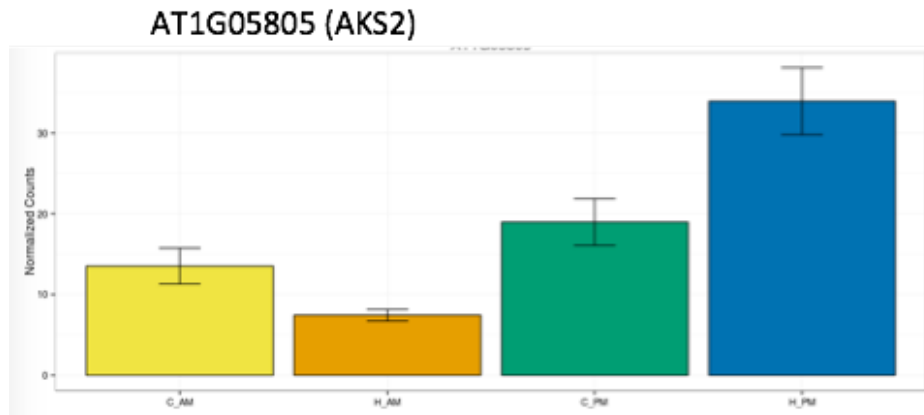


Figure 15. RNA sequencing data of transcription of genes up-regulated in the evening.

Among the genes that were up-regulated in the evening with heat stress, many of them were clock genes. From the figure above, PRR7 and GI had high responses to heat stress in the evening. The figure showed their high expression in the morning was not due to heat stress. Those two genes were clock genes and are expected to be expressed higher in the morning, which was indicated by the morning control set of plants. Upon heat stress, the expression levels of PRR7 and GI were still high. Interestingly, PRR7 and GI responded to heat stress specifically in the evening. PIF 5 also showed a very similar pattern as PRR7 and GI, high in the morning with and without heat stress and high response to heat stress in the evening. LEA76, which is one of the family members of late embryogenesis abundant proteins, that is involved in drought stress (T. Zhang et al., 2015). Genes with high evening expression patterns also included other clock genes such as LINK1 and CDF1, which were not shown, both genes had similar transcriptional responses as PRR7 and GI, although the responses in the evening were not as significant as PRR7 and GI. These results showed that some of the clock genes and some of the stress genes that responded to heat stress were time of day sensitive, particularly more responsive in the evening. This could be suggesting that the clock not only regulates the heat stress response, but it was also possible that heat stress somehow feeds back into the clock and affects the clock network, specifically at nighttime.

In addition to genes that were up-regulated in the evening due to heat stress, interestingly, we also found from the RNA sequences that there were two genes repressed by heat stress in the morning, but upregulated in response to heat in the evening. . AKS2 and DnaJ C66/HSP40 (Figure 16). AKS2 stands for ABA-responsive kinase substrate 2, which belongs to basic helix-loop-helix DNA binding superfamily. In

recent study, AKS2 was shown to be involved in ABA-dependent stomata closure, and its phosphorylation was induced by ABA (Takahashi et al., 2013). DnaJ C66/HSP40 is another heat shock protein or molecular chaperone.



Condition



Figure 16. RNA sequencing data of transcription of genes of different time of day heat stress response.

Figure 16 showed a very interesting scenario of time of day transcriptional changes due to heat stress. By just looking at the morning sets of both the control and the heat treated plants, the heat stressed plants had even lower expression for both AKS2 and DnaJ C66/HSP40 than the control plants. The heat stress seemed to reduce the expression of both genes. Especially for HSP40, which was expected to be induced by heat stress, but what we observed was the opposite. Even more interesting, the night expressions of both genes were induced by heat stress. The expression levels for AKS2 and DnaJ C66/HSP40 were higher in heat treated plants than the control plants in the evening. From these results, both two genes seemed to express both in the morning and at night, but heat treatment altered the expressions of both genes in a very peculiar way. Heat inhibited the expressions of both genes in the morning, but increased their expressions in the evening. The results showed both genes were time of day sensitive to heat stress in an unexpected way.

2.3 Plant physiological changes due to time of day heat stress

We have seen the different ways of transcriptional changes due to time of day heat stress. What would happen to the plants physiologically? How would the time of day when the heat stress is perceived affect the actual heat tolerance of the plants? Based on previously published data, drought stress, low temperature stress, and UVB stress seemed to be gated in the morning. Therefore, the expectation was that heat stress would respond in the same way, gated in the morning. To set up the experiment to test the physiological changes in *Arabidopsis* plants, the same experimental setup was used as described above for RNA sequencing analysis. The *Arabidopsis* Columbia 0

seedlings were grown in triplicates entrained in 12 hours of light and dark cycling in 23°C chamber until they reached 4-leaf stage. The plants were treated with high temperature of 42°C for 2 hours at either in the morning or at night, which were then transferred back to the 23°C chamber to recover. Pictures were taken starting from the day right after heat shock, and every 2 days afterwards until the eighth day to track the progressive changes in the seedlings. The total number of healthy and dead seedlings for all three replicates were counted and plotted in a graph to show the survival rate of time of day heat treatment. The representative seedlings pictures of one set of replicates gradual change after the heat shock is shown in figure 17. Plants were treated with 42°C heat shock for 2 hours either in the morning or at night. Yellow seedlings indicated the plants had stopped growing and dead. Green seedlings indicated the plants were still growing and remained healthy.

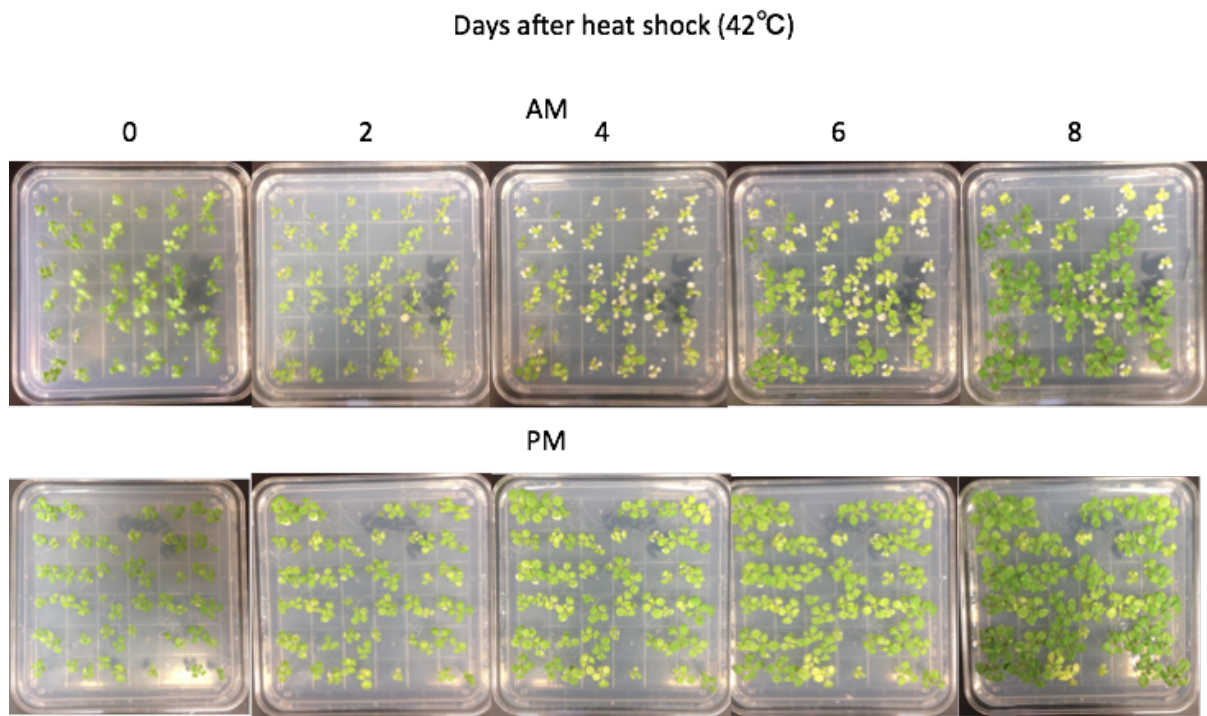


Figure 17. Pictures of *Arabidopsis* seedlings progressive physiological changes due to time of day heat stress.

From the pictures shown in figure 17, the results for physiological changes of plants responded to time of day heat shock was different from what we had expected. We expected the survival would be better in the morning than at night. However, the evening heat treated plants had more healthy seedlings than morning heat treated plants. The survival rate of the total number of seedlings upon heat shock in the morning and evening is shown below. Survival of the total number of healthy seedlings upon time of day heat shock at 42°C for 2 hours for both morning and evening.

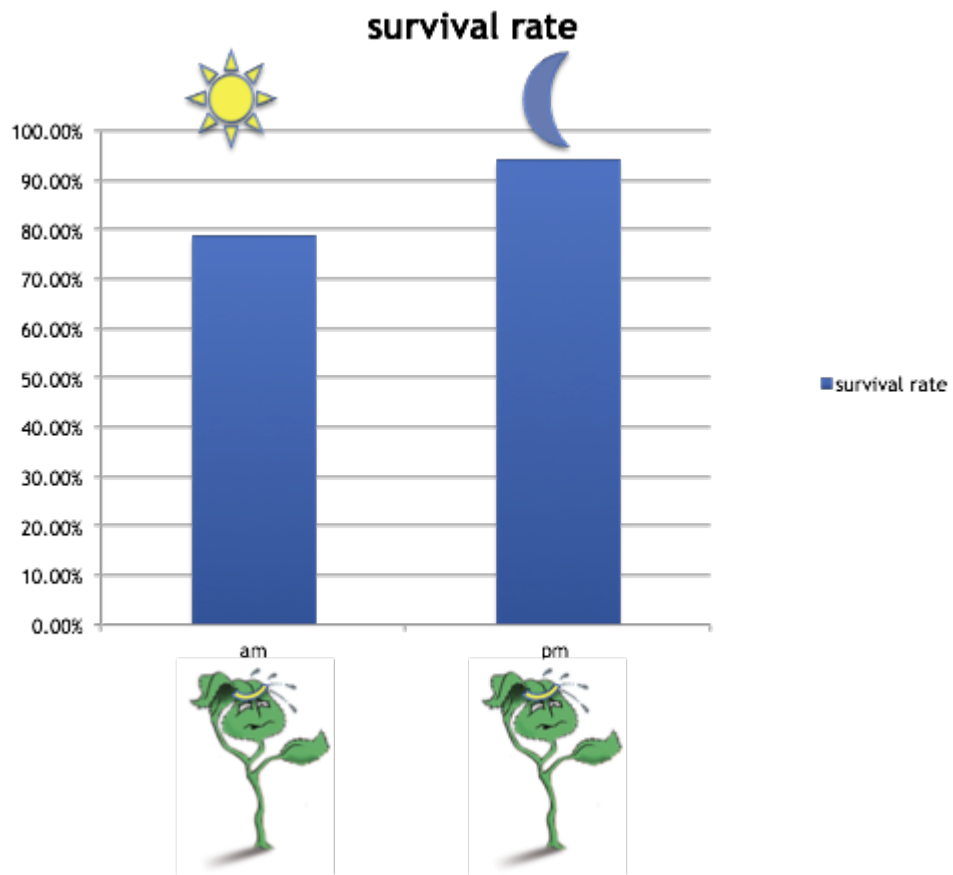


Figure 18. Survival rate of plant with time of day heat shock.

From both figure 17 and 18, physiological plant seedlings changes upon time of day heat treatment was more dramatic in the morning than in the evening. The evening heat treated plants had higher survival rate than morning heat treated plants. This result was the opposite of what we had expected, and showed that plants of the evening heat treatment survived better than the plants of the morning heat treatment.

2.4 Discussion of time of day gating of heat stress response

Since heat stress imposes a huge risk on plant and crop health and is one of the major contributors to agricultural production, and circadian clock plays an essential role in regulating most of the biological processes in plants. There has been evidences indicating that many of the abiotic stresses were gated/regulated by the circadian clock in plants, such as drought, low temperature and UVB light. Therefore, this project's goal was to investigate how time of day gate heat stress response. The results have demonstrated that like other abiotic stresses, heat stress response was affected by the time of day.

The RNA sequencing analysis experiments described here examined how the time of day gated heat stress by using the model plant organism, *Arabidopsis thaliana* wild type Columbia-0. RNA sequencing examined the transcriptional changes of *Arabidopsis* in response to time of day heat stress. From the results, many unique genes were identified, some responding to heat stress mainly in the morning, others mainly at night, and some at both times of day. When plants experience heat stress, many of the signaling transductions start to initiate the transcription of many genes and activate the downstream pathways for plants to establish thermotolerance. Heat shock proteins

(HSPs) and heat shock transcription factors/activators (HsfAs) are the major set of genes that immediately respond to heat shock both in the morning and at night. From the RNA sequencing analysis results, there were genes that were up-regulated in the morning with high fold changes in response to heat stress. These genes were grouped into a category with unique high expression in the morning upon heat stress. I picked four representative genes in this category, HSP18.2, HSP20, HSP21 and HSP17.6. The graph of normalized counts indicated the transcriptions for those genes were activated at a high level in the morning, but the levels of the expression were low in the evening. This result indicated that when experiencing time of day heat stress, some of the HSP genes were up-regulated in the morning. In addition to the RNA sequencing data, I have confirmed HSP21 and HSP17.6 were also expressed at a very high level in the morning and low level at night in response to heat shock by qRT-PCR analysis. The qRT-PCR results also show many other HSP genes had high expressions in the morning and low expressions at night, such as HSP70, HsfA2 and HSP101, though data was not shown, the results were the same. From the RNA sequencing data, it was interesting that the many genes that were up-regulated in the morning were small HSP. The results in this category showed genes that were up-regulated in the morning when experiencing heat stress and suggests that some of the important components (some HSPs and HSFs) of the heat responses were gated to respond more in the morning. The results also suggest that some of the important heat responsive genes that were gated in the morning were small HSPs, which indicated that small HSPs might play a role in heat tolerance in the morning.

RNA sequencing data showed plants activated genes that expressed in the morning and at night when experiencing heat stress, we categorized those genes into unique high expressions in both morning and night. This high expression in both morning and night category would include most genes responded both in the morning and at night upon heat stress, which would include many of the HSP genes that I have tested with qRT-PCR, such as HSP70, HsfA2 and HSP101. Although the genes in this category responded to heat stress both in the morning and at night, many of them had expression patterns with higher response to heat in the morning and a lower response at night. Therefore, the genes in this both high morning and evening could be grouped into different subcategories: the genes that had higher expression in the morning than at night, the genes that had higher expression at night than in morning or equally high in the morning and at night upon heat stress. The representative genes that I picked from this category were MBF1c, HsfA7B, HSP70 and HSP40, which covered all the subcategories in this one big category. MBF1c, was recently identified as a component that also plays an important role in heat stress tolerance in *Arabidopsis*. From the data, the results suggest that MBF1c was not time of day sensitive in response to heat stress. HSP70, on the other hand, showed different expression pattern, with higher basal level in the morning and lower level in the evening. This expression pattern also agreed with the results from qRT-PCR analysis. Although HSP70 was high at both times during the day, the response was more gated in the morning than at night upon heat stress. HsfA7B and HSP40 both responded to heat at both morning and night, but expression was lower in the morning and higher at night, which indicated that the heat response for those two genes were gated more toward at night. Since this category contained

genes that responded to heat stress at both times during a day, time of day was not involved in the heat stress response of these genes. However, with many genes like HSP70 that responded to heat more in the morning than at night, it would be reasonable to say that the time of day might play a role in regulating those genes in response to heat stress, and that the gating of heat stress response for these genes may be more likely in the morning. Also, there were some genes that responded to heat stress more at night, and there were genes whose response was not gated to either time of day.

One set of genes responded to heat stress only in the evening, thus this would be the evening responding category. Interestingly, the genes that responded in the evening to heat stress with the highest fold change, many of them were either the clock components or regulated by the circadian clock. PRR7, GI, PIF5 and LEA were the genes I picked to demonstrate the expression pattern in this category. PRR7, GI and PIF5 are involved in the circadian clock network. PRR7 and GI were expected to express high in the morning because they were morning expressed genes. Thus with the similar high expression due to heat stress in the morning for those genes, this could not be an indication of high expression in the morning was due to heat. Those three genes should not express at night, which was shown in the control evening set of plants. The interesting thing was, those genes responded to heat stress in the evening, and responded at fairly high levels. In addition, one gene, a late embryogenesis abundant protein involved in stress response, also had similar expression pattern as the other three clock genes. In addition, other genes involved in the clock like LINK1 and CDF1, also responded to heat stress in the evening. Our initial thoughts on heat stress and the

clock was that heat stress would be regulated by the clock. Although the clock can be reset by high temperature, the temperature used for this experiment was a moderate 30°C for 1 hour. It was very surprising that genes involved in the circadian clock showed increased fold change. This finding suggested some genes involved in the clock were also heat responsive in a time of day sensitive manner. Not only can the time of day effect how the response to heat stress, but time of day heat stress could also have a role in affecting the how clock behave. Although there only a small number of clock genes were affected by this time of day heat stress, based on this data, it might be possible that heat stress could somehow feedback into the clock network and affecting the expression level of clock genes and possibly downstream pathways.

Another interesting finding from RNA sequencing was that two genes that had reduced expression in response to heat stress in the morning, but had induced expression in response to evening heat stress. The two genes were AKS2 and DnaJ C66/HSP40. AKS2 are involved in ABA-signaling stomata closure. HSP40 was another heat shock protein. Both genes are normally expressed in the morning, but with heat stress, the expression levels were reduced. This suggested that time of day heat stress had an effect on the behavior of these two gene. Although they are expressed in the evening, their expression levels were increased in response to heat stress at night. The time of day clearly plays an important role in this case.

Furthermore, after looking at the transcriptional profile of circadian clock related heat stress in plants, we also looked further into how plant actually behaves physically in response to heat stress at different times of day. Another experiment was performed to investigate the physiology of the *Arabidopsis* seedlings by treating them

time of day heat shock of 42°C for 2 hours in either morning or at night. Pictures were taken every 2 days after the heat shock for the records. After experiencing heat shock, the plants treated in the morning showed more yellowing leaves and dead seedlings after 8 days. The evening heat shocked plants had less yellowing leaves and dead seedlings and looked more healthy than the morning ones. The survival rate for the total number of the green and healthy seedlings for the evening heat shocked plants was higher than the morning ones. The results indicated that the plants were more susceptible to morning heat treatment than evening treatment. The difference of this experiment was the temperature was a severe 42°C, much higher than the temperature used for RNA sequencing and qRT-PCR. In addition, from RNA sequencing results, some of the clock genes were up-regulated by the evening heat treatment, this could trigger downstream pathways that could be the possible cause for why the plants had survived better in response to heat shock in the evening.

In conclusion, my primary experiment, RNA sequencing analysis showed that many genes were activated by time of day heat stress, and thus generated different transcriptional expression pattern. Many of the heat shock genes responded to heat both in the morning and at night, with more genes responding in the morning at different degrees, and some responding to heat in the morning. Some of them also had higher response at night to heat. The data results also indicated that heat stress could also be affecting the clock. Thus the results provided evidence that heat stress in *Arabidopsis* was sensitive to the time of day. Further experiments and investigations still need to be performed to examine exactly how time of day affects heat stress

responses, and hopefully could link the dots between circadian clock heat stress in plants.

Chapter 3: Methods

3.1 Plant growth conditions and heat stress treatments

All constructs of *Arabidopsis* plant seeds were sterilized and plated on MS plates without sucrose and stratified in 4°C for 4-5 days. The plates with seeds were then transferred to a growth chamber with constant temperature of 23°C. Plants were growing in the chamber under 12 hours of light and 12 hours of dark. For the heat shock treatments, all *Arabidopsis* plants were grown in four replicates. After the plants had reached 4-leaf stage, the experimental group of plants were then moved to a 30°C chamber and heat shocked for 1 hour in the morning (0.5 hour after light came on in the chamber), and 1 hour at night (0.5 hour after light turned off in the chamber). The control group of plants were moved along with the experimental group and put back to the original 23°C chamber. Right after heat shock at 30°C morning and night, all plant seedling including the control and experimental groups were harvested in a 2 mL microcentrifuge tubes with 4 metal beads and immediately dipped in liquid nitrogen and transferred to -80°C for RNA extraction, cDNA library preparation and RNA sequencing.

3.2 RNA extraction from plants, cDNA library prep and RNA sequencing.

Metal beads were used in each sample for grinding the plant tissue. RNA extraction were performed on *Arabidopsis* plants using RNeasy Plant Mini Kit from Qiagen. NEBNext mRNA Library Prep kit for Illumina was used for mRNA library preparation. The finished cDNA library samples sent to run on a

Bioanalyzer in Genomic Sciences Laboratory (GSL) at NC State to assess the library quality. The finished samples were then sent back to GSL for RNA sequencing.

3.3 qRT-PCR analysis

DNA extractions from plant tissues was performed with DNeasy Plant Mini kit from Qiagen. qRT-PCR analysis was performed using Real-Time PCR Supermix kit with primers of different HSP and HsfA genes. IPP2 were used as control gene for normalization.

3.4 Plant growth conditions and heat shock treatments for physiology experiment

All wild type Columbia-0 *Arabidopsis* plant seeds were sterilized and plated on MS plates without sucrose and stratified in 4°C for 4-5 days. The experiment was performed with three biological replicates. The plates with seeds were then transfer to a growth chamber with constant temperature of 23°C. Plants were growing in the chamber under 12 hours of light and 12 hours of dark. For the heat shock physiology treatments, all constructs of *Arabidopsis* plants were grown in four replicates. After the plants had reached 4-leaf stage, the experimental group of plants were then moved to a 42°C chamber and heat shocked for 2 hours in the morning (0.5 hour after light came on in the chamber), and 2 hours at night (0.5 hour after light turned off in the chamber). Pictures were taken for all the plates every other day after the initial heat shock treatment until the 8th day. Number of yellowing seedlings and dead seedlings were counted for all the plates. Graph was plotted comparing the healthy seedlings for morning and evening heat shocked plants.

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