

The Molecular Interplay Between the Circadian Clock and the Plant Immune Signal, Salicylic Acid

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Dissertation submitted in partial fulfillment of
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of Philosophy in the Department of
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ABSTRACT

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Abstract

Plants have evolved the circadian clock to anticipate environmental changes and coordinate internal biological processes. Recent studies unveiled the circadian regulation on plant immune responses as well as a reciprocal effect of immune activation on the clock activity. However, it is still largely unknown how the circadian clock interacts with specific immune signals. Plant hormone salicylic acid (SA) is a key immune signal. Its accumulation is sufficient to trigger immune responses and establish broad-spectrum resistance, known as systemic acquired resistance (SAR). My dissertation work studied whether SA could interact with the circadian clock and what potential mechanisms and the biological significance are.

I first found that SA could reinforce the circadian clock through the modulation of redox state in a NONEXPRESSION OF PR 1 (NPR1)-dependent manner. The basal redox state manifested by the NADPH abundance is shown to display a circadian rhythm. Perturbation in this cellular redox rhythm caused by the immune signal SA is sensed by the master immune regulator NPR1. NPR1 then triggers defense genes expression to generate SAR as well as transcriptionally activates several clock genes to reinforce the circadian clock. Since the basal redox state, which reflects the cellular metabolic activities, is under the circadian control, the reinforced circadian clock may negate the SA-triggered redox perturbation to restore the normal redox rhythm. One of

NPR1-regulated clock components is *TIMMING OF CAB2 EXPRESSION 1 (TOC1)*. SA/NPR1-mediated increase in *TOC1* expression alone could lead to dampening of SAR through direct transcriptional repression on defense genes. Since maintenance of the immune responses is an energy-costly process, the strength and duration of SAR, a preventative defense strategy, need to be fine-tuned to reduce unnecessary energy expenditure. Therefore, both SA-dependent circadian clock reinforcement and the specific clock component *TOC1* induction help to ensure a proper immune induction and a balanced energy allocation between defense and normal metabolic activities.

Besides the SA effects on the circadian clock, the circadian clock is found to reciprocally regulate SA biosynthesis. The clock gene, *CCA1 HIKING EXPEDITION (CHE)*, and the major SA synthesis gene, *ISOCHORISMATE SYNTHASE 1 (ICS1)*, show in-phase oscillatory rhythms, indicating that CHE may contribute to generation of the circadian rhythm of the basal SA level. I found that CHE, as a transcription factor, directly binds to the promoter of *ICS1* to positively regulate its expression. After pathogen infection, CHE promotes endogenous SA biosynthesis and acts as a positive regulator of SAR. The function of the clock component CHE in activating *ICS1* not only reveals a novel transcriptional regulatory mechanism of SA accumulation but also provides a new molecular link between the circadian clock and plant immunity.

In summary, my dissertation studies identified previously unknown molecular mechanisms of how the circadian clock mediates SA biosynthesis and SA-triggered

immune responses. The interplay between the circadian clock and SA achieves a balance between activation of immune responses and maintenance of normal metabolic activities. Further studies may explore how other plant immune signals affect the circadian clock as well as how different clock components coordinately regulate the plant immunity. These future directions will broaden our understanding about the clock-immunity crosstalk.

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List of Abbreviations

ABA	abscisic acid
ABAR	ABA-related gene
AMPK	adenosine monophosphate-activated protein kinase
AzA	azelaic acid
B.C.	Before Christ
BAK1	BRASSINOSTEROID RECEPTOR 1-ASSOCIATED KINASE
BGL2	BETA-1,3-GLUCANASE 2
BR	brassinosteroid
BRI	Brassinosteroid Insensitive 1
BTH	benzothiadiazole S-methyl ester
CAB	CHLOROPHYLL a/b-BINDING PROTEIN
CBP60g	CALMODULIN-BINDING PROTEIN 60g
CC	coiled-coil
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
CCR2	COLD AND CIRCADIAN REGULATED 2
cfu	colony formation unit
CHE	CCA1 HIKING EXPEDITION
ChIP	chromatin immunoprecipitation

ChIP-seq	chromatin immunoprecipitation-sequencing
CO	CONSTANS
Col	Columbia
DA	abietane diterpenoid dehydroabietinal
DIR1	Defective in Induced Resistance 1
dpi	days post infection
EC	evening complex
eds	enhanced disease susceptibility
EE	evening element
EF-Tu	elongation factor Tu
EIL1	EIN3-LIKE 1
EIN3	ETHYLENE INSENSITIVE 3
ELF3	EARLY FLOWERING 3
ELF4	EARLY FLOWERING 4
ER	Endoplasmic Reticulum
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
FLS2	FLAGELLIN-SENSING 2
FT	FLOWERING LOCUS T

G3P	glycerol-3-phosphate
GI	GIGANTEA
GRP7	Glycine-rich protein 7
<i>Hpa</i>	<i>Hyaloperonospora</i>
HR	hypersensitive response
ICS1	ISOCHORISMATE SYNTHASE 1
INA	2, 6-dichloroisonicotinic acid
IPL	ISOCHORISMATE PYRUVATE LYASE
JA	jasmonic acid
LHCB	LIGHT-HARVESTING COMPLEX B
LHY	LATE ELONGATED HYPOCOTLY
LRR-RK	leucine-rich repeat recpetor kinase
LUC	luciferase
LUX	LUX ARRHYTHMO
MAPK	mitogen-associated protein kinase
MeSA	methyl salicylates
MKK	MAP Kinase Kinase
NDR1	NON RACE-SPECIFIC DISEASE RESISTANCE 1
nm	nanometer

NO	nitric oxide
NPR1	NONEXPRESSER OF PR1
ODE	ordinary differential equation
ONPG	2-Nitrophenyl- β -D galactopyranoside
PAD4	PHYTOALEXIN DEFICIENT 4
PAMP	pathogen-associated molecular pattern
PCD	programmed cell death
PCR	polymerase chain reaction
PIF4	PHYTOCHROME-INTERACTING FACTOR 4
PIF5	PHYTOCHROME-INTERACTING FACTOR 5
Pip	pipeolic acid
PR	pathogenesis-related
PRR	pattern recognition receptor
PRR5	PSEUDO-RESPONSE REGULATOR 5
PRR7	PSEUDO-RESPONSE REGULATOR 7
PRR9	PSEUDO-RESPONSE REGULATOR 9
PRX	Peroxiredoxin
<i>Psm</i>	<i>Pseudomonas syringae</i> pv. <i>maculicola</i>
<i>Pst</i>	<i>Pseudomonas syringae</i> pv. <i>tomato</i>

PTI	pattern-triggered immunity
qPCR	quantitative PCR
R	resistance
RIN4	RPM1-INTERACTING PROTEIN 4
ROS	reactive oxygen species
RPP4	RECOGNITION OF PERONOSPORA PARASITICA 4
RT	reverse transcription
SA	salicylic acid
SAG	SA <i>O</i> - β -glucoside
SAGT	SA glucosyltransferase
SAR	systemic acquired resistance
SARD1	SAR DEFICIENT 1
sid	salicylic acid induction-deficient
SR1	SIGNAL RESPONSIVE 1
<i>T. ni</i>	<i>Trichoplusia ni</i>
TBF1	TL1-BINDING FACTOR 1
TBS	TCP-binding site
TCP	TEOSINTE BRANCHED1, CYCLOIDEA, and PCF
TF	transcription factor

TIR	Toll/interleukin-1 receptor
TOC1	TIMMING OF CAB2 EXPRESSION 1
TRX	thioredoxin
TTFL	Transcription-translation feedback loop
TTFL	transcriptional-translational feedback loop
TTSS	type III secretion system
UBQ5	ubiquitin 5
Y1H	yeast one-hybrid
Y2H	yeast two-hybrid
ZT	zeitgeber time
ZTL	ZEITLUPE
β-gal	β-galactosidase
6-AN	6-aminonicotinamide

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1 Introduction to the circadian clock and plant immune system

As sessile organisms, plants must be adaptive and responsive to the environment. One of the adaptive mechanisms is the circadian clock. Nearly all organisms on earth from cyanobacteria to humans have evolved the circadian clock due to the earth's revolving movements around the sun to coordinate their internal physiological processes. The importance of the circadian clock cannot be more obvious than in plants whose energy comes directly from the sun. This "scheduling" mechanism is believed to reduce random expenditures of energy and increase the fitness of plants. Meanwhile, the changes in the environment, especially abiotic and biotic stresses, need to be detected and responded to accordingly by plants. Studies revealed that crosstalk between the circadian clock and the plant immune system exists. In the first two parts of this chapter, I will introduce these two systems to cover the main concepts and findings. In the third part of this chapter, I will highlight previous studies on the interplay between the circadian clock and plant immune system.

1.1 Introduction to the circadian clock in plants

1.1.1 Overview of the circadian clock

The earth rotating around its axis every 24 h results in day and night alternatively, depending on the earth's surface face towards or against the sun. The

changes in behavior, physiology and metabolism of living organisms between the day and night are obvious. The first record of the observed diurnal rhythm was in leaf movement of the tamarind trees, which dates back to the 4th century B.C. (Bretzl, 1903). However, organisms do not simply respond to the sunrise. They “anticipate” it by an underlying timekeeping mechanism. In absence of external time cues, some diurnal rhythms persist due to the presence of a biological circadian clock.

Therefore, a diurnal rhythm can be further defined as a circadian rhythm if it fulfills the following three criteria. The first characteristic of a circadian rhythm is that it is self-sustained or endogenous. When deprived of exogenous time cues (i.e., under free-running conditions), the period of the rhythm (around 24 h) displayed under the environmental changing condition (entraining condition) is maintained. The second characteristic is that a circadian rhythm can also be reset by an appropriate environmental signal, such as light and temperature, which provides a time cue. Such signals are also known as Zeitgibers (“Time givers”). Third, a circadian rhythm must have temperature compensation, that is, its period persists across a wide range of ambient temperature (Harmer, 2009; McClung, 2006). These three attributes define a circadian rhythm to be robust and responsive.

In addition to these biological characteristics, a circadian rhythm is normally described mathematically in a form of sinusoidal wave, with period, phase and

amplitude as three critical terms. Period is defined as the time to complete one cycle, which can be measured from peak to peak or from trough to trough. Phase is the time when a specific event occurs. The amplitude of a rhythm is defined as a half of peak-to-trough distance (McClung, 2006).

As biologists, we are more interested in understanding the biological meaning of these three mathematical terms associated with a circadian rhythm. In both cyanobacteria and higher plants, enhanced fitness was observed when the period of their endogenous circadian clocks match the environmental light/dark cycles (Dodd et al., 2005; Ouyang et al., 1998). Because of the obvious importance of the clock period, most genetic screens were conducted for mutants with altered period (shorter, longer or even arrhythmic) (Millar et al., 1995). This experimental design may lead to identification of genes affecting the clock output rather than the clock genes themselves. Therefore, further criteria need to be applied: Even though a clock component should display the same oscillating period as the output rhythms; altering activity of a clock component should cause arrhythmicity; and pulse expression of a clock component should generate a predictable phase shift (Kay and Millar, 1995).

The phase of a specific physiological event indicates its appropriate occurring time. Resetting the phase by an environmental signal may result in both beneficial and adverse effects. The common experience of phase resetting is jet lag, caused by flying

across several time zones. This situation can easily lead to fatigue and sleep disorder (Waterhouse et al., 2007). However, the resetting of the phase as a response to an environmental signal can benefit organisms in some cases. For example, the application of the plant hormone abscisic acid (ABA) advances the phase of the clock gene *TIMMING OF CAB2 EXPRESSION 1 (TOC1)*, which could bind to the promoter of ABA-related gene (ABAR) to facilitate ABA signal transduction (Legnaioli et al., 2009). As illustrated in this study, although light is the most common resetting signal, other types of signals can also be transduced to modulate the circadian clock.

Compared to the period and phase, understanding the biological meaning of the amplitude of circadian rhythms is limited. Previous studies on the mouse clock mutant showed that reducing the amplitude of the circadian pacemaker made the clock more vulnerable to phase resetting stimuli (Vitaterna et al., 2006). The association of amplitude with phase resetting implies the role of amplitude in determining the robustness of the clock.

Overall, we can interpret the circadian clock as a “schedule” for organisms. This self-generating biological timer interacting with various environmental factors coordinates different internal physiological processes to anticipate and adapt to environment variations.

1.1.2 Inputs of the circadian clock

In contrast to the easily perceived concept of the circadian clock, the organization of the clock system is complex, which constitutes of three components: the input, the central oscillator and the output (Harmer, 2009). Light and temperature are two predominant clock input signals, which enable to entrain the clock as Zeitgibers. The light-mediated entrainment can be achieved via transcriptional and post-transcriptional regulation of central oscillator genes. Both phytochrome and cryptochrome photoreceptors affect a cluster of central oscillator genes, including *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* (Wang and Tobin, 1998), *LATE ELONGATED HYPOCOTLY (LHY)* (Martinez-Garcia et al., 2000), *GIGANTEA (GI)* (Locke et al., 2005) and *PSEUDO-RESPONSE REGULAGOR 9 (PRR9)* (Farre et al., 2005). Light also influences the stability of *CCA1* transcripts (Yakir et al., 2007) and translation of *LHY* (Kim et al., 2003). Furthermore, light input can directly mediate the clock demonstrated by the discovery of the central oscillator component *ZEITLUPE (ZTL)* as a blue light receptor (Kim et al., 2007).

Compared to the studies of light-mediated entrainment, molecular mechanism of temperature-triggered entrainment are largely unknown due to unidentified “temperature receptors” in plants. However, some involved components have been found. The *prp7 prp9* mutant is defective of entrainment only in certain thermal cycles,

indicating that temperature has input to the clock through multiple pathways (Salome and McClung, 2005).

Recent study reveals that sugar produced through photosynthesis is another Zeitgeber in *Arabidopsis* (Haydon et al., 2013). Although the sugar production has previously been shown to be an output of the circadian clock, it is also able to entrain circadian rhythms by regulating the transcription of *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*). This study is a strong support of the idea that some clock output pathways are monitored by the clock and the status of these outputs can also serve as inputs to modulate the central oscillator components.

In addition to light, temperature and sugar, various cellular signals can modulate different parameters of the circadian clock. Plant hormones have been reported to modulate the clock, but in various ways. Cytokinin was shown to affect the phase of the clock (Zheng et al., 2006), while brassinosteroid and ABA were found to change the period and auxin only the amplitude (Covington and Harmer, 2007; Hanano et al., 2006). Meanwhile, the cellular levels of these hormones are all under circadian regulation (Harmer, 2009; McClung, 2011). Intracellular calcium level is also clock-gated and functions as an input to the clock (Dodd et al., 2007). The feedback loop established between the circadian output and input pathways is the strategy adopted by the circadian clock to process and integrate both environmental and cellular information.

My dissertation work described in Chapter 2 and Chapter 3 illustrates this concept in the context of plant immune signaling.

1.1.3 Molecular basis of the central oscillator

The central oscillator is the core of the circadian clock, which could be modulated by inputs and generate rhythmic outputs. So far, studies of the molecular mechanisms driving the clock have largely been dependent on a reliable and convenient assay of the clock outputs using clock-controlled firefly luciferase reporters. Luciferase catalyzes the oxidation of luciferin to generate detectable bioluminescence. Forward genetic screens based on this luciferase assay have turned out to be a powerful tool for identifying clock mutants (Harmer, 2009).

After decades of studies, the central oscillator of *Arabidopsis* is interpreted as three main interlocked transcription-translation feedback loops (TTFLs), a central loop, a “morning” loop and an “evening” loop termed by the time when their main components express maximally. The basic components of the central loop are two partially function redundant MYB domain-containing transcription factors, CCA1 and LHY, and a PRR-family transcription repressor TOC1 (Nagel and Kay, 2012). *TOC1* is the first clock gene cloned in *Arabidopsis*. It was identified from a forward genetic screen based on a short circadian period monitored with the luciferase reporter driven by the promoter of *CHLOROPHYLL a/b-BINDING PROTEIN (CAB)* (Strayer et al., 2000). *CAB*, also known

as *LIGHT-HARVESTING COMPLEX B (LHCB)*, is the first identified plant gene whose transcripts displaying a circadian rhythm (Kloppstech, 1985). Overexpression of *CCA1* or *LHY* causes arrhythmic expression of clock-regulated genes, indicating their important roles in the central oscillator (Schaffer et al., 1998; Wang and Tobin, 1998). Pulse expression of *CCA1* or *LHY* is able to shift the phase of the clock, which is the key feature of an oscillator component (Knowles et al., 2008). *CCA1* and *LHY* bind to the specific *cis*-element, evening element (EE), in the *TOC1* promoter to repress its expression. EEs are widespread in the promoters of evening-phased genes (Alabadi et al., 2001). *TOC1* has recently been shown to directly bind to the promoters of *CCA1* and *LHY* as a transcription repressor by chromatin immunoprecipitation-sequencing (ChIP-seq) (Huang et al., 2012). The dawn-phased *CCA1* and *LHY*, and dusk-phased *TOC1* constitute a negative feedback loop, In addition, another central loop component, *CCA1* HIKING EXPEDITION (*CHE*), was identified through an Y1H screen. Both *CCA1* and *LHY* can bind to the promoter of *CHE* to repress its expression; while *CHE* can only repress *CCA1* expression through binding to its promoter. Therefore, *CHE* is proposed to play a role in differential regulation of *CCA1* and *LHY*. Moreover, *CHE* interacts with *TOC1* and both proteins are associated with the same region of the *CCA1* promoter. (Pruneda-Paz et al., 2009).

The “morning” loop also includes the morning-phased *CCA1* and *LHY*, which positively regulate the expression of *PRR7* and *PRR9* (Farre et al., 2005). In turn, *PRR9* (early morning), *PRR7* (mid-day) and *PRR5* (late afternoon) have the sequentially binding peak to the promoters of *CCA1* and *LHY* to repress their expression (Nakamichi et al., 2010). *TOC1*, on the other hand, inhibits the expression of these three *PRRs* through directly binding to their promoters (Huang et al., 2012).

In addition to *TOC1*, *LUX ARRHYTHMO* (*LUX*) as an evening-phased component participates in the “evening” loop. *CCA1* and *LHY* can directly bind to the EE within the *LUX* promoter to inhibit its expression; and *LUX*, in turn, promotes the expression of *CCA1* and *LHY* (Hazen et al., 2005). *LUX* can also interact with *EARLY FLOWERING 3* (*ELF3*) and *EARLY FLOWERING 4* (*ELF4*) to form the evening complex (*EC*), the most prominent force in the “evening” loop (Nusinow et al., 2011). *LUX* and *ELF3* negatively regulate *PRR9*, providing a link to the “morning” loop (Dixon et al., 2011; Hazen et al., 2005). *GI*, another “evening” loop component, is repressed by *CCA1* and *LHY* and negatively regulate *TOC1* through stabilizing *ZTL*, which mediates *TOC1* degradation (Kim et al., 2007; Martin-Tryon et al., 2007).

In addition to transcription-translation based regulation, post-transcriptional and post-translational regulation, as an intrinsic part of the system, contribute to the maintenance of the rhythmic clock (Fujiwara et al., 2008; McClung, 2011). Besides these

described central oscillator components, other components also participate to complicate and strengthen the central oscillator system.

Recent studies reveal that a non-transcriptional oscillator exists independent of TTFL oscillator. The oscillation of oxidized peroxiredoxin (PRX) was first observed in human red blood cells, which lack a nucleus (O'Neill and Reddy, 2011). Furthermore, unicellular green algae *Ostreococcus tauri* also exhibits this redox-based PRX oscillation in constant darkness, when this obligate phototrophic species shuts down all transcription (O'Neill et al., 2011). The oxidized PRX oscillation turns out to be conserved in all tested model organisms, including *Arabidopsis* (Edgar et al., 2012). Therefore, a redox-related timekeeping mechanism is proposed to be ancient and its origin may date back to the Great Oxidation Event (GOE) 2.5 billion years ago (Loudon, 2012). However, little is known about the link between the redox oscillator and the TTFL oscillator. My dissertation work described in Chapter 2 provides one route from the redox input to the TTFL oscillator.

1.1.4 Outputs of the circadian clock

Output of the circadian clock is the most visible component of the whole clock system. Clock-regulated rhythmic outputs include, but not limited to, physiological processes, gene expression, cellular signaling pathways and metabolic activities.

Genome-wide microarray studies revealed that more than one third of genes in *Arabidopsis* shows circadian oscillation, which may explain the diversity of functions modulated by the clock. The central oscillator components can regulate the expression pattern of downstream genes through binding to phase-specific *cis*-elements in their promoters (Covington et al., 2008). In addition to this prevalent regulatory mechanism, rhythmic changes in chromatin structure, such as histone acetylation and methylation, have been found to be associated with the circadian rhythms in gene transcription (Gardner et al., 2011).

Among a variety of clock-modulated physiological processes, hypocotyl growth and flowering time are the most obvious; and many central oscillator mutants show altered hypocotyl length or flowering time. The molecular mechanisms regulating these two processes have also been identified. The EC, which peaks in the early night, was found to repress the expression of PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PHYTOCHROME-INTERACTING FACTOR 5 (PIF5), two positive regulators of hypocotyl growth, through directly binding to their promoters. Therefore, hypocotyl growth is inhibited in the early night but occurs later in the night because the repression by the EC is relieved (Nusinow et al., 2011). The connection from the central oscillator to photoperiod flowering is through GI and flowering regulator CONSTANS (CO) and FLOWERING LOCUS T (FT). GI could directly activate CO, which subsequently

activates FT to trigger flowering. Meanwhile, GI can also directly activate FT through binding to its promoter (Sawa and Kay, 2011; Suarez-Lopez et al., 2001).

Besides these developmental processes, the circadian clock also regulates responses to abiotic and biotic stresses. Circadian modulation of responses to cold, drought, salt and osmotic stresses has been reported (Legnaioli et al., 2009; Nakamichi et al., 2009). The clock-mediated responses to pathogen attack will be discussed in the third section of this chapter.

Plant hormones participate in regulating growth and development (the main function of auxin), responses to abiotic stresses (the main function of ABA) and responses to biotic stresses (the main function of SA and jasmonic acid (JA)). The abundances of the plant hormones, including auxin, ABA, SA and JA, show circadian rhythms (Covington and Harmer, 2007; Goodspeed et al., 2012). Some hormone-induced genes and responses are also clock-mediated (Covington and Harmer, 2007; Legnaioli et al., 2009). Therefore, the circadian clock also indirectly affect physiological processes through regulating these important plants hormones (Spoel and van Ooijen, 2013).

Circadian clock-regulated signaling pathways are not limited to hormone signaling. Redox signaling, in the form of redox-responsive gene products and redox-related metabolism, and intracellular calcium signaling are also showed to be under the circadian clock control (Dodd et al., 2007; Lai et al., 2012; Spoel and van Ooijen, 2013).

It is not surprising that metabolism is another clock-modulated output. Sugar production by photosynthesis is a key metabolic output of the circadian clock (Haydon et al., 2013). Carbohydrate availability for growth at night is also under circadian control (Graf et al., 2010). The findings of diurnal regulation of mitochondrial proteome and phase-specific expression of mitochondrial components indicates that energy producing processes may also be modulated by the circadian clock (Giraud et al., 2010).

In summary, a wide range of clock-modulated outputs manifests the importance of the circadian clock. Future studies will reveal more molecular mechanisms by which the circadian clock regulates different outputs.

1.2 Introduction to plant immune system

1.2.1 Overview of plant innate immunity

Unlike animals who have the circulatory system and mobile immune cells, plants establish a different immune system to achieve similar purposes: pathogen recognition, defense activities induction and even the “memory” of the previous pathogen attack. The first layer of plant defense is to perceive diverse pathogen-associated molecular patterns (PAMPs) of microbes to induce PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). PAMPs are molecular signatures, which allow the plant to distinguish different classes of microbes, for instance, flagellin and elongation factor Tu (EF-Tu) from bacteria, chitin and xylanase from fungi and heptaglucan from oomycetes. PAMPs

are recognized by plasma membrane-localized pattern recognition receptors (PRRs) (Zipfel, 2009). Synthetic 22 amino-acid peptide flg22, representing the most highly conserved part of N-terminus of bacterial flagellin, functions as an elicitor to induce callose formation, activate defense genes and inhibit plant growth. FLAGELLIN-SENSING 2 (FLS2), the PRR recognizing flg22, was identified through a genetic screen based on the inhibition of seedling growth and found to be a leucine-rich repeat receptor kinase (LRR-RK). FLS2 directly binds to flg22 and confers the recognition specificity (Gomez-Gomez and Boller, 2000). It has become clear that FLS2 associates with several co-receptors. The best characterized one is BRASSINOSTEROID RECEPTOR 1-ASSOCIATED KINASE (BAK1), another LRR-RK, interacting with FLS2 within two minutes after the flg22 treatment to activate PTI signaling (Chinchilla et al., 2007). BAK1 was first identified as the partner of brassinosteroid (BR) receptor, Brassinosteroid Insensitive 1 (BRI1), and was later found to interact with multiple PRRs to induce PTI (Zipfel, 2009).

The flg22/FLS2 interaction as the most characterized PAMP/PRR pair is widely used to study the downstream events following PAMP perception. The early signaling events that occur within 1-5 minutes include changes of ion fluxes across the plasma membrane, the oxidative burst and an activation of MAPK cascades. The signal transduction then leads to gene activation, receptor endocytosis and ethylene

biosynthesis within 30 min. In addition to these early responses, PTI signaling also triggers callose deposition to reinforce the cell wall as a late response (Boller and Felix, 2009).

Unlike fungal pathogens, bacterial pathogens cannot directly enter the plant tissues to initiate pathogenesis. They rely on the stomatal opening or accidental wounds. Studies in *Arabidopsis* show that the regulation of the stomatal closure is an important plant immune mechanism against bacterial invasion, which requires FLS2, NO production and the guard cell-specific OST1 kinase. ABA and SA also positively regulate the bacterial pathogen-induced stomatal closure. To fight back, some bacterial pathogens evolve specific virulence factors to trigger the stomatal reopening (Melotto et al., 2006).

The arms race between pathogens and their hosts is not limited in the regulation of the stomatal opening. Successful pathogens evolve effectors directly delivered into plant cells to interfere with PTI, causing effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Effectors of bacterial pathogens are injected through a syringe-like structure named type III secretion systems (TTSS) to promote pathogen virulence, often by suppressing functions of host immune regulators (Staskawicz et al., 2001). For example, the bacterial pathogen, *Pseudomonas syringae*, has been widely used as a model to study the plant-host interaction. Two effectors from *Pseudomonas syringae*, AvrPto and

AvrPtoB, were found to directly target FLS2 and BAK1, interfering with the FLS2-BAK1 complex formation or leading to the degradation of FLS2 to suppress PTI (Gohre et al., 2008; Shan et al., 2008).

Through co-evolution, plants have developed intracellular immune receptors, Resistance (R) proteins, to recognize the presence of effectors and activate effector-triggered immunity (ETI), which is the second and stronger layer of plant defense. R proteins in plants are structurally conserved, consisting of a variable amino terminus, including either a Toll/interleukin-1 receptor (TIR) or a coiled-coil (CC) domain, followed by a nucleotide-binding domain and a LRR domain (NB-LRR) (Spoel and Dong, 2012). More than 50 years ago, the “gene-for-gene” model was proposed, stating that one R protein specifically recognizes one effector from the pathogen (Flor, 1956). However, the number of R proteins encoded by each plant genome cannot explain the broad immune specificities against pathogens. The “Guard Hypothesis” has been proposed to describe R proteins as “guards” of a limited number of important cellular targets for the pathogen effectors. The altered status of a cellular target caused by the pathogen effector could be detected by the guarding R protein to transduce the signal and induce defense activities. RPM1-INTERACTING PROTEIN 4 (RIN4), which binds with and is guarded by two R proteins, RPM1 and RPS2, is the best-studied cellular targets. It is targeted by effectors, AvrRpm1 and AvrRpt2, produced by different strains

of *Pseudomonas syringae*. The subsequent phosphorylation or cleavage of RIN4 caused by these effectors activates its associated R proteins to trigger ETI (Kim et al., 2005). A crucial evidence supporting this “Guard Hypothesis” came from the large scale protein interactome analysis which showed that both pathogen effectors and plant R proteins bind a similar set of host “hub” proteins, which normally play important roles in plant defense (Mukhtar et al., 2011).

The hallmark of ETI is the hypersensitive responses (HR), a form of programmed cell death (PCD) occurs at the sites of attempted invasion to prevent the pathogen colonization on the whole plant. This HR associated PCD is characterized by cytoplasmic shrinkage, mitochondrial swelling, chromatin condensation, vacuolization and chloroplast disruption (Mur et al., 2008). The crucial molecular events for activating the HR and ETI include the accumulation of SA, generation of reactive oxygen species (ROS) by NADPH oxidase and activation of MAPK cascades (Coll et al., 2011).

1.2.2 Systemic acquired resistance in plants

In addition to PTI and ETI, pathogen infection in the local tissue can trigger a broad-spectrum and long-lasting resistance to the secondary infection in the systemic (uninfected) tissue. The immune responses is known as systemic acquired resistance (SAR), which is characterized by the accumulation of SA and the induction of *pathogenesis-related* (PR). SAR can also be induced by exogenous application of SA or its

analogues 2, 6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH) (Spoel and Dong, 2012).

Although it is well-known that SAR is associated with the accumulation of SA in both local and systemic tissues, SA is not the initial SAR signal. Grafting study showed that the chimeric plant with a wild-type scion and an SA-deficient rootstock could still develop SAR (Vernooij et al., 1994). Several candidates for this long-distance signal have been identified, including methyl salicylates (MeSA), a glycerol-3-phosphate (G3P)-dependent signal, the lipid-transfer protein Defective in Induced Resistance 1 (DIR1), abietane diterpenoid dehydroabietinal (DA), dicarboxylic acid azelaic acid (AzA) and the amino-acid derivative, piperolic acid (Pip) (Chanda et al., 2011; Chaturvedi et al., 2012; Maldonado et al., 2002; Park et al., 2007). Recent studies indicated that these putative SAR signals might function coordinately to achieve long-distance signal transduction (Dempsey and Klessig, 2012).

In systemic tissue, SA accumulation activates a key immune regulator, NONEXPRESSER OF PR1 (NPR1), which was identified through a genetic screen for mutants compromised in SA and INA-responsive *BETA-1,3-GLUCANASE 2* (*BGL2*, also named *PR2*) expression (Cao et al., 1994). The *npr1* mutant also showed a complete lack of *PR1* and *PR5* induction after SA, INA and avirulent pathogen treatment and failed to generate SAR (Cao et al., 1997). Continuous efforts have been made to study the

mechanism of how NPR1 responds to SA and regulates downstream defense genes. SA or pathogen infection could cause changes in cellular redox status (Mou et al., 2003). As a result of the cellular redox changes, the cysteine residues of NPR1 (C82 and C216) are reduced by thioredoxins (TRX), leading to an oligomer-to-monomer switch in NPR1 conformation and nuclear translocation of the monomer NPR1 (Tada et al., 2008). Nuclear NPR1 interacts with TGAs and NIMI-interacting (NIMIN) TFs to regulate the expression of downstream defense genes (Despres, 2003; Kesarwani et al., 2007). TGAs mainly activate NPR1-mediated genes; while NIMIN represses the expression of defense genes (Johnson et al., 2003; Zhou et al., 2000).

Genome-wide analysis through microarray suggested that SA triggers transcriptional reprogramming (10% of the whole genome) largely in an NPR1-dependent manner (Wang et al., 2006). These NPR1 targets include PR proteins, ER-resident proteins and WRKY TFs. PR proteins, the executioners of SAR, are small secreted or vacuole-targeted peptides with antimicrobial activities. ER-resident proteins contribute to the secretion of PR proteins (Wang et al., 2006). The genes encoding for these ER-resident proteins are found to be regulated by the TL1-BINDING FACTOR 1 (TBF1) TF, which plays an important role in the growth-to-defense transition. After SA treatment, TBF1 represses the genes regulating chloroplast functions and protein

translations as well as induces the genes for ER activities and defense responses (Pajerowska-Mukhtar et al., 2012).

Different from ETI in local tissues, SAR in systemic tissues is not associated with PCD. The identification of SA receptors, two NPR1 paralogs, NPR3 and NPR4, helped explain the opposing cell fates in local and systemic tissues. Both NPR3 and NPR4 could interact with Cullin3 E3 ubiquitin ligase to mediate ubiquitination and degradation of NPR1, which is a positive regulator of SAR, but a negative regulator of ETI. The binding affinity of NPR3 and NPR4 to NPR1 is determined by SA. In local tissues, the high level of SA only allows NPR3-mediated NPR1 degradation to remove its inhibition of PCD and ETI. In systemic tissues, the lower level of SA is insufficient to bring about the NPR1-NPR3 interaction but high enough to disrupt the NPR1-NPR4 interaction. Therefore, NPR1 could promote cell survival and facilitate SAR in systemic tissues (Fu et al., 2012).

1.2.3 Regulation of the key immune hormone salicylic acid

The genetic screen for *enhanced disease susceptibility (eds)* mutants and genetic screen for *salicylic acid induction-deficient (sid)* mutants identified the same sets of genes, which are important for the accumulation of SA after the pathogen infection. Two such mutants, *eds16* and *sid2*, are allelic and mutated in *ISOCHORISMATE SYNTHASE 1 (ICS1)*, which encoding a key enzyme for SA synthesis (Nawrath and Metraux, 1999;

Wildermuth et al., 2001). SA in plants can be produced in chloroplast from chorismate in two distinct pathways: (1) ICS-mediated pathway converting chorismate to isochorismate, which is then further converted to SA by ISOCHORISMATE PYRUVATE LYASE (IPL); (2) Chorismate-derived phenylalanine-dependent pathway (Vlot et al., 2009). *Arabidopsis* encodes two ICS enzymes, ICS1 and ICS2. The ICS1-mediated SA synthesis is responsible for about 90% of SA production after pathogen or UV treatment (Garcion et al., 2008; Wildermuth et al., 2001). Another pair of such mutants, *eds5* and *sid1*, has been found to be allelic (Nawrath et al., 2002; Nawrath and Mettraux, 1999). However, the function of EDS5 protein was only recently demonstrated as an SA transporter located at the chloroplast envelope to facilitate the transportation of SA from chloroplast to cytosol (Serrano et al., 2013). These two SA-deficient mutants show compromised SAR, increased susceptibility to *Pseudomonas syringae* infection and reduced *PR1* induction.

In addition to ICS1 and EDS5, EDS1, and PHYTOALEXIN DEFICIENT 4 (PAD4) also contribute to the pathogen-induced SA accumulation but function upstream of ICS1 and EDS5, as exogenous SA treatment can rescue the *eds1* and *pad4* phenotype (Falk et al., 1999; Feys et al., 2001). The lipase-like proteins EDS1 and PAD4 interact with each other and function in ETI mediated by the TIR-NB-LRR class of R proteins and in the subsequent basal resistance to biotrophic pathogens probably due to SA synthesis. In

parallel to EDS1, NONSPECIFIC DISEASE RESISTANCE 1 (NDR1), a glycosylphosphatidylinositol-anchored plasma membrane protein, positively regulates ETI triggered by the CC-NB-LRR class of R proteins and influences SA synthesis. The *ndr1* mutant exhibits compromised SAR, which can be rescued by exogenous BTH treatment, indicating that NDR1 functions upstream of SA synthesis as well (Shapiro and Zhang, 2001). The transcription of all these five SA accumulation-related genes, *ICS1*, *EDS5*, *EDS1*, *PAD4* and *NDR1*, can be induced by SA, indicating that SA has the positive feedback on these genes for the signal amplification (Ford et al., 2010; Qiu et al., 2008).

To fine-tune the level of SA, plants regulate the expression of these five SA accumulation-related genes. SAR DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60g (CBP60g) were reported to directly bind to the *ICS1* promoter to activate its transcription in response to PAMPs and *Pseudomonas syringae* (Wang et al., 2009; Wang et al., 2011a; Zhang et al., 2010). The NAC TF ANAC019 was also found to directly bind to the *ICS1* promoter but repress its expression (Zheng et al., 2012). ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE 1 (EIL1) were additional TFs detected at the *ICS1* promoter to suppress the PAMP-triggered *ICS1* induction (Chen et al., 2009). A Ca²⁺/calmodulin (CaM)-binding TF, SIGNAL RESPONSIVE 1 (SR1), represses the transcription of *EDS1* by directly binding to its promoter (Du et al., 2009). My

dissertation work described in Chapter 3 reveals that a central oscillator component participates in the transcriptional regulation of the SA synthesis gene, *ICS1*.

In planta, SA can be converted into SA *O*- β -glucoside (SAG) through SA glucosyltransferase (SAGT). SAG is an inactive SA storage form, which can be converted back to SA when necessary. Another SA inactive form is MeSA (Vlot et al., 2009). These SA metabolic processes as well as the regulation of SA synthesis ensure proper SA levels are achieved in both basal and pathogen infection conditions.

1.3 Interplay between the circadian clock and plant immunity

1.3.1 Circadian clock-mediated regulation on plant immunity

Before experimental data showed the interplay between the circadian clock and plant immunity, studies in animals had already concluded that the circadian clock does regulate the innate immunity. The best characterized studies were performed in fruit fly. Genes involved in innate immunity were found to display circadian rhythms under the constant dark condition through microarray studies (McDonald and Rosbash, 2001; Ueda et al., 2002). More convincing evidence was that the clock mutant flies showed more susceptibility to two Gram-positive bacterial pathogens compared to wild-type flies. This enhanced disease susceptibility phenotype observed in the clock mutant could be rescued with a wild-type copy of that clock gene (Shirasu-Hiza et al., 2007). Further investigation revealed that flies had different susceptibility to infections at the different

time of the day both in diurnal and free-running conditions (Lee and Edery, 2008).

Overall, endogenous circadian clock in fruit flies modulates their innate immunity.

As early as in 1997, certain pathogen-inducible genes of plant were noticed to have diurnal or circadian expression patterns (Molina et al., 1997). However, the first experimental evidence supporting the circadian control of plant immune responses was obtained only in 2011. This work was not originally intended to study the circadian clock. Wang *et al.* used *Hyaloperonospora (Hpa)*, an obligate biotrophic oomycete, to investigate the regulatory mechanisms of ETI-related defense genes. *Hpa* is one of the few pathogens which can infect *Arabidopsis* naturally to cause downy mildew disease. The Columbia (Col) ecotype of *Arabidopsis* containing the R gene, RECOGNITION OF PERONOSPORA PARASITICA 4 (RPP4), can mount ETI against the Emwa1 isolate of *Hpa*. The *rpp4* mutant, in which this R gene is knocked out, is susceptible to *Hpa* Emwa1. Through microarray analysis between Col wild-type and the *rpp4* mutant followed by enhanced disease susceptibility tests, 22 RPP4-dependent novel immune components were identified. Notably, EE is enriched in these defense gene promoters. CCA1-binding sites are also found in some of these defense gene promoters. More interestingly, RPP4 itself has two EE in its promoter and its expression shows a circadian rhythm. All these clues suggested that the central oscillator component CCA1 might regulate these defense genes. Indeed, the *cca1* mutant shows enhanced susceptible to *Hpa* Emwa1; CCA1ox

plants were more resistant. More definitive evidence about the circadian control on plant immunity came from the experiment showing that plants were more resistant to *Hpa* Emwa1 at dawn than at dusk. The *cca1* mutant was abolished in this diurnal difference in resistance. This circadian variation in plant immunity was found to be due to the pulse expression of defense genes at dawn, when the high humidity facilitates the spread of *Hpa* spores and infection is most likely to occur. Therefore, plants can “anticipate” pathogen infection by the circadian clock (Wang et al., 2011b).

This idea was further supported by another study using the bacterial pathogen *Pseudomonas syringae*. It showed that *Arabidopsis* was more resistant to the virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) in the subjective morning. Arrhythmic plants *CCA1ox* and *elf3* were compromised in this circadian variation of plant defense. Based on public available microarray data sets, this observed circadian difference in immunity was proposed to be associated with the circadian regulation of several PTI-related genes. The expression of *FLS2*, the *MAPK* cascade genes, such as *MKK5*, *MAPK3* and *MAPK6*, and the downstream *WRKY22* TF gene are all under the circadian control and peak in the morning. Consistently, callose deposition as a PTI response is significantly higher in the subjective morning and this temporal difference is abolished in *CCA1ox* plants (Bhardwaj et al., 2011). This work provides another example of the timing of plant immunity by the circadian clock. However, in all

the infection experiments performed in this work, pathogens were directly injected into plant leaves, bypassing the stomata, the natural entry sites for this bacterial pathogen. In this context, it becomes difficult to understand the biological meaning of the observed circadian regulation on PTI against *Pst* DC3000.

A recent study found that spraying *Pst* DC3000 onto *Arabidopsis* resulted in the opposing circadian difference in plant resistance compared to the earlier study in which bacteria were infiltrated, that is, plants showed more susceptibility in the subjective morning (Zhang et al., 2013). It has been known that stomatal activities are regulated by the circadian clock with the most opening in the subjective morning and the least opening in the subjective evening (Gorton et al., 1989). Therefore, spraying *Pst* DC3000 in the subjective morning led to higher susceptibility. It was proposed in this work that since bacterial pathogens had more chances to enter plants through opened stomata in the morning, it is necessary to raise the basal defense level at this time of the day. Whereas, in the evening, basal defense is less needed due to closed stomata that provide a physical barrier to the bacterial pathogen. This hypothesis was consistent with the results using the infiltration method.

This study also identified the CCA1- and LHY-mediated Glycine-rich Protein 7 (GRP7)-dependent pathway as a possible molecular mechanism by which the circadian clock regulates the stomatal activity. GRP7, also known as COLD AND CIRCADIAN

REGULATED 2 (CCR2), was previously shown to regulate stomatal aperture (Kim et al., 2008). CCA1 and LHY may accomplish this through direct binding to EE in the promoter of *GRP7*. The CCA1- and LHY-dependent pathway proposed in this study is only one of the several mechanisms (Zhang et al., 2013). Other central oscillator components, such as ELF3 and TOC1, have also been shown to affect stomatal activity (Bhardwaj et al., 2011; Legnaioli et al., 2009). Meanwhile, the stomata-independent immunity may also be under the clock control and contribute to the observed temporal differences in resistance.

Besides the oomycete *Hpa* and the bacterial pathogen *Pst*, the herbivore cabbage loopers, *Trichoplusia ni* (*T. ni*), was found to display a circadian feeding behavior, with peak feeding time in the middle of the day. The study showed that when both *Arabidopsis* and *T. ni* were entrained in-phase, plants displayed high resistance. When *Arabidopsis* and *T. ni* were entrained out-phase, that is, the subjective day of the insect was the subjective night of plant, the plant became more susceptible. Because JA is a hormone regulating insect resistance in plants, the endogenous JA level, which peaks in a few hours before the feeding peak of *T. ni* in midday, was proposed to be associated with this circadian oscillation in insect resistance. The fact that JA accumulation precedes the increase in *T. ni* feeding is consistent with the idea that the plant circadian clock is able to anticipate the herbivore attack. It was then confirmed that the observed

in-phase enhancement of *T. ni* resistance was both circadian clock- and JA-dependent, since this phenotype was abolished in arrhythmic plants or in JA-deficient mutants (Goodspeed et al., 2012). To further test the importance of the circadian clock, *T. ni* was given the choice to feed on either wild-type or arrhythmic CCA1ox plants. The tissue loss was quantified and indicated that the herbivore prefers to feed on CCA1ox plants. This choice experiment provided strong evidence that the *Arabidopsis* circadian clock is indeed advantageous for *T. ni* resistance (Goodspeed et al., 2013a).

The follow-up study performed by the same group found that even postharvest crops could be entrained to enhance their insect resistance and phytochemical cycling. Similar to the finding in *Arabidopsis*, postharvest cabbage entrained in light/dark cycles display the in-phase enhanced resistance to *T. ni* (Goodspeed et al., 2012). In addition to cabbage, diverse group of vegetables and fruits, including lettuce, spinach, zucchini, sweet potato, carrot and blueberry, can also be entrained postharvest to confer the phase-dependent herbivore resistance. Anti-herbivore metabolites, glucosinolates, including an anticancer phytochemical, 4MSO, showed the circadian rhythm in the entrained postharvest cabbage. The abundance of 4MSO in the entrained postharvest cabbage is significantly higher than the no-entrained cabbage. This clock entrainment suggests a new storage practice for vegetables and fruits to preserve their overall nutritional values (Goodspeed et al., 2013b).

Based on these studies, the circadian controls of levels of stress hormone and defense gene expression are probably the determinants of the phase-dependent plant defense variation. Although PTI and ETI lead to the opposite cell fates, that is, cell survival in PTI and cell death in ETI, they share some defense responses, including the activation of MAPKs and WRKYs, and the production of ROS. A recent study showed that CCA1 regulates ROS homeostasis and oxidative stress. Mutation of CCA1 disrupts the rhythmicity of ROS-related genes, H₂O₂ level and catalase activity, and enhances the susceptibility to oxidative damage (Lai et al., 2012). Therefore, it is reasonable to propose that temporal variation in plant defense responses may also partially due to phase-dependent cellular ROS levels.

1.3.2 Plant defense activation regulates the circadian clock

A recent study indicated that the circadian clock could also be reciprocally regulated as a result of defense activation. It was found that both the bacterial pathogen *Pseudomonas syringae* and the bacterial PAMP signal flg22 could shorten the period of the circadian clock through an unknown mechanism (Zhang et al., 2013). Since activation of plant immune response is an energy-costly process, feedback on the circadian clock may impact some clock-regulated outputs, such as metabolic activities and source redistribution, to redistribute source to balance growth and defense.

1.4 Dissertation outline

The above introduction has highlighted the circadian clock as a “scheduling” system to mediate diverse physiological processes including defenses responses. Meanwhile, defense activation can also act as an input of the circadian clock. However, our knowledge in this feedback effect of plant defense on the circadian clock is still limited.

Since SA is a central immune signal, my dissertation focuses on the interplay between SA and the circadian clock. Chapter 2 of my dissertation unveils that SA can reinforce the circadian clock and elevate the expression of some central oscillator genes. I also show an underlying molecular mechanism by which SA affects the circadian clock and the biological significance of this effect in the context of SAR.

In Chapter 3, I demonstrate that a central oscillator component transcriptionally regulates the SA synthesis gene, *ICS1*, and consequently affect SA accumulation and SAR. Although it has already been known that the endogenous SA level displays a circadian rhythm, my dissertation work identified a specific molecular link between the circadian clock and SA synthesis.

Finally, Chapter 4 summarizes all my findings and places them in perspective of our knowledge on the clock-immunity crosstalk. In addition, future research directions

and biological significance of the interplay between the circadian clock and plant immunity will be discussed.

2 SA reinforces the circadian clock through a master immune regulator

2.1 Introduction

As described in Chapter 1, plants are able to anticipate the time when pathogen infection is most likely to occur using the circadian clock (Bhardwaj et al., 2011; Goodspeed et al., 2012; Habbal and Al-Jabri, 2009; Wang et al., 2011b). Besides this daily anticipation, plants can also establish SAR in systemic tissues to anticipate and prepare for secondary pathogen infection after a local infection. The existence of two anticipation systems, the circadian clock and SAR, raises an interesting question: Do they interact? Since SA is the key immune signal in SAR, I specifically aimed to address if SA could affect the circadian clock in the context of SAR.

In *Arabidopsis*, both the circadian clock and SAR have been well-studied previously. The daily time keeping oscillator of *Arabidopsis* is driven by three interlocked transcription-translation feedback loops. The central loop consists of three transcription factors (TFs): two partially redundant morning-phased TFs, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and the evening-phased TF TIMING OF CAB2 EXPRESSION 1 (TOC1). CCA1 and LHY directly bind to the *TOC1* promoter to repress its expression whereas TOC1 also functions as a transcription repressor of *CCA1* and *LHY* (Nagel and Kay, 2012).

2.2 Results

2.2.1 SA modulates the amplitude of the circadian clock and transcription of the central oscillator gene

I first focused on the evening-phased TF gene, *TOC1*, using the *TOC1* promoter to luciferase (*TOC1p:LUC*) reporter line because *TOC1* is at the core of the circadian clock and has the most inputs to other clock components (Pokhilko et al., 2013). To measure the *TOC1p:LUC* activity, we entrained the transgenic plants under 12 hour light/12 hour dark cycles for 3 weeks and then imaged the luciferase activity under constant light conditions. The luminescence was recorded for one day before mock or SA treatment to ensure comparable baselines for all sample leaves and then followed for another three days to monitor the effect of SA. The results showed that both the amplitude and the average expression of *TOC1* were significantly up-regulated; yet, the period of the reporter expression did not change after treating plants with SA at subjective dawn (Figure 2-1). When SA was applied at subjective dusk, similar but more immediate increases in the amplitude and the average expression of the reporter were observed with no change in period (Figure 2-2).

To study the effect on the circadian clock by the endogenous SA, which has been reported to oscillate diurnally (Goodspeed et al., 2012), I crossed the *TOC1p:LUC* reporter into the SA biosynthesis mutant, *sid2* (also known as *ics1*) (Nawrath and Metraux, 1999). I found that in the absence of the endogenous SA biosynthesis, the amplitude and the average expression of *TOC1* were significantly reduced (Figure 2-3).

Collectively, my results indicate that SA could modulate the amplitude of the circadian clock and transcription of the central oscillator gene.

2.2.2 SA-mediated regulation of the central oscillator is NPR1-dependent

Transcriptional regulation of the clock gene by SA prompted us to search for the link between SA signal and the circadian clock. Previous work showed that SA induces cellular redox changes and leads to reduction of the master immune regulator NPR1, through its redox-sensitive cysteine residues (Mou et al., 2003). This results in an oligomer-to-monomer switch in NPR1 conformation and nuclear translocation of the monomer (Tada et al., 2008) to trigger genome-wide transcriptional reprogramming (Chai et al., 2006). To test whether NPR1 is involved in this SA-mediated regulation of the central oscillator, I crossed the *TOC1p:LUC* reporter into the *npr1* mutant background (Cao et al., 1997). I found that in the *npr1* mutant, the SA-triggered increases in the amplitude and the average expression of the reporter were abolished (Figure 2-4a). Similar results were obtained when SA was applied either at dawn or dusk (Figure 2-4). The findings therefore indicate that this SA-mediated regulation of the central oscillator gene is NPR1 dependent.

2.2.3 Nuclear NPR1 is a positive regulator of the *TOC1* rhythm under both basal and SA-induced conditions

It is worth noting that similar to the SA-deficient *sid2* mutant, the basal amplitude and average expression of *TOC1* were significantly lower in the *npr1* mutant

than in wild-type plants (Figure 2-5). I hypothesized that NPR1 is an intrinsic regulator of *TOC1* through the rhythmic accumulation of the endogenous SA. To examine the daily changes in NPR1 monomer levels, we collected plant samples during a two-day period under constant light conditions. Through western blotting after a non-reducing SDS-PAGE, we found a diurnal oscillatory pattern for the NPR1 monomer with a peak at night (Figure 2-6) around the same time as the peak of the endogenous SA (Goodspeed et al., 2012). Therefore, the diurnal oscillation in the endogenous SA levels may drive the rhythmic nuclear translocation of NPR1 to regulate the circadian clock genes.

The reduction of NPR1 from oligomer to monomer is catalysed by the cytoplasmic localized TRX, TRX-H3 and TRX-H5 (Tada et al., 2008). In the *trx-h3* and *trx-h5* mutants, the nuclear translocation of NPR1 is largely impaired (Tada et al., 2008). In order to confirm the requirement of NPR1 nuclear translocation in regulating *TOC1* expression, the *TOC1p:LUC* reporter was crossed into the *trx-h3* and *trx-h5* single and double mutant backgrounds. As expected, the basal oscillatory rhythm of *TOC1* was dampened in the *trx* mutants (Figure 2-7). In the *trx-h3 trx-h5* double mutant, the responsiveness of *TOC1* expression to SA was also dramatically compromised compared to wild type (Figure 2-8). These results further support our claim that nuclear NPR1 is a positive regulator of the *TOC1* rhythm under both basal and SA-induced conditions.

2.2.4 TGAs bind to the promoter of *TOC1* to activate its expression

I next searched for the specific TFs by which NPR1 regulates the *TOC1* gene expression. NPR1 has been shown to be a transcription cofactor of the TGA class of TFs in SA-induced expression of defense genes (Despres et al., 2000; Kesarwani et al., 2007). It is possible that NPR1 regulates the transcription of *TOC1* in a similar manner. Through bioinformatics analysis, two TGA-binding sites were found in the *TOC1* promoter (Figure 2-9a). The binding of the seven *Arabidopsis* TGAs to the *TOC1* promoter was examined using the yeast one-hybrid (Y1H) assay. As shown in Figure 2-11, all TGAs, except TGA3, had strong binding affinities to the *TOC1* promoter (Figure 2-10). To confirm this result, the two TGA-binding sites within the *TOC1* promoter were mutated individually or in combination (Figure 2-9a) and the effects of these mutations were tested. I found that mutating either one of the TGA-binding sites dramatically decreased the binding affinities of TGAs and mutating both binding sites completely blocked the binding (Figure 2-10).

To examine the potential effects of TGAs on the *TOC1* promoter activity *in planta*, we generated a *LUC* reporter construct of the *TOC1* promoter with the two TGA-binding sites mutated (*TOC1p (TBSm):LUC*) and transformed it into *Arabidopsis*. The wild-type *TOC1p:LUC* was transformed in parallel as a positive control. Imaging of multiple independent T1 transformants showed that mutations in the two TGA-binding sites

significantly inhibited the transcription of the reporter, indicating that TGAs are transcription activators of *TOC1* in *planta* (Figure 2-11).

2.2.5 SA/NPR1-mediated increase in *TOC1* expression dampens the immune response through direct transcriptional repression on defense genes

In a previous ChIP-seq experiment, defense-related genes were found to be significantly enriched among the *TOC1* direct transcriptional targets (Huang et al., 2012). Indeed, the *toc1* mutant was more responsive to the external application of the SA analog, BTH, with regard to defense gene induction and resistance to the bacterial pathogen *Pseudomonas syringae maculicola* (*Psm*) ES4326 (Figure 2-12, Figure 2-13). This result suggests that during pathogen challenge, SA/NPR1-mediated increase in *TOC1* expression alone could lead to dampening of the immune response through direct transcriptional repression on defense genes.

2.2.6 NPR1 positively regulate the transcription of *LHY* and *PRR7*

The complex interplay between clock components suggests that *TOC1* may not be the only clock gene regulated by NPR1. The expression pattern of *TOC1* may represent an overall effect exerted by all the regulatory inputs of NPR1 to the clock. It is known that lowering the level of *TOC1* mRNA shortens the period of the clock in constant light (Millar et al., 1995). However, this effect was not observed in the *npr1* mutant, in which *TOC1* expression was reduced. Moreover, if *TOC1* were the only circadian clock gene regulated by SA and NPR1, SA treatment at dawn should have

caused an immediate induction in *TOC1* expression instead of a 12-hour delay observed in our experiment.

To systematically search for other possible clock components that are regulated by NPR1, we performed mathematical modeling using the P2012 circadian model (Pokhilko et al., 2013), which includes most of the known components of the *Arabidopsis* circadian clock. Based on our data, we made the assumption that NPR1 is a non-competitive transcriptional activator for other clock genes as it is for *TOC1*. We systematically coupled NPR1 to *TOC1* and to two other circadian clock genes X and Y from the P2012 model (Figure 2-14). For each X, Y pair, we used nonlinear least squares fitting to find those NPR1 parameters that best fit our *TOC1p:LUC* data sets. We repeated this procedure for all X, Y pairwise combinations of the circadian clock genes.

More specifically, we first optimized the ODE system to fit the *TOC1* expression data from the *npr1* mutant. We then used heat maps to display the best least-squares fit to the data for each of the NPR1 coupling combinations (Figure 2-14a). The results from the *npr1* background showed a characteristic “crosshair” pattern, centred on *PRR7*, indicating that the basal regulation of *PRR7* by NPR1 best explains the unchanged *TOC1* period in the *npr1* mutant (Figure 2-15).

To test our model, I measured the transcript level of *PRR7* in *npr1* using quantitative PCR (qPCR). Consistent with our model, the result showed the decreased *PRR7* expression in the *npr1* mutant compared to the wild-type control (Figure 2-16).

The promoter of *PRR7* also contains two TGA-binding sites (Figure 2-9b) as in the *TOC1* promoter, indicating a potential regulatory mechanism by TGAs.

The second fitting for SA treatment involved multiple parameters. We used our fixed basal expression parameters and the NPR1 western data (Figure 2-6, Figure 2-17) to fit the SA-induced *TOC1* expression data from Figure 2-1. The resulting heat map showed the same “crosshair” pattern for *CCA1* or *LHY* (Figure 2-14b). Since *CCA1* and *LHY* are not distinguished in the P2012 model, we experimentally tested the SA effect on their expression individually using qPCR. We found that while *CCA1* expression was slightly reduced by SA treatment (Figure 2-18) probably as a result of the increased *TOC1* expression, *LHY* expression was up-regulated in its amplitude as predicted by our model (Figure 2-19). Additionally, the basal transcription level of *LHY* was lower in *npr1* compared to wild type (Figure 2-20). Because *LHY* is an antagonist of *TOC1* in the clock, induction of *LHY* by SA explains the delayed increase in *TOC1* after SA treatment at dawn (Figure 2-21) when *LHY* has the highest expression level. This network balance architecture of NPR1 affecting both the morning-phased *LHY* and the evening-phased *TOC1* ensures the maintenance of the circadian clock without period change in response to SA.

2.2.7 SA reinforces the circadian clock

Previous studies on the mouse clock mutant suggest that the amplitude of the clock determines how easily the clock can be reset. Lowering the amplitude made the

clock more vulnerable to resetting stimuli (Vitaterna et al., 2006). To study the effect of the SA-induced amplitude increase in central oscillator genes in plants, we used light as a resetting signal. It was shown previously that when applied at the appropriate strength at a certain circadian phase, light can even stop the clock (Winfree, 1970). We found that when a 40-minute light stimulus was applied, SA-treated wild-type plants still had a robust circadian rhythm under constant dark condition. In contrast, the *npr1* plants became almost arrhythmic with dramatically lengthened period (Figure 2-22). Therefore, the increased amplitude of the central oscillator genes caused by SA rendered the circadian clock more resistant to resetting stimuli; while the dampened amplitude in the *npr1* mutant made the clock more vulnerable. By increasing in the amplitudes of *TOC1* and *LHY* expression, SA reinforces the circadian clock.

2.2.8 SA perturbs NADP⁺/NADPH oscillation

The biological significance of this SA induced reinforcement of the circadian clock may lie in the fact that the circadian clock plays a major role in regulating cellular metabolic activities including the expression of key enzymes in the oxidative pentose phosphate pathway (Figure 2-23), a major NADPH biosynthesis pathway. NADPH is a major reducing power for cellular metabolic activities. To test whether NADPH oscillates in plants, we first examined the daily changes of NADPH and its oxidized form NADP⁺ under constant light conditions. Indeed, the abundance of NADPH and

NADP⁺ display a circadian oscillation with NADPH peaking before dawn and NADP⁺ peaking before dusk (Figure 2-24).

Our previous study showed that treating plants with SA could alter the cellular redox and triggers genome-wide transcriptional reprogramming (Wang et al., 2006). This induction could be blocked by the treatment of 6-aminonicotinamide (6-AN), an inhibitor of the pentose phosphate pathway (Mou et al., 2003). We hypothesized that the basal NADPH rhythm might be affected upon immune induction. To test this hypothesis, we measured the NADPH and NADP⁺ levels after treating plants with SA. We found that SA could significantly perturb the rhythms of both compounds under constant light conditions (Figure 2-24).

Therefore, the reinforcement of the clock may negate the redox perturbation triggered by SA to restore the normal redox rhythms. The perturbation in redox rhythms should not be prolonged, since mutants with abnormal redox states have been shown to have reduced fitness (Reichheld et al., 2007; Xiong et al., 2009). We propose that in *Arabidopsis* the daily redox rhythm is intrinsically linked with the basal expression of the circadian clock through NPR1. Perturbation in the cellular redox rhythms caused by the immune signal SA is sensed by NPR1 to trigger defense gene expression and at the same time reinforce the circadian clock (Figure 2-25). This interplay between the redox rhythm and the circadian clock through NPR1 ensures a proper immune induction in response to SA without compromising fitness.

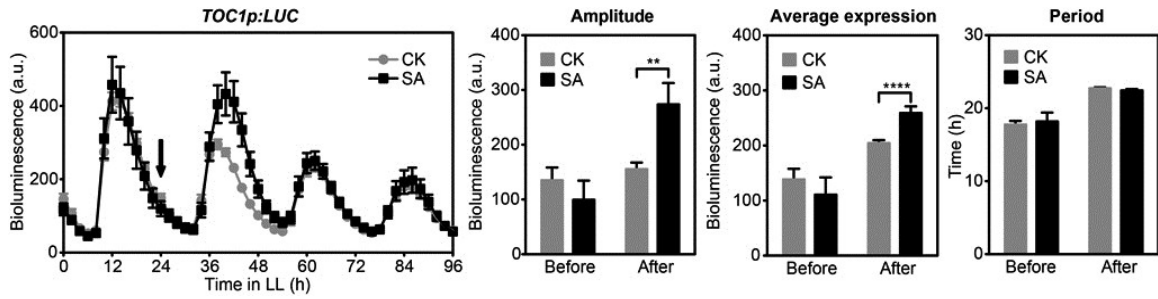


Figure 2-1: Application of SA at dawn increases the amplitude and average expression of *TOC1p:LUC* without changing the period.

The trace plot shows the *TOC1p:LUC* activity rhythms (mean \pm SEM, $n = 6$) in 3-week-old soil-grown plants treated with water (CK) or 1 mM SA at subjective dawn (black arrow). LL, constant light. The bar graphs represent the estimates of amplitude, average expression level and period of *TOC1p:LUC* (mean \pm SEM, Holm-Sidak test) respectively. Before, before treatment; After, after treatment. **, p -values < 0.01 ; ****, p -values < 0.0001 . These experiments have been repeated at least three times with similar results.

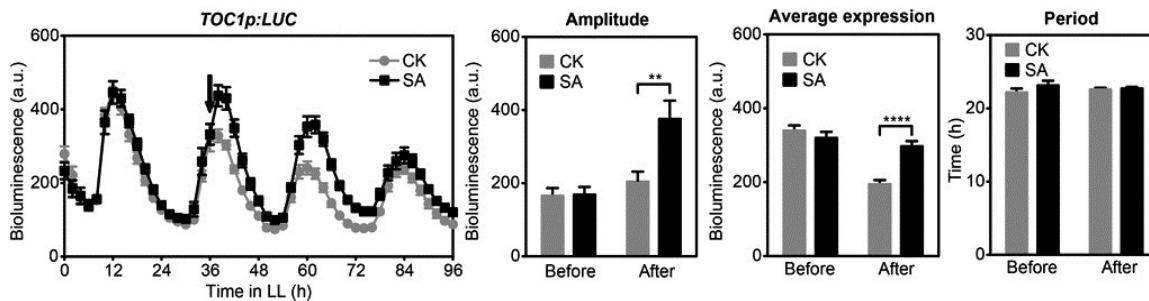


Figure 2-2: Application of SA at dusk increases the amplitude and the average expression of *TOC1p:LUC* without changing the period.

The trace plot shows the *TOC1p:LUC* activity rhythms in 3-week-old soil-grown plants treated with water (CK) or 1 mM SA at subjective dusk (black arrow) (mean \pm SEM, $n = 6$). LL, constant light. The bar graphs represent the estimates of amplitude, average expression level and period of *TOC1p:LUC* (mean \pm SEM, Holm-Sidak test) respectively. Before, before treatment; After, after treatment. **, p -values < 0.01 ; ****, p -values < 0.0001 . These experiments have been repeated at least three times with similar results.

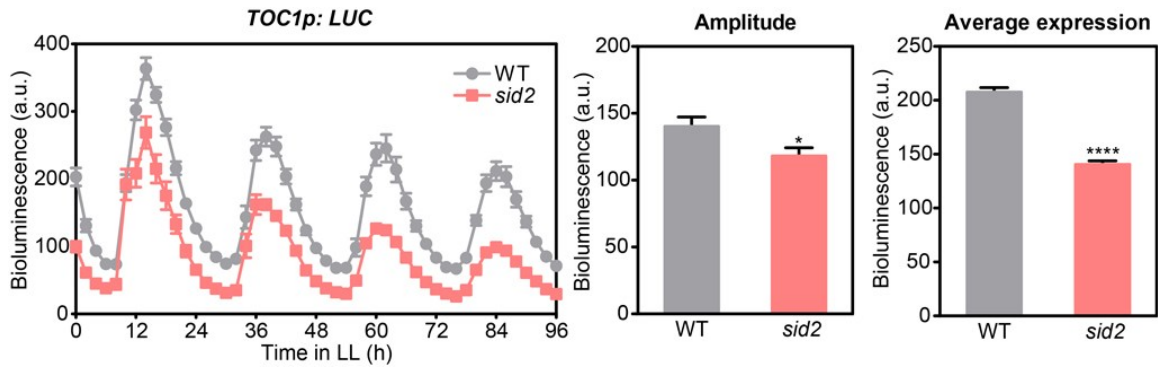


Figure 2-3: The amplitude and the average expression of *TOC1p:LUC* are lower in the SA-deficient mutant, *sid2*.

The trace plot shows the *TOC1p:LUC* activity rhythms in 3-week-old soil-grown wild-type (WT) and *sid2* plants (mean \pm SEM, $n = 6$). LL, constant light. Bar graphs represent the estimates of amplitude and average expression of *TOC1p:LUC*, respectively (mean \pm SEM, student *t*-test). *, p -values < 0.05 ; ****, p -values < 0.0001 . This experiment has been repeated twice with similar results.

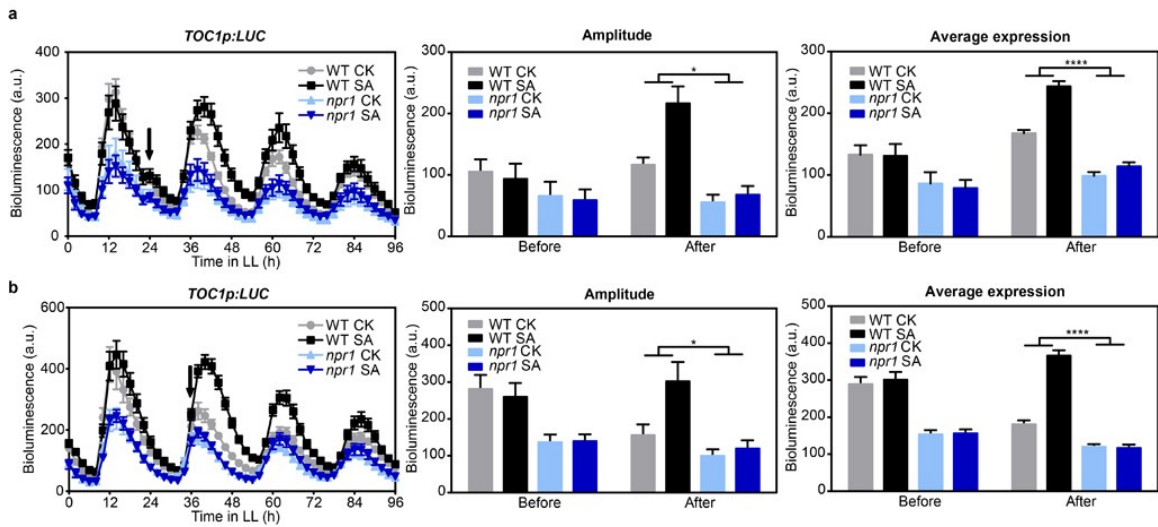


Figure 2-4: SA-triggered increases in *TOC1p:LUC* amplitude and average expression are NPR1-dependent.

The trace plots show the *TOC1p:LUC* activity rhythms in 3-week-old soil-grown wild-type (WT) and *npr1-3* plants treated with water (CK) or 1 mM SA at subjective dawn (a) and subjective dusk (b) (mean \pm SEM, $n = 6$). LL, constant light. Black arrows indicate the treatment time. The bar graphs show the estimates of amplitude and average expression level of *TOC1p:LUC*, respectively (mean \pm SEM, two-way ANOVA). Before, before treatment; After, after treatment. *, p -values < 0.05 ; ****, p -values < 0.0001 . These experiments have been repeated three times with similar results.

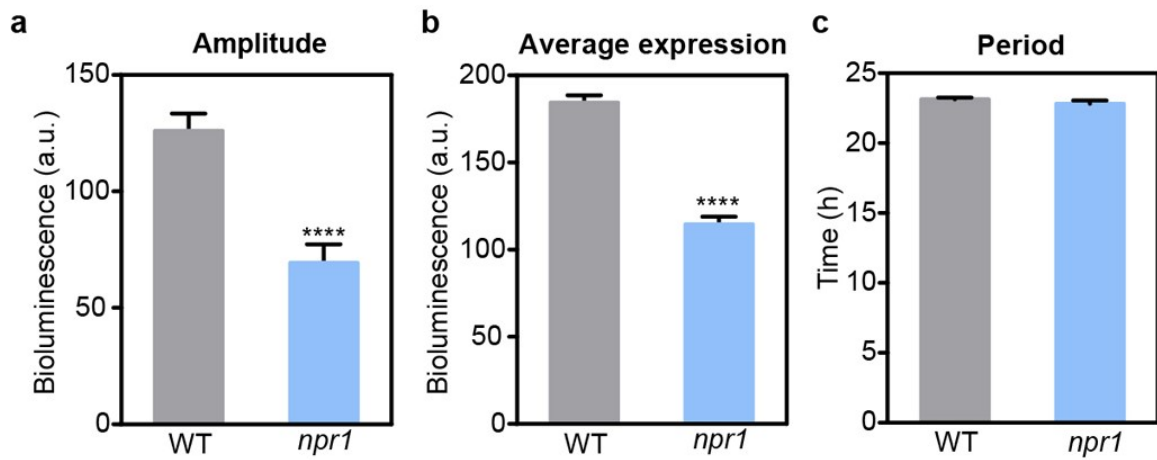


Figure 2-5: The *npr1* mutant shows decreased amplitude and average expression of *TOC1p:LUC* but maintains the period under basal conditions.

The bar graphs show the amplitude (a), average expression (b) and period (c) of *TOC1p:LUC* in wild-type (WT) and *npr1*. The error bars represent SEM. Student *t*-test was used for statistical analysis. ***, *p*-values < 0.001; ****, *p*-values < 0.0001. This experiment has been repeated more than three times with similar results.

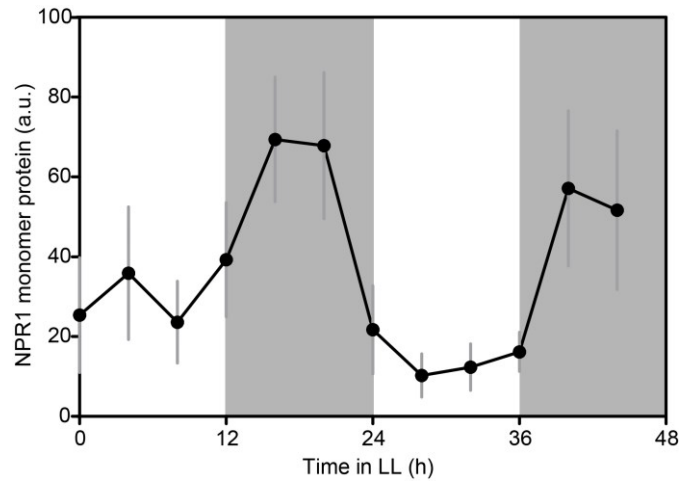


Figure 2-6: NPR1 monomer displays a circadian rhythm.

The plot shows western blot quantification of NPR1 monomer protein from 3-week-old plants grown in soil under constant light (LL) conditions. White bars represent subjective day and grey bars represent subjective night. Error bars represent SEM from three biological replicates.

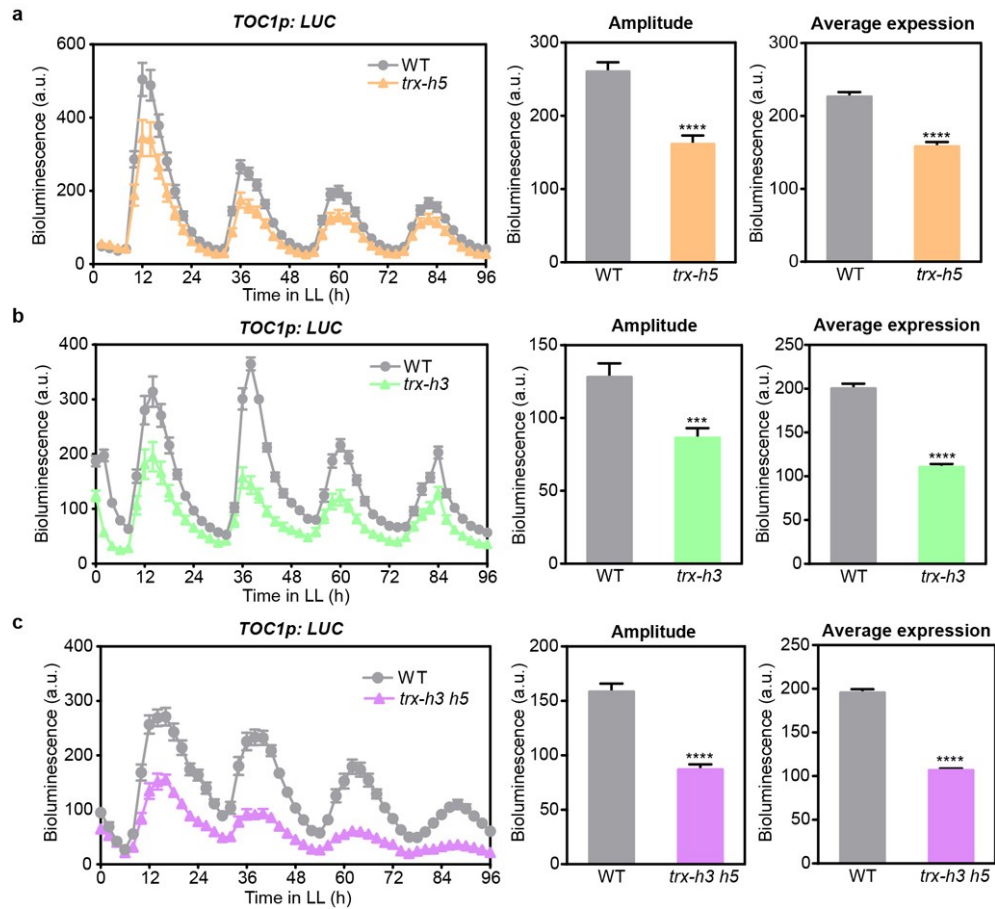


Figure 2-7: The amplitude and average expression of *TOC1p:LUC* are dampened in the *trx* mutants.

The trace plots show the *TOC1p:LUC* activity rhythms in 3-week-old soil-grown wild-type (WT) and *trx-h5* (a), *trx-h3* (b) and *trx-h3 h5* (c) (mean \pm SEM, $n = 6$). LL, constant light. The bar graphs show the estimates of amplitude and average expression (mean \pm SEM, student *t*-test). ***, p -values < 0.001 ; ****, p -values < 0.0001 . These experiments have been repeated at least twice with similar results.

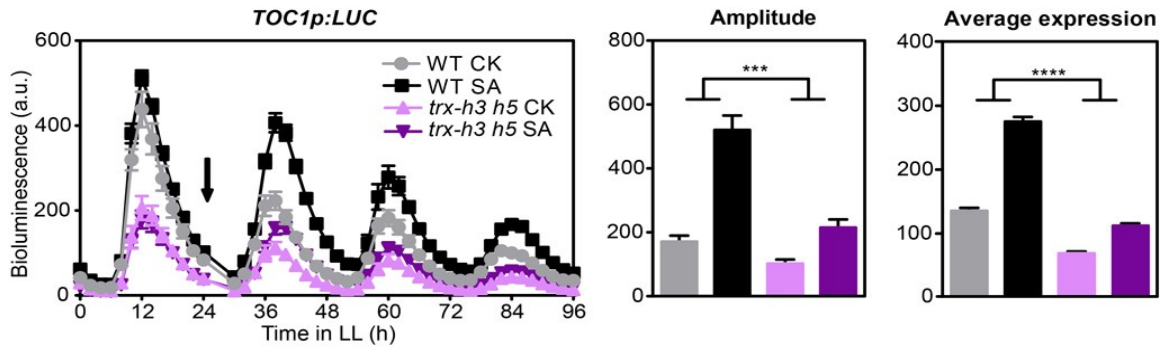


Figure 2-8: SA-triggered increases in *TOC1p:LUC* amplitude and average expression are largely abolished in *trx-h3 h5*.

The trace plot shows the *TOC1p:LUC* activity rhythms in 3-week-old soil-grown in wild-type (WT) and *trx-h3 trx-h5* plants treated with water (CK) or 1 mM SA (mean \pm SEM, $n = 6$). LL, constant light. The black arrow indicates the treatment time. The bar graphs show the estimates of amplitude and average expression level, respectively (mean \pm SEM, two-way ANOVA). ***, p -values < 0.001 ; ****, p -values < 0.0001 . This experiment has been repeated three times with similar results.

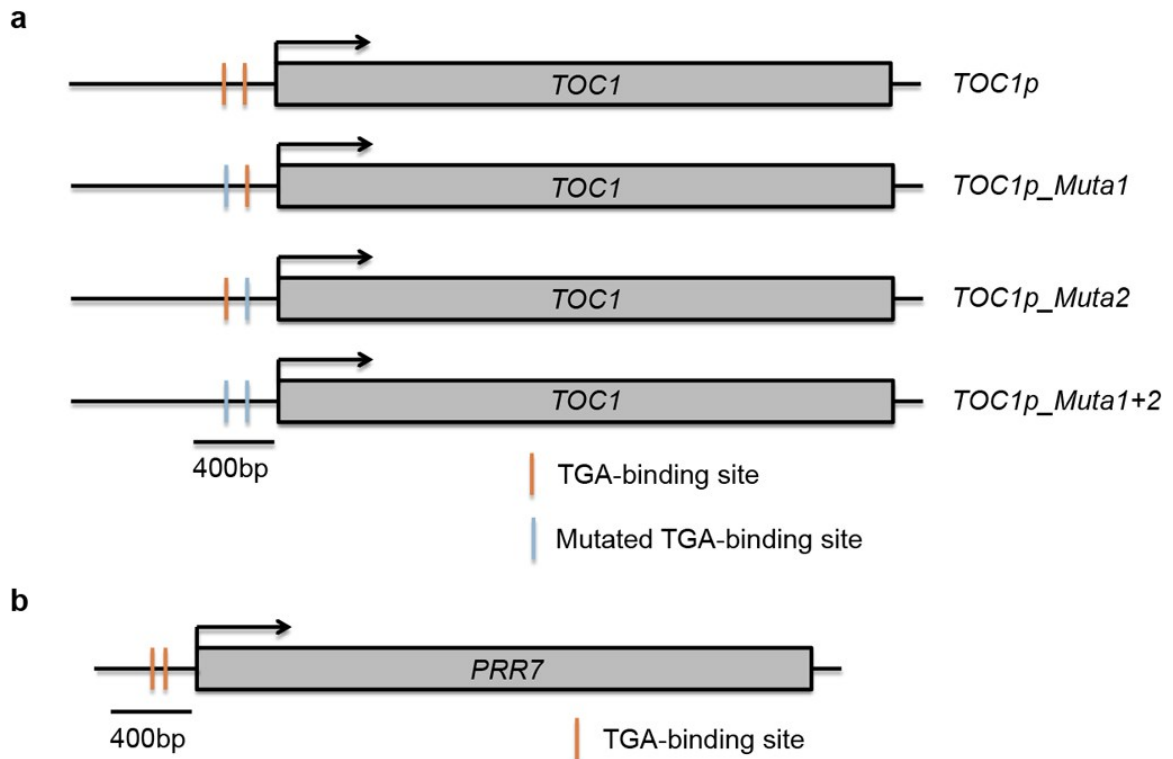


Figure 2-9: TGA-binding sites in the *TOC1* and *PRR7* promoters.

a, Schematics showing the two TGA-binding sites in the *TOC1* promoter with orange bars representing wild-type TGA-binding sites and blue bars representing the mutated TGA-binding sites. The TGA-binding sites are located from -258 to -251 bp and from -183 to -176 bp upstream of the transcription start site. **b**, A schematic showing that two TGA-binding sites in the *PRR7* promoter. The TGA-binding sites are located from -184 to -177 bp and -131 to -138 bp upstream of the transcription start site.

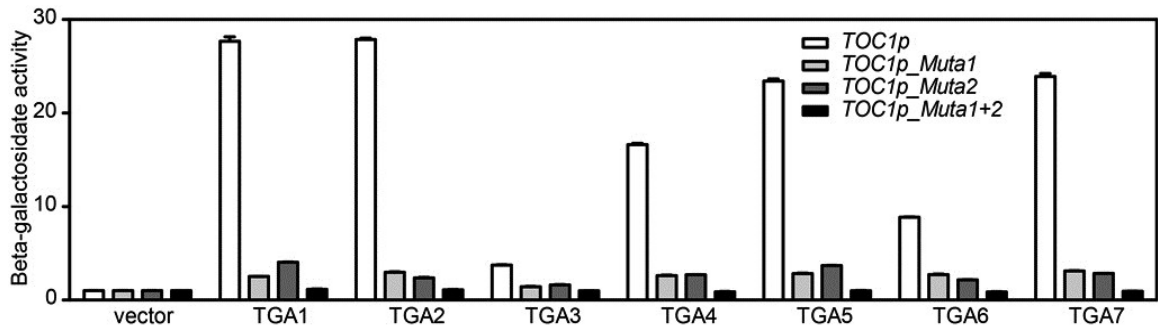


Figure 2-10: TGAs bind to the promoter of *TOC1* in yeast.

The binding of TGAs to the promoter of *TOC1* in Y1H. Beta-galactosidase reporter activities were measured using ONPG as the substrate and were normalized to the control with an empty pDEST-AD vector. Two TGA-binding sites were mutated individually or in combination. Error bars represent SEM from three technical replications. This experiment has been repeated twice with similar results.

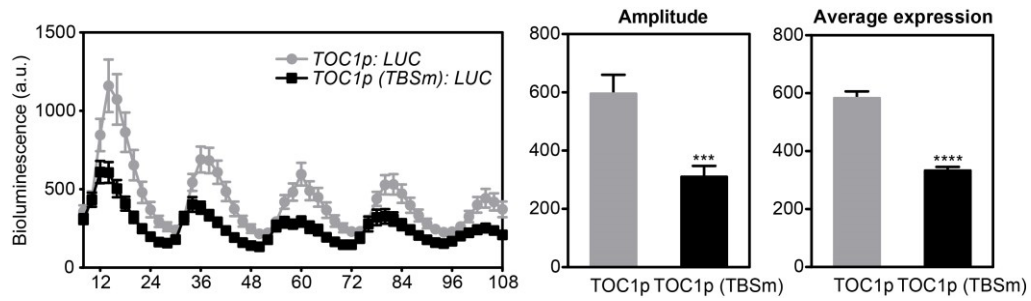


Figure 2-11: TGAs bind to the promoter of *TOC1* in *planta*.

The trace plot shows the luciferase activity rhythms in 3-week-old soil-grown plants carrying *TOC1p:LUC* and *TOC1p(TBSm):LUC* (two TGA-binding sites mutated) (mean ± SEM, n = 20, different T1 plants). The bar graphs show the estimates of amplitude and average expression (mean ± SEM, student *t*-test). ***, *p*-values < 0.001; ****, *p*-values < 0.0001. This experiment has been repeated twice from two independent transformation with similar results.

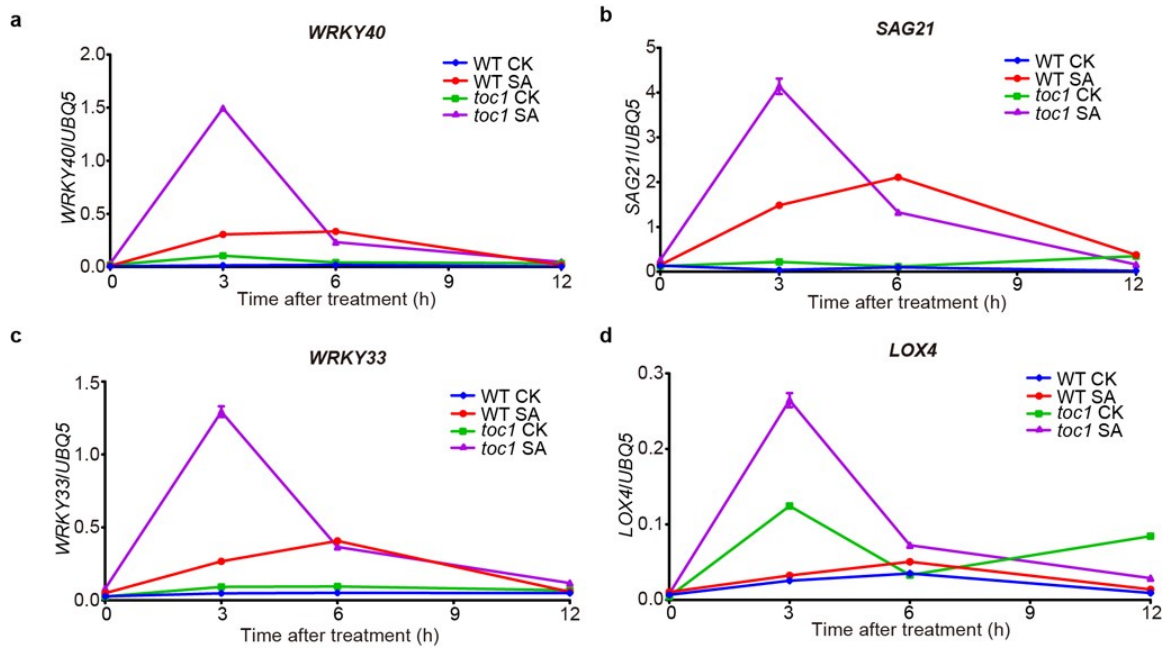


Figure 2-12: Increased defense gene expression in *toc1* after SA treatment.

The transcript levels of defense genes *WRKY40* (a), *SAG21* (b), *WRKY33* (c) and *LOX4* (d) normalized to *UBQ5*. Water (CK) or 1 mM SA was applied at dawn and samples were collected at 0, 3, 6 and 12 hours after treatment. This experiment has been repeated twice with similar results. This figure is provided by Wei Wang.

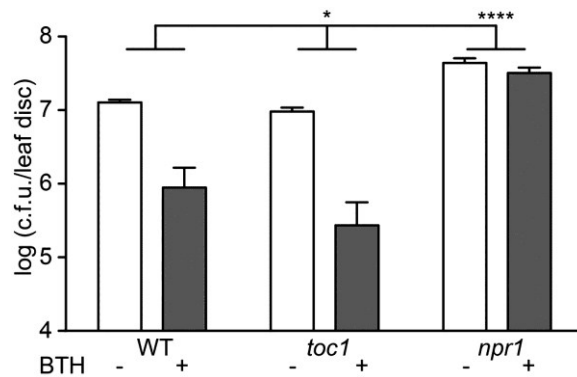


Figure 2-13: The *toc1* mutation enhances SA-induced resistance.

Three-week-old soil-grown wild-type (WT), *toc1* and *npr1* plants were sprayed with water (-) or with the synthetic analog of SA, BTH (300 μ M) (+) before infiltrated with *Psm* ES4326 ($OD_{600nm} = 0.001$). *In planta* bacterial growth was measure 3 days after. c.f.u., colony forming units. Error bars represent 95% confidence intervals, n = 8. Statistical significance determined by two-way ANOVA. *, *p*-values < 0.05; ****, *p*-values < 0.0001. This experiment has been repeated three times with similar results.

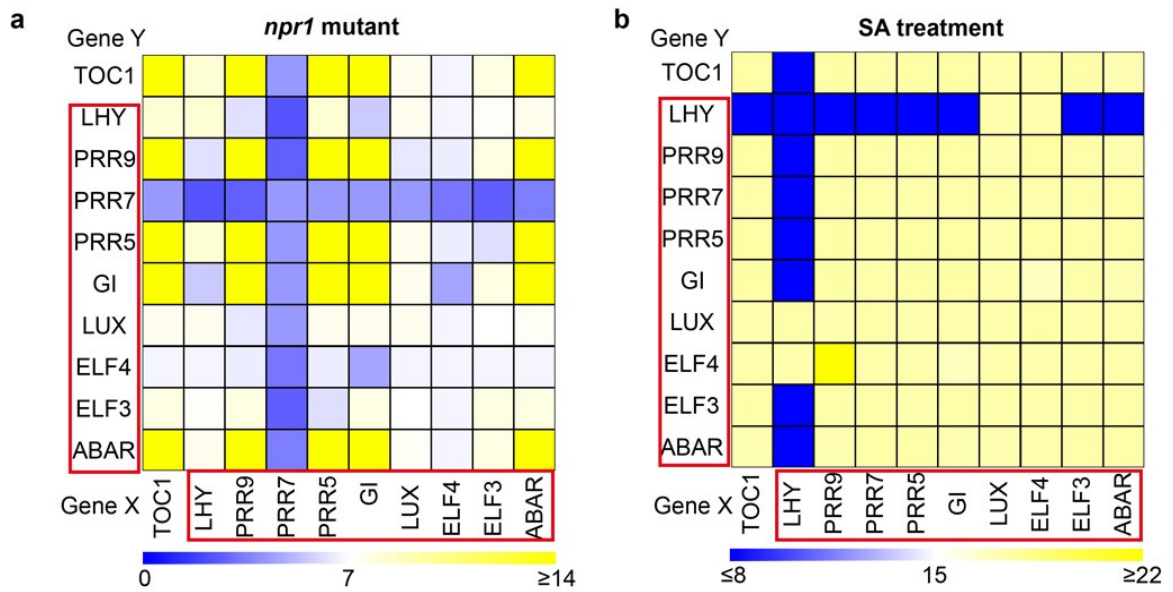


Figure 2-14: The heat maps showing the model fitness of clock genes.

The heat maps show the least squares fitting results of different query genes (X, Y) to *npr1* mutant data (a) and SA treated wild-type plants data (b). The color bars indicate the least squares residual for each gene combination, where lower residuals ('blue') indicate a better fit to the data. This figure is provided by Sargis Karapetyan.

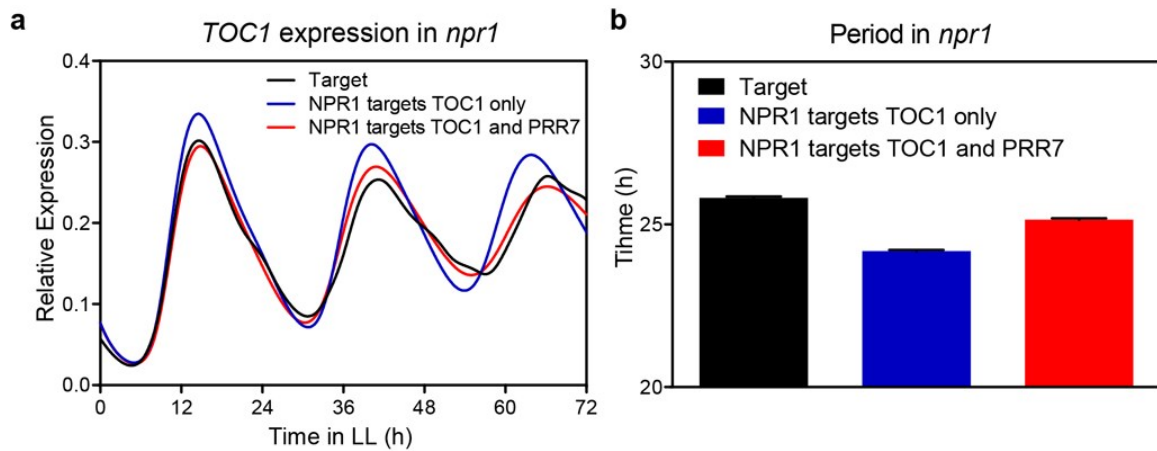


Figure 2-15: Modeling shows that *PRR7* basal-regulation by NPR1 best explains *TOC1* expression in *npr1* mutant.

a, Comparison of best-fit solutions for the *TOC1*-only and the *TOC1*-plus-*PRR7* coupling in the *npr1* mutant. **b**, Addition of *PRR7* coupling improves the fitness and mostly rescues the short period phenotype of the *TOC1*-only model. This figure is provided by Sargis Karapetyan.

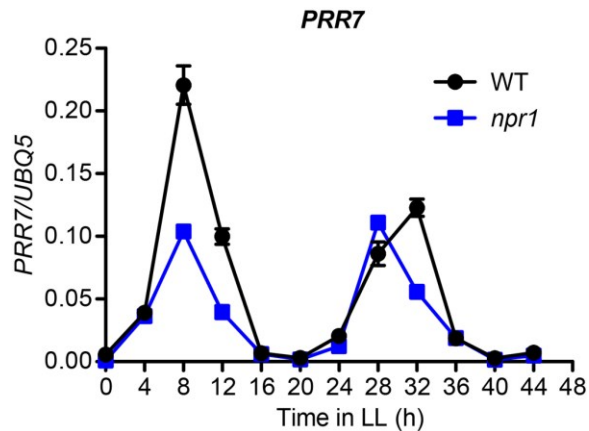


Figure 2-16: The basal expression of *PRR7* is reduced in *npr1*.

Three-week-old soil-grown wild-type (WT) and *npr1* plants were collected under constant light (LL) condition for mRNA extraction. The expression level of *PRR7* was determined using qPCR. Gene expression was normalized to the constitutively expressed *UBQ5*. Error bars represent SD of three technical replicates (n = 3). The experiments have been performed three independent times with similar results.

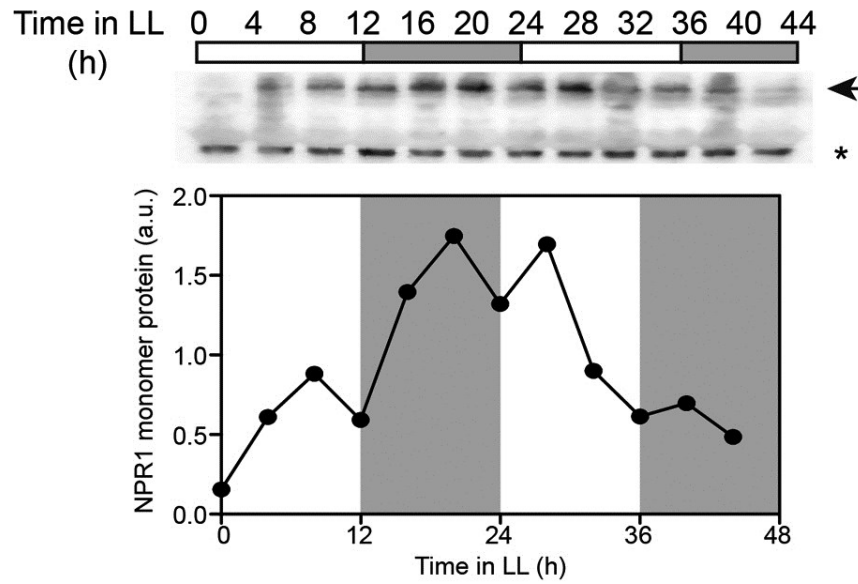


Figure 2-17: The abundance of NPR1 monomer after SA treatment.

White bars represent subjective day and grey bars represent subjective night. 1 mM SA was applied at 0 h. LL, constant light. The arrow indicates the NPR1 monomer. The star indicates a non-specific band used for normalization. The plot shows the quantification of NPR1 abundance from the western blot after normalization. This experiment has been repeated three times with similar results.

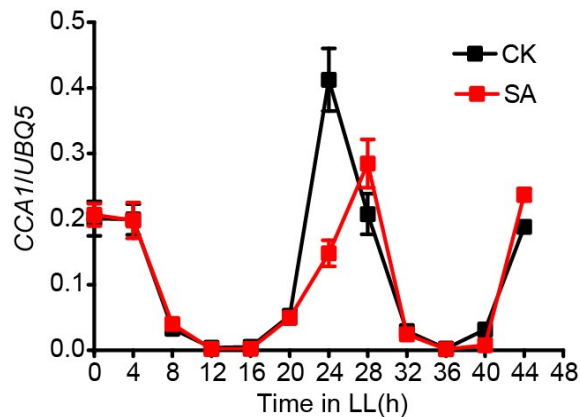


Figure 2-18: The transcription level of *CCA1* is not increased by SA.

The mRNA was extracted to analyse the transcription level of *CCA1* by qPCR.

Samples were collected under constant light (LL). Water (CK) or 1 mM SA was applied at 0 h. The expression of *CCA1* was normalized to constitutively expressed *UBQ5*. The error bars represent SEM (n = 3). This experiment has been done three times with similar results.

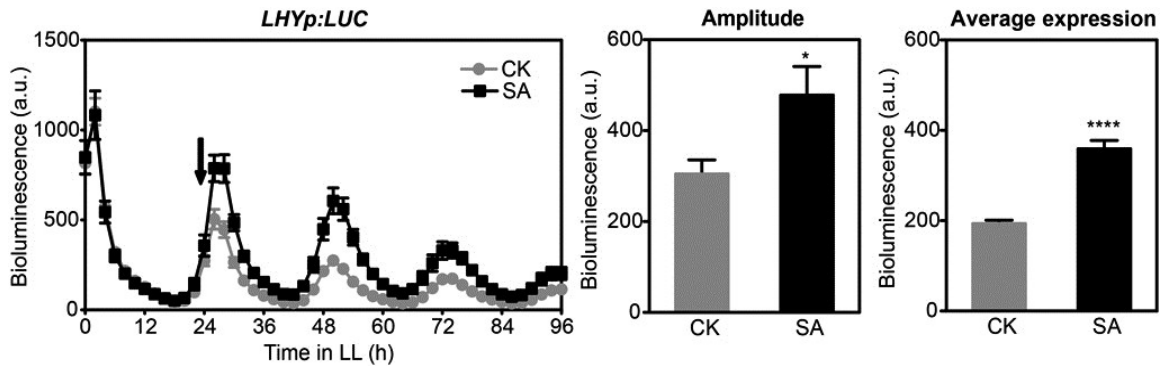


Figure 2-19: SA induces the amplitude and average expression of *LHY*.

The trace plot shows the *LHYp:LUC* activity rhythms (mean \pm SEM, $n = 6$) in 3-week-old soil-grown plants treated with water (CK) or 1 mM SA at subjective dawn (black arrow). LL, constant light. The bar graphs represent the estimates of amplitude and average expression level of *LHYp:LUC* using data after treatment (mean \pm SEM, student *t*-test). *, p -values < 0.05 ; ****, p -values < 0.0001 . This experiment has been repeated three times with similar results.

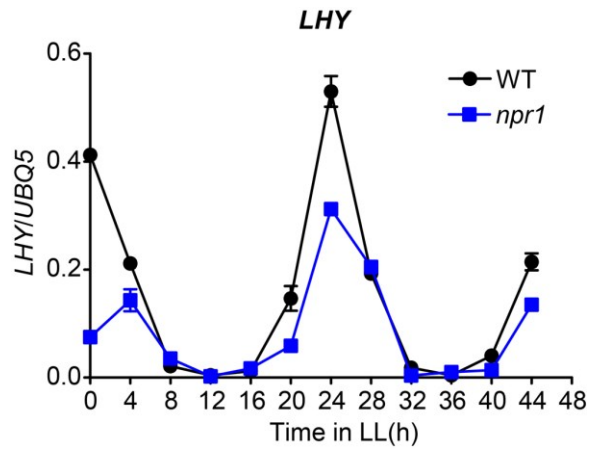


Figure 2-20: The basal expression of *LHY* is reduced in *npr1*.

Three-week-old soil-grown wild-type (WT) and *npr1* plants were collected under constant light (LL) condition for mRNA extraction. The expression level of *LHY* was determined using qPCR. Gene expression was normalized to the constitutively expressed *UBQ5*. Error bars represent SD of three technical replicates (n = 3). The experiments have been performed three independent times with similar results.

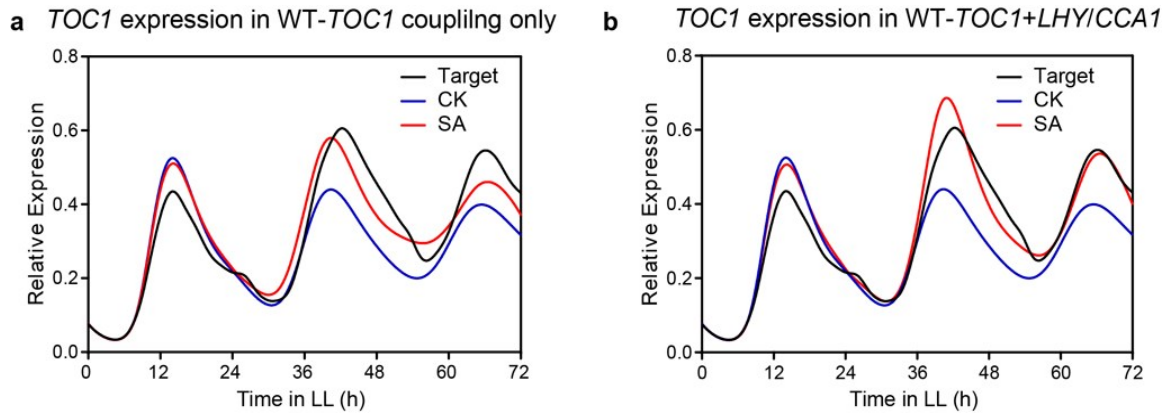


Figure 2-21: Modeling shows that *LHY/CCA1* regulation by NPR1 best explains *TOC1* expression in response to SA treatment.

Comparison of best-fit solutions for the *TOC1*-only and the *TOC1*-plus-*LHY/CCA1* coupling after SA treatment. The *TOC1*-only case shows immediate induction of *TOC1* upon addition of SA (a), while addition of *LHY* prevents the induction of *TOC1* until dusk as observed in the experiments (b). This figure is provided by Sargis Karapetyan.

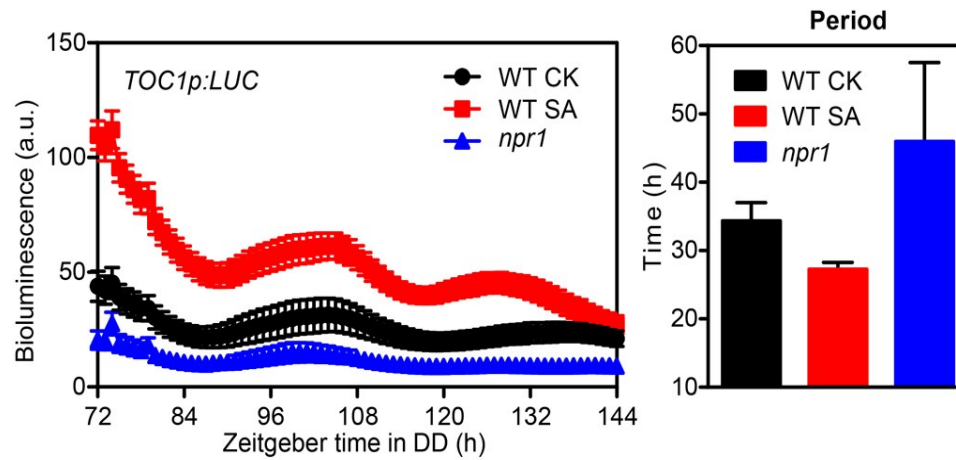


Figure 2-22: SA reinforces the circadian clock.

The trace plot shows light perturbation (a 40-min white light exposure with intensity of $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, applied at ZT57) to the *TOC1p:LUC* activity rhythms in wild-type (WT) plants treated with water (CK) or 1 mM SA at subjective dawn (ZT48) and in *npr1* mutant plants (mean \pm SEM, $n = 12$). DD, constant dark. The bar graph shows the estimate of period (mean \pm SEM). These experiments have been repeated twice with similar results.

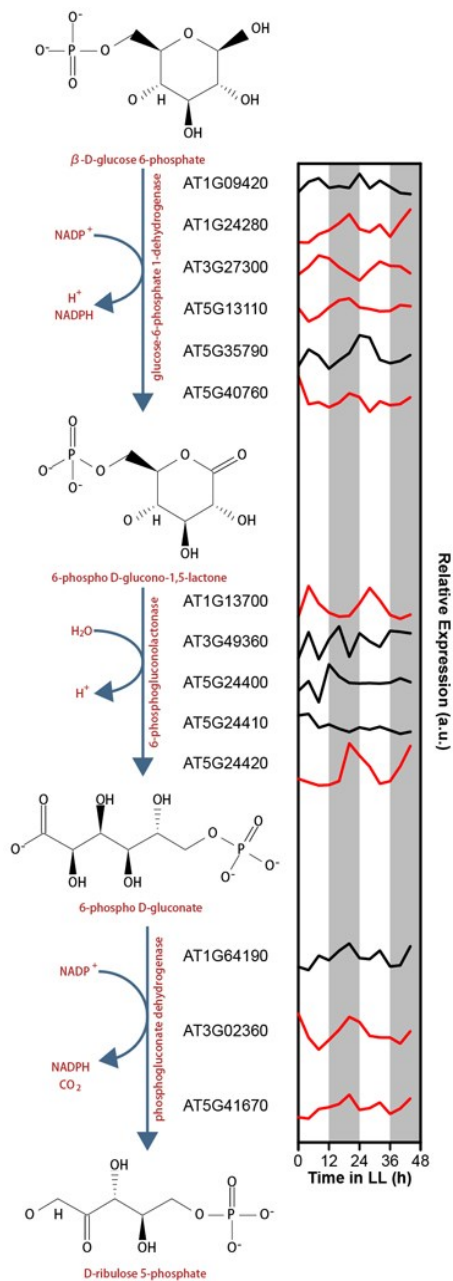


Figure 2-23: The circadian regulation of key enzyme genes in oxidative pentose phosphate pathway.

A diagram showing steps in the oxidative pentose phosphate pathway. The AGI numbers of genes encoding key enzymes in this pathway are listed. The relative

expression pattern of these genes under constant light (LL) are obtained from microarray data (<http://diurnal.mocklerlab.org/>, microarray: LL23_LDHH) (Mockler et al., 2007). The white bars represent subjective day. The grey bars represent subjective night. The expression patterns of genes with significant circadian rhythms (coefficient > 0.75) are showed in red.

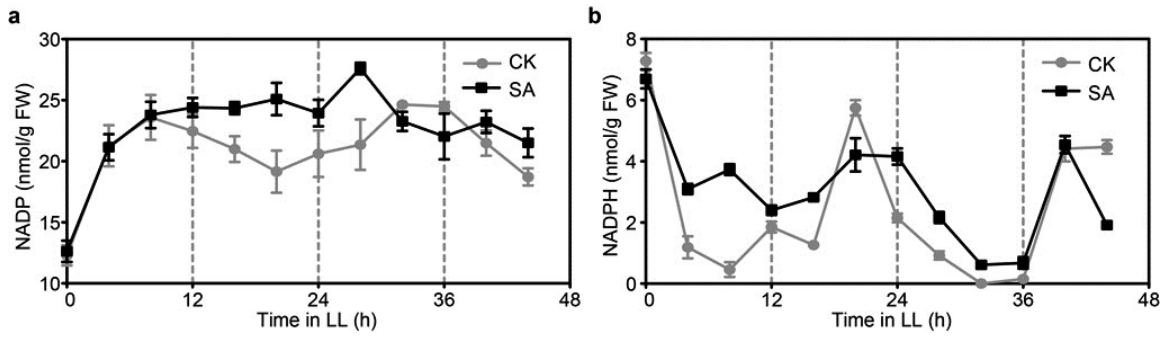


Figure 2-24: The levels of NADP⁺ and NADPH abundance under basal and SA-induced conditions.

The trace plots show NADP⁺ (a) and NADPH (b) levels in wild-type (WT) plants under constant light (LL) conditions. Water (CK, gray line) or 1 mM SA (black line) was applied at 0 h. The error bars stand for SEM (n = 3). These experiments have been repeated twice with similar results. This figure is provided by Wei Wang.

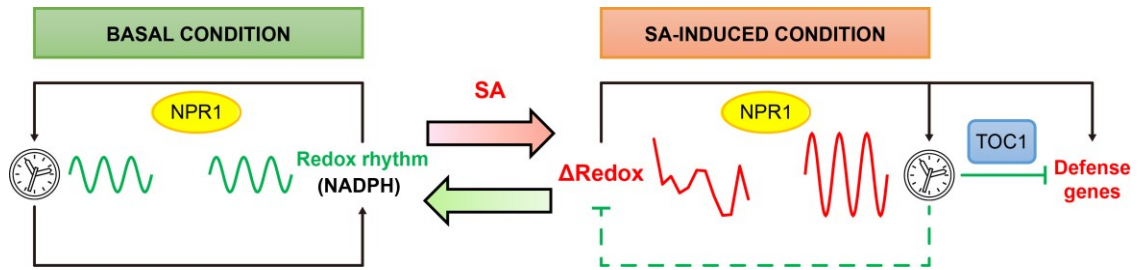


Figure 2-25: A model showing the relationship between the redox state and the circadian clock under basal and SA-induced conditions.

The daily redox rhythm is intrinsically linked with the basal expression of the circadian clock through NPR1. Perturbation in the cellular redox rhythms caused by the immune signal SA during pathogen challenge is sensed by NPR1 to trigger defense gene expression and at the same time to reinforce the circadian clock. While SA-induced *TOC1* expression represses the defense gene, reinforced clock may negate the redox perturbation to dampen the immune activation. Black arrows represent positive regulation. Green block arrows represent negative regulation. Dash line indicates a relationship that may or may not be direct.

2.3 Discussions

Besides the circadian clock, recent studies showed that non-transcriptional redox oscillators exist in human red blood cells (O'Neill and Reddy, 2011) and in the unicellular alga *Ostreococcus tauri* (O'Neill et al., 2011). This diurnal oxidation of the PRX protein was also observed in *Arabidopsis* (Edgar et al., 2012). Our finding of the oscillation of NADPH, which participates in various biosynthesis pathways and in replenishment of other reducing equivalents, implies the existence of a widespread redox or metabolic rhythm beyond the oscillation of oxidized PRX.

Mutations in PRX were shown to perturb the phase of the circadian clock, implying that the circadian clock is linked to the redox rhythm (Edgar et al., 2012). However, the mechanism by which these two oscillating systems are linked remains unknown. Our finding that the redox-sensitive NPR1 is the intrinsic transcription regulator of clock components provides the mechanism of how the redox rhythm affects the circadian clock.

The role of SA as an input to the redox rhythm is consistent with its known function in regulating respiration in plants (Rhoads and McIntosh, 1992) and in binding and inhibiting activities of a number of ROS scavenging enzymes (Chen et al., 1993; Durner and Klessig, 1995; Fu and Dong, 2013; Tian et al., 2012). Recently, SA was also found to bind and activate the human adenosine monophosphate-activated protein kinase (AMPK), which is a major energy sensor and regulator for all eukaryotes

(Steinberg et al., 2013). Collectively, our results suggest that a general redox oscillation manifested by the NADPH rhythm exists in *Arabidopsis* and perhaps also in other organisms, and this rhythm is sensitive to external perturbations.

Besides SA, other plant hormones have also been reported to modulate the circadian clock (Covington and Harmer, 2007; Hanano et al., 2006; Legnaioli et al., 2009; Zheng et al., 2006). While cytokinin affects the phase of the clock, BR and ABA modulate the period. Auxin, on the other hand, only affects the amplitude. How these hormones regulate different aspects of the circadian clock remains largely unknown. Our discovery of a general redox rhythm manifested by the oscillation of NADPH/NADP⁺ levels and its effect on the central circadian oscillator provided a possible answer. It is well-known that different plant hormone signaling pathways crosstalk and the modulation of redox status by these hormones appears to be a common event of plant hormone signaling network (Bartoli et al., 2013). Therefore, it is possible that distinct redox perturbation signals triggered by different plant hormones may influence the central oscillator in different ways. Mathematical modeling may be prudent for identifying the potential modes of connections between the other hormones and the central oscillators.

Our discovery of the reinforcement of the circadian clock by SA provides a possible explanation for the broad medicinal effects of SA and its various derivatives such as aspirin in humans with regard to treatment of type II diabetes and significant reduction in death from certain types of cancer (Rothwell et al., 2011). SA has recently

been shown to bind and activate the mammalian AMPK, which is a key metabolic sensor in all eukaryotes (Hawley et al., 2012). Activation of AMPK leads to metabolic reprogramming which also involves dramatic redox status changes (O'Neill and Hardie, 2013). In humans, the redox status may directly affect the circadian clock, since NADH and NADPH can enhance the binding affinity of human circadian clock components to their downstream target genes (Rutter et al., 2002). The amplitudes of human circadian genes are greatly reduced in tumour cells compared to healthy liver cells (Filipski et al., 2005). Moreover, colon or breast cancer patients having stronger circadian rhythms were found to survive twice as long as the patients with dampened rhythms (Levi et al., 2007). Based on the link between SA and the circadian clock in plants, we hypothesize that in humans, SA and aspirin may help fight against diabetes and cancer by reinforcing the circadian clock and consequently strengthening the metabolic rhythms.

2.4 Materials and experimental methods

2.4.1 Plant materials

The *TOC1p: LUC* (Col-0) and *LHYp: LUC* (Col-0) lines were kindly provided by Dr. Robertson McClung. The *toc1* mutant was kindly provided by Dr. Takafumi Yamashino. Mutants of *npr1-3* (Cao et al., 1997), *sid2* (Nawrath and Metraux, 1999), *trx-h3* (Tada et al., 2008) and *trx-h5* (Tada et al., 2008) were used to cross with the luciferase reporter lines. To generate different T1 lines of *TOC1p:LUC* and *TOC1p (TBSm): LUC* (*TOC1* promoter mutated in two TGA-binding sites), wild-type and mutated *TOC1*

promoters (amplified using QuikChange Lighting Multi Site-directed mutagenesis kit, Agilent Technologies) were first cloned into the pDONR207 vector (Invitrogen) through the Gateway BP reaction (Invitrogen) and then transferred to the destination vector pGWB235 (Nakagawa et al., 2007) through the Gateway LR recombination reaction (Invitrogen). *Agrobacteria*-mediated transformation of *Arabidopsis* was performed as previously described using wild-type plants (Clough and Bent, 1998). Different T1 lines were selected for the luciferase imaging experiment. All primer sequences used for transgenic plants are listed in Table 2-1.

2.4.2 NADP⁺ and NADPH measurement

Three-week-old plants grown under diurnal condition (12 h light/12 h dark) were treated with water or 1 mM SA at subjective dawn and samples were collected every 4 h for two days under constant light conditions. NADP⁺ and NADPH were measured according to Queval et al (Queval and Noctor, 2007) with some modifications. Briefly, 50 mg 3-week-old leaves were pulverized in liquid nitrogen using Genogrinder and extracted using 10 mM Tris-HCl (pH 8.0, 1 ml Tris-HCL per 100 mg tissue). The homogenate was centrifuged at 16,000 g for 10 min at 4 °C. The supernatant was separated into two 0.2 ml aliquots. To extract NADP⁺, 50 µl 1 M HCl was added to one 0.2 ml aliquot. The mixture was heated in boiling water for 1 min. Then 25 µl MES (pH5.6) was added and the pH of the extract was adjusted to 5-6 using 0.2 M NaOH. To extract NADPH, 50 µl 1 M NaOH was added to the other 0.2 ml aliquot. The mixture

was heated in boiling water for 1 min. Then 25 μ l MES (pH5.6) was added and the pH of the extract was adjusted to 7-8 using 0.2 M HCl. Three 20 μ l aliquots of the NADP⁺ and the NADPH extracts were used. Samples containing only the extraction buffer were used as blank. The measurement of the samples and the derivation of the standard curves were performed exactly according to Queval et al (Queval and Noctor, 2007).

2.4.3 Luciferase imaging and bioluminescence analysis

Plants grown in soil with entrainment at 12 h light /12 h dark cycles for three weeks were sprayed with 2.5 mM luciferin (Gold Biotechnology) in 0.02% Triton X-100 (Sigma) one day before luciferase imaging. Plants were then placed into the imaging system (Nightshade LB985) under constant light conditions and assayed for bioluminescence by acquiring images every 2 h with exposure time of 20 min. To test the SA effect, 1 mM SA (Sigma) or water (as control) was sprayed at different indicated times. Subsequent quantifications of bioluminescence intensity were performed using Image J. The 5th and 6th true leaves from each plant were selected and mean bioluminescence intensity for the selected region throughout the experiment was measured after background subtraction.

2.4.4 Analysis of circadian rhythms

The quantified time course bioluminescence data were decomposed into a line and a sine wave with exponential decaying amplitude using GraphPad 6. The intercept of the line at y-axis was considered as the average expression level. The period and

amplitude were inferred from the sine wave. The exponential decay was used to compensate the dampening of the bioluminescence over time.

2.4.5 Western blot

Three-week-old plants grown under diurnal conditions (12 h light/12 h dark) were treated with water or 1 mM SA at subjective dawn and samples were collected every 4 h for two days under constant light conditions. Detection of the NPR1 monomer protein was performed as previously described using an antibody against NPR1 (Mou et al., 2003).

2.4.6 Y1H and ONPG assay

The *TOC1* and *LHY* promoters were first cloned into the pDONR P4-P1R vector (Invitrogen) through the Gateway BP reaction. The entry clones were recombined into the destination vectors pMW#2 (Invitrogen) and pMW#3 (Invitrogen). Mutagenesis of the *TOC1* promoter was performed using QuikChange Lighting Multi Site-directed mutagenesis kit (Agilent Technologies) according to the instruction manual.

TOC1p_Muta1 (the *TOC1* promoter mutated in the 1st TGA-binding site), TOC1p_Muta2 (the *TOC1* promoter mutated in the 2nd TGA-binding site) and TOC1p_Muta1+2 (the *TOC1* promoter mutated in two TGA-binding sites) were cloned into the destination vectors pMW#2 and pMW#3 through the Gateway cloning kit (Invitrogen). The coding sequences of TGAs were cloned into pDONR207 and subsequently transferred into the pDEST-AD vector by Gateway LR reactions.

Transformation into the yeast strain YM4271 was performed as previously described (Deplancke et al., 2004). β -galactosidase (β -gal) activity was determined as described before (Pruneda-Paz et al., 2009) with some modifications. Briefly, transformed yeast was grown in 3 ml SD-His-Ura-Trp liquid medium at 30 °C for overnight. After incubation, some of the yeast cultures were inoculated to 6 ml YPD in 50 ml conical tube at 30 °C until OD_{600nm} reached between 0.6 and 0.8. After the culture was cooled down on ice, a 1 ml aliquot was used to determine the accurate OD_{600nm} using a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences). Yeast cells from three aliquots of 900 μ l culture were spun down and re-suspended in 150 μ l Z buffer, and then lysed by two freeze/thaw cycles. The enzymatic reaction was started by adding 850 μ l Z buffer/ 600 μ g ONPG (2-Nitrophenyl- β -D galactopyranoside, Sigma) and was incubated at 30 °C between 10 and 24 h. The reaction was stopped by addition of 400 μ l 1M Na_2CO_3 . After centrifugation, the supernatant was used to determine the OD_{420nm} .

All primer sequences used for Y1H are listed in Table 2-2.

2.4.7 SA-induced resistance assay

Plants were treated with water or 300 μ M BTH (Novartis) on the 8th, 11th, 15th and 18th days of growth. On the 21st day, plants were then infiltrated with *Psm* ES4326 ($OD_{600nm} = 0.001$) as previously described (Mou et al., 2003). Briefly, 8 plants/genotype/treatment were inoculated with *Psm* ES4326 into two leaves and sampling was performed 3 days post inoculation to analyse the bacterial growth.

2.4.8 RNA extraction and quantitative PCR

Three-week-old plants grown under diurnal conditions (12 h light/12 h dark) were treated with water or 1 mM SA at subjective dawn and samples were collected every 4 h for two days under constant light conditions. RNA extraction was performed as previously described (Cao et al., 1997). cDNA synthesis (SuperScript III, Invitrogen) and quantitative PCR (SYBR Green, Roche) were performed according to the manufacturer's protocols. All primer sequences used for qPCR are listed in Table 2-3.

2.4.9 Light perturbation assay

Plants grown in soil with 12 h light /12 h dark cycles for three weeks were sprayed with 2.5 mM luciferin (Gold Biotechnology) in 0.02% Triton X-100 (Sigma) one day before luciferase imaging. Plants were then placed into the imaging system (Nightshade LB985) under constant dark conditions at ZT12 (dusk) and assayed for bioluminescence by acquiring images every 1 h with exposure time of 20 min. Water or 1 mM SA were applied at ZT48 (subjective dawn). A 40-min white light perturbation ($50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was applied at ZT57.

Table 2-1: Primer sequences for transgenic plants

Primer name	Sequences
TOC1p_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAGATCGC TCGGCTCAACAA
TOC1p_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCATTGTTTTGT TTTGTCAATC
TOC1p_Muta1	ATATTTTCTCCAAGAGTCCGTGGCCTTTTCTC
TOC1p_Muta2	TTTTTATTGTCCACGGACTCTCCTTGGCCTAA

Table 2-2: Primer sequences for Y1H

Primer name	Sequences
TOC1p_P4P1R_F	GGGGACAAC TTTGTATAGAAAAGTTGGAGATCGCTCGGC TCAACAA
TOC1p_P4P1R_R	GGGGACTGCTTTTTTTGTACAAACTTGATTGTTTTGTTTTGT CAATC
TOC1p_Muta1	ATATTTTCTCCAAGAGTCCGTGGCCTTTTCTC
TOC1p_Muta2	TTTTTATTGTCCACGGACTCTCCTTGGCCTAA
TGA1_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAATTC GACATCGACACAT
TGA1_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGTTGGTTC ACGATGTCGAGT
TGA2_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTG ATACCAGTCCGAGA
TGA2_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTCTGGG TCGAGCAAGCCA
TGA3_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGA TGATGAGCTCTTCT
TGA3_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTGTGTTTC TCGTGGACGAGC
TGA4_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAATA CAACCTCGACACAT
TGA4_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGTTGGTTC ACGTTGCCTAGC
TGA5_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGGAG ATACTAGTCCAAGA
TGA5_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTCTTGG TCTGGCAAGCCA
TGA6_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTG ATACCAGTTCAAGG
TGA6_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTCTTGG CCGGGCAAGCCA
TGA7_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGATGA GTTCTTCTCTCCA
TGA7_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTTGGTTC TTGTGGACGAGC

Table 2-3: Primer sequences for qPCR

Primer name	Sequences
LHY_qP_F	CGCTGCTTCGGTCTGGCCTT
LHY_qP_R	TGTAGCAGCGGCAATGGCAGT
PRR7_qP_F	CAGTCCACGAGCGGTATCTC
PRR7_qP_R	CCAGGGCCAGATCACAGTTT
CCA1_qP_F	TGACCGGTCCTCGTGTGGCT
CCA1_qP_R	ACTGCGGCGTGCATTGGACT
SAG21_qP_F	TCTTCCGACGTGGTTATGCG
SAG21_qP_R	CGTCAATCTCGTTGGAACCG
WRKY40_qP_F	ACAACGTCTTGAGGAAGCAAC
WRKY40_qP_R	TCCGTTGAGCTACTCTCCGA
WRKY33_qP_F	AGCCGAGAATCGTAGTGCAG
WRKY33_qP_R	CGTGTGATGCTCTCTCCACA
LOX4_qP_F	GCTGATTCATCCGAAGGGG
LOX4_qP_R	GTACCAGGCTTGGAGCTCAG
UBQ5_qP_F	GACGCTTCATCTCGTCC
UBQ5_qP_R	GTAAACGTAGGTGAGTCC

2.5 Acknowledgements

I would like to thank my collaborator Dr. Wei Wang for his help on NADP/NADPH⁺ measurement and statistical analysis. I would like to thank Sargis Karapetyan and Dr. Nicolas Buchler for their work on modelling part. I also want to thank Dr. R. McClung for providing the *TOC1p:LUC*, *LHYp:LUC* lines and Dr. T. Yamashino for providing the *toc1* mutant.

3 Salicylic acid biosynthesis is regulated by a circadian clock component

3.1 Introduction

As described in Chapter 1, the plant hormone salicylic acid (SA) controls the immune response known as systemic acquired resistance (SAR). SAR is a broad-spectrum resistance that can be induced by either a local pathogen challenge or exogenous application of SA. The primary infection by pathogens leads to plant cell death in the local tissue and generation of a systemic signal. This SAR signal is then transported to the intact tissue to induce SA synthesis to establish resistance (Fu and Dong, 2013). ICS1 is a key enzyme for defense-triggered SA synthesis and several signal proteins such as NDR1, EDS1, EDS5 and PAD4 also regulate the accumulation of SA (Vlot et al., 2009). All these SA accumulation-related genes are transcriptionally induced by pathogens as well as by SA and its synthetic analog, BTH, indicating a positive feedback loop between SA and transcription of these genes (Wang et al., 2008). This also suggests that transcription regulation is an important step in controlling SA accumulation and SAR.

However, our knowledge on how these SA accumulation-related genes are activated is still limited. It is notable that the abundance of the endogenous SA shows a circadian rhythm (Goodspeed et al., 2012), but the underlying molecular mechanism of the circadian regulation is largely unknown. My study specifically aimed to identify the clock component that regulates SA accumulation.

3.2 Results

3.2.1 The expression of *CHE* is in-phase with the SA synthesis gene *ICS1*

It is reasonable to propose that the expression of some SA accumulation-related genes may be under the circadian control, consequently generating the circadian rhythm of the endogenous SA level. Through searching the gene expression patterns in the publically available time-course microarrays, I found that among these SA accumulation-related genes, only *ICS1* displayed a strong oscillation under free-running conditions (Mockler et al., 2007) (<http://diurnal.mocklerlab.org/>). Therefore, clock components may affect the accumulation of SA through regulation of *ICS1* expression.

Interestingly, the clock component, *CHE*, exhibited a similar expression pattern as that of *ICS1* based on the microarray data. A previous study showed that *CHE* functions as a TF to regulate the expression of *CCA1* (Pruneda-Paz et al., 2009). To confirm its circadian oscillation pattern as well as to assess the *ICS1* expression at the same time, I collected time-course samples under the constant light conditions for RNA extraction and gene expression analysis. The results indicated that *CHE* and *ICS1* had similar oscillatory expression patterns peaking before the subjective night (Figure 3-1). The in-phase expression patterns of *ICS1* and *CHE* raise the possibility that *CHE* is a TF regulating *ICS1* transcription.

3.2.2 CHE interacts with the *ICS1* promoter

CHE as is a TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) family TF (Pruneda-Paz et al., 2009). Through bioinformatics analysis, I found one TCP-binding *cis*-element in the *ICS1* promoter. More direct evidence for CHE regulating *ICS1* came from a Y1H screen using the *ICS1* promoter as bait (unpublished data, collaborated with Dr. Steve Kay lab). This CHE interaction with the *ICS1* promoter was further confirmed by additional Y1H analysis (Figure 3-2). To test whether CHE binds to the *ICS1* promoter through the TCP-binding site (TBS), I introduced a point mutation to the binding site and found it completely abolished the CHE binding in yeast (Figure 3-2). We then performed ChIP using CHE_{OE} (*35S:CHE-GFP*) and wild-type plants to study CHE binding *in vivo*. Our results showed that *in planta* CHE could indeed bind to TBS within the *ICS1* promoter (Figure 3-3).

3.2.3 CHE positively regulates the *ICS1* expression

Treating plants with SA can induce *ICS1* expression to facilitate more SA accumulation. This signal amplification mechanism has been hypothesized to be important for the establishment of SAR (Jirage et al., 1999; Zhou et al., 1998). I found that this SA-triggered *ICS1* induction was significantly blocked in the *che* mutants (Figure 3-4). I then further tested the effect of the *che* mutation on pathogen-triggered systemic induction of *ICS1*. *Psm* ES4326 carrying *avrRpt2* was infiltrated locally and systemic tissues were then collected for gene expression analysis. The result showed that *ICS1*

induction was repressed in the *che* mutant (Figure 3-5). Collectively, these data suggest that CHE is a positive regulator of *ICS1* expression.

3.2.4 CHE is required for the establishment of SAR

Since systemic induction of *ICS1* is blocked in the *che* mutant, it is reasonable to hypothesize that SAR is also compromised in this mutant. Consistently, I found that the SA level in systemic tissues was lower in *che* than in wild-type plants in response to *Psm* ES4326/*avrRpt2* challenge (Figure 3-6), correlating with the reduced expression of *PR1* gene, which has been widely used as a marker for SA-mediated gene expression (Figure 3-7). Subsequently, I performed the SAR test on wild-type, *che* and the SAR-deficient mutant *npr1* plants. The compromised SAR in *che* indicates that functional CHE is important for the SAR establishment (Figure 3-8). Overall, CHE binds to the *ICS1* promoter to positively regulate its transcription and consequently affect the SA synthesis and the SAR establishment.

3.2.5 CHE is required for activation of another *ICS1* positive regulator SARD1

I next investigated the possible mechanism by which CHE amplifies the SA signal. Besides CHE, SARD1 and CBP60g are the other positive regulators of *ICS1* through directly binding to its promoter. *SARD1* is transcriptionally activated by an unknown upstream regulator upon pathogen infection; while the activity of CBP60g is mostly modulated by Ca²⁺ (Zhang et al., 2010). I found that the transcription of *SARD1* is partially CHE-dependent, because both basal and pathogen-induced systemic

expression of *SARD1* partially comprised in the *che* mutant (Figure 3-9). Therefore, activation of *SARD1* may require a functional CHE.

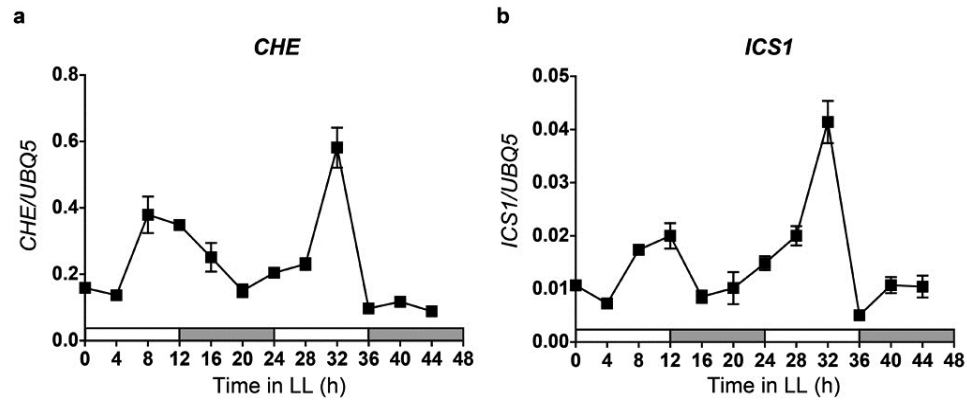


Figure 3-1: The transcript levels of *CHE* and *ICS1* under constant light conditions.

Three-week-old wild-type plants were transferred from 12 h light/12h dark cycles to constant light condition. Leaf samples were collected every 4 h for two days. RNA was extracted and used to generate cDNA for qPCR to detect the transcript levels of *CHE* (a) and *ICS1* (b). *UBQ5*, internal control. LL, constant light. This experiment has been performed twice with similar results. Error bars represent SEM; n = 3. White bars indicate subjective days and gray bars indicate subjective nights.

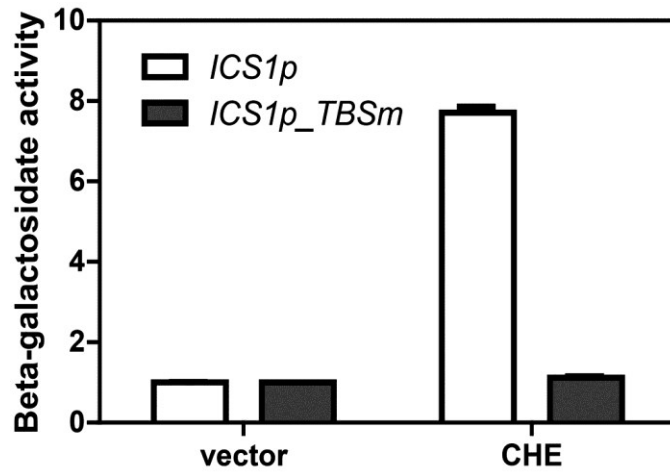


Figure 3-2: CHE binds to the TCP-binding site within the *ICS1* promoter in yeast one-hybrid assay.

Beta-galactosidase reporter activities were measured using ONPG as the substrate and normalized to the control with the empty pDEST-AD vector. The TCP-binding site (TBS) located from -152 to -145 bp upstream of transcription start site was mutated (TBSm). Error bars represent SEM from three technical replications. This experiment has been repeated three times with similar results.

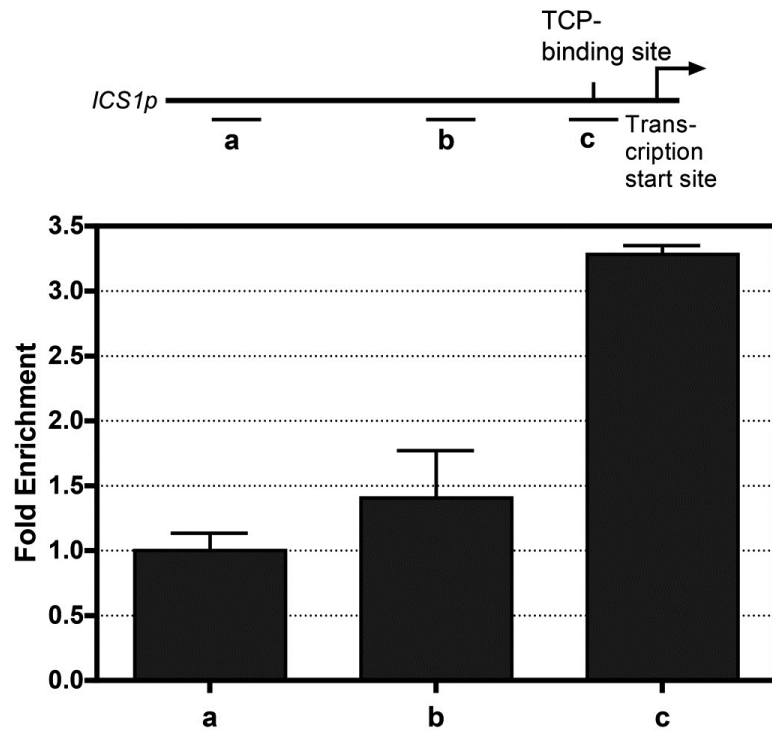


Figure 3-3: CHE binds to the TCP-binding site of the *ICS1* promoter *in vivo*.

ChIP experiments were performed using three-week-old *35S::CHE-GFP* plants.

The long horizontal line represents the *ICS1* promoter. The tick above the line represents the TCP-binding site. The short horizontal lines indicate the regions where the three sets of primers amplify. Error bars represent SD. This experiment has been repeated three times with similar results. This figure is provided by Xiao-yu Zheng.

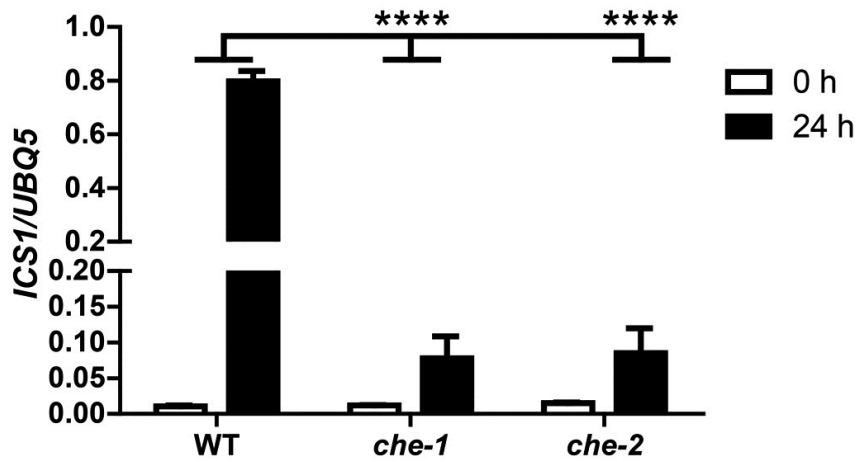


Figure 3-4: SA-triggered *ICS1* induction is largely blocked in *che* mutants.

Three-week-old plants were treated with 1 mM SA. Samples were collected at 0 h and 24 h after SA spray. RNA was extracted and used to generate cDNA for qPCR to determine the transcripts level of *ICS1*. *UBQ5*, internal control. WT, wild-type. Error bars represent SEM from three technical replications. Two-way ANOVA analysis was used to test statistical significance. ****, p -values < 0.0001. This experiment has been repeated three times with similar results.

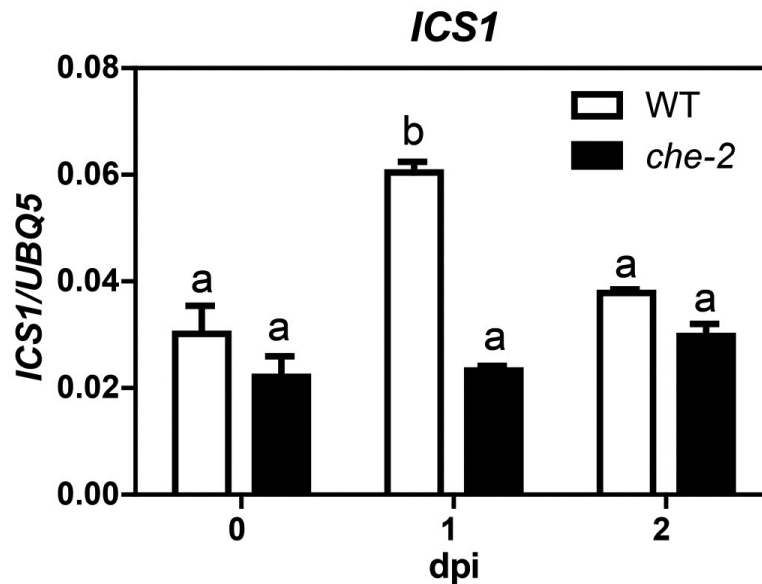


Figure 3-5: Systemic induction of *ICS1* by local infection is CHE-dependent.

Three-week-old plants were infiltrated with avirulent pathogen *Psm* ES4326/*avrRpt2*. Systemic tissues were collected at 0, 1 and 2 dpi. RNA was extracted and used to generate cDNA for qPCR to measure the transcript levels of *ICS1*. *UBQ5*, internal control. WT, wild-type. dpi, days post infection. Error bars represent SEM from three technical replications. One-way ANOVA followed by Tukey's multiple comparisons test for statistical analysis (p -values < 0.05). This experiment has been repeated three times with similar results.

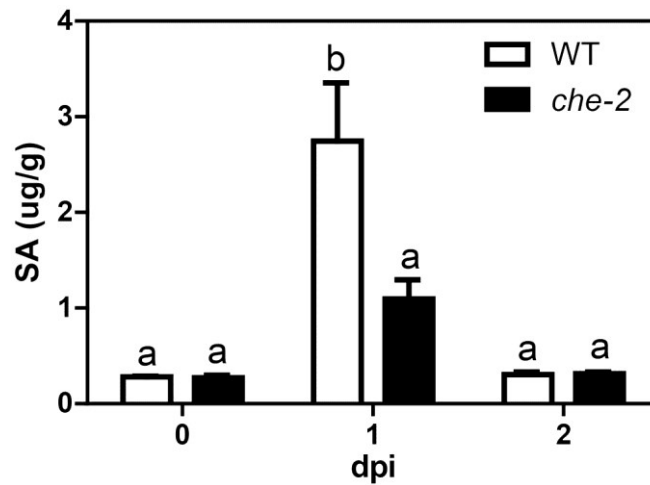


Figure 3-6: SA accumulation in systemic tissues after local avirulent pathogen infection is compromised in the *che* mutant.

Three-week-old plants were infiltrated with avirulent pathogen *avrRpt2 Psm* ES4326. Systemic tissues were collected at 0, 1 and 2 dpi for SA measurement using HPLC. WT, wild-type. dpi, days post infection. Error bars represent SEM from three technical replications. One-way ANOVA followed by Tukey's multiple comparisons test for statistical analysis (p -values < 0.05). This experiment has been repeated three times with similar results.

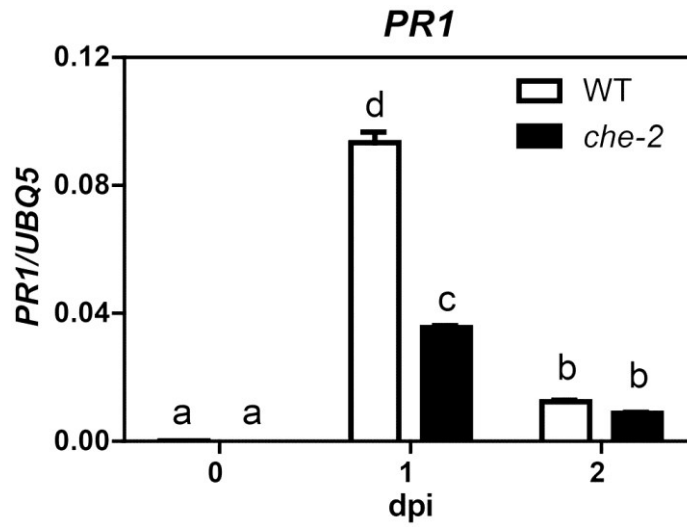


Figure 3-7: Systemic induction of *PR1* by local infection is partially CHE-dependent.

Three-week-old plants were infiltrated with the avirulent pathogen *Psm* ES4326/*avrRpt2*. Systemic tissues were collected at 0, 1 and 2 dpi. RNA was extracted and used to generate cDNA for qPCR to measure the transcript level of *ICS1*. *UBQ5*, internal control. WT, wild-type. dpi, days post infection. Error bars represent SEM from three technical replications. One-way ANOVA followed by Tukey's multiple comparisons test for statistical analysis (p -values < 0.05). This experiment has been repeated three times with similar results.

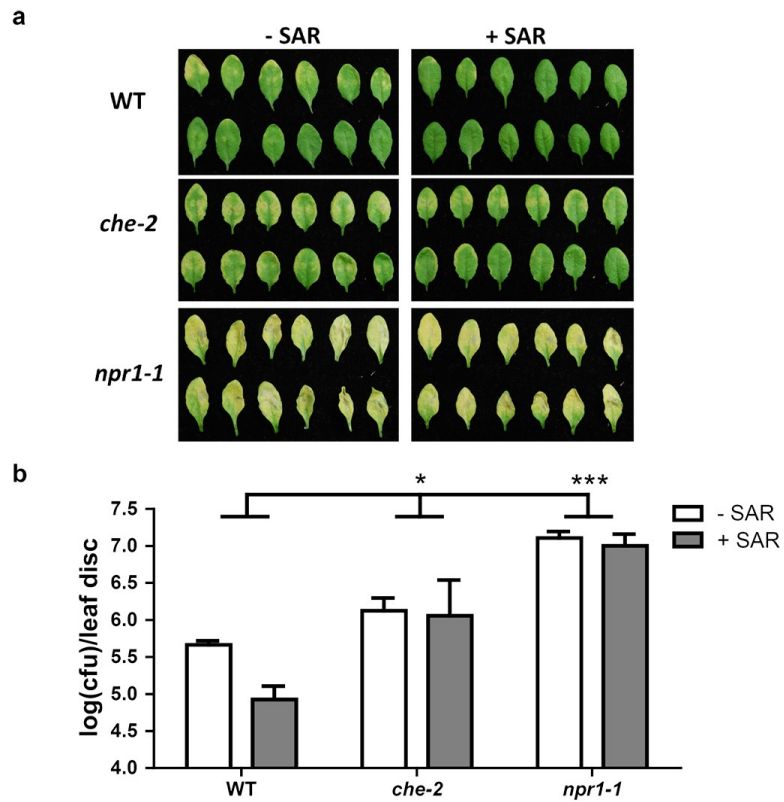


Figure 3-8: Establishment of SAR requires a functional CHE.

Three-week-old plants were infiltrated with 10 mM MgSO_4 (-SAR) or *Psm* ES4326/*avrRpt2* (+SAR). Three days later, systemic leaves were infiltrated with *Psm* ES4326. **a**, Disease symptom shown three days after the second pathogen infection. **b**, Bacterial growth in systemic leaves were measured three days after the second pathogen infection. WT, wild-type. Error bars represent 95% confidence intervals (n = 8). Two-way ANOVA analysis to test statistical significance. *, *p*-values < 0.05; ***, *p*-values < 0.001. This experiment has been repeated three times with similar results.

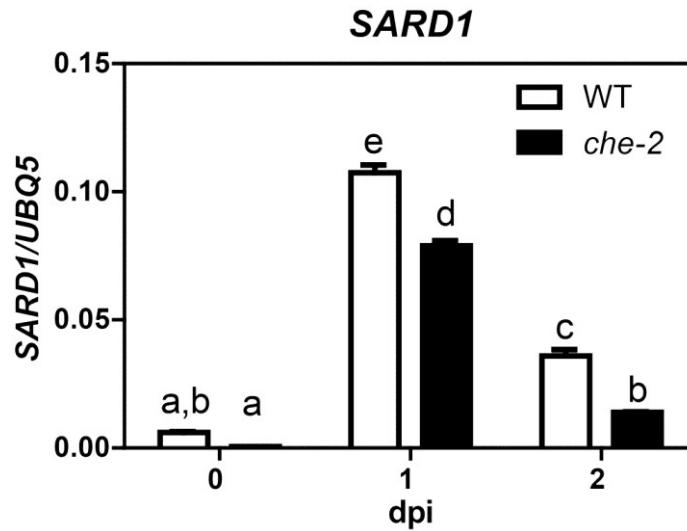


Figure 3-9: Expression of *SARD1* is reduced in the *che* mutant.

Three-week-old plants were infiltrated with the avirulent pathogen *Psm* ES4326/*avrRpt2*. Systemic tissues were collected at 0, 1 and 2 dpi. RNA was extracted and used to generate cDNA for qPCR to measure the transcript level of *ICS1*. *UBQ5*, internal control. WT, wild-type. dpi, days post infection. Error bars represent SEM from three technical replications. One-way ANOVA followed by Tukey's multiple comparisons test for statistical analysis (p -values < 0.05). This experiment has been repeated three times with similar results.

3.3 Discussion and future directions

In this study, the clock component, CHE, was identified as a positive regulator of the SA synthesis gene, *ICS1*. Unlike the previously reported SARD1, which was shown by ChIP and gel mobility shift analysis to become associated with the *ICS1* promoter only upon pathogen or UV treatment (Zhang et al., 2010), CHE can bind to the *ICS1* promoter directly in the absence of any external stimuli. This indicates that under normal conditions, the clock component CHE is responsible for the daily oscillatory expression of SA. To further confirm this hypothesis, a time-course experiment will be conducted to measure and compare the SA levels between wild-type and the *che* mutant.

Upon local pathogen infection, CHE and SARD1 can both bind to the *ICS1* promoter at different *cis*-elements to induce SA biosynthesis. Meanwhile, CHE also activates *SARD1* to amplify the SAR input signal (Alon, 2007). Therefore, CHE is involved in both basal and pathogen-induced SA synthesis. However, how pathogen further activates CHE in systemic tissues is not known. One possibility is that *CHE* is transcriptionally activated. Unfortunately, I have not been able to obtain consistent results to either confirm or disapprove this hypothesis. The RNA extraction-based method used in the study was labor-intensive and not in real-time, which was not suitable for studying this highly dynamic process. Therefore, I propose to employ the *CHEp:LUC* reporter to monitor the transcriptional activity of *CHE* as an alternative

method. Once critical time points of *CHE* induction have been identified using the reporter, the RNA extraction method can be used to confirm the results.

Another possibility mechanism by which CHE protein is modulated in systemic tissues under SAR condition is through protein modification. A previous study showed that the DNA-binding activities of the class I TCP TFs could be modulated by redox through a conserved cysteine (Viola et al., 2013). As a member of the class I TCP TFs, CHE also has this conserved cysteine. Moreover, the cellular redox status is changed after pathogen or SA treatment (Chapter 2) (Mou et al., 2003). Therefore, it is possible that the altered redox states in systemic tissues after local pathogen infection may affect the binding affinity of CHE to the *ICS1* promoter.

Beyond these hypotheses, the known regulation of CHE came from studies of its interaction with other clock components. *CHE* can be transcriptionally repressed by *CCA1* and *LHY*. In return, CHE can also repress *CCA1* expression. But this repression can be alleviated through the CHE-TOC1 protein-protein interaction (Pruneda-Paz et al., 2009). As described in Chapter 2, elevated SA level enhances expression of *LHY* and *TOC1*, which may subsequently modulate the transcription of the *CHE* gene and the activity of the CHE protein. This hypothesis can be preliminarily tested by monitoring *LHYp:LUC* and *TOC1p:LUC* in systemic tissues after SAR induction to determine whether there is a correlation between their expression patterns and that of *CHE*.

To summarize, my dissertation work unveiled a novel function of the clock component, CHE, in plant defense. We showed that CHE positively regulates SA synthesis through direct binding to the *ICS1* promoter. CHE is likely to be responsible for the oscillatory basal expression of SA and is required for systemic SA accumulation and resistance upon pathogen challenge. The significance of a clock component regulating SA synthesis may be to allow plants to time basal resistance in anticipation of pathogen threat as well as to fine-tune SA synthesis through interactions with other clock components upon SAR induction.

3.4 Materials and experimental methods

3.4.1 Plant materials and growth conditions

che-1, *che-2*, CHEox (35S:CHE-GFP #98) and *npr1-1* plants were previously described (Cao et al., 1997; Pruneda-Paz et al., 2009). Plants were grown in soil under 12 h light/12 h dark at about 60% humidity for three weeks.

3.4.2 Yeast one-hybrid and ONPG assay

The *ICS1* native promoter (1979 bp upstream of transcription start site) was first cloned into pMW#3 (Invitrogen) and integrated into the yeast strain YM4271 (MAT α). The CHE coding sequence was cloned into pDSET22 and transformed into a MAT α yeast strain. The strain containing the *ICS1* promoter was mated with the strain that containing either pDEST22-CHE or the empty vector and the zygote colonies were selected.

Mutagenesis of *ICS1* promoter was performed using the QuikChange Lighting Multi Site-directed mutagenesis kit (Agilent Technologies) according to the instruction manual. *ICS1p_TBSm* (TCP-binding site mutated in the *ICS1* promoter) were cloned into the destination vectors pMW#2 and pMW#3 using the Gateway cloning kit (Invitrogen). The coding sequence of CHE was cloned into the pDONR207 vector and subsequently transferred into the pDEST-AD vector by Gateway LR reactions. Transformation into yeast strain YM4271 was performed as previously described (Deplancke et al., 2006).

β -gal activity was determined as described before with some modifications (Pruneda-Paz et al., 2009). Briefly, transformed yeast cells were grown in 3 ml SD-His-Ura-Trp liquid medium at 30 °C overnight. After incubation, some of the yeast cultures were inoculated into 6 ml YPD in 50 ml conical tube at 30 °C until OD_{600nm} reached between 0.6 and 0.8. After the culture was cooled down on ice, a 1 ml aliquot was used to determine the accurate OD_{600nm} using spectrophotometer. Yeast cells from three aliquots of 900 μ l culture were spun down and re-suspended in 150 μ l Z buffer, and then lysed by two freeze/thaw cycles. The enzymatic reaction was started by adding 850 μ l Z buffer/ 600 μ g ONPG (2-Nitrophenyl- β -D galactopyranoside, Sigma) and was incubated at 30 °C between 10 and 24 h. The reaction was stopped by addition of 400 μ l 1M Na_2CO_3 . After centrifugation, the supernatant was used to determine the OD_{420nm} .

All the primers used for Y1H are listed in Table 3-1.

3.4.3 Chromatin Immunoprecipitation

ChIP experiment was performed as previously described (Zheng et al., 2012). Three-week-old Col wild-type and CHEox plants were used. Primers used for ChIP are listed in Table 3-2.

3.4.4 RNA extraction and quantitative PCR

RNA was extracted using TRIzol (Ambion) as previously described (Tada et al., 2008). cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). qPCR using gene-specific primers was performed using SYBR Green (Roche) on the real-time PCR machine Mastercycler realplex² (Eppendorf). *UBQ5* was used as an internal control in analyzing the qPCR data. The qPCR primers are listed in Table 3-3.

3.4.5 SA extraction and measurement

SA was extracted as previously described (Zheng et al., 2012). Briefly, SA was extracted from around 200 mg leaf tissues using 90% methanol and 100% methanol subsequently. The samples were then vacuum-dried and suspended in 5% trichloroacetic acid. SA was then extracted twice using ethyl acetate-cyclopentane (1:1). The extracts were further dried and dissolved in methanol and subjected to HPLC measurement.

3.4.6 SAR assay

SAR assay was performed as previously described (Fu et al., 2012). Briefly, two lower leaves of 3-week-old plants were pressure-infiltrated with 10 mM MgSO₄ (mock

treatment) or avirulent bacterial pathogen *Psm* ES4326 carrying *avrRpt2* ($OD_{600nm} = 0.02$). Three days later, virulent bacterial pathogen *Psm* ES4326 ($OD_{600nm} = 0.001$) was infiltrated into two upper leaves (systemic leaves). 8 plants/genotype/treatment were used. Sampling was performed 3 days post inoculation to analyze the bacterial growth.

Table 3-1: Primer sequences for Y1H

Primer Name	Sequences
ICS1p_tgttt	ATGAAATGAAAATCTTCAATTTTATGTTTCCCCTGCTAC ATCAGTCCC
ICS1p_tgtttaa	GAAATGAAAATCTTCAATTTTATGTTTAAACTGCTACAT CAGTCCCCTATTTATATC
CHE_GW_F	GGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCC GACAACGACGGAGCA
CHE_GW_R	GGGACCACTTTGTACAAGAAAGCTGGGTCACGTGGTT CGTGGTCGTCTTC

Table 3-2: Primer sequences for ChIP

Primer Name	Sequences
ICS1_a_F	AGAAATTCGTAGCATCCACAACACACA
ICS1_a_R	AAACTGAAACTAGACACGGTCCTCAGA
ICS1_b_F	AAGGAGCATGCGTGTAATGCCA
ICS1_b_R	CGTTTGATACGGAAGCGGTTTGCAC
ICS1_c_F	TGCACGACTAACTTTAGAAAAATGT
ICS1_c_R	AGGGGACTGATGTAGCAGGGGC

Table 3-3: Primer sequences for qPCR

Primer Name	Sequences
CHE_qP_F	TAATGGGTGGTGGTGGTTCTG
CHE_qP_R	GCAAAGCTCCAGACTTGTCC
ICS1_qP_F	GGCAGGGAGACTTACG
ICS1_qP_R	AGGTCCCGCATAACATT
PR1_qP_F	CTCATACACTCTGGTGGG
PR1_qP_R	TTGGCACATCCGAGTC
SARD1_qP_F	CCTCAACCAGCCCTACGTTA
SARD1_qP_R	TAGTGGCTCGCAGCATATTG
UBQ5_qP_F	GACGCTTCATCTCGTCC
UBQ5_qP_R	GTAAACGTAGGTGAGTCC

3.5 Acknowledgements

I would like to thank my collaborator Dr. Xiao-yu Zheng for performing ChIP experiment and helping in SA measurement. I would like to thank Dr. Jose Pruneda-Paz and Dr. Steve Kay for performing Y1H screen. I also want to thank Dr. Wei Wang for his help in collecting time-course samples.

4 Summary and perspectives

Study of the interplay between the circadian clock and plant immunity is an emerging research area. Through my dissertation work, I identified a molecular crosstalk between the circadian clock and the key immune signal, SA.

In the absence of pathogen infection, the circadian clock may regulate the basal SA oscillation through CHE-mediated transcriptional activation of the SA synthesis gene *ICS1*. Meanwhile, the oscillatory endogenous SA affects the cellular redox status, which subsequently modulates the redox sensitive master immune regulator NPR1. NPR1 monomers translocate into the nucleus to mediate expression of both defense and clock genes (Figure 4-1). Therefore, the rhythmic endogenous SA abundance leads to the corresponding rhythmic NPR1 monomer level, probably generating the temporal variation in basal defense.

Upon pathogen challenge, CHE positively regulates the transcription of *ICS1* to promote systemic SA accumulation to anticipate potential secondary pathogen attack. As a preventative defense strategy, the strength and duration of SAR need to be fine-tuned. My work showed that SA not only induces pathogen resistance but also reinforces the circadian clock, which leads to repression of defense genes through induction of the central clock gene *TOC1* (Figure 4-1). Therefore, the involvement of clock components in regulating SA biosynthesis and the subsequent SAR enables the plants to achieve the proper defense level without compromising fitness.

My dissertation studies focus on the interactions between plant immune signal SA and the circadian clock. As introduced in Chapter 1, plant immune system is complex with different layers of defense. Future studies may expand to other immune factors. PAMPs as extracellular signals can indirectly affect the plant circadian clock. For example, flg22 has been shown to shorten the period of the clock (Zhang et al., 2013). However, which specific clock components are indirectly targeted by flg22 are still unknown. It is reasonable to hypothesize that other PAMPs, such as elf18, may also affect different aspects of the circadian clock, that is, period, phase or amplitude. Besides PAMPs, effectors from pathogen probably influence or manipulate the plant clock. Since the circadian clock coordinates a wide range of physiological and cellular processes to maximize the fitness, some pathogen effectors may interfere the normal growth schedule by interacting with a plant clock component. Another possibility is that the altered ROS and redox states as shared downstream signaling events of both PTI and ETI could regulate clock components, which is the case in SAR condition as described in Chapter 2.

Further studies may also explore more on how different clock components regulate the plant immunity. The clock components CCA1, LHY, TOC1, CHE and ELF3 have been shown to play roles in plant defense based on previous study and my study (Bhardwaj et al., 2011; Wang et al., 2011b; Zhang et al., 2013). However, more questions arise. How these clock components coordinate with each other in regulating plant

defense? Do any other clock components also participate in the regulation of plant immunity? Since most clock components are TFs, ChIP-seq experiments will help to find the direct targets and possible binding element of the specific clock component. Furthermore, performing ChIP-seq using samples with and without pathogen infection will provide hints that if pathogen infection could recruit the clock component to different targets.

Besides studying the pathogen influence on the plant circadian clock, it is interesting to ask the question whether pathogens also have diurnal behaviors that influence their ability to infect plants. Circadian rhythms in cyanobacteria have been thoroughly studied. The central oscillator of the clock is composed of three proteins, KaiA, KaiB, and KaiC, which function through phosphorylation/dephosphorylation cycles to allow the maintenance of the clock. Homolog of KaiC protein was found in bacterial pathogen *Pseudomonas syringae*, but the function of this protein is unknown. It has become apparent that genes encoding phytochromes are also present in the genome of different bacteria. Since phytochromes participate in regulating circadian rhythms, similar light-responsive proteins found in bacteria may imply some light or even clock-dependent microbial physiological processes (Soriano et al., 2010). Although some evidence indicates that circadian rhythms may exist in non-photosynthetic bacteria, it is still unclear if bacterial pathogens, such as *Pseudomonas syringae*, have the circadian clock.

Overall, our current knowledge suggests that when studying plant defense responses one should consider the temporal factor, more specifically, the possible involvement of circadian regulation. Therefore, the time of day when experiments are performed is important for obtaining consistent results. The interaction between the plant immunity and the circadian clock reconciles the potential conflict between the normal growth schedule and the proper defense activation.

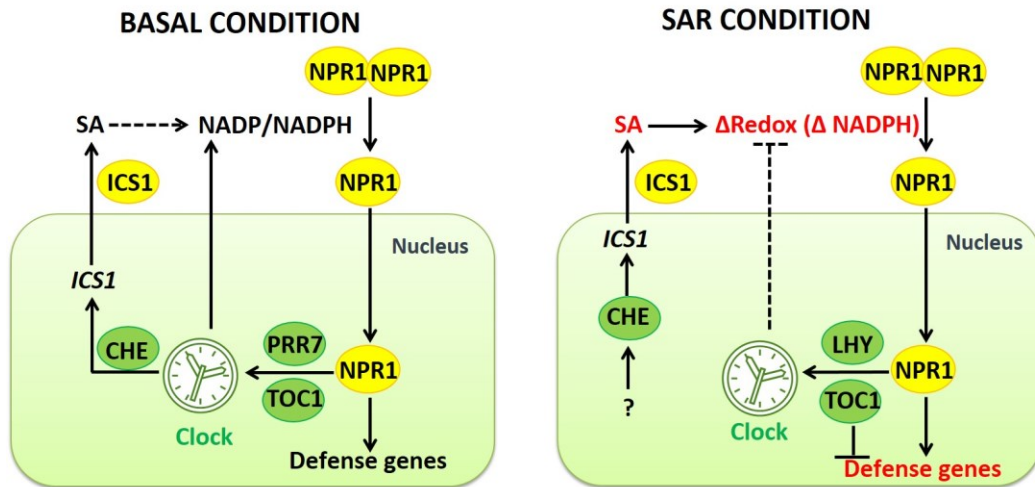


Figure 4-1: Working model of interaction between the circadian clock and SA.

In the absence of pathogen challenge, the circadian clock may regulate the basal SA oscillation through CHE, because CHE directly activate the expression of the SA synthesis gene *ICS1*. Meanwhile, the cellular redox status manifested by the abundance of NADP or NADPH has the circadian rhythm driven by the circadian clock and endogenous SA. The cellular redox status modulates the redox sensitive master immune regulator NPR1. NPR1 monomers translocate into the nucleus to regulate defense genes and clock gene *TOC1* and *PRR7* expression. Under SAR-induced condition, an unknown signal (represented by the question mark) activates CHE to trigger the transcription of *ICS1* and promote the SA accumulation. Subsequently, SA reinforces the circadian clock to negate the redox perturbation and induces *TOC1* to repress the expression of defense genes. Black arrows indicate positive regulation. Blocked arrows represent negative regulation. Dash lines indicate indirect regulation.

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Biography

Mian Zhou was born on January 9th, 1986 in Xi'an, China. I started my undergraduate study at College of Biological sciences at China Agriculture University in 2004. In 2006, I conducted my undergraduate research program in Dr. Wei-hua Wu lab, characterizing the function of an interested TF in salt stress. I completed my undergraduate thesis "Analysis of Tyrosine Phosphorylation in *Drosophila Mad*" at Dr. Cai Yu lab at Temasek Life Sciences Laboratory (TLL) in Singapore in 2008.

After obtaining my Bachelor of Science degree, I started pursue Ph.D degree at Department of Biology in Duke University in August 2008. I spent my first year doing rotations in the labs of Dr. Tai-ping Sun and Dr. Paul Magwene, learning the transient expression assay to study protein regulation of gene expression and some bioinformatics tools. After spending one and a half years in conducting a genetic screen to identify the potential temperature sensor of *Arabidopsis* in Dr. Zhen-Ming Pei lab, I became a Ph.D candidate. I joined Dr. Xinnian Dong lab in March 2011 and was attracted by plant immunity and the circadian clock interactions.

During my graduate study, I gained teaching experience as a teaching assistant in Microbiology. I also took several opportunities to communicate and present my work. I attended the 23rd and 24th Annual Plant Molecular Biology Retreat in 2009 and 2010. I also presented my study at department DCMB seminar in 2010. Supported by the NSF2013 grant, I participated in the annual joint group meeting with members from the

labs of Drs. Frederick Ausubel, Xinnian Dong and Shauna Somerville. During the graduate study, my knowledge and skills for performing and presenting science were built up, which will help me move forward in my future career.

Publications:

Zhou, M., Wang, W., Karapetyan, S., Buchler, N. and Dong, X. Perturbation in the redox rhythm reinforces the circadian clock through a master immune regulator in *Arabidopsis*. (In preparation)

Zhou, M., Zheng, X.Y., Pruneda-Paz, J. L., Kay, S. A. and Dong, X. The circadian clock component CHE positively regulate SA biosynthesis in *Arabidopsis*. (In preparation)

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2005, 2006, 2007 Outstanding Academic Performance Fellowship

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