UNIVERSITY OF CALIFORNIA SAN DIEGO

Uncovering novel pathways that regulate *NPR1* Expression in *Arabidopsis thaliana*

A Thesis submitted in partial satisfaction of the
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by

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The Thesis of Ciara Alvarez-Malo is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

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DEDICATION

This work is dedicated to my family and friends.

My commitment to my career has meant I have missed countless opportunities to share my time with you, but you have always been supportive.

I would like to extend a special thanks to my parents for always feeding my intrepid spirit and raising me to know that I can become anything I put my mind to.

Last but far from least, I want to thank my dear husband, a constant inspiration and my daily source of joy, for his generosity and spirited encouragement.

EPIGRAPH

Knowing how to think empowers you far beyond those who know only what to think.

-Neil deGrasse Tyson

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ABSTRACT OF THE THESIS

Uncovering novel pathways that regulate *NPR1* Expression in *Arabidopsis thaliana*

by

Ciara Alvarez-Malo

Master of Science in Biology

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Professor Jose L. Pruneda-Paz, Chair

Biotic stresses such as crop disease are one of the major threats to food production worldwide. Global losses due to pathogens and pests associated with wheat, rice, maize, potato and soybean reduced the output of these five major crops, which generate 50% of the global human calorie intake, by 10-40% (Savary, 2019). It has been projected that crop disease and pest incidences are expanding in a poleward direction (2.7 km annually) (Bebber, 2014). As evidence of the negative impacts of environmental shifts accumulates in combination with a market

preference for pesticide-free produce, it is expected that the main strategies to combat food insecurity will come from science and technology (Cole, 2018). Thus, scientists and policy makers alike consider increasing the pathogen tolerance of crop plants through molecular and plant breeding approaches as the most attractive and viable option to sustain food production (Cole, 2018). In this regard, disease resistant crops designed to constitutively overexpress NONEXPRESSER OF PR GENES1 (NPR1)- a master immune regulator protein homologous to many plant species- have gained popularity given their phenotypic ability to resist pathogenic attacks (Silva, 2018). However, this approach has led to undesirable side effects that contest whether *NPR1* overexpressing (NOX) plants are a viable solution to improve crop fitness (Silva, 2018). given their tendency towards growth retardation. The finer details governing the signaling cascades involved in NPR1-associated defense responses remain elusive, calling for further plant defense studies. The *Arabidopsis thaliana-Pseudomonas syringae* pathosystem has been broadly employed to understand plant-pathogen interactions (Volko, 1998). Through morning/evening infection experiments and bioluminescence imaging of *NPR1* luciferase reporter lines, we discovered that NPR1 mediates the circadian regulation of defense responses, but said regulation does not involve changes in *NPR1* transcription. Given *P. syringae*'s hemibiotrophic approach (Thaler, 2004) we investigated changes in *NPR1* expression following salicylic acid (SA) and jasmonic acid (JA) treatments, tissue wounding, and *P. syr* infection. Remarkably, we found that while *NPR1* expression increased after any of the aforementioned treatments, it expressed rhythmically after JA treatments, tissue wounding, and *P. syr* infection. From this we concluded that wounding triggers increase in *NPR1* expression and expression oscillations.

Chapter 1. Introduction

Biotic stresses such as crop disease are one of the major threats to food production worldwide. Global losses due to 137 pathogens and pests associated with wheat, rice, maize, potato and soybean reduced the output of these five major crops, which generate 50% of the global human calorie intake, by 10-40% (Savary, 2019). Annual crop loss to pests alone accounts for 20–40% of global crop losses. Managing crop diseases, epidemics and invasive insect problem costs the agriculture industry approximately $290 mn annually (FAO 2017). It has been projected that crop disease and pest incidences are expanding in a poleward direction (2.7 km annually) (Bebber, 2014). These incidences have largely been attributed to the merging of globalization's effects leading to increased plant, pest and disease movement, increase in disease vectors, climate change and global warming (FAO 2017). As evidence of the negative impacts of environmental shifts accumulates in combination with a market preference for pesticide-free produce, it is expected that the main strategies to combat food insecurity will come from science and technology (Cole, 2018). Thus, scientists and policy makers alike consider increasing the pathogen tolerance of crop plants through molecular and plant breeding approaches as the most attractive and viable option to sustain food production (Cole, 2018).

In this regard, disease resistant crops designed to constitutively overexpress NONEXPRESSER OF PR GENES1 (NPR1)- a master immune regulator protein homologous to many plant species (Dong, 2004)- have gained popularity given their phenotypic ability to resist pathogenic attacks (Silva, 2018). However, this approach has led to undesirable side effects that contest whether *NPR1* overexpressing (NOX) plants are a viable solution to improve crop fitness (Silva, 2018). For example, rice NOX plants were found to exhibit spontaneous lesions, growth retardation and smaller seeds which may lower yield productivity (Chern, 2005). Overall, NOX

plants' upregulated defense responses are linked to detrimental effects on growth and development (Chern, 2005). However, the finer details governing the signaling cascades involved in NPR1-associated defense responses remain elusive, calling for further plant defense studies.

Plant defense responses result from the orchestration of a variety of mechanisms that together ensure both the suppression of pathogen proliferation as well as the prevention of future health threats. Tightly regulated efficient defense responses are most advantageous for plants given that their high metabolic demands withhold resources that would otherwise be available for growth and reproduction (Huot, 2014). The reality of finite resources likely has influenced the evolution of precise and complex infection resistance responses to best balance growth and defense (Baldwin, 2013). Suitably, the plant defense responses are controlled by an integrated multi-layer regulatory network that involves phytohormone-mediated signaling pathways (Clarke, 2000). Of these, a pathway regulated by salicylic acid (SA) is key for plant defense responses against bacterial pathogens such as *Pseudomonas syringae* pv. tomato DC30000 (*Pst* DC3000). Upon a bacterial pathogen attack, plants swiftly induce SA production, prompting an extensive transcriptional reprogramming largely mediated by the transcription cofactor NONEXPRESSER OF PR GENES1 (NPR1). Alternatively, a phytohormone-mediated pathway regulated by jasmonic acid (JA) activates plant defense responses against herbivory and necrotrophic pathogens, both of which elicit wounding or considerable tissue damage. Upon tissue damage, plants swiftly induce a tailored JA-dependent systemic response that has been largely considered to be inversely related to the upregulation of SA that leads to *NPR1* signaling (Spoel, 2008). While the model above seems generally correct, there are exceptions and additional complexities. Several lines of evidence indicate that NPR1 mediates JA signaling (Yu,

2001), but the underlying mechanisms are not well understood. Also, *Pseudomonas syringae (P. syringae*), a gram-negative bacteria which is often considered a biotroph, should be considered a hemibiotroph (Thaler, 2004) as it infects through stomata and tissue wounds. While host cell death does not occur during the early infection stages, plant tissue chlorosis and necrosis arises at later stages of infection. Given that *P. syringae* is a bacterial pathogen that can elicit wounding (Thaler, 2004) there is a need to further investigate the complexities of the mechanisms involved in the regulation of defense responses and how these relate to NPR1's role as a master immune regulator.

Initially framed in Arabidopsis studies as a requisite element for systemic acquired resistance (SAR), a preventive defense strategy that primes defenses in healthy tissue after an initial nonpathogenic infection elsewhere on the body, NPR1 is also essential for induced systemic resistance (ISR) induced by non-pathogenic rhizobacteria. Indeed, multiple Arabidopsis *npr1* loss-of-function variants have been found to undermine innate immunity at the site of infection as compared to wild-type (WT) alleles following a virulent *P. syringae* DC3000 infection. The mutation *npr1* and the transgene *NahG*, which block SA signaling, result in loss of resistance to the biotrophic oomycete *Peronospora parasitica*, but have no effect on resistance to the necrotrophic fungus *Alternaria brassicicola*. Conversely, the *coi1* mutation, which blocks JA signaling, severely compromises resistance to the necrotrophic fungus, but has no effect on resistance to the biotrophic pathogen (Thines, 2007). Such observations led to the suggestion that plant defense responses may be tailored to the attacking pathogen, with SA-dependent defenses acting against biotrophs, and JA- (and ET-) dependent responses acting against necrotrophs (McDowell, 2000). Crosstalk between signaling networks is essential to spatial, temporal and

plant–pathogen specificity which informs resource allocation during encounters with multiple biotic and abiotic stresses (Spoel, 2008).

The molecular mechanisms underlying the temporal activation/repression of plant defense responses are considerably complex. Defense responses have been found to be modulated by the circadian clock, given that the plant's capacity to defend itself relates to the time of the day at which the onset of the biotic and abiotic stress occurs. For instance, pathogen-associated molecular pattern (PAMP)-induced callose deposition in Arabidopsis was significantly higher in morning induced plants as compared to evening induced plants (Baldwin, 2013). The rhythmicity of PAMP-induced callose deposition was shown to depend on the functional clock component CCA1 (CIRCADIAN CLOCK ASSOCIATED 1), and is probably coordinated by higher expression levels of PAMP perception and signaling components in the morning (Bhardwaj, 2011).

The circadian clock primes active plant defenses against biotrophs in the morning and daytime, when the stomata are open and the conditions are favorable for pathogen invasion (Baldwin, 2013). The clock suppresses active defense against biotrophs at night, when the closed stomata limit the possibility of pathogen invasion. The clock also tracks the accumulation of JA signaling component MYC2 during the day, and its depletion through the night. The nighttime peak of SA and the daytime peak of JA could anticipate the morning attack by biotrophs and peak herbivore activity just before dusk, respectively (Clarke, 2000). Importantly, NPR1 protein cellular localization is also modulated by the circadian clock, and a nighttime peak of NPR1 monomer-the active form of the protein- might prime active plant defenses against biotrophs in the morning (Zhou, 2015).

Importantly, *NPR1* is a well-conserved protein found across commercially significant crop species such as monocots rice and wheat, and dicots soybean and tomato. Successful heterologous expression of *NPR1* among phylogenetically distant species underlines that its molecular function and involvement in plant immune responses are highly conserved (Chern, 2001). Specifically, overexpression of Arabidopsis *NPR1* in rice and wheat grants extensive pathogen resistance (Chern, 2005), (Zhang, 2005). Similarly, soybean *NPR1* reestablishes the capacity of *npr1-1* Arabidopsis plants to induce SAR in the aftermath of an avirulent *P. syringae* infection (Silva, 2018).

Both transcriptional and posttranscriptional mechanisms were shown to play a role in the activation of defense responses against bacterial pathogens (Santer, 2009). Several lines of evidence indicate that the transcriptional upregulation of *NPR1* observed upon pathogen attack is a major step in the activation of plant defenses (Dong, 2004). First, both an exogenous SA treatment and a pathogen infection induce *NPR1* gene expression (Spoel, 2008). Second, *NPR1* overexpression confers enhanced defenses in a dose dependent manner (Chern, 2005), and *NPR1* down-regulation or loss-of-function increases susceptibility against a broad pathogen spectrum (Cao, 1997). Finally, mutations in W-box motifs in the *NPR1* promoter resulted in reduced promoter activity and a compromised disease resistance (Yu, 2001). Remarkably, despite such important role of *NPR1* upregulation for mounting plant immunity, little is known about the molecular mechanisms that regulate *NPR1* expression at the transcriptional level. Much more is known about post-transcriptional mechanisms (Withers, 2016). While unaffected plants hold NPR1 protein oligomers within the cytosolic compartment, the increased concentration of SA triggered upon pathogen encounter modifies the redox state of plant cells, reducing disulfide bonds and disrupting the cytoplasmic NPR1 oligomers (Spoel, 2003). In turn, the resulting

NPR1 monomers translocate to the nucleus where they bind TFs, modulating the expression of hundreds of SA-target genes (Zhang, 2005). Consistently, genome-wide transcript profiling indicates that most SA regulated transcriptional responses are NPR1-dependent (Zhou, 2015).

In the present study, we aimed at uncovering novel pathways that regulate *NPR1* expression in *Arabidopsis thaliana*, envisioning that it would also reveal new strategies to manipulate plant immunity. We first identified whether NPR1 mediated the circadian regulation of defense responses upon *P. syringae* infection. We then employed *NPR1* luciferase reporter lines to study whether the circadian regulation of defense responses was directed through changes in *NPR1* transcription. This not only allowed us to determine that the circadian regulation of defense did not involve *NPR1* transcription, but also led to the study of *NPR1* expression following pathogenic activation of defense. Importantly, we discovered that *NPR1* gene expression responds not only to *P. syringae* infection but also JA signaling and wounding. Furthermore, we demonstrated that all three treatments in diurnal conditions led to rhythmic *NPR1* expression, with the plant population percentage of rhythmicity increasing with the strength of the stimulus (JA treatment < wounding < *P. syr* infection).

Chapter 2. Results

The circadian clock regulates immune responses to single-leaf *P. syringae* infections

The *Arabidopsis-Pseudomonas syringae* pathosystem has extensively been employed to investigate plant responses to a bacterial infection (Glazebrook, 1999). Two main infection protocols have been the most popular. The first involves the infiltration of a bacterial inoculum into the leaf apoplast, while the second involves dipping all aerial tissues into a bacterial cell suspension. (Yao, 2013). Interestingly, the time of the day at which the infection is performed and the type of method employed influence the outcome of the infection. For instance, using the infiltration method it was shown that plants defend better, as indicated by colony forming unit (cfu) counts 3 days post infection (dpi), when the infection is performed at the beginning of the day. On the other hand, Arabidopsis plants showed better defenses upon evening infection when the dipping method was used. Despite these differences in both infection scenarios it was established that the circadian clock controls the differential outcome to morning and evening infections (Wang, 2011).

Recently, we established an additional protocol to study the interaction between clock and immune responses. Such protocol involved the infection of a single leaf, rather than all aerial tissues, into a *P. syringae* cell suspension (Li, 2018). To determine if this new protocol would be suitable to study how the circadian clock regulates plant defenses, single-leaf infections were performed at both morning and evening times in wild-type plants grown in constant light and temperature conditions. It should be noted that only clock-regulated responses will remain rhythmic in constant conditions (Pruneda-Paz, 2010). Bacterial cfu counts obtained 3 dpi were significantly higher after morning compared to evening infections (Fig. 1A). To further confirm

this result we repeated the experiment while employing a luciferase tagged *Pst* DC3000 strain (*Pst* DC3000-LUC+). Using this strain, it is possible to measure luciferase activity to estimate the bacterial population 3dpi, which provides a simpler read out to evaluate the outcome of an infection. As observed in our previous experiment, we found that luciferase activity was significantly higher in morning infected leaves compared to the evening infected ones suggesting that higher bacterial titers are present in morning infected leaves (Fig 1B). Altogether, these results indicated that single-leaf infections could be used to reveal the circadian clock regulation of plant defenses. Additionally, given that we observed an increased resistance upon evening infections, our results suggest that immunity to single-leaf infections and whole aerial tissue infections are likely regulated by the same mechanisms.

NPR1 mediates the circadian regulation of Arabidopsis defense against *Pst* DC3000

Arabidopsis defenses to a *P. syringae* infection are largely mediated by NPR1. To determine if clock regulation of plant defenses against *P. syringae* require NPR1 function, single-leaf infections were performed at both morning and evening times in npr1 mutant plants *(npr1-1)* grown in constant light and temperature conditions. Interestingly, bacterial cfu counts obtained 3 dpi were similar after morning or evening infections (Fig 1A). To further confirm this result we repeated the experiment, but using the *Pst* DC3000-LUC+ strain and observed that luciferase activity was also similar in morning or evening infected leaves (Fig 1B). Of note, *npr1-1* seedlings were overall more vulnerable than wild-type plants which is consistent with previously published results and the role of NPR1 as a master regulator of plant defense responses. Altogether, these results indicated that NPR1 mediated the rhythmic outcome of single-leaf infections observed in wild-type plants.

To investigate if the circadian clock regulates *NPR1* transcription, we evaluated if the activity of *NPR1* promoter is rhythmic in constant conditions. For that, we used Arabidopsis seedlings carrying a luciferase reporter gene (LUC+) expressed under the control of the *NPR1* promoter (*NPR1::LUC+*). Bioluminescence was quantified at regular intervals for 5 consecutive days in plants grown in a high sucrose (3%) supplemented medium in constant light and temperature conditions. Results indicated that in this conditions luciferase activity was arrhythmic suggesting that *NPR1* promoter activity was not rhythmic (Fig 2A). Given that some genes were shown to oscillate at low sucrose conditions but not at high sucrose conditions, and vice versa [21], we repeated the experiment in plants grown in a low sucrose (1%) supplemented medium. Results indicated that in this alternative condition luciferase activity was still arrhythmic (Fig 2B). Given that the master clock has been located at the shoot apex of Arabidopsis plants (Takahashi, 2015), we investigated if *NPR1* promoter driven luminescence was rhythmic at the shoot apex, however no oscillations were detected in this specific plant tissue (Fig 3). Altogether, our results indicated that *NPR1* transcription is not regulated by the circadian clock suggesting that the mechanism by which NPR1 mediates clock regulation of plant defense responses likely involves the regulation of NPR1 protein stability, function or subcellular localization (Zhou, 2015).

NPR1 promoter activity is regulated by wounding in diurnal conditions

We found that *NPR1* transcription is arrhythmic under constant light and temperature after light-dark (LD) cycle entrainment in high and low sucrose conditions (Fig 2)**,** independent of tissue specificity (Fig 3). However, previous research has shown that SA induces NPR1

promoter activity in diurnal conditions (Yu, 2001). To determine if *NPR1* promoter activity oscillates in plants grown in diurnal conditions, rather than constant conditions, we analyzed the bioluminescence of NPR1::LUC+ plants grown under light/dark cycles. To this end, we used two independent NPR1::LUC+ transgenic lines, homozygous lines created previously (Bonaldi, unpublished) and newly generated lines. Given that newly generated lines were heterozygous and due to the restricted space for bioluminescence imaging, we decided to first select lines and transfer them to an organized array prior to imaging. For the homozygous lines created previously plants were plated and germinated in the desired arrayed format such that no plant handling was involved throughout the experiment. Unexpectedly, analysis of the bioluminescence time course indicated that while the undisturbed old line displayed no rhythms, ~50% of all individuals for the new lines displayed measurable circadian rhythms (as indicated by relative amplitude values) (Figure 4A).

Based on this result we hypothesized the circadian luciferase activity could be due to either the genomic location of the NPR1:LUC+ reporter construct in new versus old lines, or to the handling that we performed to array the new lines prior imaging. To test these hypotheses, we decided to repeat the experiment using the original NPR1::LUC+ reporter lines, that did not show rhythmic luciferase activity in our prior experiment, but now we arrayed plants prior to the bioluminescence detection step. In this experiment, we observed that while plants that remained undisturbed did not display rhythmic luciferase activity (Figure 4C and 4D), 50% of the plants that were re-arrayed prior to imaging showed measurable circadian rhythms (Figure 4B). These results indicated that handling the plants was enough to induce circadian rhythms in *NPR1* promoter activity in a significant portion of all plants analyzed. To this end, we hypothesized that wounding responses could be triggered during plant handling. To test this idea, we performed the

same experiments but using a well-established mechanical wounding procedure (Onkokesung, 2010). Consistent with our prior results, we found that *NPR1* promoter activity in unwounded plants was less likely to exhibit circadian rhythms in comparison to wounded plants (Figure 5). The highest proportion of single wounded plants found to be circadianlly oscillating was only 30% (Figure 5C) for morning wounded plants. Double wounding morning treatment, which induces more JA, led to an onset of de novo oscillations in 40% of all treated plants. These results indicated that *NPR1* promoter activity begins to oscillate in a rhythmic fashion in response to tissue damage.

To further explore the mechanisms involved, we considered that tissue damage results in increased production of SA and JA, two phytohormones that antagonistically regulate plant defense responses (Rayapuram, 2007). To analyze if either hormone may trigger circadian rhythms in NPR1::LUC+ activity, seedlings carrying the reporter were grown undisturbed and were sprayed with SA or JA solutions prior to bioluminescence imaging. Results revealed that, as previously described, SA leads to an overall increase in *NPR1* promoter activity (Figure 6A). However, no significant increase in circadian oscillations were observed (Figure 6C).

Interestingly, we found that JA treatment also resulted in an overall increase in *NPR1* promoter activity (Figure 6B), suggesting that JA had similar, rather than antagonizing, effects as SA regarding the regulation of the *NPR1* promoter activity. More importantly, we observed that JA sprayed plants were more likely to exhibit a rhythmic NPR1::LUC+ activity (Figure 6C). These results suggested that the increase in NPR1::LUC+ oscillation observed after wounding are likely mediated by the upregulation of JA levels.

Given that NPR1 protein function is important to control plant infections against the bacterial pathogen *P. syringae*, and that the infection process was shown to cause tissue damage

(Thaler, 2004) we decided to explore if *Pst* DC3000 infection leads to similar phenotypes as the ones observed upon JA treatment. To this end, we analyzed the NPR1::LUC+ activity for several days after single-leaf *Pst* DC3000 infections in plants grown under light/dark cycles. Our results analysis indicated that indeed the infection of a single leaf resulted in a robust rhythmicity in *NPR1* promoter activity that was maintained for 2.5 days (Figure 7A). It should be noted that ~70 % of the all infected plants exhibited this LUC+ expression pattern (Figure 7B), suggesting that this is a robust response in the infection context.

Altogether, these experiments revealed a previously unexplored fluctuation of the *NPR1* promoter activity after infection. Most efforts to understand how defense responses vary depending on the time of infection have considered the plant status prior infection, however our results suggest that the dynamics of gene expression after the infection should be considered as well. In addition, these results suggest an unprecedented transcriptional regulatory mode where genes that are not normally expressed rhythmically, could begin to oscillate in a stress condition. Considering such response will be critical to understanding the molecular mechanisms that regulate plant stress responses.

Chapter 3. Discussion

As a critical component of plant defense responses, NPR1 has been found to sophisticatedly govern multiple immune responses to both biotrophic and/or necrotrophic pathogens (Thaler, 2004)**.** While it is known that bacterial pathogen infections and SA treatments induce NPR1 expression and immune defense responses (Li, 2018), the genetic mechanisms responsible for NPR1 upregulation remain elusive. In the present study, we tackled the identification of differential immune responses involving NPR1 as related to the circadian clock. We found that the defense responses for Arabidopsis plants infected with a bacterial pathogen (*Pst* DC3000) in the morning as compared to the evening were not equal. Morning infected plants fared better than evening infected plants, highlighting a contiguous relationship between plant immunity and anticipatable environmental changes (Bhardwaj, 2011).

Circadian-regulated plant defense responses to pathogen attack have been previously identified (Roden, 2009). For instance, pathogen-associated molecular pattern (PAMP)-induced callose deposition in Arabidopsis was significantly higher in morning induced plants as compared to evening induced plants (Baldwin, 2013). The rhythmicity of PAMP-induced callose deposition was shown to depend on the functional clock component CCA1 (CIRCADIAN CLOCK ASSOCIATED 1), and is probably coordinated by higher expression levels of PAMP perception and signaling components in the morning (Bhardwaj, 2011).

Importantly, our experiments show that the inequality of defenses observed for morning/evening-infected plants ceased when the experiments were performed using Arabidopsis mutants (npr1-1) expressing a nonfunctional NPR1 protein (Cao, 1997). Since the pathogenic bacteria thrived in *npr1-1* plants regardless of the time of infection, our results show

that the circadian regulation of defense responses requires the presence of functional NPR1 protein.

The circadian regulation of defense responses via NPR1 could be exercised via two potential mechanisms of action: transcriptional and post-translational. Notably, Wang and colleagues found that a variety of genes involved in pathogen-driven defense responses contain clock-related elements in their promoters and show rhythmic expression patterns (Wang, 2007). Furthermore, published work showed that NPR1 protein levels and sub-cellular localization exhibit daily rhythms (Zhou, 2015). However, the post-transcriptional regulation, at least in terms of the changes in protein levels, could be due to changes at the transcriptional level. Given that very little is known about the transcriptional mechanisms that regulate *NPR1* expression, we focused on the first possibility.

After numerous experiments using *NPR1* promoter luciferase reporter lines in entrained conditions yielded no changes to *NPR1* transcription, our focus on circadian regulation of *NPR1* promoter transcription shifted towards exploring conditions in which *NPR1* promoter transcription would be altered. We found that in light-driven conditions, substantial changes in *NPR1* expression result in response to mechanical wounding treatments. In specific, the transcriptional response was sensitive to the time of tissue damage and to the amount of tissue damage (Figure 5).

Given that our results show a greater *NPR1* response is elicited after morning wounding treatments we anticipate our findings are related to the upregulation of jasmonic acid (JA)-related defense in the evening. Previous research has identified JA-regulated circadian-controlled defense pathways (Staswick, 2004) which have been related to sharp diurnal rhythms in basal concentrations of JA in leaves (Wasternack, 2007). The rhythms peak at midday, anteceding the

peak expression of JA-regulated genes and of MYC2, an important transcriptional regulator of JA-mediated transcripts, by sunset (Staswick, 2002). Experiments inducing JA defense responses at different times of the day have shown that leaf nocturnal damage doubled JA levels as compared to day-time damage (Xie, 1998). Furthermore, *Arabidopsis* plants treated with methyl-JA (MeJA)showed responsiveness to JA was highest at dawn, which is also the peak expression time for coronatin-insensitive 1 (COI1) protein, a primary JA-receptor (Staswick, 2002).

Taken together, these examples demonstrate that the levels of various NPR1 defense-related processes are not homogeneously expressed, but rather fluctuate in diurnal rhythms and are partly controlled by the circadian clock (Baldwin, 2013). From imaging experiments, we discovered that MeJA (Figure 6C), wounding (Figure 5), and *P.syr* DC3000 infection (Figure 7) treatments induced de novo circadian oscillations in *NPR1* promoter activity. However, these findings were in sharp contrast with the fact that SA treatments failed to induce the same (Figure 6C).

As previously discussed, under basal conditions, JA levels as well as JA-regulated defense pathways exhibit diurnal rhythms. Under wounding or herbivory conditions, JA levels are upregulated by several orders of magnitude and maintained (Schaller, 2008), upregulating defense. While our observation that *NPR1* promoter activity is induced by JA treatment is consistent with the defense framework that implies a relationship between both phytohormone-mediated pathways and NPR1 (Staswick, 2002), the induction of circadian oscillations in *NPR1* promoter activity following JA treatment is likely dependent upon intermediary molecules activated by the sustained increase in JA concentration (Lorenzo, 2005).

Candidate rhythm-inducing molecules can be identified in the JA-dependent pathway. Following plant tissue damage, JA accumulation leads to conjugate jasmonoyl isoleucine

(JA-Ile) biosynthesis (Wasternack, 2007), (Schaller, 2008). After JA-Ile binds to COI1 (Thines, 2007), (Xie, 1998), the COI1–JAZ co-receptor complex leads to ubiquitination and 26S proteasome-dependent degradation of jasmonate ZIM-domain repressor proteins (JAZ) (Thines, 2007), which are normally bound to transcription factors, such as MYC proteins (Schaller, 2008). JAZ degradation and release of MYC proteins from transcriptional repression immediately activate gene transcription (Chini, 2007).

The induction of circadian oscillations in *NPR1* promoter activity following JA treatment could be induced by the availability of COI1. Diurnal changes in COI1 concentration would influence the activation of defense-related genes by leading to variations in the availability of the COI1–JAZ co-receptor complex without which there is no JAZ degradation and consequently no MYC protein-dependent transcriptional activation. If COI1 concentrations circadianly oscillate following plant tissue damage or *P.syr* infection, it could lead to rhythmic MYC protein release, which in turn could induce the circadian oscillations in *NPR1* promoter activity we observed in this study.

The observed induction of de novo circadian oscillations in *NPR1* promoter activity not only grants a new role to JA-mediated pathway, but could also change how Arabidopsis infection experiments are performed. In specific, our results suggest that quantifying after the desired treatment is completed could not be as informative as quantifying before, during and after treatment.

In terms of Future Directions, we will continue our investigation into the induction of circadian oscillations in *NPR1* promoter activity and how they relate to the circadian clock's regulation of defense in infected conditions. Firstly, we will need to refine our experiments around the main input driving the onset of these oscillations, which will hopefully increase the

percentage of treated oscillating plants closer to 100% or closer to the entire population. We need to procure this goal while balancing the SA-JA-pathway crosstalk (Spoel, 2008), (Clarke, 2000) and limiting variables that may disturb our study.

We also are working to identify TFs that mediate *NPR1* promoter expression upon wounding or JA treatment. Our lab has already identified TFs that not only bind to the *NPR1* promoter, but also modify *NPR1* promoter activity and defense (Bonaldi, unpublished). Given that we are interested in a developing a strategy that can regulate defense by modifying *NPR1* promoter activity through a finer approach than *NPR1* overexpression, which does increase disease resistance, but leads to undesirable side-effects (Chern, 2005). We are currently working on modifying NPR1 promoter TF BS availability through CRISPR technology (described in materials and methods) to influence plant defense responses with the goal of not only advancing our understanding of plant defense, but also designing novel disease-resistant crops.

Chapter 4. Materials and Methods

Plant materials and growth conditions

Infection Experiments

Arabidopsis thaliana (Arabidopsis) seedlings used in this work were from the Columbia ecotype (Col-0). NPR1 mutant plants (npr1-1) were previously described (Dong, 2004). NPR1 overexpression (NOX) lines were generated by backcrossing NOX(CCA1::LUC+) lines developed previously (Li, 2018) to the Arabidopsis Col-0 background.

For Pseudomonas syringae infection assays, sterile seeds were stratified in 1% agar medium for 2-3 days at 4°C. Seeds were planted in autoclaved soil (Sunshine professional mix, Sungro) and incubated under 12hr light (100 mmol.m-2.s-1) / 12hr dark cycles (LD) for 14 days at 22°C. At the beginning of day 14, plants were either kept under the LD regime or transferred to constant light (60 mmol.m-2.s-1, 22°C) (LL), with single leaf infections were performed 24h (ZT24) and 36h (ZT36) after.

NPR1 Transcription Experiments

For NPR1 transcription studies, sterile NPR1::LUC+ seeds were placed on 60mm plates (36 seeds/plate) containing 1x Murashige & Skoog basal salts (MS) medium (Caisson Labs) supplemented with 3% or 1% sucrose and stratified for 2-3 days at 4°C. Plates were incubated for 10 days under 12hr light (100 $\mu mol.m^{-2}.s^{-1}$)/12hr dark cycles (LD) at 22°C. At the beginning of the 10^{th} day, plants were transferred to constant light (60 $\mu mol.m^{-2}.s^{-1}$, 22°C) (LL) for bioluminescence imaging and remained in LL for the entire imaging period. For experiments fully conducted in light/dark (LD) driven conditions, the plants were transferred to the imaging

chamber at the beginning of the 10th day, but remained in 12hr light (100 μmol.m^{-2}.s^{-1})/12hr dark cycles (LD) at 22°C until the end of the imaging period.

Recombinant DNA constructs

NPR1 Promoter Reporter Lines

To create the first NPR1::LUC+ Arabidopsis reporter line, the intergenic region upstream of the NPR1 protein coding sequence was PCR amplified (forward primer: 5'-CACCCTCTTAAATAATATATTAGTTAATA-3' and reverse primer: 5'-CAACAGGTTCCGATGAATTG-3') and cloned in the pENTR/D-TOPO vector (Life Technologies). The NPR1 promoter was transferred from pENTR-NPR1pr to a gateway compatible version of the pZPXomegaLUC+ binary vector (Schultz, 2001) using LR Clonase II according to manufacturer's instructions (Life Technologies). Finally, the pZPXomega-NPR1::LUC+ construct was transformed into wild-type Arabidopsis plants via GV3101 Agrobacterium mediated transformation (Zhang, 2006). GV3101 cells carrying the pMDC32-NPR1 plasmid were cultivated overnight at 37°C in liquid Lysogeny Broth (LB) medium enriched with kanamycin (50mg/L) and gentamycin (30mg/L). Afterwards cells were harvested by centrifugation (3220 x g for 10min at room temperature) and resuspended in 5% sucrose solution containing 0.02% Silwet L-77 (Lehle seeds). Flourishing Arabidopsis inflorescences for the aforementioned reporter lines were dipped into the agrobacterium cell suspension for 45sec, and dipped plants were wrapped with a plastic film and incubated horizontally in a growth room for 24hr. Finally, the plastic covering was removed, and the plants were returned to the normal upright growth position and incubated in a growth chamber until seed collection (~1.5 months).

To create the second NPR1::LUC+ Arabidopsis reporter line (NPR1::LUC+**), the same process was followed except for the NPR1 promoter transfer from pENTR-NPR1pr to a Gibson Assembly compatible version of the pZPXomegaLUC+ binary vector using LR Clonase II according to manufacturer's instructions (Life Technologies). Finally, the pZPXomega-NPR1::LUC+ construct was transformed into wild-type Arabidopsis plants via AGL0 Agrobacterium mediated transformation as described above.

LUC+ Bacterium

Details for the construction of this strain will be provided upon request.

CRISPR

To create the precise targeted *NPR1* promoter transcription factor (TF) binding site (BS) deletion Arabidopsis lines, TF binding sites were identified on the intergenic region upstream of the NPR1 protein coding sequence, and primers including the appropriate guide-RNA (gRNA) sequence were designed. Three fragments were PCR amplified using two gRNAs. The first 462bp fragment (forward primer: 5'-tactgaattgggggatccCGACTTGCCTTCCGCACAATAC-3' and reverse primer: 5'-N_{19}CAATCACTACTTCGACTCTAGCTG-3'), the second 599bp fragment (forward primer: 5'-GN_{19}GTTTTAGAGCTAGAAATAGCAAG-3' and reverse primer: 5'-N_{19}CAATCTCTTAGTCGACTCTACC-3'), and the third 306bp fragment (forward primer: 5'-GN_{19}GTTTTAGAGCTAGAAATAGCAAG-3' and reverse primer: 5'-tctagaactagtggatccTATTGGTTTATCTCATCGGAACTG -3') were amplified from the pJJJ4 vector, and assembled into a Gibson Assembly compatible vector pJJJ2 (UBQ10::pcoCAS9) or p3J1 (DDR45::pcoCAS9) according to manufacturer's instructions (Life Technologies). Finally,

the construct was transformed into wild-type Arabidopsis plants via AGL0 Agrobacterium mediated transformation as described above.

Single leaf Pseudomonas syringae infection

Pst DC3000 and *Pst* DC3000-LUC+ liquid cultures (King's B medium: 2% Proteose peptone No.3, 1% Glycerol, 8.6mM K2HPO4 and 6mM MgSO4) were grown in the dark at 28°C (shaking at 200 rpm) until OD600 between 0.5 and 0.6 was reached (serial dilutions were started to assure that a suitable culture was available at the time of infection treatment). Bacteria from the liquid culture (OD600 between 0.5 and 0.6) were harvested by centrifugation at 3220 x g for 2min, resuspended in sterile water (LabChem), and harvested by centrifugation at 3220 x g for 3min. The bacterial pellet was resuspended in water (LabChem) adjusting OD600 to 0.2 (1x108 cfu), and Silwet L77 (Lehle seeds) was added to a final concentration of 0.025%. About half of a single leaf was dipped into this *Pst* DC3000 cell suspension or a *Pst* DC3000-LUC+ cell suspension for 1min. After treatment, excess inoculum was blot-dried from the leaf surface using a sterile filter paper strip and plants were returned to LL for tissue collection or bioluminescence imaging. Infected leaves were detached 3.5 days after infection and imaged to quantify leaf surface. Bacterial loads in each infected leaf were quantified by cfu counting (*Pst* DC3000) or bioluminescence detection (*Pst* DC3000-LUC+).

***Pst* DC3000 Bacterial Quantification**

Inoculated leaves were harvested after 3.5 days for seedlings from Columbia ecotype (Col-0), npr1-1, and npr1 overexpression lines (NOX). After grinding leaf tissue in 500uL of water and preparing a 1:5 dilution series by transferring 20uL, 5uL samples were spotted on

Lysogeny Broth (LB) agar rifampicin plates in octuplet. After a 24hr incubation at 28°C, bacterial colonies were counted. The CFU/cm^2 was then calculated for each leaf tissue.

To normalize CFU data by leaf size, plated leaf images were captured prior to tissue grinding with a Samsung Galaxy Note8's dual 12-megapixel camera and formatted to 1200 pixels per centimeter (ppcm). Total image size was set at 12.5cm x 12.5cm (Adobe Photoshop, https://www.adobe.com/products/photoshop.html) After leaf size in ppcm was individually determined, it was employed to normalize its respective CFU data. Statistical analyses of normalized CFU data were performed using GraphPad Prism version 6 (GraphPad Software, http://www.graphpad.com)

***Pst* DC3000-LUC+ Bioluminescence detection**

One day before infection, soil-grown plants were sprayed with sterile 5mM of D-luciferin potassium salt (in 0.01% Triton X-100 solution) and, at the same time, that 5mM D-luciferin potassium salt (in water solution) was added to the soil (3ml per plant). Infected leaves were detached from each plant and placed upside-down on a plate containing Murashige-Skoog (MS) medium supplemented with 1% sucrose. Leaf detachment and plating prior to bioluminescence acquisition minimized pixel count bias and unequal signal bleeding due to the plant's rhythmic leaf movements. Bioluminescence was quantified for 17 min using a Hamamatsu CCD Camera (Hamamatsu Photonics). Bioluminescence images were processed using the MetaMorph image analysis software (Molecular Devices) to determine bioluminescence counts per infected leaf. Leaf images were formatted to 1200 pixels per centimeter (ppcm) using Adobe Photoshop (, https://www.adobe.com/products/photoshop.html) and leaf surface (in pixels) was calculated. Normalized bacterial concentration per leaf area was calculated as

bioluminescence/pixel leaf area for each infected leaf. Statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, http://www.graphpad.com)

Plant wounding, SA and MeJA treatments

One day before treatment, NPR1::LUC+ MS grown seedlings were sprayed with 5mM of D-lucifernin potassium salt (in 0.01% Triton X-100 solution). At the beginning of the 10th day, plants were either mechanically wounded, sprayed with 0.2mM of SA solution, or sprayed with 50mM of MeJA solution. Mechanical damage was inflicted with a surface-sterilized hemostat clamp, usually injuring about 50% of the leaf lamina surface. For single-wounded plants, only one leaflet per plant was wounded, whereas for double-wounded plants, two leaflets per plant were wounded. Single-wounded plants were treated at two independent timepoints: morning (ZT1) and evening (ZT10). After wounding, whole plant bioluminescence images were acquired every 2.5hr for 96hr using a digital Pixis 1024 CCD camera (Princeton Instruments).

NPR1::LUC+ Bioluminescence Detection and Data Analysis

One day before the imaging period started, plants were sprayed with 5mM of D-lucifernin potassium salt (in 0.01% Triton X-100 solution). For soil grown plants, 5mM D-lucifernin potassium salt (in water solution) was also added to the soil at the same time (3ml per plant). Bioluminescence was quantified every 2.5hr for soil grown WT, npr1-1, and NPR1 overexpression lines seedlings, and for NPR1::LUC+ MS grown seedlings using a Pixis 1024 CCD camera (Princeton Instruments).

Bioluminescence images were processed using the MetaMorph image analysis software (Molecular Devices) to determine bioluminescence counts (for plate and soil grown plants) and

number of bioluminescent pixels (for soil grown plants) per plant for a specific tissue section. To visualize changes in plant gene expression across an entire time course experiment, the experimental pixel count data was graphed using GraphPad Prism version 8 (GraphPad Software, http://www.graphpad.com). Bioluminescence counts (for plate grown seedlings) for each experiment were analyzed by Fast Fourier Transform-Non Linear Least-squares (FFT-NLLS) using the interface provided by the Biological Rhythms Analysis Software System (BRASS) (Li, 2018).

Quantification and Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 8. Details of statistical tests applied are indicated in figure legends including statistical methods, number of biological replicates, number of individuals, mean and error bar details, and statistical significances.

Accession Numbers

Gene models in this article can be found in The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org) with the following accession numbers: NPR1, AT1G64280.

REFERENCES

1. Baldwin IT., Meldau S. (2013). Just in time: circadian defense patterns and the optimal defense hypothesis. Plant Signal Behav. doi:10.4161/psb.24410

2. Bebber DP, Holmes T, Gurr SJ. (2014). The global spread of crop pests and pathogens. Global Ecol Biogeogr. 23(12):1398–1407. doi:10.1111/geb.12214.

3. Bender CL., Alarcon-Chaidez F., Gross DC. (1999). Pseudomonas syringae phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol. Mol. Biol. Rev. 63 : 266 – 92

4. Bhardwaj V, Meier S, Petersen LN, Ingle RA, Roden LC. (2011). Defence responses of Arabidopsis thaliana to infection by Pseudomonas syringae are regulated by the circadian clock. PLoS ONE. doi: 10.1371/journal.pone.0026968. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

5. Bonaldi, K., Li, Z., Kang, S.E., Breton, G., & Pruneda-Paz, J.L. (2017). Novel cell surface luciferase reporter for high-throughput yeast one-hybrid screens. Nucleic Acids Research, 45(18). doi: 10.1093/nar/gkx682.

6. Butt A., Mousley C., Morris K., Beynon J., Can C. (1998). Differential expression of a senescence-enhanced metallothionein gene in Arabidopsis in response to isolates of Peronospora parasitica and Pseudomonas syringae . Plant J. 16 : 209 – 21

7. Cao, H., Glazebrook, J., Clarke, J. D., Volko, S., & Dong, X. (1997). The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9019406

8. Cao H, Li X, Dong X (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc Natl Acad Sci U S A 95: 6531-6536.

9. Cheong J.J., Choi Y.D. (2003). Methyl jasmonate as a vital substance in plants. Trends Genet. 19:409–413. [PubMed] [Ref list]

10. Chern MS, Fitzgerald HA, Yadav RC, Canlas PE, Dong X, Ronald PC. (2001). Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis. Plant J 27: 101-113.

11. Chern, M., Fitzgerald, H. A., Canlas, P. E., Navarre, D. A., & Ronald, P. C. (2005). Overexpression of a rice NPR1 homolog leads to constitutive activation of defense response and hypersensitivity to light. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/15986920

12. Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, J. M., Lorenzo, O., . . . Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/17637675

13. Clarke JD., Volko SM., Ledford H., Ausubel FM., Dong X. (2000). Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in Arabidopsis. The Plant Cell. 12, 2175–2190.

14. Cole, B., Augustin, M., Robertson, M., Manners, J. (2018). "The Science of Food Security." Nature News, Nature Publishing Group. www.nature.com/articles/s41538-018-0021-9.

15. Curtis MD, Grossniklaus U. (2003). A Gateway Cloning Vector Set for High-Throughput Functional Analysis of Genes in Planta. Plant Physiology. 133(2): 462– 469. doi: 10.1104/pp.103.027979

16. Devoto A., Turner J.G. (2005). Jasmonate-regulated Arabidopsis stress signalling network. Physiol. Plant. 123:161–172. [Ref list]

17. Dong X. (2004). NPR1, all things considered. Curr Opin Plant Biol 7: 547-552.

18. Eckstein, A., Zięba, P., & Gabryś, H. (2012). Sugar and Light Effects on the Condition of the Photosynthetic Apparatus of Arabidopsis thaliana Cultured in vitro. Retrieved from https://link.springer.com/article/10.1007/s00344-011-9222-z

19. Espinosa A., Alfano JR. (2004). Disabling surveillance: bacterial type III secretion system effectors that suppress innate immunity. Cell Microbiol. 6 : 1027 – 40

20. FAO. The future of food and agriculture | FAO | Food and Agriculture Organization of the United Nations. (2017). Available at: http://www.fao.org/publications/fofa/en/

21. Farmer E.E. (2007). Plant biology: Jasmonate perception machines. Nature. 448:659–660. [PubMed] [Ref list]

22. Fürstenberg-Hägg, J., Zagrobelny, M., & Bak, S. (2013). Plant defense against insect herbivores. International Journal of Molecular Sciences, Molecular Diversity Preservation International (MDPI). Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/23681010

23. Glazebrook, J. (1999). Genes controlling expression of defense responses in Arabidopsis. Current Opinion in Plant Biology, 2(4), 280–286. https://doi.org/https://doi.org/10.1016/S1369-5266(99)80050-8

24. Grones P., Friml J. (2015). Auxin transporters and binding proteins at a glance. Journal of Cell Science. 128, 1–7.

25. Hazen SP., Borevitz JO., Harmon FG., Pruneda-Paz JL., Schultz TF., Yanovsky MJ., Liljegren SJ., Ecker JR., Kay SA. (2005). Rapid array mapping of circadian clock and developmental mutations in Arabidopsis. Plant Physiol. 138(2):990-907.

26. Huot B., Yao J., Montgomery BL., He SY. (2014). Growth–defense tradeoffs in plants: a balancing act to optimize fitness. Molecular Plant. 7, 1267–1287.

27. Kieffer M., Neve J., Kepinski S. (2010). Defining auxin response contexts in plant development. Current Opinion in Plant Biology. 13, 12–20.

28. Li, Z., Bonaldi, K., Uribe, F., and Pruneda-Paz, J.L. (2018). A Localized Pseudomonas syringae Infection Triggers Systemic Clock Responses in Arabidopsis. Curr. Biol.

29. Lorenzo O., Solano R. (2005). Molecular players regulating the jasmonate signalling network. Curr. Opin. Plant Biol. 8:532–540. [PubMed] [Ref list]

30. Muhammad N., Martin K., Thomas D., (2015). The nexus between growth and defence signalling: auxin and cytokinin modulate plant immune response pathways. Journal of Experimental Botany.

31. McDowell JM., Dangl JL. (2000). Signal transduction in the plant immune response. Trends Biochem. Sci. 25: 79 – 82

32. Onkokesung, N., Gális, I., Von Dahl, C. C., Matsuoka, K., Saluz, H., & Baldwin, I. T. (2010). Jasmonic acid and ethylene modulate local responses to wounding and simulated herbivory in Nicotiana attenuata leaves. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2879812/

33. Oh Y., Baldwin I.T., Gális I. (2012). NaJAZh regulates a subset of defense responses against herbivores and spontaneous leaf necrosis in Nicotiana attenuata plants. Plant Physiol. 159:769–788. [PMC free article] [PubMed] [Ref list]

34. Pruneda-Paz JL*1, Breton G*, Nagel DH, Kang SE, Bonaldi K, Doherty C, Ravelo S, Galli M, Ecker JR, Kay SA1 (2014). A Genome Scale Resource for the Functional Characterization of Arabidopsis Transcription factors. Cell Reports 8: 1-11. PMID: 25043187. (*co-first authors, 1co-corresponding authors).

35. Pruneda-Paz JL., and Kay SA. (2010). An expanding universe of circadian networks in higher plants. Trends Plant Sci. 15(5):259-265.

36. Rayapuram C1, Baldwin IT. (2007). Increased SA in NPR1-silenced plants antagonizes JA and JA-dependent direct and indirect defenses in herbivore-attacked Nicotiana attenuata in nature. Plant J. 52(4):700-15.

37. Roden LC., Ingle RA. (2009). Lights, rhythms, infection: the role of light and the circadian clock in determining the outcome of plant-pathogen interactions. Plant Cell 21: 2546-2552.

38. Santner A, Estelle M . (2009). Recent advances and emerging trends in plant hormone signalling. Nature 459, 1071–1078.

39. Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., & Nelson, A. (2019). The global burden of pathogens and pests on major food crops. Nature Ecology and Evolution, 3(3), 430-439. https://doi.org/10.1038/s41559-018-0793-y

40. Schaller GE., Bishopp A., Kieber JJ. (2015). The yin-yang of hormones: cytokinin and auxin interactions in plant development. The Plant Cell. 27, 44–63.

41. Schaller A., Stintzi A. (2008). Jasmonate Biosynthesis and Signaling for Induced Plant Defence against Herbivory. In: Schaller A., editor. Induced Plant Resistance to Herbivory. Springer Science+Business Media; Berlin, Germany. pp. 349–366. [Ref list]

42. Schultz TF., Kiyosue T., Yanofsky M., Wada M., Kay SA. (2001). A role for LKP2 in the circadian clock of Arabidopsis. Plant Cell. 13: 2659-2670.

43. Schulze B., Ryan L., Mesmin Mekem S., Annik S., Wilhelm B. (2005). Profiling of structurally labile oxylipins in plants by in situ derivatization with pentafluorobenzyl hydroxylamine. Analytical Biochemistry. [PubMed] [Ref list]

44. Silva KJP, Mahna N, Mou Z, Folta KM. (2018). NPR1 as a transgenic crop protection strategy in horticultural species. Hortic Res. doi: 10.1038/s41438-018-0026-1.

45. Spoel SH, Dong X. (2008). Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe 3: 348-351.

46. Spoel, S. H., Koornneef, A., Claessens, S. M., Korzelius, J. P., Pelt, J. A., Mueller, M. J., . . . Pieterse, C. M. (2003). NPR1 Modulates Cross-Talk between Salicylate- and Jasmonate-Dependent Defense Pathways through a Novel Function in the Cytosol. Retrieved from http://www.plantcell.org/content/15/3/760

47. Staswick P.E., Tiryaki I. (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isolecuine in Arabidopsis. Plant Cell. 16:2117–2127. [PMC free article] [PubMed] [Ref list]

48. Staswick P.E., Tiryaki I., Rowe M.L. (2002). Jasmonate response locus JAR1 and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell. 14:1405–1415. [PMC free article] [PubMed] [Ref list]

49. Swingle, B., Bao, Z., Markel, E., Chambers, A., & Cartinhour, S. (2010). Recombineering using RecTE from Pseudomonas syringae. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2916495/

50. Takahashi, N., Hirata, Y., Aihara, K., & Mas, P. (2015). A hierarchical multi-oscillator network orchestrates the Arabidopsis circadian system. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/26406375

51. Thaler JS., Owen B., Higgins VJ. (2004). The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. Plant Physiol. 135: 530 – 38

52. Thines B., Katsir L., Melotto M., Niu Y., Mandaokar A., Liu G.H., Nomura K., He S.Y., Howe G.A., Browse J. (2007) JAZ repressor proteins are targets of the scfcoi1 complex during jasmonate signalling. Nature. 448:661–662. [PubMed] [Ref list]

53. Thomma BPHJ., Eggermont K., Penninckx IAMA., Mauch-Mani B., Vogelsang R., (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA 95: 15107 – 11

54. Volko SM, Boller T, Ausubel FM (1998) Isolation of new Arabidopsis mutants with enhanced disease susceptibility to Pseudomonas syringae by direct screening. Genetics 149: 537-548.

55. Wang W., Barnaby JY., Tada Y., Li H., Tör M., Caldelari D., (2011). Timing of plant immune responses by a central circadian regulator. Nature. 470:110–4. doi: 10.1038/nature09766.

56. Wang D., Pajerowska-Mukhtar K., Culler AH., Dong X. (2007). Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Current Biology. 17, 1784–1790.

57. Wasternack C. (2007). Jasmonates: An update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann. Bot. 100:681–697. [PMC free article] [PubMed] [Ref list]

58. Withers J., Dong X. (2016). Posttranslational Modifications of NPR1: A Single Protein Playing Multiple Roles in Plant Immunity and Physiology. PLoS Pathog 12: e1005707.

59. Xie D.X., Feys B.F., James S., Nieto-Rostro M., Turner J.G. (1998). Coi1: An Arabidopsis gene required for jasmonate-regulated defense and fertility. Science. 280:1091–1094. [PubMed] [Ref list]

60. Yao, J., Withers, J., & He, S. Y. (2013). Pseudomonas syringae infection assays in Arabidopsis. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/23615988

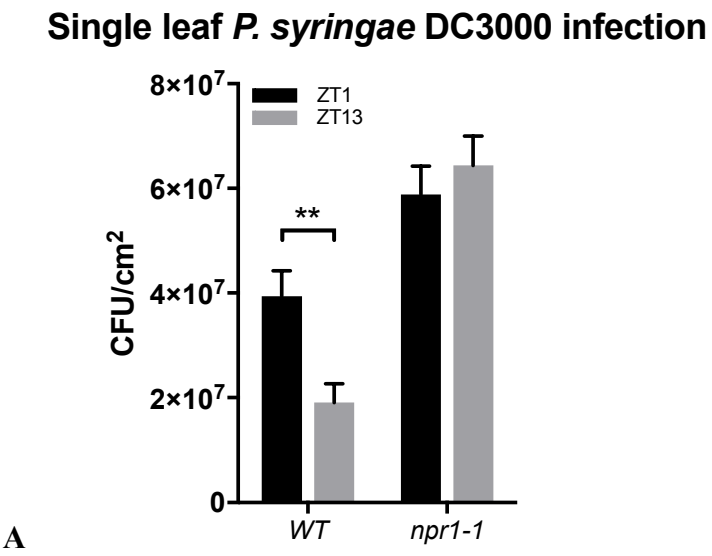
61. Yu, D., Chen, C., & Chen, Z. (2001). Evidence for an Important Role of WRKY DNA Binding Proteins in the Regulation of NPR1 Gene Expression. Retrieved from http://www.plantcell.org/content/13/7/1527

62. Zhang, H., & Cai, X. (2005). Nonexpressor of pathogenesis-related genes 1 (NPR1): A key node of plant disease resistance signalling network. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/16176083

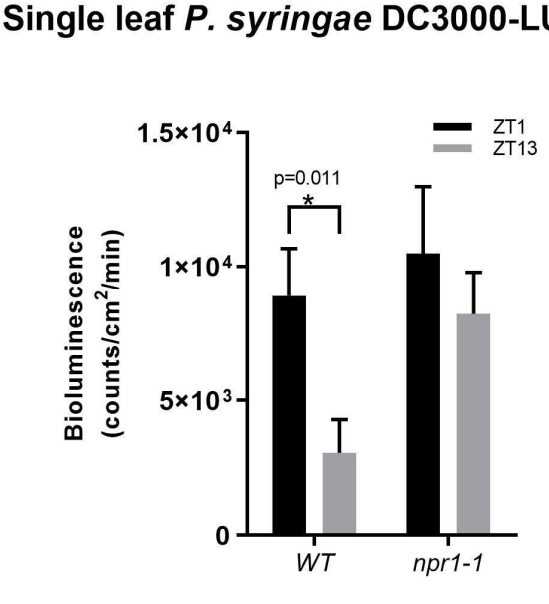
63. Zhang Y., Goritschnig S., Dong X., Li X. (2003). A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of npr1-1, constitutive 1. The Plant Cell. 15, 2636–2646.

64. Zhang X., Henriques R., Lin SS., Niu QW., Chua NH. (2006). Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nat Protoc 1: 641-646.

65. Zhou, M., Wang, W., Karapetyan, S., Mwimba, M., Marqués, J., Buchler, N. E., & Dong, X. (2015). Redox rhythm reinforces the circadian clock to gate immune response. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4526266

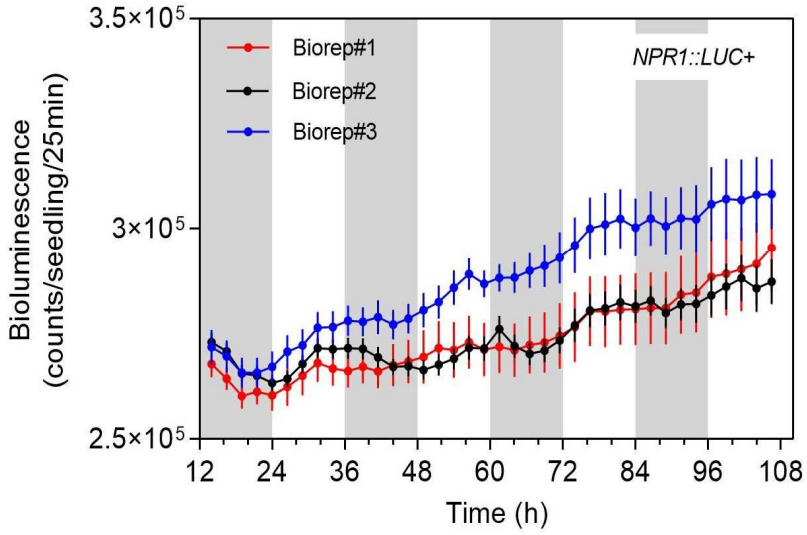


A



B

Figure 1. NPR1 Mediates Circadian Regulation of Defense Responses Triggered by Single-Leaf *P. syringae* Infection (A and B) Bacterial cell density (normalized by plant size) in soil-grown 14-day-old wild-type (WT) and *npr1-1* seedlings after morning (ZT1, black) and evening (ZT13, grey) single-leaf *P. syringae DC3000* (A) and *P. syringae DC3000-LUC+* (B) dipping infection. Results indicate mean values (±SEM; n=16) and are representative of 3 independent experiments.



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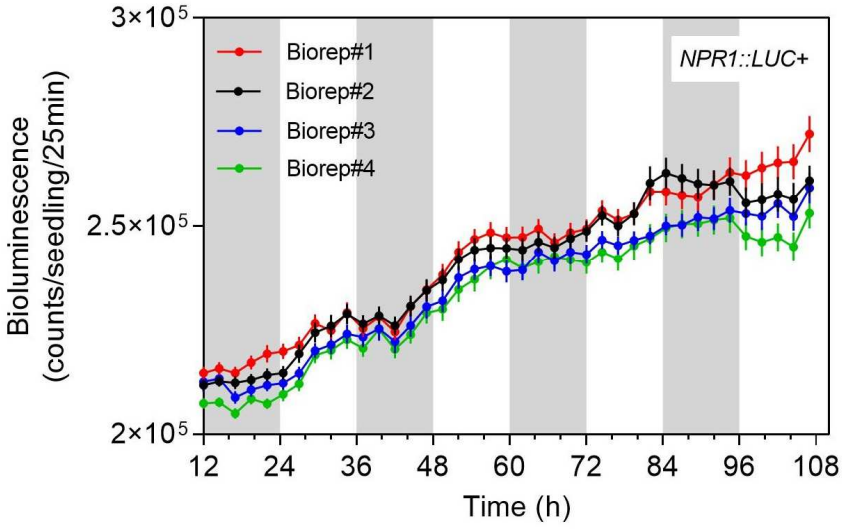


B

Figure 2. *NPR1* Gene Expression Does Not Exhibit Circadian Oscillations in High or Low Sucrose Conditions (A and B) Bioluminescence data from 10-day-old NPR1::LUC+ seedlings grown on MS enriched with 1% sucrose (A) and 3% sucrose (B). Results indicate mean values (±SEM; n=16) and are representative of 3-4 independent biological replicates.

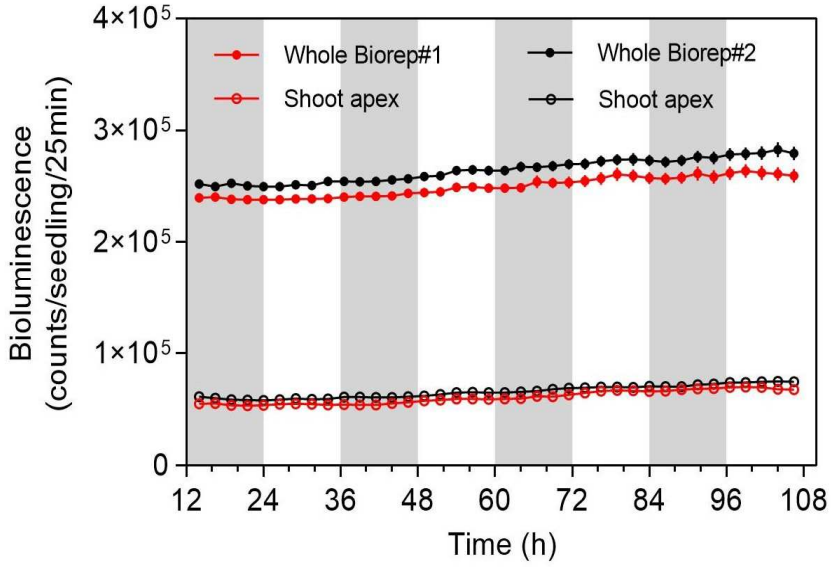


Figure 3. *NPR1* Gene Expression's Lack of Circadian Oscillations is Not Tissue-specific
Bioluminescence data from 10-day-old NPR1::LUC+ seedlings grown on MS enriched with 3% sucrose in 12hr light/dark cycles collected from the whole plant (empty circle) and the shoot apex plant region (filled circle). Results indicate mean values (±SEM; n=16) and are representative of 2 independent biological replicates (red, black).

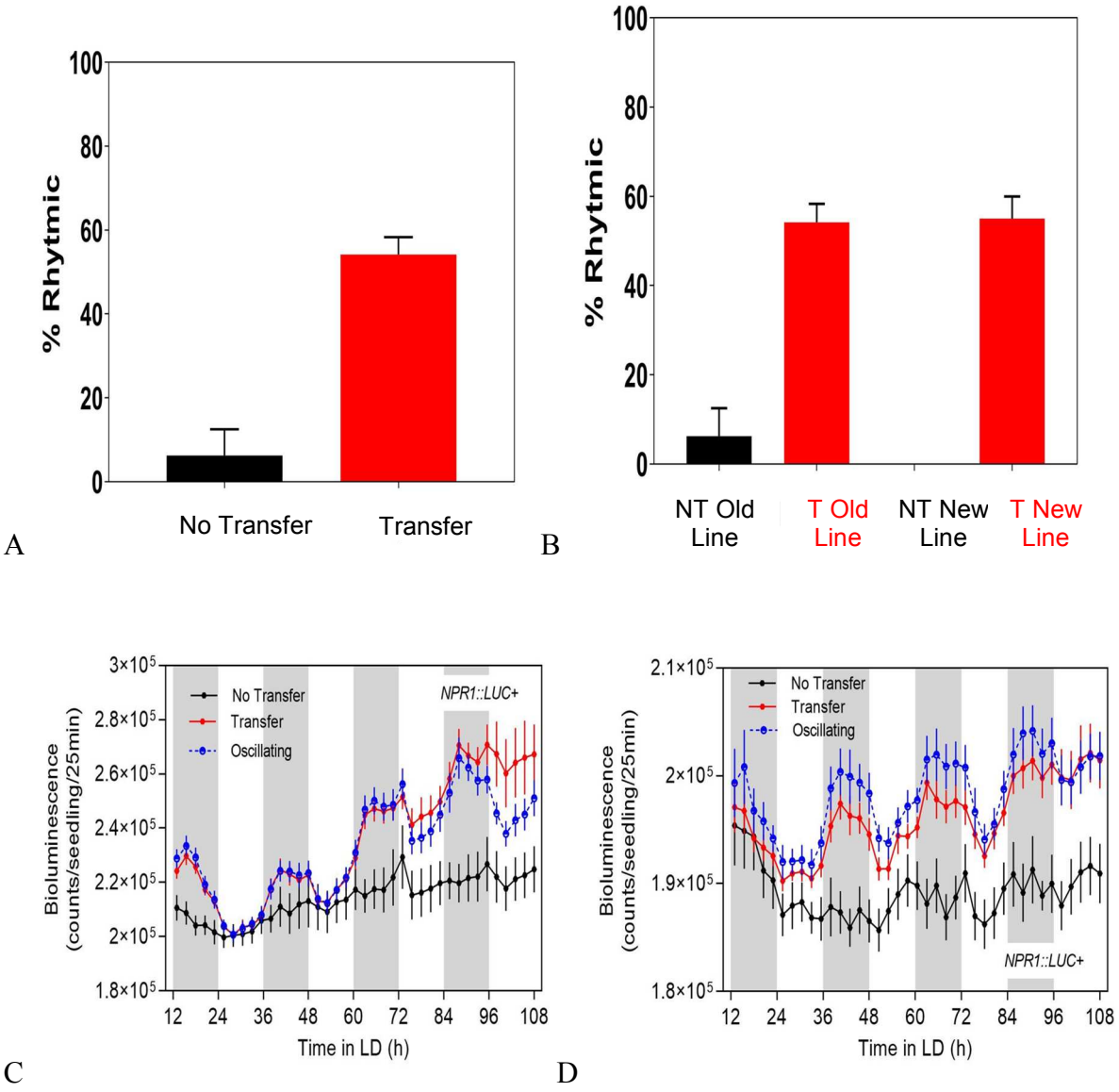


Figure 4. *NPR1* Gene Expression Triggered by Handling Exhibits Circadian Oscillations
Bioluminescence data from 10-day-old NPR1::LUC+ seedlings grown on MS enriched with 3% sucrose in 12hr light/dark cycles. Results indicate mean values (±SEM; n=16) and are representative of independent biological replicates. (A) Old undisturbed transgenic lines (black) and newly generated transferred transgenic line (red). (B) Untransferred old and new lines (black) and transferred old and new lines (red). (C and D) display traces for old and new lines, respectively. Oscillating *NPR1* gene expression results as percentage of rhythmic plants when 100% equates to total plant population.

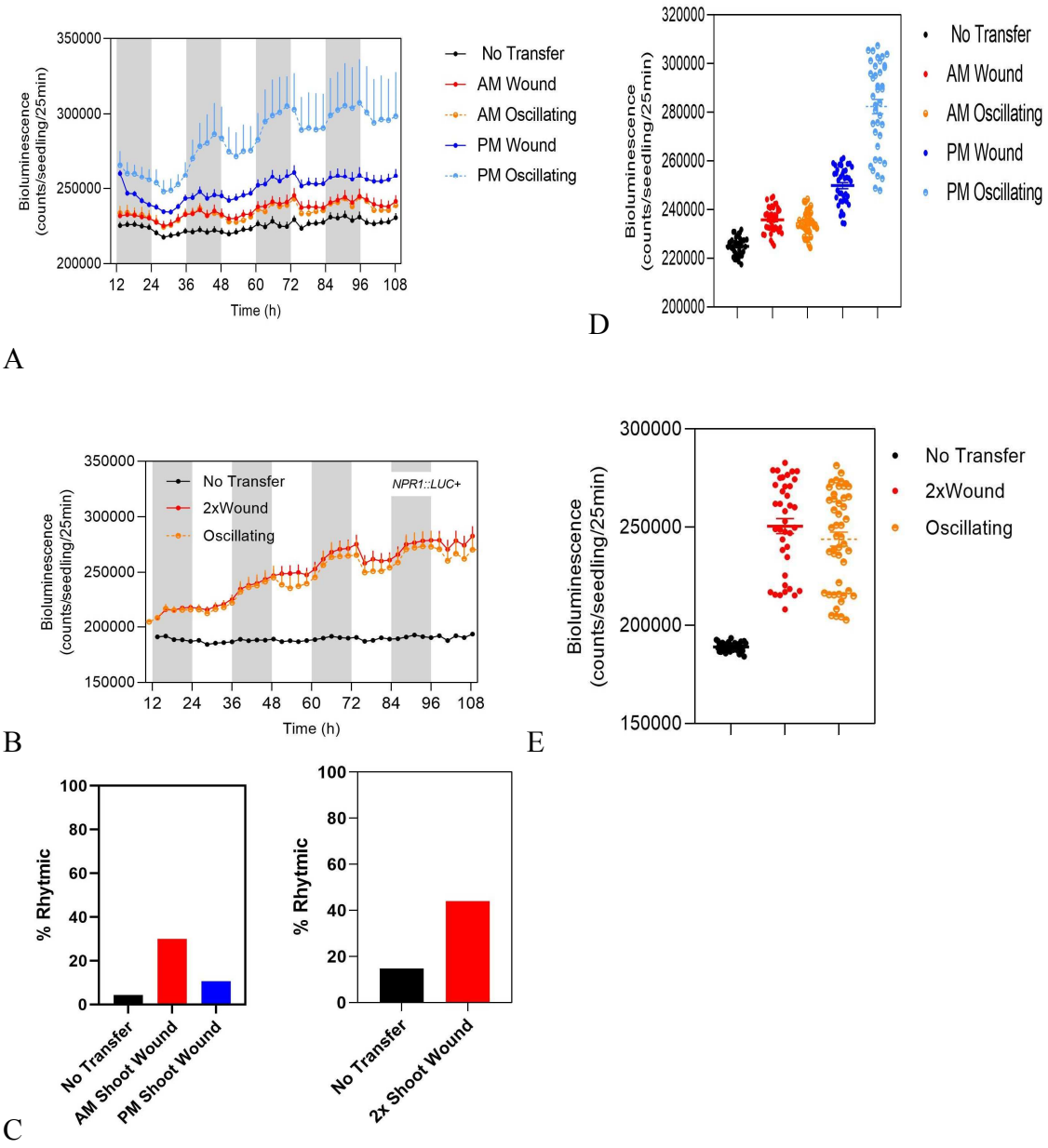


Figure 5. *NPR1* Gene Expression Triggered by Mechanical Wounding Exhibits Circadian Oscillations

Bioluminescence data from 10-day-old NPR1::LUC+ seedlings grown on MS enriched with 3% sucrose in 12hr light/dark cycles after single (A) and double (B) wounding treatments. Results indicate mean values (±SEM; n=16) and are representative of independent biological replicates. Oscillating *NPR1* gene expression results mapped as variations in bioluminescence light intensity (D and E) are representative of sharp differences between peak and trough. Oscillating *NPR1* gene expression results as percentage of rhythmic plants (C) when 100% equates to total plant population.

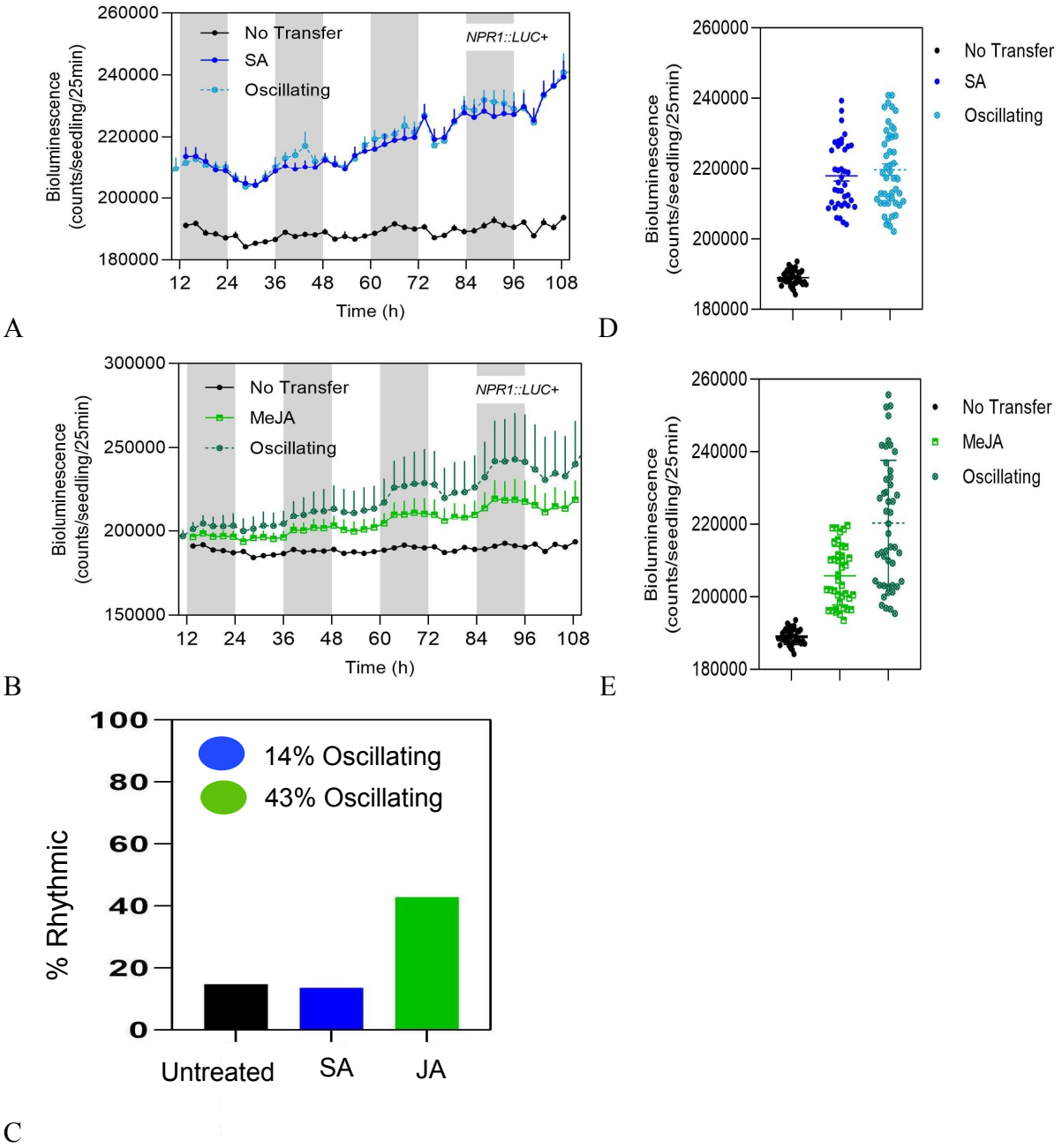
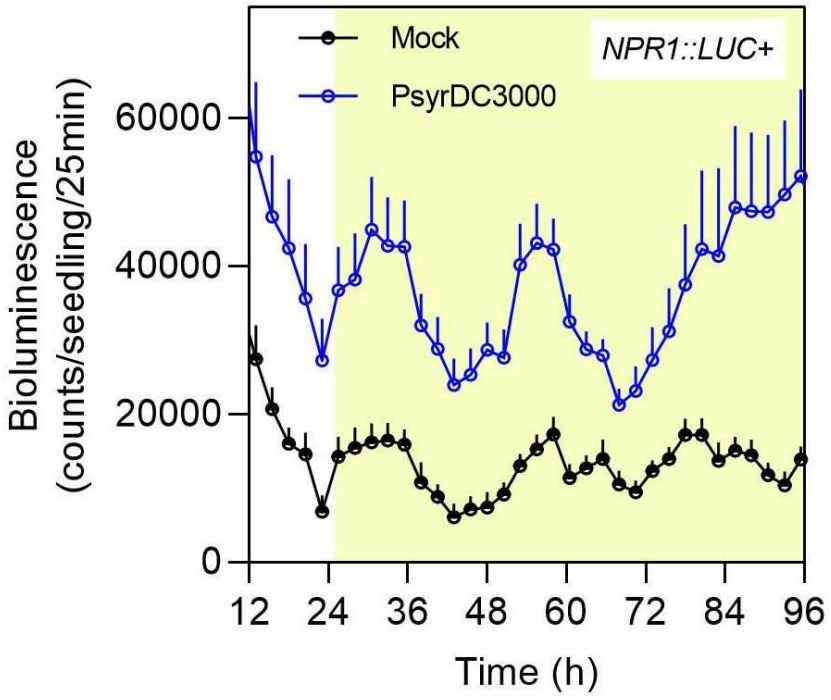
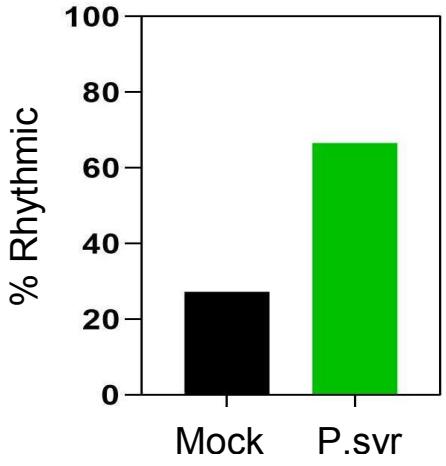


Figure 6. *NPR1* Gene Expression Triggered by both Salicylic Acid (SA) and Jasmonic Acid (JA) Exhibits Circadian Oscillations Only After Jasmonic Acid Treatment
(A and B) Bioluminescence data from 10-day-old NPR1::LUC+ seedlings grown on MS enriched with 3% sucrose in 12hr light/dark cycles after SA (blue) and JA (green) treatments. Results indicate mean values (±SEM; n=16) and are representative of independent biological replicates. Oscillating *NPR1* gene expression results mapped as variations in bioluminescence light intensity (D and E) are representative of sharp differences between peak and trough. Oscillating *NPR1* gene expression results as percentage of rhythmic plants (C) when 100% equates to total plant population.



A



B

Figure 7. *NPR1* Gene Expression Triggered by Single-Leaf *P. syringae* Infection Exhibits Circadian Oscillations

(A) Bioluminescence data from 14-day-old NPR1::LUC+ seedlings grown on MS enriched with 3% sucrose after morning single-leaf mock (black) and *P. syringae DC3000* (blue) dipping infection. Results indicate mean values (±SEM; n=16) and are representative of independent biological replicates. Oscillating *NPR1* gene expression results as percentage of rhythmic plants (B) when 100% equates to total plant population.