TEMPERATURE REGULATION OF PIPECOLIC ACID-MEDIATED PLANT SYSTEMIC IMMUNITY IN ARABIDOPSIS THALIANA

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TEMPERATURE REGULATION OF PIPECOLIC ACID-MEDIATED PLANT SYSTEMIC IMMUNITY IN ARABIDOPSIS THALIANA

by
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BSc Biology, Wilfrid Laurier University, 2020

THESIS
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Abstract

Significant crop losses are caused by pathogenic infections annually, which are exacerbated by increasing global temperatures due to climate change. One way by which plants respond to pathogenic attacks is through the activation of pattern-triggered immunity (PTI), effector-triggered immunity (ETI), and systemic acquired resistance (SAR), which lead to production of the central defence phytohormone salicylic acid (SA). Accompanying SA release is the putative mobilization of pipecolic acid (Pip), which acts as an immune regulatory plant metabolite that works with and independently from SA. As demonstrated in the model plant *Arabidopsis thaliana* following infection with the model bacterial pathogen *Pseudomonas syringae* pv. tomato (*Pst*) DC3000, Pip and its hydroxylated derivative *N*-hydroxypipecolic acid (NHP) accumulate in local and distal tissues to amplify the plant immune response and prime the plant for future infections. Previous studies have only shown that increased temperature negatively impact PTI, ETI and SA production in the local/primary sites of infection. However, how temperature affects plant systemic immunity has not been fully explored. In this thesis, I showed that systemic immunity in *Arabidopsis* to *Pst* DC3000 was significantly reduced at elevated temperatures. Elevated temperature decreased expression of the SAR-associated Pip-NHP biosynthetic genes *AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1)* and *FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1)* in systemically primed leaf tissues. Remarkably, exogenous Pip application via local leaf infiltration or root-drench restored immunity to *Pst* DC3000 at elevated temperature; however, local leaf infiltration did not restore immunity in systemic leaves. I have also shown how Pip-induced gene expression locally and systemically were affected by temperature. Finally, because of the interlinked regulation between SA and Pip/NHP by the master transcription factor *CAM-BINDING PROTEIN 60-LIKE G (CBP60g)*, I have shown that *Arabidopsis* plants constitutively expressing *CBP60g (35S:CBP60g)* exhibited SAR at
both normal and elevated temperatures. My results suggest that \textit{CBP60g} controls the temperature-sensitivity of plant systemic immunity by modulating NHP biosynthesis. Overall, this thesis contributes to understanding the signaling pathways regulating local and systemic plant immune responses in our warming climate.
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List of Abbreviations

This is not an exhaustive list, only abbreviations used three or more times throughout the thesis are included.

**ACT1** - ACTIN 1

**ALD1** - AGD2-LIKE DEFENCE PROTEIN 1

**AvrRpt2** - Avirulent resistance to *P. syringae pv. tomato*

**Aza** - Azelaic acid

**BAK1** - BRI1-ASSOCIATED KINASE 1

**CAMTA** - CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR

**CBP60g** – CALMODULIN-BINDING PROTEIN 60 G

**CNLs** - Coiled-coil NLRs

**CPK5** - Calcium-dependent protein kinase 5

**EC** – Evening complex

**EDS1** - ENHANCED DISEASE SUSCEPTIBILITY 1

**EDS5** - ENHANCED DISEASE SUSCEPTIBILITY 5

**ELF3** - EARLY FLOWERING 3

**ETI** – Effector-triggered immunity

**FLS2** - FLAGELLIN-SENSITIVE 2

**FMO1** - FLAVIN-DEPENDENT MONOOXYGENASE 1

**G3P** - Glycerol-3-phosphate

**GBPL3**- GUANYLATE BINDING PROTEIN-LIKE 3

**HR** - Hypersensitive response

**HSP** - Heat shock protein

**ICS1** - ISOCHORISMA TE SYNTHASE 1

**MAPK**- Mitogen-activated protein kinase

**NB-LRR** – Nucleotide-binding leucine-rich repeat

**NHP** – N-hydroxypipeolic acid

**NO** – Nitric oxide
NPR1 - NON-EXPRESSOR OF PR GENES 1
PAD4 - PHYTOALEXIN DEFICIENT 4
PAMP – Pathogen-associated molecular pattern
PBS3 - AVRPPHB SUSCEPTIBLE 3
PCR1 - PLANT CADMIUM RESISTANCE 1
PIF4 - PHYTOCHROME INTERACTING FACTOR 4
Pip – Pipecolic acid
PP2AA3 – PROTEIN PHOSPHATASE 2A SUBUNIT A3 PROTEIN
PR1 – PATHOGENESIS-RELATED PROTEIN
PRR – Pattern recognition receptor
Pst – Pseudomonas syringae
PTI – Pattern-triggered immunity
RLK – Receptor-like kinases
RLP – Receptor-like proteins
ROS – Reactive oxygen species
RT-PCR – Reverse transcriptase – polymerase chain reaction
SA – Salicylic acid
SAR – Systemic acquired resistance
SARD1 – SAR DEFICIENT 1
SARD4 – SAR DEFICIENT 4
TGA - TGACG SEQUENCE-SPECIFIC BINDING PROTEIN
TIR - Toll/interleukin 1 receptor
Ch 1. General Introduction and Literature Review


1.1 Plant Disease

Plant diseases cause significant crop losses, which impact society at the individual, household, national, and global levels (Savary et al., 2019). Crop losses are often due to pathogenic infections that compromise plant health, making it critical to better understand the mechanisms underpinning plant-pathogen interactions with the goal of improving worldwide food security (Laflamme et al., 2016). This is important due to the ever-growing demand on the agricultural industry for increased crop productivity as the global population continues to increase (Bailey-Serres et al., 2019). In addition to reduced agricultural yields, disease management is often costly and has long-lasting impact on the natural environment, which can lead to further problems.

The development of plant diseases is greatly influenced by the world’s changing climate, as various climatic factors affect a pathogen’s ability to cause disease in plants (Velásquez et al., 2018; Chaloner et al., 2021; Burdonid and Zhanid, 2020). Global carbon dioxide concentrations are increasing, which leads to increased temperature and altered water availability in certain regions (Velásquez et al., 2018). Therefore, the environment plays a major role in shaping plant growth, immunity, and overall health.

Within any given environment, plants will experience a wide range of biotic and abiotic stressors. Examples of biotic factors include fungi, bacteria, phytoplasmas, oomycetes, nematodes, and viruses (Nejat and Mantri, 2017; Wang et al., 2022).
Changing abiotic/environmental conditions, such as temperature, lead to significant agricultural losses, especially when plants become more susceptible to disease (Velásquez et al., 2018; Cohen and Leach, 2020; Desaint et al., 2021). Apart from elevated temperature, abiotic stressors also include drought, salinity, mechanical wounding, high light, or any heavy metals that could influence plant health (Nejat and Mantri, 2017; Zhang et al., 2021; Zhu, 2016). The relationship between the plant, pathogen, and the environment can be described by the “disease triangle” paradigm (Stevens, 1960) in which optimal development of plant disease requires a virulent pathogen, a susceptible host, and favourable environmental conditions (Francl, 2001). Plant disease can be prevented if any of these criteria are not met (Francl, 2001).

One of the most prominent plant pathogens is the model bacterial species *Pseudomonas syringae*. In order for *P. syringae* to successfully infect a host, it must transition from an epiphytic phase when the bacteria are living on plant surfaces, typically leaves, stems, and fruits, to an endophytic phase when the bacteria enter the plant tissues and begin colonizing the intercellular space or apoplast (Xin et al., 2018). Once the bacteria have entered into the plant tissue, typically through natural openings such as the stomata, aggressive multiplication within the apoplast will take place, leading to plant disease (Xin et al., 2018). However, plants have developed a sophisticated innate immune system comprised of numerous complex pathways and signaling molecules to limit pathogenesis (Nejat and Mantri, 2017; Jones and Dangl, 2006; Zhou and Zhang, 2020; Dodds and Rathjen, 2010; Boller and He, 2009).

### 1.2 Plant Immune System

Recent advances have deepened the understanding of the mechanisms needed for the activation of plant immune receptors and signaling pathways (Zhou and Zhang, 2020). A highly sophisticated immune system is important for plants to effectively...
differentiate between pathogens, commensal microbes, and beneficial microbes so that the appropriate defence mechanisms are employed at the correct time (Zhou and Zhang, 2020). The best characterized plant immune system is that of the model organism *Arabidopsis thaliana* due to its genetic tractability and the availability of diverse genomic resources (Nishimura and Dangl, 2010). Based on research in *Arabidopsis* and other plant species, it is known that the plant immune system relies on numerous and complex signaling pathways following pathogen recognition (Bigeard et al., 2015).

Plants respond to pathogenic attack through the recognition of conserved pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Ausubel, 2005; DeFalco and Zipfel, 2021; Wan et al., 2019; Figure 1). PRRs allow the plant to recognize an entire class of potentially pathogenic organisms (Zhou and Zhang, 2020). Numerous forms of immunogenic signals are recognized by PRRs to activate the immune response known as pattern-triggered immunity (PTI), also referred to as plant basal defence (Zhou and Zhang, 2020). These may be pathogenic epitopes generated by lytic enzymes from the host plant (Buscaill et al., 2019), immunogenic peptides known as phytocytokines produced by the plant, or plant molecules derived from their own cellular damage called damage-associated molecular patterns (DAMPs). Pathogens can damage plant tissues, leading to the degradation of the cell wall, and the release of extracellular ATP and/or nicotinamide adenine dinucleotide (NAD), which are recognized as DAMPs by PRRs, leading to the activation of the immune response (Gust et al., 2017; Bacete et al., 2018). PRRs can be classified as receptor-like kinases (RLK) with both extracellular and intracellular domains or as receptor-like proteins (RLP) that lack the cytosolic signaling domain (Zhou and Zhang, 2020).

Following PAMP detection by PRRs, many cellular events will be activated (Zhou and Zhang, 2020). PRRs exist as protein complexes that are in a resting state prior to PAMP binding (Zhou and Zhang, 2020). RLKs interact with the co-receptor
BRI1-ASSOCIATED KINASE 1 (BAK1), while RLPs interact with both adaptor kinase SUPPRESSOR OF BIR1-1 (SOBIR1) and the co-receptor BAK1 as the RLPs do not have their own kinase domain (Chinchilla et al., 2007; Gao et al., 2009; Liebrand et al., 2013; Zhou and Zhang, 2020). Apart from activation, there is also negative regulation of these receptors (Zhou and Zhang, 2020). When no pathogen is detected, BIR1 will sequester the BAK1 protein and only release it when cellular changes occur so that it can then interact with the appropriate PRR (Li et al., 2002; Nam and Li, 2002; Zhou and Zhang, 2020). When a PRR complex is formed with BAK1, the resulting immune signaling complex can interact with additional complexes ultimately leading to the integration of multiple signals (Zhou and Zhang, 2020). For example, the alteration of cell wall integrity and cell growth are detected and can activate defense mechanisms (Zhou and Zhang, 2020). When PRRs are activated, phosphorylation of receptor-like cytoplasmic kinases (RLCKs) will occur rapidly, followed by an influx of calcium across the cell membrane and a burst of reactive oxygen species (ROS) (Tang et al., 2017). These processes eventually lead to the activation of calcium-dependent protein kinases (CPKs) and mitogen-activated protein kinase (MAPK) cascades (Romeis et al., 2001; Tang, et al., 2017). Additional induced defence mechanisms include stomatal closing (Melotto et al., 2008), restriction of nutrient transfer from the cytosol to the apoplast to limit bacterial multiplication (Sattelmacher, 2001; Hoefle and Hückelhoven, 2008; Wang et al., 2012), and production of antimicrobial compounds (Denoux et al., 2008). These compounds include camalexin, and defence-related proteins and peptides (such as PATHOGENESIS-RELATED PROTEIN 1 (PR1)) (O’Brien, et al., 2012; Figure 1).

PTI may not always be efficient because some pathogens can overcome PTI by secreting effectors into and outside of the cell, therefore requiring a second line of plant defence known as effector-triggered immunity (ETI) (Figure 1) (Thordal-Christensen, 2020; Saur et al., 2020; Cui et al., 2015; Jones et al., 2016). ETI is the immune response
to specific pathogens and occurs in response to specific elicitor molecules known as effectors (Toruño et al., 2016; Wang et al., 2022; Thordal-Christensen, 2020). Plant pathogens produce effectors that enhance their ability to colonize, grow, and reproduce, allowing them to overcome the host plants’ defences (Mukhtar et al., 2011; Thordal-Christensen, 2020). Effector molecules can manipulate the host’s cell structure and function and may be toxic (Thordal-Christensen, 2020). Additionally, effectors may act alone or in combination with other molecules to suppress host PTI. Pathogenic bacteria such as *P. syringae* have a Type III secretion system that has a syringe-shaped injectisome with a needle-like extracellular projection that can span both the outer and inner bacterial membrane (Jin and He, 2001; Wei and Collmer, 2018). Once the bacterium successfully injects the projection into the host cell, the PRRs cannot recognize the intracellular effectors (Wei and Collmer, 2018).

ETI-associated Resistance (R) proteins will provide resistance to individual pathogens by recognizing strain-specific effectors (Wei and Collmer, 2018). The majority of R proteins are intracellular nucleotide-binding leucine-rich repeat (NB-LRR) receptors (Zhou and Zhang, 2020). These effectors are detected by NB-LRR receptors to activate various downstream responses (Thordal-Christensen, 2020; Saur et al., 2020; Cui et al., 2015; Jones et al., 2016). There are two classes of NB-LRR receptors called TNLs and CNLs (Zhou and Zhang, 2020). These two classes are structurally different in terms of their protein domains, their activation mechanisms, and their downstream signaling pathways (Zhou and Zhang, 2020). The classification is based on their N-terminal domain, either Toll/interleukin 1 receptor (TIR) NLRs (TNLs) or coiled-coil NLRs (CNLs) (Martin et al., 2020; Bi et al., 2021; Wang et al., 2019; Ma et al., 2020). In both classes, oligomerization of the N-terminal domain will lead to cell death in the area of infection and the expression of resistance genes (Martin et al., 2020; Bi, et al., 2021). TNLs are activated by the tetramerization of the domains to bring the TIR
domains close together so that they can interact and trigger the hypersensitive response (HR) (Martin et al., 2020). CNLs form pentameric resistosomes when activated and this structure is required to trigger cell death and for disease resistance to be initiated (Wang et al., 2019). ETI activated by TNLs or CNLs often leads to localized programmed cell death, which is referred to as HR, at the site of infection to limit pathogen progression (Bednarek, 2012; Mur, 2008) (Figure 1). In terms of required proteins for effective downstream signaling, TNLs typically require ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), PHYTOALEXIN DEFICIENT 4 (PAD4), SENESCENCE-ASSOCIATED GENE 101 (SAG101), N REQUIREMENT GENE 1 (NRG1), and ACTIVATED DISEASE RESISTANCE 1 (ADR1), whereas CNLs typically require NON-RACE SPECIFIC DISEASE RESISTANCE-1 (NDR1) (Martin et al., 2020; Wang et al., 2019; Sun et al., 2021; Wagner et al., 2013; Pruitt et al., 2021; Dongus and Parker, 2021; Lapin et al., 2020). ETI is both more accelerated and amplified than PTI, and it involves increasing ROS levels and activating hormone signaling pathways, such as salicylic acid or SA (Thordal-Christensen, 2020) (Figure 1). Recent data has shown that major components in PTI and ETI are required for both pathways. Particularly, PTI and ETI reinforce each other, which leads to more robust defense responses against pathogen infections, contrary to the previous thought that PTI and ETI were separate pathways (Pruitt et al., 2021; Ngou et al., 2021; Tian et al., 2021; Yuan et al., 2021).
Figure 1. Major signaling events occurring in the plant immune system.

When a plant senses a pathogen, several mechanisms and signaling pathways are induced. The mechanisms by which the plant protects itself are shown in a representative diagram of a single plant cell. The first line of inducible defence in plants is pattern triggered immunity or PTI. PTI is triggered through the detection of non-self-microbial signatures, which are called pathogen-associated molecular patterns or PAMPs. PAMPs are recognized by Pattern recognition receptors, or PRRs. Upon PAMP perception, PRRs initiate downstream immune signaling, including a rapid burst of calcium ions and reactive oxygen species, expression of defence genes and the production of the phytohormone salicylic acid, or SA. The second layer of inducible defense is effector triggered immunity or ETI. ETI is activated by the intracellular recognition of pathogen effector molecules by nucleotide-binding leucine-rich repeat receptors (NLRs). This also leads to an ion flux, the activation of resistance genes and SA biosynthesis. ETI is both more accelerated and amplified than PTI, although recent studies show their mutual potentiation. Adapted from Zhou and Zhang, 2020; Created with BioRender.com.
1.3 Plant Systemic Immunity

Sustained immune activation at the local site of infection can induce a state of readiness to respond to future stress in unaffected systemic tissues, which is known as systemic acquired resistance (SAR) (Vallad and Goodman, 2004; Vlot et al., 2021; Zeier, 2021; Durrant and Dong, 2004; Shine et al., 2019; Kachroo and Kachroo, 2020) (Figure 2). SAR is typically induced following primary exposure to virulent, avirulent, or nonpathogenic microbes (Vallad and Goodman, 2004). SAR is widely conserved across the plant kingdom, and it is one of two types of systemic immunity in plants that depend on two parallel and interconnected pathways (Vlot, et al. 2021). SAR (as the first type) is dependent on the hormone SA and is induced by pathogens that interact with the leaves (Pieterse et al. 2009). The second type is induced systemic resistance (ISR), which is induced by beneficial microbes interacting with the roots and is mediated by the hormones jasmonic acid (JA) and ethylene (ET) (Pieterse et al. 2009).

During SAR, broad-spectrum host defence mechanisms are deployed, such as rapidly generating mobile signals at the site of infection and transporting them throughout the plant foliage to prime distal tissues against future infection (Schneider et al., 1996; Vlot et al., 2021; Zeier, 2021; Durrant and Dong, 2004; Kachroo and Kachroo, 2018; Shine et al., 2019; Kachroo and Kachroo, 2020). Several potential key chemical inducers of SAR have been identified, including methyl SA, azelaic acid (AzA), glycerol-3-phosphate (G3P), dehydroabietinal (DA), nitric oxide (NO), reactive oxygen species (ROS), pipelicolic acid (Pip) and N-hydroxypipelicolic acid (NHP) (Singh et al., 2017; Wendehenne et al., 2014; Chen et al., 2018; Hartmann et al., 2018). The complete nature of the mobile SAR signal/s remain(s) unconfirmed (Ding et al. 2016); interestingly, the SAR-mediating DIR1 protein has been shown to move to distant tissues (Champigny et al., 2013; Carella et al., 2016).
The phytohormone SA is an integral component of SAR and one of the most potent inducers of broad-spectrum resistance in plants (Kachroo et al., 2020; Klessig et al., 2018; Zhang and Li, 2019; Ding and Ding, 2020; Saleem et al., 2021; Peng et al., 2021). However, little is known about how other signaling molecules work with, and independently from SA (Wang et al., 2018). It is known that SA is systemically mobile; however, it has been established that its long-distance mobility is not solely responsible for SAR establishment (Vernooij et al., 1994; Lim et al., 2020). Based on this, it is proposed that SA is required and contributes to systemic propagation of defence signaling alongside other molecules (Gaffney et al., 1993; Vernooij et al., 1994; Pallas et al., 1996).

Recently, other candidate long-distance signals have been suggested, including piperolic acid (Pip) and its derivative N-hydroxypiperolic acid (NHP) (Návarová et al., 2012; Chen et al., 2018; Hartmann et al., 2018) (Figure 3). Prior to Pip being shown to be a key signal in the plant immune system, it has long been detected in numerous plant species (Zacharius et al., 1954; Yatsu and Boynton, 1959). Pip and NHP treatment have been shown to induce SAR, and studies have shown that blocking Pip or NHP biosynthesis will result in complete loss of SAR (Song et al., 2004; Mishina and Zeier, 2006; Jing et al., 2011). This suggests that Pip and NHP play a role in both local defences and long-distance signaling (Huang et al., 2020).

As previously mentioned, three potential inducers of SAR are AzA, G3P, and SA (Lim et al., 2016). It was suggested by Lim et al. (2016) that AzA and G3P undergo symplastic transport through channels called plasmodesmata, which is different from SA movement via the extracytosolic apoplast (Lim et al., 2016). AzA is produced from the hydrolysis of fatty acids (FAs) derived from the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (Yu et al., 2013). MGDG and DGDG biosynthesis is catalyzed by plastidial enzymes (Yu et al., 2013). When C18 FAs are cleaved at the double bond at C9, the conversion to AzA will occur (Yu et al., 2013).
AzA accumulation in response to SAR induction promotes the biosynthesis of G3P, another well-known SAR regulator (Yu et al., 2013; Shine et al., 2019). In a study done by Shine et al. (2019), it was determined through analysis of endogenous G3P accumulation that G3P mediates, in part, a root-shoot-root signaling, resulting in protection in the plant’s foliage and roots (Shine et al., 2019). G3P synthesis occurs via the phosphorylation of glycerol by glycerol kinase encoded by GLI1, or through the reduction of dihydroxyacetone phosphate by G3P dehydrogenase encoded by GLY1 (Jung et al., 2009; Yu et al. 2013). Pathogen–inducible G3P accumulation and downstream signalling require the lipid transfer protein (LTP) DIR1 (defective in induced resistance) and the LTP-like AZI1 (AzA-insensitive) (Jung et al., 2009; Yu et al. 2013), which suggests that SAR regulation requires a G3P-DIR1 feedback loop (Gao et al., 2014).

Of note, the SAR-inducing molecules G3P and AzA cannot induce SAR in dir1-1 mutants, indicating that one or more of these small molecules may be physiological ligands of DIR1 (Maldonado et al., 2002; Champigny et al., 2013). Previous studies have suggested that DIR1 travels to the induced leaf through the phloem (Champigny et al., 2013). To show this, DIR1-GFP accumulation was monitored in phloem exudates using an estrogen-SAR assay (Champigny et al., 2013). In this assay, the same leaf was first treated with estrogen, which induced DIR1-GFP expression; this was then followed by SAR induction in the same leaf of dir1-1 (Champigny et al., 2013). DIR1-GFP was identified in exudates collected from local and systemic leaves of SAR-induced plants using both DIR1 and GFP antibodies (Champigny et al., 2013). This provides strong evidence that DIR1 moves through the phloem to distant leaves to initiate priming (Champigny et al., 2013). To further uncover how DIR1 enters the phloem, plant lines with compromised cell-to-cell movement were used (Champigny et al., 2013). The cell-to-cell
movement in these plants was affected by overexpression of plasmodesmata-located proteins; more importantly, these plants were also defective for SAR, and DIR1 was not observed in the phloem of systemic leaves, further supporting that cell-to-cell movement of DIR1 through plasmodesmata is vital for the movement of SAR signals (Champigny et al., 2013). Overall, DIR1 encodes a putative apoplastic LTP that is thought to interact with a lipid-derived molecule to promote long-distance signalling (Champigny et al., 2013).

Additional important SAR regulators are NO and ROS. Like SA, NO accumulation is required for SAR and the synthesis of these molecules involves regulation through the same galactolipids (Gao et al., 2014). Additionally, these galactolipids can regulate the SAR mediators AZA and G3P, which function downstream of NO (Gao et al., 2014).
Figure 2. Model of the molecular mechanisms governing SAR.

Following pathogen infection in the local leaf there will be a production of immune signals that will result systemic acquired resistance – or SAR. The proposed mobile mediator of SAR is Pipecolic acid, or Pip. The three critical genes required for Pip and its metabolically active form NHP are \textit{ALD1}, \textit{SARD4}, and \textit{FMO1}. Adapted from Vlot et al., 2021; Created with BioRender.com.
Figure 3. Schematic model of the NHP pathway and its role in inducing SAR in *Arabidopsis*.

Following pathogen infection, NHP is generated through the three-step process shown involving the three pathogen-inducible genes *ALD1*, *SARD4*, and *FMO1*. The action of *ALD1* leads to intermediates which are reduced by *SARD4* to produce Pip. Then, *FMO1* catalyzes the conversion of Pip to NHP. It is thought that NHP moves from the local to the systemic leaves after pathogen attack. Adapted from Shan and He, 2018; Created with BioRender.
1.4 **Pipecolic Acid/NHP and Plant Immunity**

Pipecolic acid (Pip) is an important metabolite that occurs universally throughout the plant kingdom (Rossner et al., 2017; Hartmann and Zeier, 2018). Pip is a non-protein amino acid associated with SAR due to its ability to induce SA accumulation when applied to leaves (Návarová et al., 2012; Berndorff et al., 2016; Yildiz et al., 2021). Following pathogenic infection, Pip and its hydroxylated derivative NHP have been found to accumulate in both local and distal leaves (Návarová et al., 2012; Berndorff et al., 2016; Yildiz et al., 2021; Hartmann et al., 2018). The Pip-NHP biosynthetic pathway is inducible by pathogens and leads to the production of NHP, which is the SAR-activating metabolite (Figure 4; Hartmann et al., 2018). Some characterized roles of NHP include its ability to induce the expression of defence genes, amplification of the resistance response, synergistic action with SA, promotion of the hypersensitive response, and contribution to SAR by priming the plant against future infection (Hartmann et al., 2018).

The Pip/NHP pathway has three consecutive enzymatic reactions (Hartmann and Zeier, 2018). First, L-lysine undergoes α-transamination through the action of the aminotransferase AGD2-LIKE DEFENCE PROTEIN 1 (ALD1), which leads to the production of cyclic dehydropipecolic (DP) intermediates (Zeier, 2013). The ALD1 gene is strongly induced systemically in plant foliage following pathogenic attack in a local leaf (Song et al., 2004; Návarová et al., 2012; Cecchini et al., 2015). The DP intermediates are then reduced to Pip via the action of the reductase SAR-deficient 4 (SARD4) (Hartmann et al., 2017; Ding et al., 2016). Like ALD1, the SARD4 gene is also induced systemically in the foliage of *Arabidopsis* following local pathogenic attack (Hartmann et al., 2017). Following Pip production, Pip is then N-hydroxylated to NHP by flavin-dependent monooxygenase 1 (FMO1) (Hartmann et al., 2018; Chen et al., 2018). Again, like ALD1 and SARD4, the expression of FMO1 is also strongly induced systemically.
throughout the plant’s foliage in response to local pathogen attack (Mishina and Zeier. 2006).

**Figure 4. Enzymatic steps in the NHP biosynthetic pathway.**

The NHP biosynthetic pathway begins with L-Lysine catabolism via *ALD1*, followed by the dehydrative cyclization to intermediates (1,2-dehydropipecolic acid (DP)). *SARD4* reduces the intermediates to Pip. Finally, *FMO1* *N*-hydroxylates Pip to NHP. Accumulation of NHP is essential for SAR. Adapted from Hartmann and Zeier, 2018; Created with BioRender.com.
Pip/NHP signaling has been shown to function in parallel with SA-derived signaling to induce SAR (Wang et al., 2018; Zeier, 2021; Shields et al., 2022). There is a relationship between Pip/NHP and the SA/G3P-derived parallel signaling pathways, in which Pip/NHP primarily functions upstream of the NO-ROS-AzA-G3P branch of the SAR pathway (Wang et al., 2018; Vlot et al., 2021; Figure 2). When a virulent pathogen successfully infects a host plant, independent signaling events lead to the accumulation of SA and NO in the locally infected leaves (Yu et al., 2013). The accumulation of NO then triggers the synthesis of ROS leading to a feedback loop occurring between NO and ROS (Yu et al., 2013). Additionally, this infection leads to Pip accumulation in the local tissue, which as mentioned previously, induces the accumulation of NO, ROS, AzA, and G3P (Wang et al., 2018; Figure 2). It has been suggested that SA and G3P are then transported to systemic tissue, where Pip will be induced, and this will reactivate the NO, ROS, and AzA cascade leading to more G3P biosynthesis (Wang et al., 2018; Figure 2). When Pip is absent, the biosynthesis of SAR signals acting upstream of Pip are not altered (Wang et al., 2018). Overall, there are many important chemical signals associated with SAR establishment in the systemic tissues which are tightly coordinated with Pip/NHP (Wang et al., 2018).

1.5 Crosstalk between Pipecolic Acid/NHP and Salicylic Acid

The Pip/NHP pathway is regulated by SA, and SA cooperates with NHP in SAR induction (Hartmann et al., 2018). SA reduces excess accumulation of NHP, that is, SA can negatively regulate the conversion of Pip to NHP (Hartmann et al., 2018; Yildiz et al., 2021). It has been suggested that Pip acts as a mediator of defence amplification and priming, by conditioning plants for effective biosynthesis of SA and expression of defence-related genes (Bernsdorff et al., 2016).
Like the Pip-NHP biosynthetic pathway, the synthesis of SA is well-characterized in *Arabidopsis* plants. SA can be produced via the isochorismate (IC) pathway (Wildermuth et al., 2001), where the ISOCORISMATE SYNTHASE 1 (ICS1) enzyme, also known as SALICYLIC ACID INDUCTION DEFICIENT 2 (SID2), catalyzes the committed step of pathogen-induced SA synthesis (Zhang and Li, 2019). To determine the relationship between Pip and SA, plants deficient in Pip (*ald1* mutant) and deficient in SA induction (*sid2* mutant) plus the *sid2 ald1* double mutant were examined (Bernsdorff et al., 2016). The *sid2* plants have a mutation in the ICS1 coding region, which results in a complete loss of ICS1 function and reduced SA production (Bernsdorff et al., 2016). This reduced SA production subsequently leads to reduction but not complete loss of SAR with partial defence transcriptional response in the systemic tissues, revealing SA-independent signaling pathways that contribute to SAR responses (Bernsdorff et al., 2016). In the experiments performed by Bernsdorff et al. (2016), there was significantly higher bacterial growth in the *sid2* mutant compared to the *ald1* mutant, suggesting that the relative contribution of SA greater than the contribution of Pip in basal immunity. Additionally, leaf-inoculated *sid2 ald1* showed the least resistant phenotype of all investigated lines where there was significantly higher bacterial multiplication than what was observed in Col-0, *ald1*, and *sid2* plants. This suggests that SA and Pip provide additive contributions to basal immunity against *Pst*.

Additionally, the results suggest that both SA-dependent and SA-independent pathways are regulated by Pip. In the absence of inducible SA biosynthesis, *Arabidopsis* can switch on Pip/NHP to induce a moderate SAR response through the action of *FMO1* (Bernsdorff et al., 2016).

In the *Arabidopsis* immune system, the protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), and one of its signaling partners, PHYTOALEXIN DEFICIENT 4 (PAD4), play a role in plant basal immunity and ETI through their
involvement in promoting SA accumulation (Joglekar et al., 2018). Recent studies have found that these genes are also important for the induced expression of the Pip-NHP biosynthetic genes *ALD1* and *FMO1* (Joglekar et al., 2018). This further shows that the SA and Pip-NHP pathways during plant disease resistance share common overlapping regulators.

Following a local infection, NHP accumulates in the distal leaves and plays a role in establishing SAR (Ding et al., 2016). Yildiz et al. (2021) recently discovered that treating *Arabidopsis* with NHP exogenously resulted in a response similar to SAR. In this response, key metabolites needed for the immune response and signal transduction such as NHP, are thought to become mobile (Yildiz et al., 2021). Additionally, treatment with exogenous NHP results in an amplified response to pathogens due to increased SA production, accumulation of the phytoalexin camalexin and the expression of immune response genes (Yildiz et al., 2021). SA accumulation amplifies NHP-triggered SAR and drives the appropriate transcriptional and defence priming responses (Yildiz et al., 2021).

For either NHP or SA to properly activate immune responses, the function of the transcriptional coregulator and SA receptor NON-EXPRESSOR OF PR GENES 1 (NPR1) is essential (Cao et al., 1997; Ding et al., 2018; Liu et al., 2020; Wu et al., 2012; Návarová et al., 2012; Yildiz et al., 2021; Nair et al., 2021). It is thought that NHP functions as a mobile immune regulator capable of moving independently of active SA signaling from leaf-to-leaf to systemically activate defence responses (Yildiz et al., 2021). However, demonstration of systemic NHP transport has yet to be shown. Interestingly, the authors also discovered that exogenous NHP treatment led to local upregulation of more than 1,500 SAR-related genes in *Arabidopsis*, potentially priming the plants for an enhanced defense response (Yildiz et al., 2021).
1.6 Transcriptional Regulation of Plant Immunity

For successful immune signaling in *Arabidopsis*, two members of the calmodulin-binding protein 60 (CBP60) gene family are required: *CBP60g* and *SYSTEMIC AQUIRED RESISTANCE DEFICIENT 1 (SARD1)* (Wang et al., 2011; Wan et al., 2012; Wang et al., 2009; Sun et al., 2015; Zhang et al., 2010; Sun et al., 2018; Lu et al., 2018). *CBP60g* and *SARD1* encode master immune transcription factors, controlling both SA and Pip/NHP production (Figure 5; Hartmann et al., 2018; Wang et al., 2011; Wan et al., 2012; Wang et al., 2009; Sun et al., 2015; Zhang et al., 2010; Sun et al., 2018). Analysis of *CBP60g* and *SARD1*-dependent gene promoter sequences revealed frequent occurrence of a GAAATTT motif, suggesting that *CBP60g* and *SARD1* may directly control expression of numerous defence genes, including those essential for both local and systemic immunity (Wang et al., 2011). The SA biosynthetic genes *ICS1*, *ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5)*, and *AVRPPHB SUSCEPTIBLE 3 (PBS3)* and the Pip/NHP biosynthetic genes *ALD1*, *SARD4*, and *FMO1* are all regulated by *SARD1* and *CBP60g* (Huang et al., 2020). When they are expressed, there will be increased levels of SA and NHP (Figure 5) (Hartmann et al., 2018). However, whether *CBP60g* and *SARD1* participate during downstream Pip/NHP signaling is still unknown.
Figure 5. Transcriptional regulation of SARD1/CBP60g and SA/NHP biosynthesis.

The SA biosynthetic genes \( ICS1/EDS5/PBS3 \) and NHP \( ALD1/SARD4/FMO1 \) are all regulated via the master transcription factors SARD1 and CBP60g. Central immune regulators ENHANCED DISEASE SUSCEPTIBILITY 1 \( (EDS1) \) and PHYTOALEXIN DEFICIENT 4 \( (PAD4) \) both mediate pattern-triggered immunity and effector-triggered immunity and are also needed for SA and NHP accumulation. Expression of these genes lead to increased levels of SA and NHP. The SA receptor NPR1 is directly activated by SA and indirectly activated by NHP. Figure: Shields et al., 2022; Created with BioRender.com.
Apart from the CBP60 transcription factor family, another group of transcription factors important for SA-mediated plant immunity are the TGACG SEQUENCE-SPECIFIC BINDING PROTEINS (TGAs), which specifically bind to variants of the palindrome TGACGTCA (Xiang et al., 1997). TGA1 and TGA4 function to ultimately modulate plant SA and Pip/NHP production (Li et al., 2018), since they are required for full expression of SARD1 and CBP60g (Zhang and Li, 2019). Homologous transcription factors TGA2, TGA3, TGA5, and TGA6 also belong to the same TGA family (Kesarwani et al., 2007). They are essential for the plant's response to SA and NHP, since higher-order tga mutant plants are SA-insensitive and SAR-deficient (Zhang et al., 2003; Kesarwani et al., 2007). The requirement of these TGAs for SA- and NHP-mediated transcriptional reprogramming is expected since TGAs recruit the master coactivator and SA receptor NPR1, which is required for SA- and NHP-responsive expression (Ding et al., 2018; Nair et al., 2021).

A study by Sun et al. (2020) has outlined additional transcription factors that are negative regulators of both SA and Pip/NHP accumulation. The CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR (CAMTA) family recognizes the CGCG box in target gene promoter regions (Bouché et al., 2002). CAMTA1, CAMTA2, and CAMTA3 were investigated to elucidate the underlying mechanisms. Different mutant plants were used; for example, camta3 (loss-of-function mutant) was found to have increased bacterial resistance, increased SA levels and lower growth, compared to the wild-type plants, as well as exhibiting autoimmunity (Du et al., 2009). In another study, analysis indicated that the sard3 mutant contains mutations in the 12th exon of At2g22300, which encodes CAMTA3 (Bouché et al., 2002). Further analysis confirmed that sard3 carries a gain-of-function mutation of CAMTA3. For consistency with the genetic mutant nomenclature, sard3 was renamed as camta3-3D (Jing et al., 2011). The camta3-3D (or sard3) gain-of-
function mutant has lower bacterial resistance and no SAR, compared to wild-type plants (Jing et al., 2011).

Altogether, a working model was proposed regarding the negative regulation of SA and NHP biosynthesis by CAMTA1/2/3 (Sun et al., 2020). In detail, CAMTA1/2/3 redundantly negatively regulate the expression of SARD1 and CBP60g to reduce SA and NHP accumulation, with mutual amplification between SA and NHP signaling pathways leading to robust immune responses (Sun et al., 2020; Shields et al., 2022).

### 1.7 The Role of CBP60g and SARD1 in Plant Immunity

As mentioned previously, CBP60g and SARD1 make up a partially redundant pair of proteins that are required for SA accumulation as well as other defense responses (Wang et al., 2011). CBP60g and SARD1 regulate the SA pathway genes *ICS1, EDS5*, and *PBS3* and the NHP biosynthetic genes *ALD1, SARD4*, and *FMO1* (Wang et al., 2011; Sun et al., 2015; Huang et al., 2020). CBP60g plays a more important role earlier in the defense response and SARD1 plays an important role later (Wang et al., 2011). Therefore, it is important to consider the other various roles of these two master immune transcription factors.

These two transcription factors are activated by various extracellular signals, including plant hormones, biotic stresses, and abiotic stresses, which collectively elicit changes in cellular calcium (Ca$^{2+}$) concentration (Reddy et al., 2002). Calcium ions are universal secondary messengers in plants (Yang and Poovaiah, 2003). In vascular plants, there are three major families of Ca$^{2+}$ sensors; these are calmodulins (CaMs) and CaM-like proteins (CMLs), the calcineurin B-like proteins (CBLs), and the Ca$^{2+}$-dependent protein kinases (CDPKs) (Bouché et al., 2005; DeFalco et al., 2009; Galon et al., 2010; Luan et al. 2002; Reddy et al. 2002; Yang and Poovaiah. 2003). Of note, CaM binds Ca$^{2+}$ ions and is a highly conserved, small acidic protein found in eukaryotes (Luan et al., 2002;
Reddy et al., 2002; Yang and Poovaiah, 2003). Importantly, CaM binds to CBP60g (Wang et al., 2009; Zhang et al., 2010). The CBP60g CaM binding domain is located near its N-terminus, with CaM binding dependent on Ca$^{2+}$ and essential for CBP60g function in plant defense signaling (Wang et al., 2009).

The CaM-binding protein 60 (CBP60) family has eight members in A. thaliana, with SARD1 being the most similar to CBP60g (Zhang et al., 2010; Wang et al., 2011). However, SARD1 does not bind CaM unlike most other CaM-binding proteins (Zhang et al., 2010; Wang et al. 2011). Wang et al. (2011) tested CaM binding by CBP60g and SARD1. This was done using a glutathione-S-transferase (GST) with CBP60g or SARD1 that was then checked if it successfully bound to biotinylated calmodulin. It was found that GST–CBP60g bound to CaM, but GST–SARD1 did not. These results indicated that SARD1 is not a CaM-binding protein (Wang et al., 2011). This difference in CaM binding is likely crucial for the downstream responses mediated by CBP60g and SARD1 following a pathogen infection (Lecourieux et al., 2006; Gust et al., 2007).

Cytosolic Ca$^{2+}$ levels spike as a part of the immune response (Aslam et al., 2008), which could lead to CBP60g activation through CaM binding, thereby leading to increased SA levels (Wang et al., 2011; Sun et al., 2022). Since SARD1 does not bind CaM, SARD1 may become primarily responsible for the activation of SA signaling once the Ca$^{2+}$ flux has subsided (Wang et al., 2011).

A recent study has found that CBP60g and SARD1 expression can be promoted by transcription factors WRKY54 and WRKY70 (Chen et al., 2021). WRKY70 plays a complex role in plant immunity and its expression is upregulated by SA accumulation (Wang et al., 2006). This study tested if WRKY54 and WRKY70 are necessary for the induced expression of SARD1 and CBP60g by testing sid2, wrky54 wrky70 and sid2 wrky54 wrky70 mutants after Psm ES4326 infection (Chen et al., 2021). It was found that SARD1 and CBP60g expression in response to Psm ES4326 was significantly
lower in *sid2* mutants and was reduced further in *sid2 wrky54 wrky70* mutants, compared to wild-type plants, suggesting that WRKY54 and WRKY70 contribute to the SID2-independent expression of *SARD1* and *CBP60g* during pathogen infection (Chen et al., 2021). Additionally, *Arabidopsis snc2-1D* mutants were used, which carry a gain-of-function mutation in a receptor-like protein, leading to constitutively activated immune responses (Zhang et al., 2010). The requirement of WRKY54 and WRKY70 for *SARD1* and *CBP60g* expression in *snc2-1D*-mediated autoimmunity and during *Psm ES4326* infection suggest that WRKY54 and WRKY70 positively regulate *SARD1* and *CBP60g* expression (Chen et al., 2021). Chromatin immunoprecipitation (ChIP) analysis by Sun et al. (2015) revealed that many plant defence regulators including *WRKY70* are direct binding targets of *SARD1* and *CBP60g* (Sun et al., 2015). ChIP is a technique used to show direct interactions between proteins and DNA in the cell (Dasgupta and Chellappan, 2007). This suggests that *SARD1* and *CBP60g* function as master regulators of plant defence responses and that WRKY54/WRKY70 and SARD1/CBP60g form an amplification loop to promote each other's expression (Sun et al. 2015; Chen et al., 2021).

Beyond this, additional roles of *CBP60g* have been studied, including its positive and negative regulatory roles in various *Arabidopsis* defence-/stress-related responses. Earlier studies have shown that *CBP60g* overexpression caused elevated SA accumulation, increased expression of defense genes, and enhanced resistance to *Pseudomonas syringae* (Wan et al., 2012). In addition to enhanced defense responses, *CBP60g* overexpression lines showed hypersensitivity to the stress hormone abscisic acid (ABA) and enhanced drought stress tolerance (Wan et al., 2012). It was also found that ABA treatment and drought stress lead to a higher expression of the SA biosynthetic gene *ICS1* in *35S::CBP60g* plants (Wan et al., 2012). These results suggest that *CBP60g*
serves as a molecular link that positively regulates ABA- and SA-mediated pathways (Wan et al., 2012).

Expanding on its various roles in plant immunity, CBP60g can also act as a negative regulator under certain circumstances. In a study by Zou et al. (2017), they determined that CBP60g represses anthocyanin accumulation induced by drought, and sucrose or kinetin treatment (Zou et al., 2017). Anthocyanins are pigments produced by the flavonoid biosynthetic pathway and exist in most plants, giving colour to petals and fruits (Boss et al., 1996). It was revealed that the anthocyanin biosynthetic genes CHALCONE SYNTHASE (CHS), CHALCONE FLAVANONE ISOMERASE (CHI) and DIHYDROFLAVONOL 4 REDUCTASE (DFR), as well as genes encoding (ARABIDOPSIS THALIANA PRODUCTION OF ANTHOCYANIN PIGMENT 1) PAP1 (a MYB transcription factor) and TRANSPARENT TESTA 8 (TT8 (a basic helix-loop-helix (bHLH) transcription factor)), were downregulated by CBP60g (Zou et al., 2017).

Furthermore, an additional study analyzed the roles of CBP60g and SARD1 in terms of SAR (Zhang et al., 2010). SAR was negatively impacted to a lesser degree in cbp60g or sard1 single mutants, compared to the cbp60g sard1 double mutant (Zhang et al., 2010). Furthermore, in the wild-type Col-0, cbp60g sard1 mutants displayed increased susceptibility to P. syringae, and decreased levels of SA, expression of SA-related genes and NHP production (Wang et al., 2011; Chen et al., 2021). Interestingly, it was found that the signaling node defined by CBP60g and SARD1 lies in the SA sector downstream of the PAD4/EDS1 node, which affects expression of gene sets that overlap with the gene sets affected by the ETI-related NDR1 and SA biosynthetic enzyme PBS3 (Wang et al., 2011).

The role of Ca²⁺ signaling in relation to CBP60g/SARD1 was previously mentioned in terms of local PAMP-induced immune responses. However, the role of Ca²⁺ is less clear during late systemic signaling and the switch to SAR establishment (Guerra et al.,
2019). Regulatory calcium-binding proteins under the transcriptional control of SARD1/CBP60g may depend on temporally and spatially distinct intracellular calcium conditions compared to the initiating local calcium burst (Truman and Glazebrook, 2012; Aldon et al., 2018; Guerra et al. 2019). Interestingly, other Ca\(^{2+}\)/calmodulin-dependent transcriptional regulators CAMTA3 and CBP60a can negatively regulate long-term defense transcriptional reprogramming (Galon et al., 2008; Truman and Glazebrook, 2012; Sun et al., 2015). This implies that intracellular calcium level is also essential in SAR (Guerra et al., 2019). Additionally, in local basal resistance, the calcium-dependent protein kinase CPK5 functions upstream of SA synthesis, perception, and signaling, while it leads to systemic accumulation of the SAR-inducing metabolite NHP, and SAR marker genes (including SARD1) (Guerra et al., 2019). Without SARD1, high NHP concentrations are not sustained, thereby diminishing SAR (Guerra et al., 2019).

1.8 Plant Temperature Sensing Mechanisms

Thermosensing is the primary step when a thermosensor decodes the perceived stimulus into cellular signaling by altering its own structure and/or activity, or by interacting with other molecular components that lead to downstream responses (Zhu, 2016). In plants, various thermosensing mechanisms have been experimentally demonstrated but their direct link to plant immunity remains unclear (Castroverde and Dina, 2021).

Phytochromes (Phy) are photoreceptor proteins that control many physiological processes in plants; however, these light sensors may also be involved in thermal responses. In Arabidopsis, five phytochromes (PhyA-E) perceive red and far-red light (Hillman, 1967). Bright sunlight contains more red light compared to far-red light, and chlorophyll strongly absorbs red light, which allows plants to sense and respond to both the intensity and duration of natural light (Hillman, 1967). These light receptors are activated by light and a conformational change (photoconversion) takes place (Rockwell...
et al., 2006). Recent studies have found that certain plant photosensors may be temperature-sensitive, which allows them to respond appropriately to environmental changes. The best characterized of these is phytochrome B (phyB) (Jung et al., 2016; Legris et al., 2016).

Downstream of phyB receptors, multiple signaling pathways are integrated to regulate the activity of the transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) (Li et al., 2018). PIF4 plays a central role in mediating daytime plant growth under both normal and high temperatures (Li et al., 2018). PIF4 protein stability is regulated by red light-activated phyB which phosphorylates PIF4 leading to proteasome-mediated PIF4 degradation (Lorrain et al. 2008). When *Arabidopsis* is exposed to red light, phyB is converted from its inactive form P$_r$ to the active P$_{fr}$ state, which is a homodimer that translocates to the nucleus, blocking the activity of PIF4 and PIF7 (Hayes et al., 2020). Increased temperatures promote phyB reversion to its inactive state, allowing PIF4 and PIF7 to transcribe thermomorphogenesis (temperature-sensitive growth/development)-promoting genes (Hayes et al., 2020). However, phyB and PIFs do not contribute to temperature-modulated immunity and disease resistance in *Arabidopsis* plants (Huot et al., 2017; Kim et al., 2022).

Because plants naturally encounter different temperatures during the day and throughout the night, numerous genes are regulated by the plant circadian clock to ensure synchronization with changing light availability and temperature (Hayes et al., 2020). The circadian clock-associated evening complex (EC) is a transcriptional repressor that responds to temperature (Jung et al., 2020). It is made up of core components that are most highly expressed in the early evenings and is one of the key ways in which light and temperature are sensed by the plant’s internal clock (Ezer et al., 2017).

There are three components that make up the Evening Complex. The first is EARLY FLOWERING 3 (ELF3), which is a large scaffold protein and is key in temperature
sensing (Box et al., 2015; Ezer et al., 2017; Jung et al., 2020). Next is ELF4, which is a small α-helical protein whose function is unknown, and the third component is LUX ARRYTHMO (LUX), which is a DNA-binding protein necessary for recruiting the evening complex to transcriptional targets (Nusinow et al., 2011; Huang et al., 2016). The EC has various roles including conveying temperature information to the circadian clock, this is done through the temperature-dependent binding of the EC to DNA (Ezer et al., 2017; Silva et al., 2020). At elevated temperatures, the EC binds DNA less strongly than at cooler temperatures (Ezer et al., 2017). Besides regulation of the internal clock, the EC also represses the expression of thermomorphogenesis-promoting genes (including PIF4) to limit the amount of temperature-induced growth (Box et al., 2015). Interestingly, phytochrome activity (e.g. phyB) plays a role in regulating the EC function (Ezer et al., 2017; Huang et al., 2016). Therefore, temperature-sensitivity of EC DNA binding may be connected to increased thermal reversion of phyB (Legris et al., 2016; Klose et al., 2020). However, Kim et al. (2022) recently showed that thermosensing via the EC component ELF3 is not involved in SA-mediated plant immunity.

An additional way by which plants sense changes in temperature is through changes in the membrane fluidity (Knight et al., 1996; Königshofer et al., 2008). This can affect both the structure and activity of membrane-localized proteins, as some protein conformational changes are coupled to temperature-induced changes of their biochemical environment (Cournia et al. 2015). Heat can induce a rapid influx of calcium into the cell through the control of heat-sensitive membrane-associated calcium channels (Finka et al., 2012). Increased concentrations of Ca\(^{2+}\) lead to the induction of temperature-responsive gene expression, resulting in the heat stress response (Saidi et al., 2009). However, the mechanistic connections between membrane fluidity, Ca\(^{2+}\) signalling and temperature-regulated immune responses remain largely unexplored.
Finally, to prevent any damage from elevated temperatures and to maintain homeostasis, plants undergo the heat stress response through the expression of heat shock proteins (HSPs) (Hayes et al. 2020). One of the better characterized HSPs is HSP90, which promotes the stability of the auxin hormone receptor TIR1 which, in turn, will promote root and shoot elongation at elevated temperature (Wang et al., 2016). HSP expression in response to high temperatures is promoted by transcription factors called heat shock factors (HSFs) (Scharf et al. 2012). It remains to be seen how HSPs and HSFs contribute to temperature-sensitive immunity and disease resistance in plants.

1.9 Temperature Regulation of Plant Immunity

Recent research is emerging that climate change-associated elevated temperatures can mechanistically affect plant immune responses. Additionally, a changing environment can also change microbial populations and function (Cavicchioli, et al., 2019). The outcome of plant-pathogen interactions, or disease triangle is determined by the arms race between the plant's immune system and the pathogen's ability to survive the attempted defence, resulting in disease if the pathogen is successful, or in resistance if the plant immune system is successful, as well as the effect of the environment on the pathogen (Jones and Dangl, 2006). Plants experiencing different temperatures also have fundamentally different immune responses, sometimes resulting in loss of disease resistance (Cheng et al., 2013).

For every plant-pathogen interaction, there is an optimal temperature that will allow for disease development (Velásquez et al., 2018). Previous studies have shown that elevated temperature increases the susceptibility of A. thaliana plants to the bacterial pathogen P. syringae pv. tomato (Pst) DC3000 due to the increased translocation of bacterial effector proteins into plant cells and decreased biosynthesis of the defence hormone SA (Huot et al., 2017). Elevated temperature downregulates expression of the
SA biosynthetic gene *ICS1* (Huot et al., 2017), which is known to be directly controlled by the thermosensitive master transcription factors CBP60g and SARD1 (Kim et al., 2022).

It has been newly uncovered that suppression SA accumulation in *Arabidopsis* at 28°C occurs due to reduced formation of GUANYLATE BINDING PROTEIN-LIKE 3 (GBPL3) defence-activated biomolecular condensates (GDACs) at elevated temperature (Huang et al., 2021; Kim et al., 2022). Biomolecular condensates are compartments in eukaryotic cells that are involved in numerous processes, including RNA metabolism, the DNA damage response, and signal transduction (Banani et al., 2017). In plants, intranuclear GDACs are formed in response to defence signals during biotic stress (Huang et al., 2021). Recently, Kim et al., (2022) found that elevated temperature-mediated suppression of GBPL3 recruitment/binding does not occur at all GBPL3 target genes; however, elevated temperature suppresses GBPL3 recruitment to *CBP60g* and *SARD1*, which are crucial for SA biosynthesis and signaling.

Apart from suppressing host SA production, elevated temperatures can also affect earlier components of PTI and ETI (Alcázar and Parker, 2011; Cheng et al., 2019; Janda et al., 2019). In *Arabidopsis*, Cheng et al., 2013 showed that PTI is activated within a temperature interval of 23 °C–32 °C with the peak response occurring at 28 °C. To detect MAPK activity, 10-day-old seedlings were transferred to water overnight and then treated with flg22 or H$_2$O (Cheng et al., 2013). Following extraction, immunoblotting was performed using antibodies to detect phosphorylation status of MPK3 and MPK6 (Cheng et al., 2013). For the treatments done at different temperatures, the seedlings were pre-treated at their respective temperatures for 15 minutes before being treated with flg22 or H$_2$O (Cheng et al., 2013). The MAPK activation analysis showed that PTI responses are preferentially activated at elevated ambient temperatures (23–32°C) vs. lower temperatures (10–23°C), as the elevated temperature is more optimal for bacterial growth (Cheng et al., 2013).
In terms of ETI, the same study by Cheng et al. (2013) determined that bacterial effector-triggered responses are preferentially activated at low ambient temperatures but are suppressed at elevated ambient temperatures (Cheng et al., 2013). Strikingly, SA production downstream of both PTI and ETI activation is suppressed at warmer temperatures (Kim et al., 2022). Nonetheless, in certain exceptional cases with some dominant R genes, resistance may be maintained or enhanced at high temperatures (Venkatesh and Kang. 2019). The mechanism that allows for enhanced ETI at higher temperatures is not yet known, but there could be temperature-induced alternative R gene splicing (Chen, et al., 2018). Additionally, previous studies have shown ETI-associated hypersensitive response in Arabidopsis induced by the type III effectors avirulent resistance to P. syringae pv. tomato (AvrRpt2), avirulent resistance to P. syringae pv. maculicola (AvrRpm1), and avirulent resistance to P. syringae pv. tomato pisi (AvrRps4) is inhibited at elevated temperature compared to normal temperature (Goel et al., 2008; Freeman and Beattie, 2009; Wang et al., 2009; Cheng et al., 2013; Menna et al., 2015). Overall, higher temperatures broadly impact the plant immune system by targeting PTI, ETI and SA biosynthesis.

1.10 Rationale

Although SA and Pip/NHP have a close functional and regulatory relationship (Návarová et al., 2012; Chen et al., 2018; Hartmann et al., 2018; Hartmann and Zeier, 2018; Shields et al., 2022), it remains unexplored how Pip/NHP-mediated systemic immunity is affected by changing temperature conditions. In addition, previous research has exclusively characterized temperature regulation of PTI, ETI and SA pathways in local pathogen-infected tissues. Thus, there are major knowledge gaps in the relationship between SAR and Pip/NHP signaling pathways at varying temperatures, and on the impact of elevated temperature on plant systemic immunity. Overall, my MSc thesis
focused on these two major unanswered questions: (1) “Do changing temperature conditions impact the successful deployment of plant systemic immunity via SAR?” and (2) “If so, what molecular mechanisms underpin this temperature-regulation?”

1.11 Objectives and Hypotheses

Based on the rationale in the previous section, the overarching goal of my thesis was to investigate the temperature regulation of Pip/NHP-mediated plant systemic immunity. In detail, my M.Sc. thesis aimed to answer the following research questions:

(1) Does temperature affect SAR?; (2) Does temperature affect Pip/NHP biosynthesis?; (3) Does temperature affect Pip-induced immunity and/or Pip-induced signaling?; (4) Is temperature-regulated SAR governed by the thermosensitive master immune regulator CBP60g?

The first objective determined plant systemic resistance to bacteria following Pst pathogen treatment at elevated temperature. Numerous studies have characterized SAR showing a reduction of bacterial levels in systemic tissues at 23°C following an initial priming infection (compared to mock), but the effect of elevated temperature on SAR is not yet known. I hypothesized that any SAR-induced priming will be lost or reduced at 28°C, as elevated temperature has been shown to downregulate the local pathogen induced immune responses in plants (Huot et al., 2017; Cheng et al., 2013; Kim et al., 2022).

The second objective was to determine if temperature impacts Pip/NHP production. Previous studies in local leaves have shown that elevated temperature increases the susceptibility of Arabidopsis to Pst by promoting the translocation of bacterial effector proteins into plant cells, which suppress SA biosynthesis (Huot et al., 2017). Based on this, I hypothesized that these same effects will be occurring in systemic
leaves as well and that the Pip/NHP biosynthetic genes will be more highly induced in plants at 23°C versus 28°C.

The third objective aimed to explore the causation of both SAR and Pip/NHP downregulation at elevated temperatures by direct Pip supplementation to the plant. Based on previous studies showing that Pip treatment can induce SAR at normal temperatures (Návarová et al., 2012), I hypothesized that Pip treatment would reduce the systemic bacterial levels at 23°C (compared to mock), but this Pip-induced protection will be lost or reduced at 28°C. Additionally, a molecular approach was taken for this objective. Previous studies also showed that exogenous Pip/NHP application leads to systemic immunity by upregulating Pip-biosynthetic and response genes at normal temperature (Návarová et al., 2012). Based on this, I hypothesized that Pip treatment would induce systemic Pip/NHP gene expression at 23°C (compared to mock) but not at 28°C.

The fourth objective was aimed to answer what controls downregulation of Pip/NHP-mediated SAR at elevated temperatures. In this objective, I used 35S:CBP60g OE17 plants which constitutively express CBP60g. It has been shown that CBP60g (and its partially redundant homolog SARD1) is a master transcription factor in plant immunity, and controls pathogen-inducible SA and NHP biosynthesis (Sun et al., 2015; Hartmann and Zeier, 2018; Ding and Ding, 2020). Because CBP60g gene expression is warm temperature-downregulated (Kim et al., 2022), 35S:CBP60g plants with temperature-resilient CBP60g gene expression were used to determine the genetic and molecular mechanisms of temperature-regulated Pip/NHP biosynthesis and SAR signaling under different temperatures. Similar to what was observed for local immunity (Kim et al., 2022), I hypothesized that constitutive CBP60g over-expression would facilitate temperature resiliency in terms of SAR and Pip/NHP biosynthetic gene expression.
Ch 2. Materials and Methods

2.1 The Arabidopsis-Pseudomonas Model Pathosystem

*Arabidopsis thaliana* is a well-established model plant worldwide for numerous reasons (Wienkoop et al., 2010). *Arabidopsis* is a small plant with a generation time of approximately six weeks and grows well in laboratory conditions (Masson, 2001). It has a small nuclear genome of 125Mb, and numerous collections of T-DNA insertion lines have been generated and are available for research (Masson, 2001). There is a wide variety of resources available, such as the entire genome sequence, numerous natural variants, and molecular tools used for research (Wienkoop et al., 2010).

The bacterial pathogen *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 can cause disease in *Arabidopsis*, which contributes to its popular use in studying plant-pathogen interactions (Xin and He, 2013). In 1991, a *Pst* strain called DC3000 was found to infect both its natural host (tomato) and *Arabidopsis* in a laboratory setting (Xin and He, 2013). This key finding has led to the characterization of the molecular mechanisms by which this strain causes disease in plants. Notably, there are two main virulence systems by which *Pst* DC3000 causes disease: (1) the phytotoxin coronatine (COR) and (2) the type III secretion system, which will translocate bacterial effectors into the host plant cells (Xin and He, 2013). *Pst* DC3000 locally infects the leaves and fruits; under suitable conditions, aggressive multiplication can cause plant cells to die, and the infected tissue may show signs of necrosis (Hirano and Upper, 2000).

The use of *Pst* DC3000 only provides subtle results in terms of resistance, so an avirulent strain, known as *Pst* DC3000(AvrRpt2), is used to activate a stronger ETI response in *Arabidopsis* (Lim and Kunkel, 2004). AvrRpt2 is an effector protein, which activates resistance in *Arabidopsis* plants naturally harbouring the resistance protein RESISTANT TO *P. SYRINGAE* 2 (*RPS2*) (Lim and Kunkel. 2004). This gene encodes an
intracellular immune receptor that was first discovered based on its ability to trigger pathogen recognition in resistant host plants (Dong et al., 1991; Whalen et al., 1991).

2.2 Plant Material and Growth Conditions

Soil was prepared by mixing one-part Promix-PGX soil (Plant Products, Ancaster, Ontario), one-part Turface (Turface Athletics, Buffalo Grove, IL), and one-part Vermiculite Prolite (Therm-O-Rock, New Eagle, PA) with enough deionized water to completely moisten the soil. Each batch was autoclaved in a 30-minute liquid cycle, and cooled soil was packed down into individual pots (10cm x 10cm). A solution of approximately 100mL of distilled water and Miracle-Gro (The Scotts Company, Mississauga, ON) was poured over top (1L of distilled water with 4 grams of Miracle-Gro).

Three days before planting, Arabidopsis thaliana Col-0 and/or 35S::CBP60g (Wan et al., 2012) seeds were sterilized for 10 minutes in microcentrifuge tubes containing 500μL of 70% ethanol. After pipetting out the ethanol, 500μL of autoclaved MilliQ water were used to wash the seeds three times. Then, 500μL of sterile 0.1% agarose were pipetted into the microcentrifuge tube containing the seeds, and the tube was wrapped in aluminum foil and stratified at 4°C for three days.

Four seeds were sown (using a pipette) onto each corner of the previously prepared pots. Distilled water was sprayed on top of the soil, and the pots were placed on plastic flat trays and covered with a plastic dome to increase the humidity and placed in the growth chamber at 23°C. The growth chamber conditions used were 23°C with relative humidity (~60%) on a light cycle with 12 hours of light (80-100 μmol m⁻² s⁻¹) followed by 12 hours of dark. Plants were watered with distilled water ensuring the flats stayed moist, and they were watered with 0.5X nutrient water weekly (Table 1). Extra plants were grown to maturation to produce siliques for seed collection.
Hoagland’s solution (Hoagland and Arnon, 1950) was used weekly at 0.5X strength to provide additional nutrients for plant growth. Five separate bottles were prepared for the macronutrients, while the micronutrients were prepared by combining them in one bottle. Macronutrients and micronutrients were combined based on Table 1.

Table 1: Nutrient Water Components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Solution</th>
<th>mL Stock per 1 L</th>
<th>mL Stock per 20 L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2M Potassium nitrate, KNO₃</td>
<td>202 g/L</td>
<td>2.5</td>
<td>50</td>
</tr>
<tr>
<td>1M Potassium dihydrogen phosphate, KH₂PO₄ (pH to 6.0)</td>
<td>136 g/L</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>2M Calcium nitrate tetrahydrate, Ca(NO₃)₂•4H₂O</td>
<td>236 g/0.5 L</td>
<td>2.5</td>
<td>50</td>
</tr>
<tr>
<td>Iron(III)-EDTA or Iron chelate, Fe-EDTA or Fe-EDDHA</td>
<td>15 g/L</td>
<td>1.5</td>
<td>30</td>
</tr>
<tr>
<td>2M Magnesium sulfate heptahydrate, MgSO₄•7H₂O</td>
<td>493 g/L</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boric acid, H₃BO₃</td>
<td>2.86 g/L</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Manganese chloride tetrahydrate, MnCl₂•4H₂O</td>
<td>1.81 g/L</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Zinc sulfate heptahydrate, ZnSO₄•7H₂O</td>
<td>0.22 g/L</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Copper sulfate pentahydrate, CuSO₄•5H₂O</td>
<td>0.08 g/L</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Molybdic acid monohydrate, H₂MoO₄•H₂O OR</td>
<td>0.09 g/L</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Sodium molybdate dihydrate, Na₂MoO₄•2H₂O</td>
<td>0.12 g/L</td>
<td>1</td>
<td>20</td>
</tr>
</tbody>
</table>

2.3 Bacterial Culture and Inoculation for Disease Assay

The bacterial infiltration and disease assay was based on a previously published protocol (Huot et al., 2017). Twenty-four hours before primary inoculations, plants were domed to increase humidity, allowing the stomata to open. Plants were infiltrated with either Pst DC3000, Pst DC3000(AvrRpt2), or a mock treatment of 0.25 mM MgCl₂ using a needleless syringe. Pst was cultured through the growth of a stock plate by quadrant streaking from a glycerol stock. An individual colony was streak-plated; 24 hours later,
this was spread-plated and incubated at room temperature for an additional 24 hours before inoculation. *Pst* DC3000 was plated onto 100 µg/mL rifampicin-containing LM media (Table 2). This media was used to isolate for *Pst* DC3000 as this strain is naturally resistant to Rifampicin. *Pst* DC3000/AvrRpt2 was plated onto 100 µg/mL rifampicin-containing and 60 µg/mL spectinomycin-containing LM media (Table 2). These two antibiotics allow for the isolated growth of *Pst* DC3000/AvrRpt2 as this strain is a *Pst* DC3000 strain transformed with a plasmid vector to express AvrRpt2. That plasmid contains a spectinomycin resistance gene as a selection marker. The inoculum was prepared by collecting approximately a 1cm x 1cm area of *Pst* from the streak plate in an inoculating loop and vortexed vigorously in 10mL of MgCl₂. The absorbance was measured, and dilutions were done to prepare the desired concentration. Approximately 2–4 × 10⁷ CFU mL⁻¹ (absorbance OD₆₀₀ = 0.001 or 0.02) in 0.25 mM MgCl₂ of *Pst* DC3000 or AvrRpt2 was prepared and used as the inoculum. Plants were incubated at either 23°C (control temperature) or 28°C (elevated temperature). Tissues were harvested at 2 days post-inoculation (See 2.7 Tissue Harvest for Molecular Analysis) or inoculated a second time for analysis of systemic tissues (see 2.4 SAR priming Assays).

### 2.4 SAR Priming Assay

Two days after primary inoculation with mock (MgCl₂) or *Pst* DC3000 (absorbance at 600 nm = 0.02) or *Pst* DC3000 AvrRpt2 (absorbance at 600 nm = 0.02) in lower leaves, the plants were then inoculated with *Pst* DC3000 in upper, systemic leaves (Figure 6) using a needleless syringe. *Pst* DC3000 (absorbance at 600 nm = 0.001) in 0.25 mM MgCl₂ was prepared. Plants were infiltrated with *Pst* DC3000 and then incubated at 23°C and 28°C. Tissues were harvested at 3 days post-inoculation. Microcentrifuge tubes were prepared containing three 3mm zirconium oxide beads (MilliporeSigma, Burlington, MA) and 250 µL of sterile 0.25mM MgCl₂ buffer. A biopsy punch was used to excise two discs
per leaf (6 discs from 3 leaves total = 0.7536 cm²), and all discs from one plant were placed in a tube. Samples were homogenized in the Tissue Lyser II (Qiagen) at 30 beats per second for 1.5 minutes; then, the adapters were inverted, and homogenization was repeated. An additional 500 µL of sterile 0.25mM MgCl₂ buffer were added to each tube and then vortexed vigorously. Serial dilutions (10⁻¹ to 10⁻⁸ dilutions) for each sample were prepared using a 96-well plate and 10 µL of each dilution were plated onto 100 µg/mL rifampicin-containing LM media (Table 2). There were four biological replicates (individual plants) per treatment. Plates were left to dry and then incubated in an inverted position at room temperature (21°C - 23°C). Colonies were visible and counted 48 hours later.

Table 2: LM Media Components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount for 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ water</td>
<td>1L</td>
</tr>
<tr>
<td>Bacto-Tryptone</td>
<td>10g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>6g</td>
</tr>
<tr>
<td>K₂HPO₄ (Dipotassium phosphate)</td>
<td>1.2g</td>
</tr>
<tr>
<td>NaCl (Sodium Chloride)</td>
<td>0.6g</td>
</tr>
<tr>
<td>MgSO₄ • 7H₂O (magnesium sulfate heptahydrate)</td>
<td>0.4g</td>
</tr>
<tr>
<td>Agar</td>
<td>15g</td>
</tr>
</tbody>
</table>
Figure 6: Schematic diagram of *Arabidopsis* showing local and systemic leaves.

The priming treatment is applied to the lower (local) leaves, and the secondary infiltration is applied to the upper (systemic) leaves. Created with Biorender.com.
2.5 Leaf-based Pipecolic Acid Infiltration

For primary inoculations, plants were domed 24 hours prior to increase humidity, allowing the stomata to open. Plants were then infiltrated with either 1 mM L-Pipecolic Acid (Pip; Sigma Aldrich, St. Louis MO) or autoclaved MilliQ water (mock treatment). Five to six leaves of intermediate growth and seemingly good health per plant were labeled at the petiole using a Sharpie. The needleless syringe was filled with the prepared solution and pressed firmly against the underside (abaxial) of the leaf. The solution was gently infiltrated into the leaf until it was evenly distributed throughout the leaf blade. Plants were returned to the growth chamber at their designated temperature (23°C or 28°C) for tissue collection or further treatments. Tissues for molecular analysis were collected according to the method described in Section 2.7.

2.6 Root-based Pipecolic Acid Application

Pip was also supplied in plants by pipetting 1 mL of 1mM Pip onto the base of the plant for root uptake. Similarly, as above, plants were returned to the growth chamber at their designated temperature (23°C or 28°C) for tissue collection or further treatments. Tissues for molecular analysis were collected according to the method described in Section 2.7.

2.7 Tissue Harvest for Molecular Analysis

Forceps were used to harvest three leaves from each plant (minimal petiole was collected), which were then placed into 2-mL impact resistant tubes (USA Scientific, Ocala, FL), each containing three 3mm zirconium oxide beads (MilliporeSigma, Burlington, MA). Plant tissues inside the tubes were flash-frozen immediately with liquid nitrogen. The flash-frozen plant tissues were stored at -80°C until further analyses.
2.8 Pip Priming Assay

Two days after mock (autoclaved MilliQ water) or 1mM Pip treatments via the leaves or roots and incubation at either 23°C or 28°C, the plants were inoculated with *Pst* DC3000 in upper, systemic leaves using a needleless syringe. *Pst* DC3000 (absorbance at 600 nm = 0.001) in 0.25 mM MgCl₂ was prepared. Plants were infiltrated with the *Pst* DC3000 preparation in the same leaves that were initially treated with Pip (after primary Pip treatment via the leaves) or in three intermediate leaves of seemingly good health (after primary Pip treatment via the roots), or in systemic leaves (after primary Pip treatment via the leaves). Tissues were harvested at 3 days post-inoculation. Bacterial quantification was performed as previously detailed in Section 2.4 (SAR Priming Assay).

2.9 RNA Extraction

Before RNA extraction (a minimum of two hours), TissueLyser adapters were pre-cooled in the -80°C freezer. RNA extraction was done using the Qiagen Plant RNeasy Mini Kit (Qiagen, Toronto, ON). The extraction buffer was freshly prepared in the fume hood by combining 10 µL beta-mercaptoethanol (β-ME) per 1 mL Qiagen Buffer RLT. The frozen samples were placed and balanced into the TissueLyser adapters, and these were homogenized at 25 beats/s for a minute. The adapters were inverted, and the homogenization was repeated. Afterwards, 450 µL of extraction buffer were added to each sample, then vortexed and spun for 15 seconds. The lysate was transferred to the labelled QIAshredder Spin Column inside a microcentrifuge tube and centrifuged for two minutes at maximum speed. Around 400 µL of flowthrough supernatant were collected and pipetted into a microcentrifuge tube. This was followed by addition of 200 µL of 95% ethanol and mixing by pipetting. The contents of this tube were then transferred to an RNeasy Spin Column placed inside a collection tube. This was centrifuged for 15 sec at
10,000 rpm. The flowthrough was discarded, and the column was repositioned into the tube. Subsequently, 350 µL Buffer RW1 were added, and the tube was centrifuged for 15 sec at 10,000 rpm. The flowthrough was once again discarded, and the column was repositioned into the tube. A previously prepared DNase I incubation mix (Kunitz units) was pipetted directly to the membrane of the RNeasy Spin Column and incubated for 15 minutes at room temperature. After the incubation period, 350 µL of Buffer RW1 were added to column, and then centrifuged for 15 sec at 10,000 rpm. The flowthrough was discarded, and the column was repositioned into the tube. Afterwards, 500 µL of Buffer RPE were added to the RNeasy Spin Column, and this was centrifuged for 15 sec at 10,000 rpm. The flowthrough was discarded, and the column was repositioned into the tube. Another 500 µL of Buffer RPE were added to the RNeasy Spin Column, which was centrifuged for 2 min at 10,000 rpm. The flowthrough was discarded, and the column was repositioned into a new collection tube. Then the RNA extracts were centrifuged at maximum speed for 1 min. The column was transferred to a new microcentrifuge tube and 50 µL RNase-free water was added directly to the spin column membrane. After a 10-minute incubation, it was centrifuged for 1 min at 10,000 rpm to elute the RNA. The eluted RNA was pipetted directly onto the spin column again and left to incubate for an additional 10 minutes. This was centrifuged for 1 min at 10,000 rpm to elute the final RNA extracts. The RNA was diluted to 30 ng/µL (following measuring the initial concentration using a DeNovix spectrophotometer (DeNovix, Wilmington, DE)). RNA quality was also determined by measuring the A260/A280 and A260/A230 ratios. Both the undiluted and diluted extracts were labeled and stored at -80°C.

2.10 cDNA Synthesis

cDNA synthesis was performed with diluted RNA samples (30 ng/µL) using qScript™ cDNA SuperMix (QuantaBio, Beverly, MA). Two sets of tubes were prepared
for each set of samples, one using qScript™ cDNA SuperMix (+RT; with cDNA synthesis) and one with RNase free water in its place (-RT; without cDNA synthesis). The samples with water were used as a control for genomic DNA contamination. For each reaction, 4 µL of the qScript™ cDNA SuperMix and 6 µL of RNase free water were prepared as a master mix (+RT tubes). Ten µL of the prepared master mix were added to a 0.2 mL PCR tube with 10 µL of the previously prepared diluted RNA. Additionally, 10 µL of RNase free water were added to a second set of 0.2 mL PCR tubes and 10 µL of diluted template RNA were added (-RT tubes). These samples were vortexed gently to mix the components and then centrifuged briefly. The reactions were placed into the thermocycler at 25ºC for 5 minutes followed by 30 minutes at 42ºC. The temperature was then raised to 85ºC for 5 minutes to inactivate the reaction, then held at 4ºC until the tubes were removed and stored at -20ºC. For further analysis of cDNA by qPCR, samples were diluted to 1/20th (95 µL of RNase free water with 5 µL cDNA) based on a previously established procedure (Huot et al., 2017).

2.11 qPCR Analyses

The qPCR protocol is based on the procedure from Huot et al. (2017) with certain modifications. A master mix was prepared containing 5 µL of PowerTrack SYBR Green master mix (Life Technologies), 0.25 µL of 10 µM forward primer, 0.25 µL of 10 µM reverse primer and 2.5 µL nuclease-free water for each individual reaction. Eight µL of the master mix was combined with 2 µL of template cDNA (~1.5ng total cDNA). The plate was sealed and briefly spun down before running using the ΔΔC_T Method in Standard Mode with the PCR conditions outlined in Table 3. The resulting qPCR solutions were assayed using the Applied Biosystems QuantStudio3 platform (Life Technologies), and individual C_T values were determined for both the target genes (ALD1, FMO1, PR1, etc.) and the well characterized internal control gene (PP2AA3). Transcripts of interest had
their expression normalized using the equation $2^{-\Delta CT}$, where $\Delta CT$ is $CT$ target gene$-CT$ PP2AA3.

qPCR was carried out with three technical replicates for each biological replicate, and with each treatment having four biological replicates (individual plants). The primers used for qPCR are shown in Table 4.

**Table 3: qPCR Standard Mode Conditions and Stages used for the $\Delta\Delta CT$ Method.**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp (°C)</th>
<th>Time (sec)</th>
<th># Reps</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>15</td>
<td>40</td>
</tr>
<tr>
<td>3 (dissociation stage)</td>
<td>60</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>15</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4: List of qPCR Primers.** The Table includes gene names, the lab primer code, the sequence of the primer, length, annealing temperature, and the expected qPCR product size (Amp in bp).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Position (F/R)</th>
<th>Lab Primer Code</th>
<th>Sequence (5'-3')</th>
<th>Length</th>
<th>Tm °C</th>
<th>Amp</th>
<th>Source</th>
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<td>ALD1_qRT_F</td>
<td>BH114</td>
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<td>19</td>
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<td>61</td>
<td>Huot et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>ALD1_qRT_R</td>
<td>BH115</td>
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<td>CBP60g</td>
<td>CBP60g_qRT_F</td>
<td>BH116</td>
<td>TCGTTGGACGCCAACAACAAACA</td>
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<td>61.9</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBP60g_qRT_R</td>
<td>BH117</td>
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<tr>
<td>FMO1</td>
<td>FMO1_qRT_F</td>
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<td></td>
<td>FMO1_qRT_R</td>
<td>BH113</td>
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</tr>
<tr>
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<td>sid2_qRT_F2</td>
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<td>57.3</td>
<td>94</td>
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</tr>
<tr>
<td></td>
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<td>BH6</td>
<td>ACACAAACATTCGTACATATACG</td>
<td>25</td>
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<td></td>
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<tr>
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<td>117</td>
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</tr>
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<tr>
<td>PP2AA3</td>
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<td>82</td>
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<tr>
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<tr>
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<td>BH3</td>
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</table>
2.12 Conventional PCR Amplification

PCR was performed by combining the Taq FroggaBio Mix (Froggabio, North York, ON), (contains 0.25 U/μL Taq DNA polymerase, 2X PCR buffer, 0.4 mM dNTPs, 3.2 mM MgCl₂, 0.02% bromophenol blue), with the forward and reverse primers (10 μM) to amplify the gene of interest (Table 5), and the template cDNA for the samples of interest. All the components were thawed on ice and gently mixed prior to the preparation of the mix. A master mix was prepared using the Taq mix, the primers, and ultra-pure water, 18μL were aliquoted into 200μL PCR tubes and 2μL of template DNA or cDNA were added. PCR was performed using the standard conditions (Table 6) with 30 cycles at the annealing temperature which was dependent on each set of primers. The ACT1 housekeeping gene served as the internal control gene when measuring expression of different genes of interest.
Table 5: List of PCR Primers. The Table includes gene names, the personal primer code, the sequence of the primer, length, annealing temperature, and the expected PCR product size (Amp in bp).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Position</th>
<th>Personal Primer Code</th>
<th>Sequence (5' → 3')</th>
<th>Length</th>
<th>Tm °C</th>
<th>Amp</th>
<th>Source</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>IDT50</td>
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<tr>
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<td>IDT55</td>
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<td>22</td>
<td>51.1</td>
<td>227</td>
<td>Danve Castroverde, Wilfrid Laurier University</td>
</tr>
<tr>
<td></td>
<td>ALD1_RT_R2</td>
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<tr>
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<td>52.9</td>
<td>167</td>
<td>Bethany Huot, Michigan State University</td>
</tr>
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<td></td>
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<td>BH4</td>
<td>TCTCGTTCACATAATTCCAC</td>
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<td></td>
</tr>
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<td>GCCTGGAATGTCTGATAAGTG</td>
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<td>61.7</td>
<td>291</td>
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<tr>
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<td>IDT13</td>
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Table 6: Conditions used for PCR amplification. The annealing temperature was variable and determined based on the primer pair being used (see Table 5).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th># of Cycles</th>
</tr>
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<td>3 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 seconds</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>Variable (based on primer sequences)</td>
<td>30 seconds</td>
<td>25-35</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>

2.13 Gel Electrophoresis

To make a 1% agarose gel, 0.5g of agarose was added to 50mL of 1X Tris-Acetate-EDTA (TAE) buffer and swirled to mix in a flask. A Kimwipe (Kim Technologies,
Bridgewater, NJ) was used to cover the mouth of the flask, and the agarose was dissolved in the TAE buffer by heating in the microwave oven for approximately 45 seconds. The agarose was completely dissolved when the solution was completely clear, and no shiny flecks were left. While the agarose/ TAE solution cooled, the gel mold was set up with the appropriate combs and stoppers. Once the solution cooled to room temperature, 2.5µL of RedSafe dye (Froggabio, North York, ON) was added and mixed in by swirling. The solution was poured into the previously prepared mold and allowed to solidify for about 20 or 25 minutes. Once the gel was solidified, the stoppers and combs were removed, and the gel tray was transferred into the running tank filled with 1X TAE buffer. As reference, 5 µL of the DNA ladder (GeneDireX, Taoyuan, Taiwan) was loaded onto the first lane of any row where samples were loaded. Subsequently, 10 µl of each sample were loaded according to a predetermined template, and the cover will be repositioned on the tank. Samples were electrophoresed at 100V until they reached about ¾ of the way to the bottom of the gel. The samples moved towards the positive (red) electrode, and away from the negative (black) electrode.

2.14 Visualization of PCR and RT-PCR Bands

Gel bands were visualized with the VersaDoc (Bio-Rad, Mississauga, ON) equipped with Quantity One software (Bio-Rad, Mississauga, ON). To view the images, the gel was centered in the lens area, and the lens was cleaned previously with a Kimwipe (Kim Technologies, Bridgewater, NJ) and 70% ethanol. Imaging was done by selecting the option for Radiant Red nucleic acid stain. Exposure was adjusted until a clear image was obtained and image files were exported to a USB drive.
2.15 Calculations and Statistics

Multiple treatments and two temperatures were compared in my thesis. Previous studies have shown that Log-transformed bacterial numbers follow normal distribution (Huot et al., 2017; Kim et al., 2022). For all gene expression experiments, homoscedasticity plots and QQ plots were examined to confirm homology of variance and normal distribution of data. After meeting these statistical criteria, gene expression values and bacterial numbers were analyzed with a two-way ANOVA followed by Tukey’s HSD (honestly significant difference) test at 95% confidence interval with an alpha threshold of 0.05. Four biological replicates (individual plants) were sampled per treatment per temperature in all experiments, unless otherwise specified. Experiments were performed independently at least twice to ensure reproducibility. Exceptions to this, and number of replicates are shown in Table 7.
Table 7: Summary of Experiments. Summary of all experiments included in this thesis outlining the corresponding Figure panels, a brief description, the priming treatment and the number of experimental replicates; each experimental replicate consists of 4 biological replicates.

<table>
<thead>
<tr>
<th>Figure Number</th>
<th>Panel</th>
<th>Description</th>
<th>Priming treatment/Treatment</th>
<th>Experimental Replicates</th>
<th>Experiment ID(s)</th>
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<td>0.001 Pst DC3000</td>
<td>3</td>
<td>SAR 1, 2, 3</td>
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<tr>
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<td>C</td>
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<td>0.02 Pst DC3000</td>
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<td>SAR 4, 6, 7, 8</td>
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<tr>
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<td>E</td>
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<td>SAR 9, 11, 12</td>
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<td>DC1</td>
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<td>E-F</td>
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<td>C</td>
<td></td>
<td>Pip – Root Application</td>
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<tr>
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<td>E</td>
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<td>Pip – Local</td>
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<tr>
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<td>C-D</td>
<td></td>
<td>Pip – Systemic</td>
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<td>PIP4</td>
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<tr>
<td>11</td>
<td>A-B</td>
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<td>Pip – Local</td>
<td>3</td>
<td>PIP5, 7, 9</td>
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<tr>
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<td>C-D</td>
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<td>Pip – Systemic</td>
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<td>PIP4, 6, 8</td>
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<tr>
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<tr>
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</tr>
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<tr>
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<tr>
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<td>F</td>
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<tr>
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<tr>
<td></td>
<td>C</td>
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<td>0.02 Pst DC3000</td>
<td>3</td>
<td>DC11, 12, 13</td>
</tr>
<tr>
<td>16</td>
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<td>C-D</td>
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<td>B</td>
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<td>0.02 Pst DC3000/AvrRpt2</td>
<td>3</td>
<td>DC9, 10, 14</td>
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</tbody>
</table>

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Ch 3. Results

3.1 Impact of temperature on systemic acquired resistance in Arabidopsis

Previous studies have shown that local resistance and pathogen-induced expression of various defence-related genes at the primary (local) site of infection are downregulated at elevated temperature (Huot et al., 2017; Kim et al., 2022). However, it remained unclear how uninfected tissues distal from the site of infection (i.e. systemic acquired resistance and systemic immune gene expression) were affected by elevated temperatures. To investigate this, SAR assays were conducted using four-week-old Arabidopsis Col-0 plants (Appendix, Figure 24). As shown in Figure 7A, local priming with virulent Pst DC3000 (absorbance at 600nm = 0.001) led to no significant decrease in bacterial CFUs in systemic leaves after a secondary infection. I observed no decrease in systemic bacterial levels after the initial infection in the plants acclimated to 23°C (p=0.843). In the plants acclimated to 28°C, systemic bacterial growth was also not affected in the Pst DC3000-primed plants compared to the mock-treated plants(p=0.203). The resulting symptoms as shown in Figure 7B revealed that elevated temperature led to more severe disease symptoms. This initial result indicated that OD$_{600}$ of 0.001 Pst DC3000 was insufficient to elicit significant SAR at both temperatures.

Since a lower concentration of Pst DC3000 suspension was not sufficient to elicit SAR under our lab conditions, I then tested local priming with a higher concentration of virulent Pst DC3000 (absorbance at 600nm = 0.02). As shown in Figure 7C, initial infection led to a significant decrease in bacterial CFUs by 5.3-fold following the priming treatment, showing effective SAR at 23°C (p=0.001). In the plants grown at 28°C, there was a loss of systemic protection since bacterial levels were similar with or without initial Pst DC3000 priming (p=0.983); this was reflected by a 1.2-fold decrease in bacterial CFUs between the mock primed and Pst primed samples. In agreement, Figure 7D shows more chlorotic systemic leaves without priming treatment (mock solution; 0.25mM MgCl$_2$),
compared to the plants with a *Pst* priming treatment. In addition, elevated temperature amplified the symptoms in general, although there are slightly less severe symptoms in the *Pst* DC3000-primed leaves based on qualitative visual inspection. These findings show that SAR protection is subtle but significant at 23°C using a higher concentration of virulent *Pst* DC3000 to induce SAR, but at 28°C this protection was lost.

In addition to virulent *Pst* DC3000 priming, SAR can also be induced (in a stronger manner) by ETI-activating avirulent pathogens like *Pst* DC3000/AvrRpt2 (Mur et al., 2008). SAR at 23°C and 28°C was also tested after local priming with this strain (absorbance at 600nm = 0.02). As shown in Figure 7E, I observed a 2.6-fold decrease in bacterial CFUs in the *Pst* DC3000/AvrRpt2-primed plants acclimated to 23°C (p=0.019). At 28°C, there was no protection following an initial infection (p=0.084). Figure 7F shows slight chlorosis in the unprimed (mock) plants at 23°C, and this was amplified in the unprimed plants at 28°C. *Pst* DC3000/AvrRpt2-primed plants showed no systemic leaf symptoms at 23°C, but they were slightly chlorotic at 28°C. Experiments with avirulent *Pst* DC3000/AvrRpt2 revealed that SAR is effective at 23°C, but systemic protection is decreased significantly at 28°C.

Overall, SAR experiments after priming with both virulent and avirulent (ETI-activating) bacterial pathogen strains suggest that elevated temperature (28°C) negatively impacts SAR.
Figure 7. *Arabidopsis* systemic acquired resistance phenotypes at normal and elevated temperatures. Four-week-old *Arabidopsis* Col-0 plants were infiltrated in five-six lower leaves with 0.25mM MgCl$_2$ mock solution (A-F) or *Pst* bacterial suspension (DC3000 OD$_{600}$=0.001 in A-B, DC3000 OD$_{600}$=0.02 in C-D, DC3000/AvrRpt2 OD$_{600}$=0.02 in E-F.)
in E-F). Plants were then incubated at either 23°C or 28°C. Two days later, upper systemic leaves in both mock- and *Pst*-treated plants were infiltrated with *Pst* DC3000 suspension (OD$_{600}=0.001$) and placed back at their respective temperature. Three days after systemic infiltration (3 dpi), bacterial levels and leaf symptoms were evaluated as detailed in the Materials and Methods.

A. Systemic *Pst* DC3000 bacterial levels in log$_{10}$[CFU(cm$^{-2}$)] at 3 dpi after local priming with *Pst* DC3000 OD$_{600}=0.001$ at 23°C or 28°C.

B. Resulting symptoms at 3 dpi after local priming treatment with *Pst* DC3000 OD$_{600}=0.001$ at 23°C or 28°C.

C. Systemic *Pst* DC3000 bacterial levels in log$_{10}$[CFU(cm$^{-2}$)] at 3 dpi after local priming with *Pst* DC3000 OD$_{600}=0.02$ at 23°C or 28°C.

D. Resulting symptoms at 3 dpi after local priming treatment with *Pst* DC3000 OD$_{600}=0.02$ at 23°C or 28°C.

E. Systemic *Pst* DC3000 bacterial levels in log$_{10}$[CFU(cm$^{-2}$)] at 3 dpi after local priming with *Pst* DC3000/AvrRpt2 OD$_{600}=0.02$ at 23°C or 28°C.

F. Resulting symptoms at 3 dpi after local priming treatment with *Pst* DC3000/AvrRpt2 OD$_{600}=0.02$ at 23°C or 28°C.

Results shown in A, C, and E are the means ± S.D. (A, n=12 from 3 independent experiments; C, n=16 from 4 independent experiments; E, n=12 from 3 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test ($p < 0.05$). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 8.
3.2 Impact of temperature on SAR-associated NHP biosynthesis in Arabidopsis

Having observed that elevated temperature negatively impacts SAR, it was important to understand the molecular mechanisms underlying this heat-mediated SAR suppression. SAR is mediated by the plant immunity metabolites Pip and its N-hydroxylated form NHP (Návarová et al., 2012; Hartmann et al., 2018). Based on this, I investigated whether elevated temperature negatively regulates the NHP pathway by quantifying NHP biosynthetic gene expression. Gene expression analyses were conducted using systemic tissues from four-week-old Arabidopsis Col-0 plants (Appendix, Figure 25). As shown in Figure 8A and B, local infection with virulent Pst DC3000 (absorbance at 600nm = 0.001) led to no increase in ALD1, and no increase in FMO1 expression in systemic leaves at 23°C compared to the mock treated plants; (ALD1, p=0.236; FMO1, p=0.488). At 28°C, both ALD1 and FMO1 expression after local priming with Pst DC3000 were comparable to those after mock treatment (ALD1, p=>0.999; FMO1, p=>0.999). These findings show that local priming with virulent Pst DC3000 (absorbance at 600nm = 0.001) leads to no change in expression of SAR associated NHP biosynthetic genes at normal temperature, but these genes are downregulated at elevated temperature. Results were consistent with conventional gel-based PCR analyses (Appendix Figure 20).

Based on the trends observed in Figure 8A and B, I next used a higher priming concentration of virulent Pst DC3000 (absorbance at 600nm = 0.02) to hopefully induce NHP biosynthetic gene expression in the systemic tissue. As shown in Figure 8C, ALD1 expression in systemic tissue following a local infection led to no change at 23°C (p=0.482). At 28°C, ALD1 expression was extremely low in both treatment types (p=0.999). As shown in Figure 8D, FMO1 expression in systemic tissues increased 4.7-fold in the Pst DC3000-primed plants compared to the mock-infiltrated samples at 23°C.
(\(p=0.007\)). Consistent with my previous experiments, \(FMO1\) expression at 28°C was downregulated (\(p=0.999\)) (Appendix, Figure 26). This data shows that a treatment with a higher concentration of virulent \(Pst\) DC3000 (absorbance at 600nm = 0.02) surprisingly leads to no induction of \(ALD1\) consistent with the lower concentration of priming treatment but leads to increased expression of \(FMO1\) at 23°C. Consistently the expression of both genes of interest are downregulated by elevated temperature.

To further determine the effects of elevated temperature on systemic NHP-biosynthetic gene expression, analyses were done following local infection of ETI-activating avirulent pathogen \(Pst\) DC3000/AvrRpt2 (absorbance at 600nm = 0.02). Figure 8E and D show that this treatment led to a 4.4-fold increase in systemic \(ALD1\) and a 3.9-fold increase in systemic \(FMO1\) gene expression after local \(Pst\) DC3000/AvrRpt2 infection compared to the mock-infiltrated plants at 23°C (\(ALD1\), \(p<=0.0001\); \(FMO1\), \(p=0.039\)). At elevated temperature, systemic \(ALD1\) gene expression was lost in both systemic leaves following mock or \(Pst\) DC3000/AvrRpt2 treatments. However, \(FMO1\) expression systemically was slightly increased in the mock-treated plants but was completely downregulated after local pathogen priming at elevated temperature (Appendix, Figure 26).

Overall, since \(ALD1\) and \(FMO1\) are both critical in the biosynthesis of the SAR metabolites Pip and NHP, my results indicate that the systemic Pip/NHP biosynthetic pathway is targeted and downregulated by elevated temperature. This is consistent with the reanalysis of the SAR-regulated transcriptome which also shows downregulation of SAR at elevated temperature (Appendix, Figure 22).
Figure 8. Gene expression analysis of NHP biosynthetic genes by qRT-PCR of Arabidopsis systemic tissues following mock or pathogen (Pst DC3000) treatment at 23°C or 28°C. Four-week-old Arabidopsis Col-0 plants were infiltrated in five-six lower
leaves with 0.25mM MgCl2 (mock) or Pst bacterial suspension (DC3000 OD600=0.001 in A-B, DC3000 OD600=0.02 in C-D, DC3000/AvrRpt2 OD600=0.02 in E-F). Plants were then incubated at either 23°C or 28°C. Two days later, upper systemic leaves in both mock- and Pst-treated plants were harvested for gene expression analysis as detailed in the Materials and Methods.

A. Systemic ALD1 gene expression 2 dpi after local priming with Pst DC3000 OD600=0.001 at 23°C or 28°C.
B. Systemic FMO1 gene expression 2 dpi after local priming with Pst DC3000 OD600=0.001 at 23°C or 28°C.
C. Systemic ALD1 gene expression 2 dpi after local priming with Pst DC3000 OD600=0.02 at 23°C or 28°C.
D. Systemic FMO1 gene expression 2 dpi after local priming with Pst DC3000 OD600=0.02 at 23°C or 28°C.
E. Systemic ALD1 gene expression 2 dpi after local priming with Pst DC3000/AvrRpt2 OD600=0.02 at 23°C or 28°C.
F. Systemic FMO1 gene expression 2 dpi after local priming with Pst DC3000/AvrRpt2 OD600=0.02 at 23°C or 28°C.

Results shown in A-E are the means ± S.D. (A and B, n=4 from 1 independent experiment; C and D, n=12 from 3 independent experiments; E and F, n=8 from 2 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test (p < 0.05). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 9.
3.3 Impact of temperature on Pip/NHP-induced immunity in Arabidopsis

After determining that expression of Pip/NHP biosynthetic genes ALD1 and FMO1 are downregulated at elevated temperature, I next used a physiological approach to determine the impact of exogenous Pip priming at both normal and elevated temperature. Based on previous studies (Návarová et al., 2012), Pip treatment prior to Pst DC3000 exposure at 23°C led to higher disease resistance. However, it remained unknown if this Pip-mediated protection persisted at elevated temperature.

To investigate this, Pip-protection assays were conducted using four-week-old Arabidopsis Col-0 plants (Appendix, Figure 26). In these experiments, leaves were infiltrated with either mock (water) or Pip priming treatment, which was followed by Pst DC3000 infection in the same leaves. As shown in Figure 9A, Pip application at 23°C and 28°C led to a statistically significant decrease in bacterial CFUs compared to the mock treatment; at 23°C there was a 3.3-fold change, and at 28°C there was a 3-fold change ($p<0.0001$; $p<0.0001$). As shown in Figure 9B, symptoms were mild in both the mock- and Pip-infiltred leaves at 23°C. At 28°C, the Pip treatment prior to Pst DC3000 infection seems to have slightly reduced chlorosis compared to the mock treatment at this temperature (based on qualitative observations). These findings show that a local Pip priming via infiltration through stomata restores protection in the same tissues at both 23°C and 28°C.

Previous studies have shown successful protection following Pip application via the roots at normal temperature (Návarová et al., 2012). Figure 9C shows a 7.1-fold decrease in leaf bacterial CFUs at normal temperature, and a 3.8-fold decrease at elevated temperature after Pip application compared to mock root drench ($p=0.0002$; $p=0.0161$). This shows that Pip priming via the roots restores systemic disease protection at both 23°C and 28°C.
Finally, I determined the impact on systemic tissues (untreated, upper leaves) following local Pip priming via syringe infiltration. Figure 9D shows that there was no decrease in systemic bacterial CFUs at either temperature; at 23°C there was a 2.0-fold decrease, and at 28°C there was a 3.3-fold decrease after Pip application compared to mock \( (p=0.6001; p=0.1506) \). As shown in Figure 9E, at 23°C symptoms were mild in both the mock- and Pip-infiltrated leaves, but the leaves treated with Pip are overall less symptomatic. At 28°C, both the mock- and Pip-primed leaves were very chlorotic and their symptoms are exaggerated. These findings show that local Pip priming via the stomata cannot provide significant systemic protection at both 23°C and 28°C under our laboratory conditions.

Altogether, both local and root applications of Pip lead to disease protection following \( Pst \) DC3000 infection at both normal and elevated temperatures. However, Pip treatment in the local tissue cannot induce future disease resistance in the systemic tissue.
Figure 9. Bacterial growth assay in *Arabidopsis* systemic tissues following mock or Pip treatment at 23°C or 28°C. Four-week-old *Arabidopsis* Col-0 plants were infiltrated in five lower leaves with water (mock) or 1mM Pip solution (A-B, D-E), or treated via the roots (C). Plants were then incubated at either 23°C or 28°C. Two days later, the
same leaves (A-B), or the upper systemic leaves (C-E) in both mock- and Pip-treated plants were infiltrated with *Pst* DC3000 suspension (OD$_{600}$=0.001) and placed back at their respective temperature. Three days after systemic infiltration (3 dpi), bacterial levels and leaf symptoms were evaluated as detailed in the Materials and Methods.

A. Local *Pst* DC3000 bacterial levels in log$_{10}$[CFU(cm$^{-2}$)] at 3 dpi after local priming with 1mM Pip at 23°C or 28°C.

B. Resulting symptoms in local leaves at 3 dpi after local priming treatment with 1mM Pip at 23°C or 28°C.

C. Leaf *Pst* DC3000 bacterial levels in log$_{10}$[CFU(cm$^{-2}$)] at 3 dpi after root drench priming with 1mM Pip at 23°C or 28°C.

D. Systemic *Pst* DC3000 bacterial levels in log$_{10}$[CFU(cm$^{-2}$)] at 3 dpi after local priming with 1mM Pip at 23°C or 28°C.

E. Resulting symptoms in systemic leaves at 3 dpi after local priming treatment with 1mM Pip at 23°C or 28°C.

Results shown in A, C, and E, are the means ± S.D. (A, n=12 from 3 independent experiments; C, n=16 from 4 independent experiments; D, n=8 from 2 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test ($p < 0.05$). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 10.
3.4 Impact of temperature on Pip/NHP-induced signaling in Arabidopsis

Additional experiments were performed to gain molecular insight to the effects of exogenous Pip treatment and further understand how increased temperatures regulate Pip/NHP-induced gene expression. To explore this, gene expression analyses were conducted using both local and systemic tissues from four-week-old Arabidopsis Col-0 plants (Appendix, Figure 26). Here PR1 (PATHOGENESIS RELATED 1) and PCR1 (PLANT CADMIUM RESISTANCE 1) gene expression were measured, as both are SAR marker genes and their expression is upregulated systemically following pathogen infection (Bernsdorff et al., 2016). Here, PR1 and PCR1 were measured to gain an understanding of Pip/NHP signalling and response under different temperatures. As shown in Figure 10, local (A and B) and systemic (C and D) tissues after Pip priming via syringe-infiltration were analyzed in terms of increased PR1 and PCR1 expression. Figures 10A and C show that local and systemic PR1 expression was not affected in Pip-primed samples at 23°C ($p=0.4883; p=0.3143$). However, both local and systemic PR1 expression was very low at 28°C ($p=0.9746; p=0.9998$). At 28°C, in the local tissue there was a 7.8-fold change and in the systemic tissue there was a 7.3-fold change between the mock and Pip infiltrated samples in terms of PR1 expression. PCR1 in the local tissue was lowly expressed in all samples (Figure 10B). In the 23°C samples there was no change in PCR1 expression in the Pip-treated samples compared to mock ($p=0.6996; p=0.5104$). In the systemic tissue, PCR1 gene expression (Figure 10D) did not change in response to Pip priming at 23°C ($p=0.0651$), but induction was significantly reduced at 28°C ($p=>0.9999$). Transcript levels were very low, and the 28°C samples also showed no change in PCR1 induction in the Pip-treated samples compared to mock. Therefore, no changes were observed in the Pip/NHP signalling genes in systemic or local tissue.
Since elevated temperature downregulated Pip/NHP signalling genes in systemic tissues (Figure 8), I next investigated how NHP biosynthetic genes (ALD1 and FMO1) respond to exogenous Pip treatment under changing temperatures. Similarly, both local and systemic tissues from four-week-old Arabidopsis Col-0 plants were used for gene expression analyses. Figure 11A and B show the expression of ALD1 and FMO1 (respectively) in locally Pip-treated leaves. At both 23°C and 28°C, in the local leaves (11A) ALD1 expression is not affected in the Pip-treated samples compared to mock (p=0.9955; p=0.3916). As well, at both in the local leaves (Figure 11B) FMO1 expression is not affected by Pip-treatment (p=0.9841; p=0.2536). Figure 11C and D show systemic expression of ALD1 and FMO1 (respectively). Here, systemic ALD1 expression was not affected and FMO1 expression increased 0.99-fold following local Pip priming at 23°C (p=0.0855; p=0.0197), but they were downregulated at elevated temperature (Appendix, Figure 27). At 28°C following Pip treatment both ALD1 and FMO1 expression was not affected (p=>0.9999; p=0.9629). Pip-induced expression of the NHP biosynthetic genes ALD1 and FMO1 were very low across all samples. These results show elevated temperature impacts Pip-induced FMO1 expression systemically but not locally.

There is extensive crosstalk between the NHP and SA pathways, so the next gene of interest is the critical SA biosynthetic gene ICS1. (Návarová et al., 2012; Chen et al., 2018; Hartmann et al., 2018; Hartmann and Zeier, 2018). Again, four-week-old Arabidopsis Col-0 plants were infiltrated with Pip via stomata and both local and systemic tissues were used for gene expression analysis. SA biosynthesis is mediated by ICS1, with its transcript levels in local and systemic leaves respectively shown in Figure 12A and B. In the local tissue (Figure 12A), there was no change in ICS1 expression following Pip treatment compared to mock conditions at 23°C and 28°C (p=0.4530; p=0.5757). In the systemic tissue (Figure 12B), ICS1 expression is not affected following Pip priming at
23°C. In contrast, Pip-regulated \textit{ICS1} transcript levels were downregulated at 28°C, where there was a 11.4-fold change between the Pip treated samples at 23°C and 28°C.

These results together show that Pip does not impact the NHP- and SA-associated genes in the local tissue at both normal and elevated temperatures. Interestingly, systemic gene upregulation after Pip priming only occurs at 23°C but not at 28°C. Results were consistent with conventional gel-based PCR analyses (Appendix Figure 21). Additionally, it has been shown that several Pip induced genes are temperature sensitive (Appendix Figure 23).
Figure 10. Gene expression analysis of Pip signalling and response genes by qRT-PCR of *Arabidopsis* local and systemic tissues following mock or Pip treatment at 23°C and 28°C. Four-week-old *Arabidopsis* Col-0 plants were infiltrated in five lower leaves with water (mock) or 1mM Pip solution. Plants were then incubated at either 23°C or 28°C. Two days later, lower local (A and B), and upper systemic (C and D) leaves in both mock- and Pip-treated plants were harvested for gene expression analysis as detailed in the Materials and Methods.

A. Local *PR1* gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C.
B. Local *PCRI* gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C
C. Systemic *PR1* gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C.
D. Systemic *PCRI* gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C.
Results shown in A-D are the means ± S.D. (A and B, n=8 from 2 independent experiments; C and D, n=4 from 1 independent experiment). Data were analyzed with two-way ANOVA and Tukey's multiple comparisons test (p < 0.05). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 11.
Figure 11. Gene expression analysis of NHP biosynthetic genes by qRT-PCR of Arabidopsis local and systemic tissues following mock or Pip treatment at 23°C and 28°C. Four-week-old Arabidopsis Col-0 plants were infiltrated in five-six lower leaves with water (mock) or 1mM Pip solution. Plants were then incubated at either 23°C or 28°C. Two days later, lower local (A and B), and upper systemic (C and D) leaves in both mock- and Pip-treated plants were harvested for gene expression analysis as detailed in the Materials and Methods.

A. Local ALD1 gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C.
B. Local FMO1 gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C
C. Systemic ALD1 gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C.
D. Systemic FMO1 gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C
Results shown in A-D are the means ± S.D. (n=12 from 3 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test ($p < 0.05$). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 12.
Figure 12. Gene expression analysis of the SA biosynthetic gene by qRT-PCR of *Arabidopsis* local and systemic tissues following mock or Pip treatment at 23°C and 28°C. Four-week-old *Arabidopsis* Col-0 plants were infiltrated in five-six lower leaves with water (mock) or 1mM Pip solution. Plants were then incubated at either 23°C or 28°C. Two days later, lower local (A), and upper systemic (B) leaves in both mock- and Pip-treated plants were harvested for gene expression analysis as detailed in the Materials and Methods.

A. Local *ICS1* gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C.

B. Systemic *ICS1* gene expression 2 dpi after local priming with 1mM Pip at 23°C or 28°C.

Results shown in A-B are the means ± S.D. (n=8 from 2 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test (p < 0.05). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 13.
3.5 Impact of temperature on SAR-induced systemic SA biosynthesis in *Arabidopsis*

My previous findings (Figures 7-12) showed that elevated temperatures negatively impacted SAR and the Pip/NHP biosynthetic pathway. As mentioned previously, because the NHP and SA pathways are tightly interconnected, I next investigated if elevated temperature downregulated the SA biosynthetic gene *ICS1* following pathogen priming. To investigate this, gene expression analyses were conducted using systemic tissues from four-week-old *Arabidopsis* Col-0 plants (Appendix, Figure 26; Appendix, Figure 28). As shown in Figure 13A, local infection with virulent *Pst* DC3000 (absorbance at 600nm = 0.001) led to 6.5-fold increase in systemic *ICS1* transcript levels at 23°C after *Pst* DC3000 priming compared to the mock-treated plants (*p*<0.0001). At 28°C, *ICS1* expression was downregulated regardless of treatment. In accordance with my previous results, Figure 13B shows that a higher concentration of virulent *Pst* DC3000 (absorbance at 600nm = 0.02) priming also led to a 2.0-fold increase in systemic *ICS1* expression following a local infection compared to the mock samples at 23°C (*p*=0.0139). At 28°C, *ICS1* expression was low and not induced. To further confirm the negative impact of elevated temperature on systemic SA biosynthetic gene expression, analyses were done after local infection of ETI-activating avirulent pathogen *Pst* DC3000/AvrRpt2 (absorbance at 600nm = 0.02). Figure 13C shows a 3.4-fold increase of systemic *ICS1* expression after *Pst* DC3000/AvrRpt2 priming at normal temperature (*p*=0.0032) but not at high temperature (*p*=0.9986). Interestingly, the lowest concentration of *Pst* DC3000 induced the highest *ICS1* expression, and the lowest *ICS1* expression was observed following treatment with *Pst* DC3000/AvrRpt2.
Overall, these results suggest that systemic SA biosynthesis after virulent or avirulent pathogen priming in *Arabidopsis* plants is negatively targeted by elevated temperature by downregulating the critical *ICS1* gene.
Figure 13. Gene expression analysis of the SA biosynthetic gene by qRT-PCR of *Arabidopsis* systemic tissues following mock or pathogen (Pst DC3000) treatment.
at 23°C or 28°C. Four-week-old Arabidopsis Col-0 plants were infiltrated in five-six lower leaves with 0.25mM MgCl$_2$ (mock) or Pst bacterial suspension (DC3000 OD$_{600}$=0.001 in A, DC3000 OD$_{600}$=0.02 in B, DC3000/AvrRpt2 OD$_{600}$=0.02 in C). Plants were then incubated at either 23°C or 28°C. Two days later, upper systemic leaves in both mock- and Pst-treated plants were harvested for gene expression analysis as detailed in the Materials and Methods.

A. Systemic $ICS1$ gene expression 2 dpi after local priming with Pst DC3000 OD$_{600}$=0.001 at 23°C or 28°C.

B. Systemic $ICS1$ gene expression 2 dpi after local priming with Pst DC3000 OD$_{600}$=0.02 at 23°C or 28°C.

C. Systemic $ICS1$ gene expression 2 dpi after local priming with Pst DC3000/AvrRpt2 OD$_{600}$=0.02 at 23°C or 28°C.

Results shown in A-C are the means ± S.D. (A, n=4 from 1 independent experiment; B, n=12 from 3 independent experiments; C, n=8 from 2 independent experiments). Data were analyzed with two-way ANOVA and Tukey's multiple comparisons test ($p < 0.05$). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 14.
3.6 Impact of temperature on systemic expression of master immune regulatory genes CBP60g and SARD1

Regulation of the SA pathway genes ICS1, EDS5, and PBS3 and the NHP biosynthetic genes ALD1, SARD4, and FMO1 occur via two partially redundant master transcription factors SARD1 (SAR DEFICIENT 1) and CBP60g (CALMODULIN-BINDING PROTEIN 60-LIKE G) (Wang et al., 2011; Sun et al., 2015; Huang et al., 2020). Following pathogen infection, both SARD1 and CBP60g expression are induced leading to increased SA and NHP levels (Hartmann and Zeier, 2018; Huang et al., 2020). To determine if temperature affects SARD1 and CBP60g gene expression, analyses were conducted using systemic tissues from four-week-old Arabidopsis Col-0 plants (Appendix, Figure 26; Appendix, Figure 29). As shown in Figure 14A and B, local priming with virulent Pst DC3000 (absorbance at 600nm = 0.001) at 23°C resulted in a 5.7-fold increase in systemic CBP60g transcript levels (p=0.0172) and a 6.3-fold increase in SARD1 expression (p=0.0001) compared to the mock-treated plants. At elevated temperature, both CBP60g and SARD1 expression in systemic tissues were very low regardless of treatment.

To potentially elicit higher expression of these master immune regulatory genes in the systemic tissues, I used a higher concentration of virulent Pst DC3000 (absorbance at 600nm = 0.02) to infect the local leaves. As shown in Figure 14C, CBP60g expression in systemic tissue following a local infection led to a 2.8-fold increase at normal temperature in the Pst DC3000 infected samples (p=0.0053) but not at 28°C (p=0.9998). Similarly, as shown in Figure 14D, systemic SARD1 expression increased 4.5-fold after local Pst DC3000 priming compared to the mock infiltrated samples at 23°C (p=<0.0001) but induction was absent at elevated temperature (p=0.9851). Strikingly, a lower concentration of Pst DC3000 (absorbance OD<sub>600</sub>=0.001) led to higher SARD1 induction;
however, a higher \textit{Pst} DC3000 concentration (absorbance OD$_{600}$=0.02) unexpectedly led to higher \textit{CBP60g} induction.

Subsequently, the effects of elevated temperature on systemic \textit{CBP60g} and \textit{SARD1} expression were determined after local priming with the ETI-activating avirulent pathogen \textit{Pst} DC3000/AvrRpt2 (absorbance at 600nm = 0.02). Figure 14E and F show that pathogen priming led to a 2.2-fold increase in \textit{CBP60g} and no change in \textit{SARD1} compared to the mock-infiltrated samples at normal temperature (\textit{CBP60g}, $p=0.0009$; \textit{SARD1}, $p=0.0041$). At 28°C shown in Figure 14E and F, \textit{CBP60g} and \textit{SARD1} gene expression were not induced after initial pathogen infection ($p=>0.9999$; $p=0.6364$). \textit{CBP60g} expression in both the mock- and \textit{Pst}-primed samples at 28°C were comparable to that with the mock condition at 23°C. \textit{SARD1} gene expression also followed similar trends at elevated temperature.

Overall, these results show that the master immune regulatory genes \textit{CBP60g} and \textit{SARD1} are upregulated in the systemic tissue by primary infection with virulent \textit{Pst} DC3000 at normal temperature. However, elevated temperature negatively affects the expression of these critical genes.
Figure 14. Gene expression analysis of master immune regulatory genes by qRT-PCR of *Arabidopsis* systemic tissues following mock or pathogen (*Pst DC3000*) treatment at 23°C or 28°C. Four-week-old *Arabidopsis* Col-0 plants were infiltrated in five-six lower leaves with 0.25mM MgCl₂ (mock) or *Pst* bacterial suspension (DC3000...
OD_{600}=0.001 in A-B, DC3000 OD_{600}=0.02 in C-D, DC3000/AvrRpt2 OD_{600}=0.02 in E-F). Plants were then incubated at either 23°C or 28°C. Two days later, upper systemic leaves in both mock- and Pst-treated plants were harvested for gene expression analysis as detailed in the Materials and Methods.

A. Systemic *CBP60g* gene expression 2 dpi after local priming with Pst DC3000 OD_{600}=0.001 at 23°C or 28°C.

B. Systemic *SARD1* gene expression 2 dpi after local priming with Pst DC3000 OD_{600}=0.001 at 23°C or 28°C.

C. Systemic *CBP60g* gene expression 2 dpi after local priming with Pst DC3000 OD_{600}=0.02 at 23°C or 28°C.

D. Systemic *SARD1* gene expression 2 dpi after local priming with Pst DC3000 OD_{600}=0.02 at 23°C or 28°C.

E. Systemic *CBP60g* gene expression 2 dpi after local priming with Pst DC3000/AvrRpt2 OD_{600}=0.02 at 23°C or 28°C.

F. Systemic *SARD1* gene expression 2 dpi after local priming with Pst DC3000/AvrRpt2 OD_{600}=0.02 at 23°C or 28°C.

Results shown in A-E are the means ± S.D. (A and B, n=4 from 1 independent experiment; C and D, n=16 from 4 independent experiments; E n=12 from 3 independent experiments, and F, n=8 from 2 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test (p < 0.05). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 15.
3.7 Effect of constitutive CBP60g gene expression on plant systemic immunity at elevated temperature

In wild-type *Arabidopsis* Col-0 plants, I observed warm temperature-downregulation of systemic NHP and SA biosynthetic genes which are controlled by the functionally redundant master immune regulators CBP60g and SARD1, both of which displayed reduced expression at high temperature. Therefore, I next explored what would occur if CBP60g gene expression is constitutively expressed in *Arabidopsis* plants. For this, I examined transgenic plants constitutively expressing CBP60g through a temperature-insensitive gene promoter (35S::CBP60g OE17) for their SAR phenotypes and gene expression profiles at elevated temperature (Appendix, Figure 24). As shown in Figure 15A, local priming with virulent *Pst* DC3000 (absorbance at 600nm = 0.02) led to no protection to secondary *Pst* DC3000 infection at both 23°C and 28°C. Figure 15B shows the systemic leaf symptoms, wherein *Pst* DC3000-primed samples were less chlorotic than without priming at both 23°C and 28°C. To observe a stronger SAR response, local priming at 23°C and 28°C was also performed with the ETI-activating *Pst* DC3000/AvrRpt2 strain (absorbance at 600nm = 0.02). As shown in Figure 15C, SAR was observed in 35S::CBP60g plants at both normal and elevated temperature. At normal temperatures, systemic bacterial CFUs were decreased by 3.1-fold and at elevated temperature systemic bacterial CFUs were decreased by 2.7-fold in the *Pst* DC3000/AvrRpt2-primed samples compared to the mock treated samples (*p*=0.0020; *p*=0.0091). Figure 15D shows similar symptoms in the systemic leaves at 23°C and 28°C, reflecting the resiliency of 35S::CBP60g plants to temperature.

Overall, these findings show that SAR priming with avirulent bacterial pathogens persist in plants constitutively expressing CBP60g at both normal and elevated temperatures. This contrasts with wild-type plants wherein SAR is downregulated by elevated temperature.
Arabidopsis system acquired resistance phenotypes at normal and elevated temperatures in constitutively expressing CBP60g plants. Four-week-old Arabidopsis 35S:CBP60g OE17 plants were infiltrated in five-six lower leaves with 0.25mM MgCl₂ (mock) or Pst bacterial suspension (DC3000 OD₆₀₀=0.02 in A-B, DC3000/AvrRpt2 OD₆₀₀=0.02 in C-D). Plants were then incubated at either 23°C or 28°C. Two days later, upper systemic leaves in both mock- and Pst-treated plants were infiltrated with Pst DC3000 suspension (OD₆₀₀=0.001) and placed back at their respective temperature. Three days after systemic infiltration (3 dpi), bacterial levels and leaf symptoms were evaluated as detailed in the Materials and Methods.

A. Systemic Pst DC3000 bacterial levels in \( \log_{10}[\text{CFU(cm}^{-2})] \) at 3 dpi after local priming with Pst DC3000 OD₆₀₀=0.02 at 23°C or 28°C.
B. Resulting symptoms at 3 dpi after local priming treatment with \textit{Pst} DC3000 \textit{OD}_{600}=0.02 at 23°C or 28°C.

C. Systemic \textit{Pst} DC3000 bacterial levels in \textit{log}_{10}[\textit{CFU}(\text{cm}^{-2})] at 3 dpi after local priming with \textit{Pst} DC3000/AvrRpt2 \textit{OD}_{600}=0.02 at 23°C or 28°C.

D. Resulting symptoms at 3 dpi after local priming treatment with \textit{Pst} DC3000/AvrRpt2 \textit{OD}_{600}=0.02 at 23°C or 28°C.

Results shown in A and C are the means ± S.D. (A, n=12 from 3 independent experiments; C, n=24 from 6 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test ($p < 0.05$). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 16.
3.8 Effect of constitutive CBP60g gene expression on systemic NHP and SA biosynthetic gene expression at elevated temperature

Having observed that SAR is restored at elevated temperature in plants constitutively expressing CBP60g, the underlying molecular mechanisms were then explored (Appendix, Figure 26). Since SAR is mediated by Pip/NHP pathway, I first investigated whether the NHP pathway is restored in Arabidopsis 35S::CBP60g plants by quantifying NHP biosynthetic gene expression. Based on my previous experiments, I analyzed the expression of ALD1 and FMO1 in the systemic leaves following primary infection with both virulent Pst DC3000 (Figure 16A and B) and avirulent Pst DC3000/AvrRpt2 (Figure 16C and D) absorbance at 600nm = 0.02.

As shown in Figure 16A, ALD1 expression increased 7.9-fold in the systemic leaves following Pst DC3000 priming compared to the mock-treated 35S::CBP60g plants at 23°C (p=<0.0001). However, this induction was still lost at 28°C, and ALD1 gene expression was downregulated (Appendix, Figure 30). After priming with Pst DC3000/AvrRpt2, as shown in Figure 16C, systemic ALD1 expression was not impacted (p=0.3430). FMO1 expression was also measured as shown in Figure 16B. No increase in FMO1 expression was observed in the systemic leaves following Pst DC3000 infection compared to the mock-treated plants at 23°C. At 28°C, there was statistically significant 4.6-fold FMO1 induction after Pst DC3000 priming (p=0.0007). Following a Pst DC3000/AvrRpt2 infection as shown in Figure 16D, there were no changes across all four treatments. Overall, these results show that NHP biosynthetic gene ALD1 is induced systemically at 23°C; however, systemic ALD1 is downregulated by elevated temperature in 35S::CBP60g plants following treatment with virulent Pst DC3000.

It is well known that the NHP and SA pathways are tightly interconnected (Návarová et al., 2012; Chen et al., 2018; Hartmann et al., 2018; Hartmann and Zeier, 2018), so I next measured expression of the SA biosynthetic gene ICS1 following
pathogen infection of 35S::CBP60g plants with both virulent and avirulent Pst DC3000. As shown in Figure 17A, local infection with virulent Pst DC3000 (absorbance at 600nm = 0.02) led to 2.1-fold increase in ICS1 in systemic leaves compared to mock treated plants at 23°C (p=0.0012), while its induction compared to mock treated plants at 28°C was not changed (p=0.1672). Analyses were also done after local infection with ETI-activating avirulent pathogen Pst DC3000/AvrRpt2 (absorbance at 600nm = 0.02). Figure 17B shows systemic ICS1 expression at both normal temperature and elevated temperature after Pst DC3000/AvrRpt2 priming. Based on this, a local infection of avirulent Pst DC3000/AvrRpt2 (absorbance at 600nm = 0.02) did not change systemic ICS1 expression to a statistically significant degree at either temperature (p=0.9194; p=0.9991).

Together, these results (Figure 16 and 17) suggest that constitutive expression of CBP60g does not restore ALD1-mediated NHP biosynthesis and ICS1-mediated SA biosynthesis in terms of gene expression in systemic tissues at elevated temperature.
Figure 16. Gene expression analysis of NHP biosynthetic genes by qRT-PCR of 35S::CBP60g OE17 Arabidopsis systemic tissues following mock or pathogen (Pst DC3000) treatment at 23°C or 28°C. Four-week-old Arabidopsis 35S::CBP60g OE17 plants were infiltrated in five-six lower leaves with Pst bacterial suspension (DC3000 OD<sub>600</sub>=0.02 in A-B, DC3000/AvrRpt2 OD<sub>600</sub>=0.02 in C-D). Plants were then incubated at either 23°C or 28°C. Two days later, upper systemic leaves in both mock- and Pst-treated plants were harvested for gene expression analysis as detailed in the Materials and Methods.

A. Systemic ALD1 gene expression 2 dpi after local priming with Pst DC3000 OD<sub>600</sub>=0.02 at 23°C or 28°C.

B. Systemic FMO1 gene expression 2 dpi after local priming with Pst DC3000 OD<sub>600</sub>=0.02 at 23°C or 28°C.

C. Systemic ALD1 gene expression 2 dpi after local priming with Pst DC3000/AvrRpt2 OD<sub>600</sub>=0.02 at 23°C or 28°C.

D. Systemic FMO1 gene expression 2 dpi after local priming with Pst DC3000/AvrRpt2 OD<sub>600</sub>=0.02 at 23°C or 28°C.
Results shown in A–D are the means ± S.D. (A–D, n=12 from 3 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test (p < 0.05). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 17.
A. Systemic ICS1 gene expression 2 dpi after local priming with Pst DC3000 OD<sub>600</sub>=0.02 at 23°C or 28°C.

B. Systemic ICS1 gene expression 2 dpi after local priming with Pst DC3000/AvrRpt2 OD<sub>600</sub>=0.02 at 23°C or 28°C.

Results shown in A-B are the means ± S.D. (A and B, n=12 from 3 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test (p < 0.05). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 18.
3.9 Effect of constitutive CBP60g gene expression on systemic expression of master immune regulatory genes at elevated temperature

As previously mentioned, regulation of the SA and NHP pathways occurs via the two master transcription factors CBP60g and SARD1 (Wang et al., 2011; Sun et al., 2015). I first tested whether CBP60g gene expression is temperature-resilient in 35S::CBP60g plants. As shown in Figure 18A and C, gene expression analyses confirmed that these plants are overexpressing this master immunity gene regardless of temperature.

To determine if constitutive CBP60g expression also affects SARD1 gene expression, transcript levels were quantified using systemic tissues from four-week-old Arabidopsis 35S::CBP60g plants (Appendix, Figure 26). As shown in Figure 18B, systemic expression of SARD1 following local infection with virulent Pst DC3000 (absorbance at 600nm = 0.02) increased 10-fold compared to the mock-treated samples at 23°C ($p<0.0001$). Remarkably at 28°C, SARD1 expression in systemic tissues increased 9.0-fold in the Pst DC3000-primed samples compared to mock conditions ($p=0.0118$) (Appendix, Figure 31). Additionally, systemic SARD1 expression in 35S::CBP60g plants was determined after local infection with avirulent Pst DC3000/AvrRpt2 (absorbance at 600nm = 0.02). Figure 18D shows that this resulted in no change in SARD1 expression in the Pst DC3000/AvrRpt2-primed systemic samples compared to the mock treatment at normal temperatures or at elevated temperature ($p=0.1506; p=0.6072$).

In summary, these results show that 35S::CBP60g plants are successfully overexpressing CBP60g in all tested conditions and this leads to systemic SARD1 upregulation following infection with virulent and Pst DC3000 at normal and elevated temperature.
Figure 18. Gene expression analysis of master immune regulatory genes by qRT-PCR of 35S::CBP60g OE17 Arabidopsis systemic tissues following mock or pathogen (Pst DC3000) treatment at 23°C or 28°C. Four-week-old Arabidopsis 35S::CBP60g OE17 plants were infiltrated in five-six lower leaves with Pst bacterial suspension (DC3000 OD₆₀₀=0.02 in A-B, DC3000/AvrRpt2 OD₆₀₀=0.02 in C-D). Plants were then incubated at either 23°C or 28°C. Two days later, upper systemic leaves in both mock- and Pst-treated plants were harvested for gene expression analysis as detailed in the Materials and Methods.

A. Systemic CBP60g gene expression 2 dpi after local priming with Pst DC3000 OD₆₀₀=0.02 at 23°C or 28°C.

B. Systemic SARD1 gene expression 2 dpi after local priming with Pst DC3000 OD₆₀₀=0.02 at 23°C or 28°C.

C. Systemic CBP60g gene expression 2 dpi after local priming with Pst DC3000/AvrRpt2 OD₆₀₀=0.02 at 23°C or 28°C.
D. Systemic *SARD1* gene expression 2 dpi after local priming with *Pst* DC3000/AvrRpt2 OD$_{600}$=0.02 at 23°C or 28°C.

Results shown in A-D are the means ± S.D. (A-D, n=12 from 3 independent experiments). Data were analyzed with two-way ANOVA and Tukey’s multiple comparisons test ($p < 0.05$). Statistically significant values are indicated by different letters. The detailed ANOVA interaction analysis shown in Appendix Table 19.
Ch 4. Discussion

A plant’s ability to thrive is highly dependent on its external environmental conditions. As global temperatures increase, there is an overall negative impact on the plant immune system and a positive impact on certain pathogens’ ability to cause disease (Huot et al., 2017; Velásquez et al., 2018; Desaint et al., 2021). These plant immune mechanisms work more effectively under normal (optimal) temperatures. However, we are beginning to understand the detrimental effects of elevated temperature on the plant immune system. For example, components of pattern-triggered immunity (PTI) at the local site of infection can be downregulated by elevated temperature by suppressing cell surface immune receptor levels, such as FLS2 (Janda et al., 2019). As well, warm temperature can also suppress effector-triggered immunity (ETI) by negatively regulating intracellular NLR immune receptors (Mang et al., 2012). Additionally, downstream pathways are targeted at elevated temperatures, including immunity-induced calcium signaling (Hilleary et al., 2020) and biosynthesis of the defence hormone salicylic acid (Malamy et al., 1992; Huot et al., 2017; Kim et al., 2022), leading to decreased disease resistance. Previous studies have shown that elevated temperature negatively affects R protein-mediated disease resistance (Tsuda et al., 2008; Cheng et al., 2013). At elevated temperature, 28 °C, loss of R protein nuclear localization contributes to compromised R-mediated defence (Zhu et al., 2010).

At the local site of infection, there are major negative impacts to the plant’s health and ability to thrive when exposed to increased temperatures. At normal temperature when plants experience immune activation at the local site of infection, systemic acquired resistance (SAR) can activate a state of readiness to respond to future pathogen attack in unaffected systemic tissues (Vallad and Goodman, 2004; Kachroo and Kachroo, 2020; Shine et al., 2019; Durrant and Dong, 2004; Zeier, 2021; Vlot et al., 2021). How warm temperatures impact SAR has not been explored, and the molecular mechanisms
governing temperature-regulation of SAR are unknown. A summarized model for the findings in this thesis is shown in Figure 19.
Figure 19. Overview of findings in this thesis.

A. Results from disease assays suggest that SAR is negatively impacted by elevated temperatures. Through molecular analysis of CBP60g and Pip/NHP biosynthetic genes, it appears that elevated temperatures are correlated with downregulated systemic biosynthesis of the central SAR metabolite NHP.

B. Through the application of Pip either locally or via the roots, there is disease protection (shown through lesser bacterial growth compared to mock-treated plants) at elevated temperatures. From this, it can be suggested that Pip biosynthesis is temperature-sensitive, while transport and resulting disease protection seem to be temperature-resilient.

C. Through molecular analyses of 35S::CBP60g OE17 plants, it appears that the temperature-vulnerability of SAR in Arabidopsis is controlled by CBP60g through the regulation of Pip/NHP biosynthesis.

Adapted from: Vlot et al., 2020 and Huang et al., 2020. Created with Biorender.com.
4.1 Elevated temperature suppresses Arabidopsis systemic acquired resistance

To investigate the effect of elevated temperature on SAR, it is important to understand the genetic and metabolic regulation of pathogen-infected (local) and distal (systemic) tissues. Specifically, it is important to understand the role of signals that centrally govern SAR priming and/or establishment under different temperatures. For the establishment of functional SAR, crosstalk between the two immune-regulatory metabolites (SA and NHP) is required (Hartmann et al., 2018; Shields et al., 2022). Both SA and NHP accumulate in systemic leaves following pathogenic attack; however, the long-distance mobility of SA alone is not solely responsible for the establishment of SAR (Hartmann and Zeier, 2018; Vernooij et al., 1994; Lim et al., 2020). Therefore, SA contributes to long-distance signaling with other signaling molecules, including NHP, and NHP functions as a critical endogenous regulator of biologically induced SAR in Arabidopsis (Yildiz et al., 2021). A previous study showed that SA production at the site of local infection is temperature-sensitive (Huot et al., 2017).

Consistent with temperature-sensitive local immune responses and SA biosynthesis in pathogen-infected tissues (Huot et al., 2017; Kim et al., 2022), my thesis research showed that SAR was also negatively affected by elevated temperature (28°C) compared to normal temperature (23°C). After local priming with both virulent and ETI-activating avirulent Pst pathogens, SAR assays resulted in increased bacterial pathogen growth systemically at 28°C compared to 23°C, and therefore SAR was suppressed at elevated temperatures (Figure 7).

A priming treatment of virulent Pst DC3000 (absorbance at 600nm = 0.001) was not sufficient to elicit a significant SAR response (Figure 7A and B), so a higher concentration of virulent Pst DC3000 (absorbance at 600nm = 0.02) was used (Figure 7C and D). Here, SAR was successfully elicited at 23°C but there was a loss of systemic disease protection
at 28°C. Additionally, local priming with the ETI-activating avirulent pathogen *Pst* DC3000/AvrRpt2 (absorbance at 600nm = 0.02) elicited SAR at 23°C, but again this systemic protection was negatively affected by elevated temperature. Although it is expected that ETI-activating pathogens induce SAR in a stronger manner (Thordal-Christensen, 2020), local priming with both virulent and avirulent pathogens (absorbance at 600nm of 0.02) led to a decrease in systemic bacterial growth at normal temperature.

It was surprising that ETI-activating *Pst* DC3000/AvrRpt2 did not elicit a stronger SAR at 23°C compared to virulent DC3000 (Figure 7). It is possible that the *Pst* DC3000/AvrRpt2 strain I used was not inducing SAR optimally based on our lab conditions. It is also possible that, since the response in the local leaves was so amplified, the local tissues may have been undergoing strong HR leading to cell death, potentially resulting in suboptimal transmission of SAR signals throughout the plant.

Although my thesis focused on the impact of warm temperatures on SAR and its associated signals SA and NHP, there are other environmental factors and signals that can be considered (Vlot et al., 2021). For example, previous studies have shown that exposure to light influences SAR, and phytochrome-mediated recognition of the ratio of red:far-red light may be crucial to this process (Zeier et al., 2004; Griebel and Zeier, 2008). Specifically, light-regulated monoterpenes promote SAR immune responses, and it is known that monoterpenes act downstream of Pip/NHP, suggesting that the Pip/NHP pathway in SAR may be light-dependent (Griebel and Zeier, 2008; Riedlmeier et al., 2017; Wenig et al., 2019). This reliance on light is contrary to the action of another long-distance signal, methyl salicylate (MeSA), which accumulates in the local tissues following pathogen infection (Park et al., 2007). In tobacco, potato, and *Arabidopsis*, SAR is associated with MeSA, and in *Arabidopsis*, it appears that the role of MeSA in SAR is modulated by light exposure; plants exposed to light for longer periods
immediately following infection reduces the importance of MeSA for SAR (Vlot et al., 2008; Park et al., 2009; Liu et al., 2011). Based on this, perhaps light and other environmental factors additionally influence SAR pathways, which will be crucial to investigate as SAR-like systemic immunity has been observed in numerous plants, including maize, barley, wheat, and banana (Wu et al., 2013; Yang et al., 2013; Balmer et al., 2013a; Dey et al., 2014). The ability to perceive various environmental parameters allows plants to have flexible global immune response under changing conditions. However, it will be important to learn more about SAR in monocots, such as barley or wheat, before applying SAR signaling components derived from Arabidopsis towards protection of cereal crops (Vlot et al., 2021).

4.2 Elevated temperature downregulates SAR-associated NHP biosynthesis in Arabidopsis

After determining that SAR was negatively impacted by elevated temperature, I then wanted to elucidate the underlying mechanisms. An important candidate to investigate is the SAR metabolite NHP, considering its functional linkage with the temperature-sensitive defense hormone SA (Shields et al., 2022; Hartmann et al., 2018; Zeier, 2021). Previous studies have shown that in locally infected tissues, Pip/NHP biosynthetic gene expression is downregulated at elevated temperature (Kim et al., 2022). Kim and co-authors showed that pathogen-induced expression of the NHP biosynthetic genes ALD1/FMO1 as well as the SA biosynthetic gene ICS1 were downregulated at elevated temperature in local tissues.

In my thesis, I further showed that systemic expression of NHP biosynthetic genes, SA response genes, and master immune regulatory genes are all downregulated when exposed to elevated temperatures. In the NHP biosynthetic pathway, the
aminotransferase ALD1 is the crucial primary step leading to the production of cyclic intermediates before NHP synthesis (Zeier, 2013). Following pathogenic attack at normal temperatures, it is known that the ALD1 gene is strongly induced systemically in the plant’s foliage (Song et al., 2004; Cecchini et al., 2015; Návarová et al., 2012). Figure 8A, C, and E shows induction of ALD1 to a significant level in the Pst-primed systemic tissues at 23°C but not at 28°C. In the final step in the NHP biosynthetic pathway, FMO1 N-hydroxylates Pip to NHP (Hartmann et al., 2018; Chen et al., 2018). Again, like ALD1, in response to pathogens at normal temperatures, the expression of FMO1 is also strongly systemically induced throughout the plant’s foliage (Mishina and Zeier, 2006). In terms of the temperature effect, the same trend is shown to occur in systemic tissues as well, as Figure 8B, D, and F shows significant FMO1 induction in Pst-primed systemic tissues at 23°C; however, at 28°C there is little to no induction of FMO1.

In addition to monitoring the expression of two major NHP biosynthetic genes, it was important to also examine the functionally related SA pathway in systemic tissues. The local conversion of Pip to NHP can be negatively regulated by SA but systemic Pip/NHP signaling is boosted by SA (Hartmann et al., 2018, Zeier, 2021), which is primarily produced via the isochorismate (IC) pathway (Wildermuth et al., 2001). In this pathway, the enzyme Isochorismate Synthase 1 (ICS1) catalyzes the synthesis of pathogen-induced SA (Zhang and Li, 2019). As shown in Figure 13, ICS1 expression in systemic tissues were induced significantly after local Pst infection at 23°C but not at 28°C.

The expression of the two master transcription factor genes CBP60g and SARD1, which control both SA and Pip/NHP production (Hartmann et al., 2018; Zhang et al., 2010; Sun et al., 2015; Wang et al., 2009; Wan et al., 2012; Wang et al., 2011), were also analyzed in this thesis. CBP60g and SARD1 are both necessary for successful immune signalling in Arabidopsis, and their activation leads to increased SA and NHP levels.
(Zhang et al., 2010; Sun et al., 2015; Wang et al., 2009; Wan et al., 2012; Wang et al., 2011; Hartmann et al., 2018; Lu et al., 2018). Consistent with other defence genes, Figure 14 shows significant induction of CBP60g and SARD1 in the systemic tissues following Pst-priming at 23°C but not at 28°C. Overall, these gene expression results suggest that elevated temperature downregulates Pip/NHP levels due to decreased Pip/NHP biosynthetic gene expression.

Based on the results showing increased Pip/NHP biosynthetic gene expression at 23°C but not at 28°C, it can be hypothesized that Pip/NHP levels would follow the same trend as well. Following future quantification of Pip and NHP metabolite levels using liquid chromatography–mass spectrometry (LC-MS), it is anticipated that Pip and NHP levels will reflect the trends observed in Pip-NHP biosynthetic gene expression. Further metabolite analyses with mock- and SAR-primed plants at both normal and elevated temperatures are required to draw conclusions.

Temperature-regulation of Pip/NHP gene expression levels in Arabidopsis is consistent with how pathogen-induced biosynthesis of SA is greatly impacted by elevated temperatures (Huot et al., 2017). Remarkably, in addition to SA, other plant hormones are also regulated by temperature (Castroverde and Dina, 2021). Examples of warm temperature-upregulated or -activated hormones include auxin, brassinosteroid and gibberellin (Gray et al., 1998; Bellstaedt et al., 2019; Li et al., 2016). This increased hormone signalling leads to thermomorphogenesis (i.e. temperature-sensitive growth and development), as well as temperature regulation at genomic, transcriptional, post-transcriptional, and post-translational levels (Quint et al., 2016; Casal and Balasubramanian, 2019). This demonstrates the extensive crosstalk and linkages between plant hormone pathways and thermosensing mechanisms (Ferrero et al., 2019; Li et al., 2016; Castroverde and Dina, 2021).
Temperature also impacts how plants communicate with one another, and various studies have shown how plants can notify neighbouring plants to activate defenses for an expected attack (Liu and Brettell, 2019; Markovic et al., 2019). This process is mediated by low-molecular weight volatile organic compounds (VOCs), which easily evaporate at room temperature (Pennerman et al., 2016). The type of external stimulus will determine which blend of VOCs will be emitted by the plant; examples include mechanical damage, insect feeding, pathogen infection, and/or abiotic stresses like drought and extreme temperature (Brilli et al., 2019). Previous studies have shown that upon SAR activation in *Arabidopsis* by avirulent *Pseudomonas syringae* bacteria, plant-emitted VOCs include monoterpenes that can induce ROS accumulation and expression of SA- and SAR-associated genes, including *CBP60g*, in the receiver plants (Riedlmeier et al., 2017). Additionally, changes in intracellular Ca²⁺ levels after infection lead to the phosphorylation of the ROS-producing enzyme RESPIRATORY BURST HOMOLOG D (RBOHD) (Dubiella et al., 2013). This leads to elevated ROS levels in the form of apoplastic hydrogen peroxide (H₂O₂) (Miller et al., 2009). ROS generation is triggered by various environmental stressors such as elevated temperature (Tripathy and Oelmüller, 2012).

As mentioned previously, there are numerous SAR signalling molecules in addition to the well-characterized metabolites SA and Pip/NHP (Vlot et al., 2009; Návarová et al., 2012). Other SAR signaling components, including MeSA, AzA, DIR1, G3P, monoterpenes, dihydroabietinal (DA), and potentially more, might be functionally redundant as long-distance signals. Their crosstalk in systemic tissues may explain how different signals could further promote SAR against numerous pathogens in various environmental conditions. Since SAR is overall temperature sensitive, it is likely that temperature has some effect on the other molecules involved in SAR.
4.3 Exogenous Pip restores Arabidopsis systemic immunity at elevated temperature

The findings so far demonstrate a correlation between both downregulated SAR and Pip/NHP biosynthesis at elevated temperatures. However, these correlative results are not sufficient to conclude that temperature-sensitive Pip/NHP biosynthesis is the primary, rate-limiting step that leads to temperature-suppressed SAR in Arabidopsis. To clarify whether reduced Pip/NHP levels causes the loss of SAR at elevated temperature, I pre-treated plants with Pip before infection with bacterial pathogen under both normal and elevated temperatures. Subsequently, bacterial pathogen levels were quantified as a reflection of immunity or disease resistance.

Pip-induced immunity experiments were conducted using three approaches. First, Pip priming in the same leaves as subsequent Pst DC3000 infection showed Pip-mediated reduction in pathogen levels at both normal and elevated temperatures. Because exogenous Pip can restore disease protection at both temperatures, these results indicate that heat-mediated suppression of SAR is majorly controlled at the level of Pip/NHP production.

Second, Pip irrigation of plant roots (compared to mock treatment) led to reduced Pst DC3000 levels in leaves at both 23°C and 28°C. These results suggested that the temperature-sensitive suppression of the Pip-NHP pathway is controlled at the biosynthetic level rather than systemic transport. Therefore, Pip/NHP biosynthesis is temperature-sensitive, while transport seems to be temperature-resilient.

Third, Pip treatment in lower leaves and bacterial infection in upper leaves unexpectedly led to no significant disease protection at either 23°C or 28°C. This was an interesting result considering previous studies have shown that Pip application in lower leaves led to effective systemic protection against Pst DC3000 in upper leaves (Wang et
al., 2018). It is important to consider the optimal timepoint and Pip concentration for Pip-induced systemic immunity. Wang et al. (2018) determined that SAR was strongest in plants infiltrated with concentrations of 500 to 1000 μM Pip, while lower (e.g. 100 μM) or higher concentrations (e.g. 2000 μM Pip) led to significantly weaker SAR. It is possible that the Pip concentration (1000 μM) used in my study may have been slightly more potent than the optimal 500 to 1000 μM Pip range (Wang et al., 2018) due to supplier differences. Additionally, perhaps the 2-day length between local Pip priming and systemic pathogen infection and/or the 3-dpi assessment of bacterial levels may not have been optimal. However, my assay was consistent with the protocol used by Wang et al. (2018) although there could have been differences in actual Pip potency and/or virulence of our bacterial pathogen stock cultures. With further optimization, it would be exciting to produce effective systemic SAR following a local Pip treatment. Nonetheless, the conclusions are suggestive that warm temperature-suppressed SAR is likely primarily controlled via temperature-sensitive Pip/NHP biosynthesis, as shown by disease protection after local Pip priming or root treatment.

The SA receptor and transcriptional coactivator NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) acts as a central regulator of SAR, as it regulates major sectors of SAR signalling (Fu and Dong, 2013; Yildiz et al., 2021). Numerous downstream responses during a pathogen infection are dependent on this SA receptor (Fu et al., 2012; Wu et al., 2012). Previous studies have shown that the SA-insensitive and SAR-defective npr1 mutant exhibits delayed Pip accumulation in the primarily infected leaves, suggesting that NPR1 positively regulates Pip biosynthesis (Návarová et al., 2012; Peng et al., 2021). Interestingly, the npr1 mutants were highly sensitive to heat stress and showed abnormal expression of SA-responsive pathogenesis-related genes (Larkindale et al., 2005). Accumulation of SA and Pip/NHP
are both interdependent and synergistic, therefore SAR relies on their mutual amplification, as well as on the action of NPR1 which transduces SA- and NHP-activated immune signals (Yildiz et al., 2021). Previous studies have shown that NPR1 monomerization, which is associated with NPR1 function (Mou et al., 2003), was similar at both 23°C and 28°C (Kim et al., 2022). This showed that there is a mechanism other than NPR1 monomerization that is responsible for suppressing **CBP60g/SARD1** transcription and systemic SA/NHP production at elevated temperature (Kim et al., 2022).

Altogether, my results demonstrate that Pip treatment before bacterial pathogen infection leads to disease protection under both normal and elevated temperatures. These findings were observed using either local Pip infiltration in leaves or Pip application to the plant roots. Because of the central importance of NPR1 in Pip/NHP-induced disease protection, my results also suggest that NPR1 still functions at higher temperatures.

### 4.4 Temperature regulation of Pip-induced signaling in Arabidopsis

Although Pip-induced protection was observed at both temperatures (local and root application), there was still increased pathogen levels in Pip-treated plants at elevated temperature (compared to normal temperature), suggesting additional temperature-sensitive mechanisms downstream of Pip/NHP accumulation. Previous studies have shown that Pip treatment in local tissue leads to increased expression of defence genes in distal/systemic tissues (Návarová, et al., 2012; Hartmann et al., 2018; Yildiz et al., 2021). To determine the impact of temperature on Pip/NHP-induced signalling, we measured defence gene expression following Pip treatment.

During pathogen infection, the plant SA and NHP pathways are triggered and the master SAR regulator NPR1 is activated, which leads to the expression of **PR**
(PATHOGENESIS RELATED) genes, including PR1 (Van Loon and Van Strien, 1999; Durrant and Dong, 2004; DeLaure et al., 2008). Induced expression of PR1 is a useful marker of SAR (van Loon et al., 2006). Additionally, PLANT CADMIUM RESISTANCE1 (PCR1) encodes a member of the plant cadmium resistance (PCR) protein family that acts as a Ca$^{2+}$ transporter; like PR1, PCR1 is another SAR marker gene as it is upregulated systemically following pathogen infection (Bernsdorff et al., 2016). Figure 10 shows that Pip treatment does not significantly induce expression of PR1 and PCR1 in local or systemic tissue at 23°C and 28°C.

Next, Pip-induction of ALD1 and FMO1 in both the local and systemic tissues were also measured to determine the positive feedback amplification of Pip/NHP biosynthetic genes (Figure 11). ALD1 and FMO1 gene expression in the local tissue was not induced at 23 °C or 28 °C following Pip treatment. Similarly to the locally Pip-treated leaves, ALD1 expression in systemic tissue was not affected following Pip treatment at either temperature (Figure 11C). However, although systemic FMO1 levels were quite low in all samples, it was still observed that there was higher gene expression at 23°C compared to 28°C after Pip treatment.

Lastly, the induction of ICS1 in both the local and systemic tissues was measured following Pip treatment to determine how temperature influences the mutual amplification of SA and Pip/NHP pathways (Figure 12). In the local tissue (Figure 12A), there was no statistical significance between ICS1 expression in any of the samples at either temperature, indicating no effect of Pip on ICS1 induction locally. In previous studies by Hartmann et al., 2018 and Yildiz et al., 2021, the RNA-seq data revealed that induction of ICS1 after Pip or NHP treatment is more dramatic than the levels seen in this thesis. In the study done by Yildiz et al., 2021, there was a 4.9-fold increase in ICS1 expression following NHP treatment, compared to no change in expression I reported in Figure 12A following Pip treatment. The lack of effect I observed may be due to the method, or
amount of Pip/NHP applied to the soil. Yildiz et al., used 10 mL of a 1 mM aqueous NHP solution pipetted onto the soil or infiltrated into three rosette leaves per plant. It would be intriguing to determine the gene expression following application of NHP via the roots. For leaf infiltration, although 1mM Pip was used, I infiltrated between five or six leaves per plant. Therefore, there was a possibility that the effect was actually detrimental to the plant’s health, leading to less optimal induction of immune genes.

Overall, my results suggest a negative impact of warm temperature on the systemic feedback loop amplification and potentiation of the NHP and SA pathways. This systemic downregulation at elevated temperature of these critical NHP and SA biosynthetic genes after exogenous Pip application suggest additional temperature-modulated mechanisms downstream of Pip/NHP production. Although one can potentially rule out Pip transport based on the physiological results shown in Figure 7, this temperature-sensitivity occurring at the molecular level has yet to be defined.

4.5 CBP60g governs the temperature-vulnerability of Arabidopsis SAR

After determining that suppression of SAR at elevated temperature is likely mediated by reduced expression of certain NHP biosynthesis and signaling genes, I next investigated the molecular mechanisms underpinning heat-mediated NHP pathway suppression. CBP60g was chosen as the first candidate gene since, along with its partially redundant homolog SARD1, it acts as a master transcription factor regulating expression of the SA pathway genes ICS1, EDS5, and PBS3 and the NHP biosynthetic genes ALD1, SARD4, and FMO1 (Wang et al., 2011; Sun et al., 2015; Huang et al., 2020). Remarkably, CBP60g/SARD1 transcription is downregulated by elevated temperature in both pathogen-induced tissues (Kim et al., 2022) and SAR-primed systemic tissues (shown in this thesis; Figure 14).
To test this hypothesis, I characterized 35S::CBP60g transgenic plants with temperature-insensitive CBP60g transcription (Kim et al., 2022). The 35S promoter (derived from the cauliflower mosaic virus) is a strong constitutive promoter that facilitates increased levels of RNA transcription in various plants (Odell et al., 1985; Fromm et al., 1985). Specifically, SAR phenotypes and NHP biosynthetic gene expression levels were examined in this thesis. Based on the results in Figure 15, it seems that CBP60g controls the temperature-sensitivity of systemic immunity because 35S::CBP60g plants (with temperature-resilient CBP60g gene expression) exhibited SAR at both normal and elevated temperatures. Local priming with virulent Pst DC3000 bacteria (absorbance at 600nm = 0.02) elicited modest SAR in 35S::CBP60g plants at 23°C and 28°C, although the results were not statistically significant (Figure 15A and B). As a follow-up, local priming was performed with the ETI-inducing avirulent bacteria Pst DC3000/AvrRpt2 (absorbance at 600nm = 0.02) (Figure 15C and D). Remarkably, Pst DC3000/AvrRpt2 elicited SAR in 35S::CBP60g plants at both 23°C and 28°C.

Previous transcriptome analyses by Kim et al. (2022) showed that 35S::CBP60g plants have restored expression of the NHP biosynthetic genes ALD1 and FMO1 in the local pathogen-infected tissues. Indeed, 35S::CBP60g plants have temperature-resilient basal immunity (Kim et al., 2022), which is strikingly reminiscent of my current findings on the temperature-resilient SAR of these transgenic lines. Therefore, in addition to the SAR disease assays, this thesis extended the molecular/gene expression analyses to the systemic tissues of 35S::CBP60g plants (shown in Figures 16, 17, and 18), since these were not conducted by Kim et al. (2022).

Figure 16A shows significant ALD1 induction in systemic tissues at 23°C but not at 28°C after local Pst DC3000 infection. A similar trend is seen in Figure 16C in the ETI-activating Pst DC3000/AvrRpt2-primed samples, although there was no statistical significance due to the spread of the individual datapoints in the 23°C pathogen priming
treatment. Figure 16B and D show that \textit{FMO1} was induced after virulent \textit{Pst} DC3000 priming at both temperatures, but induced gene expression was less apparent after avirulent \textit{Pst} DC3000/AvrRpt2 priming. Unlike systemic \textit{ALD1} gene expression, there was no clear trend in the temperature-sensitivity of systemic \textit{FMO1} gene expression in 35S::\textit{CBP60g} plants. Next, Figure 17 shows systemic \textit{ICS1} expression in 35S::\textit{CBP60g} plants. Again, there was higher induction in the \textit{Pst} DC3000-primed samples at both temperatures compared to mock, but not in the \textit{Pst} DC3000/AvrRpt2-primed samples. The differences in gene expression temperature-sensitivity of \textit{ALD1} vs. \textit{FMO1} after \textit{Pst} DC3000 priming may be due to the very low levels of \textit{FMO1}. The gene expression data may be showing basal levels of \textit{FMO1}. Additionally, there are numerous outliers leading to large error bars which could be skewing the data.

Together with the NHP and SA biosynthetic gene expression analyses of 35S::\textit{CBP60g} plants, the resulting expression analyses of the two master transcription factor genes \textit{CBP60g} and \textit{SARD1} were conducted. Figure 18A and C shows \textit{CBP60g} expression, which expectedly had high values since these plants are constitutively expressing \textit{CBP60g}. Figure 18B shows increased \textit{SARD1} expression systemically at both temperatures following \textit{Pst} DC3000 infection. It appears that systemic \textit{SARD1} is more resilient to temperature changes compared to systemic \textit{ALD1} and \textit{ICS1}. However, Figure 18D shows no systemic \textit{SARD1} induction after \textit{Pst} DC3000/AvrRpt2 priming. \textit{CBP60g} and \textit{SARD1} make up a partially redundant pair of proteins that are required for SA activation and additional defence responses (Wang et al., 2011). In plants where \textit{CBP60g} is constitutively expressed, there is a possibility that their SAR resiliency lessens the temperature-sensitivity of \textit{SARD1} as well.

It was surprising to observe higher defence gene induction after infiltration with \textit{Pst} DC3000 compared to \textit{Pst} DC3000/AvrRpt2 in these experiments. Typically, the use of \textit{Pst} DC3000 provides modest resistance, so the avirulent strain \textit{Pst} DC3000/AvrRpt2
is used to activate a stronger ETI response in *Arabidopsis* (Lim and Kunkel, 2004). A possibility that caused the conflicting results is the strength of the avirulent priming treatment. The local infection treatment could have been too strong and the HR occurred before the SAR signals were transported to the distal tissue, potentially resulting in lower levels of systemic immune gene expression.

Overall, overexpression of *CBP60g* resulted in SAR restoration at elevated temperatures. This is potentially supported by recent findings by Kim et al. (2022) in the local tissue showing temperature-resilient expression of SA biosynthetic gene *ICS1* and Pip/NHP biosynthetic genes *ALD1* and *FMO1* in local pathogen-infected tissues of 35S:*CBP60g* plants. It is reasonable to suggest that elevated temperature does not affect de novo Pip/NHP biosynthesis at the local site of pathogen attack in 35S:*CBP60g* plants that constitutively express the master immune regulatory gene *CBP60g*. Intriguingly, 35S:*CBP60g* plants do not restore induction of the SA and Pip/NHP biosynthetic genes in systemic uninfected tissues, suggesting that temperature-resilient SAR in 35S::*CBP60g* plants does not absolutely require de novo biosynthesis of NHP systemically. Because systemic *ALD1* and *ICS1* expression is still temperature sensitive in plants constitutively expressing *CBP60g* (with restored SAR at elevated temperature), one can suggest that Pip and/or NHP is a mobile metabolite (Návarová et al., 2012) transported throughout the plant during SAR under different temperatures. If Pip/NHP is produced in the local tissues of 35S:*CBP60g* and transported to the systemic tissue, there would be less requirement for these plants to produce Pip/NHP in distal leaves de novo. These gene expression profiles (Figures 16-18) and disease protection results (Figure 15) support the systemic mobility of pathogen induced NHP in local tissues in successfully conferring SAR in *Arabidopsis* plants (Yildiz et al., 2021).
Ch 5. Limitations of the Thesis

My thesis demonstrated for the first time the temperature-vulnerability of plant systemic immunity and elucidated the central involvement of CBP60g-regulation of the NHP pathway under changing temperatures. However, it is important to note certain technical and conceptual limitations. For example, only two strains of one model pathogen and only one model plant species were used. The virulent bacterial strain *Pst* DC3000 and the ETI-inducing avirulent strain *Pst* DC3000/AvrRpt2 were used to induce Pip/NHP production/signaling and SAR, which may not universally extend to all pathogens. However, major discoveries and advances using this bacterial species have been broadly applicable to other pathogens in several instances, highlighting its strong usefulness as a model pathogen (Xin and He, 2013).

Additionally, I only used *A. thaliana* as the host plant species for this thesis, so my discoveries and conclusions may not universally extend to all plant species. However, *Arabidopsis* is an important model organism and has already led to greater understanding across the plant kingdom. Many discoveries in *Arabidopsis* have been broadly applicable in other plant taxa (Koornneef and Meinke, 2010). For example, it has been shown that temperature-downregulated SA pathways persist in various plants, including both monocots and dicots (Kim et al., 2022). Additionally, the master transcription factor CBP60g has putative orthologs throughout the plant kingdom (Zheng et al., 2022; Kim et al. 2022; Amani et al., 2022). Furthermore, NHP biosynthesis and immune function have been demonstrated in various plant species, highlighting its evolutionary conservation (Schnake et al., 2020). Thus, it would not be surprising that temperature-mediated suppression of NHP and SAR could be broadly conserved across diverse plant taxa.

Lastly, although highly useful and reflective of the overall plant responses, this thesis only analyzed selected Pip/NHP and/or SA biosynthetic genes. Genes such as *ALD1*, *FMO1*, *CBP60g*, *SARD1*, and *ICS1* are informative in defining the immune and
physiological responses of plants under pathogen infection. However, a global transcriptome analysis through RNA sequencing and/or proteome/metabolome profiling of *Arabidopsis* plants after either pathogen infection or Pip/NHP treatment would eventually be needed. These integrative multi-omic analyses could reveal a more expansive and comprehensive plant immune landscape (i.e. shedding light on new important genes) under changing climate conditions.
Ch 6. Future directions

Based on our understanding of temperature-modulated SA signaling and basal disease resistance (Kim et al., 2022), this study focused on investigating the role of the master immunity transcription factor CBP60g in temperature-sensitive systemic immunity. Since *CBP60g* gene expression is temperature-sensitive in pathogen-infected tissues (Kim et al., 2022) and primed systemic tissues (shown in this study), plants constitutively expressing *CBP60g* (*35S::CBP60g*) were hypothesized to restore heat-mediated defects in the Pip/NHP pathway and systemic immunity. Similarly to what was shown by Kim et al. (2022) that *35S::CBP60g* restores SA biosynthesis, basal disease resistance and effector-triggered immunity, my thesis demonstrated that *35S::CBP60g* plants also have restored systemic acquired resistance and pathogen-induced NHP biosynthetic gene expression.

However, *CBP60g* was selected using a candidate gene approach based on the foundational Kim et al. (2022) study, and there are probably additional immunity-related genes and transcription factors involved in downstream Pip/NHP-induced responses under changing temperatures. In this case, future untargeted genetic screens of a mutant plant population could be performed to identify novel genes involved in temperature-modulated Pip/NHP signaling and/or systemic immune responses. Additionally, it would be interesting to perform this study in *35S::SARD1* plants and *cbp60g sard1* double mutants to fully unravel the key players in the temperature sensitivity of plant immunity in terms of Pip/NHP biosynthesis and signaling.

Additionally, this thesis quantified gene expression levels using qPCR, and assumptions about Pip biosynthesis and signalling were made based on the upregulation or downregulation of these well-characterized genes. However, to further solidify the conclusions made, it will be critical to perform LC-MS to quantify the actual levels of Pip
and/or NHP in each sample to ensure actual metabolite levels are consistent with the findings based on gene expression analyses.
Ch 7. Integrative nature of this thesis

Integrative biology can be defined as a conceptual and technical framework. Conceptually, integrative biology means taking a holistic approach and asking biological questions with the intention of investigating through various lenses. It means merging all or numerous fields of biology, including ecology, evolution, physiology, toxicology, cell and molecular biology, and genetics, to gain a deeper understanding of nature. In a technical sense, integrative biology means using several (and sometimes complementary) methods to determine complete answers to our biological questions. For example, this can even include aspects of other sciences, such as chemistry and physics.

My thesis project conceptually aligns with my definition of integrative biology. By investigating the influence of elevated temperature on plant-pathogen interactions to further understand the vast impacts of climate change, I was using an ecological perspective. By characterizing how plants were negatively impacted by pathogens and examining physiological effects of elevated temperature on systemic acquired resistance, my thesis involved aspects of physiology. Lastly, numerous molecular and genetic analyses were conducted, which reflect the integration of cell/molecular biology and genetics to my thesis.

In terms of technologies used, this project was also integrative. Plants were grown, bacteria were cultured, plants were inoculated with pathogens/metabolites, and molecular analyses were performed. Additionally, additional disciplines were involved, although further experiments are still required to draw conclusions. For example, I have been collaborating with the Horsman Lab in using a biochemistry approach (HPLC-mass spec) to directly determine the amount of Pip/NHP in SAR-primed plants at normal and elevated temperatures.

Altogether, looking at biology as an integrative field has numerous benefits. Instead of an extremely narrow lens, an integrative approach allows all or most
perspectives to be explored. This offers space for better collaboration with other scientists, reduces rigidity of our thinking/paradigms, and opens numerous opportunities on how we think and learn about the natural world.
Ch 8. Summary

In this thesis, I investigated if changing temperature conditions also influence the successful deployment of plant systemic immunity via SAR, and I also elucidated the molecular mechanisms underlying this temperature-regulation. I have demonstrated that elevated temperature suppresses *Pst* DC3000 pathogen-induced SAR and downregulates systemic biosynthesis of the central SAR metabolite NHP in *Arabidopsis* plants. Remarkably, I show that temperature-suppressed SAR can be rescued following exogenous application of the NHP precursor pipecolic acid (Pip) or by constitutively expressing the master immunity transcription factor gene *CBP60g*. Overall, my findings indicate that the temperature-vulnerability of SAR in *Arabidopsis* is controlled by CBP60g through the regulation of Pip/NHP biosynthesis.

Our warming global temperatures will continue impacting the plant immune system (locally and systemically) because of the negative effect on the major plant defence hormone SA (Huot et al., 2017; Kim et al., 2022) and on the central SAR metabolite NHP. Therefore, the findings of this thesis have led to a deeper understanding of the intricacies of the plant immune landscape in relation to rising temperatures. This is key to understanding and hopefully minimizing the negative impacts of a warming climate on plant health and productivity.
Literature Cited


temperature on plant defence and bacterial virulence in *Arabidopsis*. *Nature Communications*, 8(1), 1808.


Sun, T., Busta, L., Zhang, Q., Ding, P., Jetter, R., and Zhang, Y. (2018). TGACG-BINDING FACTOR 1 (TGA1) and TGA4 regulate salicylic acid and pipoecolic acid biosynthesis by modulating the expression of SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60g (CBP60g). The New Phytologist, 217(1), 344–354.


Temp. Regulation of Pip in Plant Immunity.

A Shields.


Appendix

Figure 20. RT-PCR analysis of Arabidopsis systemic tissues following mock or pathogen (DC3000) treatment at 23°C or 28°C. Four-week-old Arabidopsis plants were infiltrated with mock (MgCl2) or DC3000 (absorbance at 600 nm = 0.001) in three local leaves and incubated at either 23°C or 28°C. Two days after infiltration, systemic tissues were collected and analyzed through RT-PCR and visualized through gel electrophoresis. For RT-PCR, ACT1 was used as a control target gene. Figures 20a-d show one biological replicate each for one experiment.
Figure 21. Analysis of *Arabidopsis* local tissue through RT-PCR following mock or Pipecolic acid treatment at 23°C or 28°C.

Four-week-old *Arabidopsis* plants were infiltrated with mock (water) in three local leaves or a 1mM solution of Pip in three local leaves. 24 hours after the infiltration the local tissues were collected and analyzed through RT-PCR and visualized through gel electrophoresis. For RT-PCR ACT1 was used as a control target gene. Figures 21a-d show one biological replicate each.
Figure 22. Transcriptome analysis of SAR-regulated genes at elevated temperature.

SAR transcriptome analysis done using genes from Hartmann et al., 2018 interfaced with the temperature RNA-Seq done at Michigan State University (Kim et al., 2022). The number of genes upregulated, downregulated, and unaffected at elevated temperature are shown (fold change cutoff > 2).
Figure 23. Transcriptome analysis of Pip-regulated genes at elevated temperature.

Pip transcriptome analysis using genes from Hartmann et al., 2018 interfaced with the temperature RNA-Seq done at Michigan State University (Kim et al., 2022). The number of genes upregulated, downregulated, and unaffected at elevated temperature are shown (fold change cutoff > 2).
Figure 24. Schematic Experimental Design of SAR Assays.

To physiologically determine the impact of SAR, an initial priming treatment of \textit{Pst} was infiltrated in the local leaves. Two days later the systemic tissue was inoculated with \textit{Pst DC3000}. Three days after that, the systemic tissue was collected, and a disease assay was done. From there, the number of colonies grown from each sample were counted and analyzed.
**Figure 25. Schematic Experimental Design of Molecular Analysis Experiments.**

All gene expression analysis experiments were completed following local inoculation of *Pst* or local infiltration of Pip. Two days later the systemic tissue was collected. In that two-day period plants were kept at either 23°C or 28°C. Using qPCR genes of interest were amplified.
Figure 26. Schematic Experimental Design of Pip Protection Assays.

To determine the impact of exogenous Pip, an initial priming treatment of Pip was infiltrated in the local leaves or applied to the roots. Two days later the systemic tissue was inoculated with *Pst* DC3000. Three days after that, the systemic tissue was collected, and a disease assay was done. From there, the number of colonies grown from each sample were counted and analyzed.
Table 8. ANOVA tables showing significance corresponding to Figure 7.

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### Table 9. ANOVA tables showing significance corresponding to Figure 8.

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**FM01** (0.02 Pet DC3000)

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<tr>
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<td>0.1672</td>
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<td>F (1, 12) = 5.035</td>
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<td>F (1, 12) = 12.42</td>
<td>F (1, 12) = 12.42</td>
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<td>F (1, 12) = 2.706</td>
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**ALD1** (0.02 Pet DC3000/AvrRpt2)

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<td>F (1, 28) = 31.33</td>
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<td>F (1, 28) = 7.62</td>
<td>F (1, 28) = 7.62</td>
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<td>F (1, 28) = 32.41</td>
<td>F (1, 28) = 32.41</td>
<td>P = 0.0001</td>
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<tr>
<td>Residual</td>
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**FM01** (0.02 Pet DC3000/AvrRpt2)

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<td>F (1, 28) = 31.33</td>
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<td>0.02944</td>
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<td>F (1, 28) = 32.41</td>
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Table 10. ANOVA tables showing significance corresponding to Figure 9.

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<tr>
<td>Interaction</td>
<td>0.005208</td>
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<td>0.005208</td>
<td>F(1, 44) = 0.08943</td>
<td>P=0.7083</td>
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<tr>
<td>Temperature</td>
<td>12.51</td>
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<td>12.51</td>
<td>F(1, 44) = 214.7</td>
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<td>3.050</td>
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<td>F(1, 44) = 52.37</td>
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<td>Interaction</td>
<td>0.3025</td>
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<td>Temperature</td>
<td>13.32</td>
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<td>F(1, 60) = 47.78</td>
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<tr>
<td>Temperature</td>
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<td>F(1, 28) = 63.47</td>
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<tr>
<td>Pathogen</td>
<td>1.351</td>
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<td>F(1, 28) = 5.925</td>
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<td>Residual</td>
<td>6.433</td>
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<td>0.2287</td>
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Table 11. ANOVA tables showing significance corresponding to Figure 10.

Local *PR1*

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<td>12.29</td>
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<td>F (1, 12) = 2.129</td>
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<td>Pathogen</td>
<td>233.5</td>
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<td>233.5</td>
<td>F (1, 12) = 6.374</td>
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<td>Residual</td>
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<td>12</td>
<td>37.22</td>
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Local *PCR1*

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<tr>
<td>Interaction</td>
<td>2.152</td>
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<td>F (1, 12) = 0.3209</td>
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<td>Temperature</td>
<td>80.71</td>
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<td>F (1, 12) = 12.63</td>
<td>P = 0.0046</td>
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<tr>
<td>Pathogen</td>
<td>14.36</td>
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<td>F (1, 12) = 2.141</td>
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Systemic *PR1*

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<td>Temperature</td>
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<td>F (1, 12) = 2.654</td>
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<tr>
<td>Pathogen</td>
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<td>360.1</td>
<td>F (1, 12) = 1.787</td>
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Systemic *PCR1*

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<td>Interaction</td>
<td>459.2</td>
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<td>459.2</td>
<td>F (1, 12) = 3.521</td>
<td>P = 0.0711</td>
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<td>Temperature</td>
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<td>F (1, 12) = 7.453</td>
<td>P = 0.0183</td>
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<tr>
<td>Pathogen</td>
<td>459.8</td>
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<td>459.8</td>
<td>F (1, 12) = 4.005</td>
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Table 12. ANOVA tables showing significance corresponding to Figure 11.

**Local ALD1**

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<tbody>
<tr>
<td>Interaction</td>
<td>1.522</td>
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<td>1.522</td>
<td>F (1, 28) = 0.9651</td>
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<td>F (1, 28) = 0.9272</td>
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<td>2.692</td>
<td>F (1, 28) = 1.701</td>
<td>P = 0.2028</td>
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<td>44.15</td>
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**Local FMO1**

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<tbody>
<tr>
<td>Interaction</td>
<td>0.05436</td>
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<td>0.05436</td>
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<td>F (1, 28) = 1.247</td>
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**Systemic ALD1**

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<tbody>
<tr>
<td>Interaction</td>
<td>0.1361</td>
<td>1</td>
<td>0.1361</td>
<td>F (1, 43) = 3.034</td>
<td>P = 0.0087</td>
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<td>F (1, 43) = 10.62</td>
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<td>F (1, 43) = 3.029</td>
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**Systemic FMO1**

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<td>F (1, 43) = 6.129</td>
<td>P = 0.0173</td>
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<td>F (1, 43) = 7.204</td>
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<td>F (1, 43) = 3.156</td>
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Table 13. ANOVA tables showing significance corresponding to Figure 12.

#### Local ICS1

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<td>0.01537</td>
<td>F (1, 28) = 4.4731</td>
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<td>Temperature</td>
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<td>0.03418</td>
<td>F (1, 28) = 1.052</td>
<td>P=0.3138</td>
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<td>0.05622</td>
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#### Systemic ICS1

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<th>MS</th>
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<th>P value</th>
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<td>0.01483</td>
<td>F (1, 14) = 0.9079</td>
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Table 14. ANOVA tables showing significance corresponding to Figure 13.

ICS1 (0.001 Pst DC3000)

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<th>P value</th>
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<td>7.343</td>
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<td>F (1, 12) = 28.74</td>
<td>P&lt;0.0002</td>
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<td>12.24</td>
<td>F (1, 12) = 47.91</td>
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ICS1 (0.02 Pst DC3000)

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<td>15.04</td>
<td>F (1, 14) = 32.61</td>
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ICS1 (0.02 Pst DC3000/AvrRpt2)

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<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>1.128</td>
<td>1</td>
<td>1.128</td>
<td>F (1, 29) = 6.872</td>
<td>P=0.0160</td>
</tr>
<tr>
<td>Temperature</td>
<td>2.749</td>
<td>1</td>
<td>2.749</td>
<td>F (1, 29) = 16.69</td>
<td>P=0.0003</td>
</tr>
<tr>
<td>Pathogen</td>
<td>1.327</td>
<td>1</td>
<td>1.327</td>
<td>F (1, 29) = 5.984</td>
<td>P=0.0982</td>
</tr>
<tr>
<td>Residual</td>
<td>4.595</td>
<td>29</td>
<td>0.1641</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 15. ANOVA tables showing significance corresponding to Figure 14.

### CBP60g (0.001 Pst DC3000)

<table>
<thead>
<tr>
<th>Source of Variation</th>
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<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.4369</td>
<td>1</td>
<td>0.4369</td>
<td>(1, 12) = 6.402</td>
<td>P = 0.0264</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.9518</td>
<td>1</td>
<td>0.9518</td>
<td>(1, 12) = 12.63</td>
<td>P = 0.0049</td>
</tr>
<tr>
<td>Pathogen</td>
<td>0.4372</td>
<td>1</td>
<td>0.4372</td>
<td>(1, 12) = 6.407</td>
<td>P = 0.0264</td>
</tr>
<tr>
<td>Residual</td>
<td>0.8189</td>
<td>12</td>
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</table>

### SARD1 (0.001 Pst DC3000)

<table>
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<th>Source of Variation</th>
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<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>5.403</td>
<td>1</td>
<td>5.403</td>
<td>(1, 12) = 22.31</td>
<td>P = 0.0005</td>
</tr>
<tr>
<td>Temperature</td>
<td>10.16</td>
<td>1</td>
<td>10.16</td>
<td>(1, 12) = 41.40</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Pathogen</td>
<td>5.403</td>
<td>1</td>
<td>5.403</td>
<td>(1, 12) = 22.08</td>
<td>P = 0.0005</td>
</tr>
<tr>
<td>Residual</td>
<td>2.938</td>
<td>12</td>
<td>0.2449</td>
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</table>

### CBP60g (0.02 Pst DC3000)

<table>
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<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>7.984</td>
<td>1</td>
<td>7.984</td>
<td>(1, 60) = 3.13</td>
<td>P = 0.0147</td>
</tr>
<tr>
<td>Temperature</td>
<td>24.85</td>
<td>1</td>
<td>24.85</td>
<td>(1, 60) = 19.60</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Pathogen</td>
<td>7.221</td>
<td>1</td>
<td>7.221</td>
<td>(1, 60) = 7.00</td>
<td>P = 0.0020</td>
</tr>
<tr>
<td>Residual</td>
<td>76.90</td>
<td>60</td>
<td>1.285</td>
<td></td>
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### SARD1 (0.02 Pst DC3000)

<table>
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<th>Source of Variation</th>
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<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>3.651</td>
<td>1</td>
<td>3.651</td>
<td>(1, 60) = 16.76</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Temperature</td>
<td>12.86</td>
<td>1</td>
<td>12.86</td>
<td>(1, 60) = 69.01</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Pathogen</td>
<td>4.588</td>
<td>1</td>
<td>4.588</td>
<td>(1, 60) = 21.06</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>13.07</td>
<td>60</td>
<td>0.2179</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### CBP60g (0.02 Pst DC3000/AvrRpt2)

<table>
<thead>
<tr>
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<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.4059</td>
<td>1</td>
<td>0.4059</td>
<td>(1, 44) = 8.216</td>
<td>P = 0.0063</td>
</tr>
<tr>
<td>Temperature</td>
<td>1.309</td>
<td>1</td>
<td>1.309</td>
<td>(1, 44) = 26.50</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Pathogen</td>
<td>0.4301</td>
<td>1</td>
<td>0.4301</td>
<td>(1, 44) = 7.06</td>
<td>P = 0.0061</td>
</tr>
<tr>
<td>Residual</td>
<td>2.174</td>
<td>44</td>
<td>0.04930</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### SARD1 (0.02 Pst DC3000/AvrRpt2)

<table>
<thead>
<tr>
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<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.01498</td>
<td>1</td>
<td>0.01498</td>
<td>(1, 28) = 3.304</td>
<td>P = 0.0790</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.05777</td>
<td>1</td>
<td>0.05777</td>
<td>(1, 28) = 12.30</td>
<td>P = 0.0013</td>
</tr>
<tr>
<td>Pathogen</td>
<td>0.00567</td>
<td>1</td>
<td>0.00567</td>
<td>(1, 28) = 12.29</td>
<td>P = 0.0016</td>
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<tr>
<td>Residual</td>
<td>0.1269</td>
<td>28</td>
<td>0.004533</td>
<td></td>
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</tbody>
</table>
Table 16. ANOVA tables showing significance corresponding to Figure 15.

**SAR (0.02 Pet DC3000)**

<table>
<thead>
<tr>
<th>ANOVA table</th>
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<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.1200</td>
<td>1</td>
<td>0.1200</td>
<td>F (1, 44) = 0.1481</td>
<td>P=0.7022</td>
</tr>
<tr>
<td>Temperature</td>
<td>16.80</td>
<td>1</td>
<td>16.80</td>
<td>F (1, 44) = 20.74</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Pathogen</td>
<td>4.813</td>
<td>1</td>
<td>4.813</td>
<td>F (1, 44) = 5.940</td>
<td>P=0.0189</td>
</tr>
<tr>
<td>Residual</td>
<td>35.65</td>
<td>44</td>
<td>0.8103</td>
<td>F (1, 44) = 20.74</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

**SAR (0.02 Pet DC3000/AvrRpt2)**

<table>
<thead>
<tr>
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<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.02344</td>
<td>1</td>
<td>0.02344</td>
<td>F (1, 92) = 0.1107</td>
<td>P=0.7401</td>
</tr>
<tr>
<td>Temperature</td>
<td>10.47</td>
<td>1</td>
<td>10.47</td>
<td>F (1, 92) = 49.45</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Pathogen</td>
<td>5.088</td>
<td>1</td>
<td>5.088</td>
<td>F (1, 92) = 24.03</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>19.48</td>
<td>92</td>
<td>0.2117</td>
<td>F (1, 92) = 24.03</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 17. ANOVA tables showing significance corresponding to Figure 16.

**ALD1 (0.02 Pst DC3000)**

<table>
<thead>
<tr>
<th>Source</th>
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<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.003963</td>
<td>1</td>
<td>0.003963</td>
<td>F (1, 44) = 9.323</td>
<td>P = 0.0038</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.004384</td>
<td>1</td>
<td>0.004384</td>
<td>F (1, 44) = 13.35</td>
<td>P = 0.0007</td>
</tr>
<tr>
<td>Pathogen</td>
<td>0.005316</td>
<td>1</td>
<td>0.005316</td>
<td>F (1, 44) = 16.18</td>
<td>P = 0.0002</td>
</tr>
<tr>
<td>Residual</td>
<td>0.0002289</td>
<td>44</td>
<td>0.0002289</td>
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<td></td>
</tr>
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</table>

**FMO1 (0.02 Pst DC3000)**

<table>
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<tr>
<th>Source</th>
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<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>1.597e-005</td>
<td>1</td>
<td>1.597e-005</td>
<td>F (1, 44) = 1.693</td>
<td>P = 0.1961</td>
</tr>
<tr>
<td>Temperature</td>
<td>1.599e-005</td>
<td>1</td>
<td>1.599e-005</td>
<td>F (1, 44) = 2.509</td>
<td>P = 0.1536</td>
</tr>
<tr>
<td>Pathogen</td>
<td>0.0001654</td>
<td>1</td>
<td>0.0001654</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>0.0002045</td>
<td>44</td>
<td>0.0002045</td>
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</tr>
</tbody>
</table>

**ALD1 (0.02 Pst DC3000/AvrRpt2)**

<table>
<thead>
<tr>
<th>Source</th>
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<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.001294</td>
<td>1</td>
<td>0.001294</td>
<td>F (1, 44) = 1.897</td>
<td>P = 0.1753</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.0004951</td>
<td>1</td>
<td>0.0004951</td>
<td>F (1, 44) = 0.0095</td>
<td>P = 0.910</td>
</tr>
<tr>
<td>Pathogen</td>
<td>5.888e-006</td>
<td>1</td>
<td>5.888e-006</td>
<td>F (1, 44) = 0.006704</td>
<td>P = 0.9261</td>
</tr>
<tr>
<td>Residual</td>
<td>0.02579</td>
<td>44</td>
<td>0.02579</td>
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**FMO1 (0.02 Pst DC3000/AvrRpt2)**

<table>
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<tr>
<th>Source</th>
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<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>7.363e-006</td>
<td>1</td>
<td>7.363e-006</td>
<td>F (1, 44) = 0.0195</td>
<td>P = 0.9051</td>
</tr>
<tr>
<td>Temperature</td>
<td>8.254e-005</td>
<td>1</td>
<td>8.254e-005</td>
<td>F (1, 44) = 0.0892</td>
<td>P = 0.4109</td>
</tr>
<tr>
<td>Pathogen</td>
<td>8.786e-005</td>
<td>1</td>
<td>8.786e-005</td>
<td>F (1, 44) = 0.7336</td>
<td>P = 0.3903</td>
</tr>
<tr>
<td>Residual</td>
<td>0.002270</td>
<td>44</td>
<td>0.002270</td>
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</tbody>
</table>
Table 18. ANOVA tables showing significance corresponding to Figure 17.

### ICS1 (0.02 Pst DC3000)

<table>
<thead>
<tr>
<th>ANOVA table</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.2553</td>
<td>1</td>
<td>0.2553</td>
<td>F (1, 44) = 1.620</td>
<td>P=0.184</td>
</tr>
<tr>
<td>Temperature</td>
<td>2.247</td>
<td>1</td>
<td>2.247</td>
<td>F (1, 44) = 10.46</td>
<td>P=0.0002</td>
</tr>
<tr>
<td>Pathogen</td>
<td>2.578</td>
<td>1</td>
<td>2.578</td>
<td>F (1, 44) = 18.89</td>
<td>P=0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>6.000</td>
<td>44</td>
<td>0.1365</td>
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</table>

### ICS1 (0.02 Pst DC3000/AvrRpt12)

<table>
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<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>0.001241</td>
<td>1</td>
<td>0.001241</td>
<td>F (1, 45) = 0.000252</td>
<td>P=0.9824</td>
</tr>
<tr>
<td>Pathogen</td>
<td>0.07041</td>
<td>1</td>
<td>0.07041</td>
<td>F (1, 45) = 0.1278</td>
<td>P=0.7224</td>
</tr>
<tr>
<td>Residual</td>
<td>24.88</td>
<td>45</td>
<td>0.5511</td>
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</tr>
</tbody>
</table>
Table 19. ANOVA tables showing significance corresponding to Figure 18.

**CBP60g (0.02 Pst DC3000)**

<table>
<thead>
<tr>
<th>ANOVA table</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>222.4</td>
<td>1</td>
<td>222.4</td>
<td>F (1, 44) = 0.6769</td>
<td>P=0.4151</td>
</tr>
<tr>
<td>Temperature</td>
<td>8.004</td>
<td>1</td>
<td>8.004</td>
<td>F (1, 44) = 0.02619</td>
<td>P=0.8722</td>
</tr>
<tr>
<td>Pathogen</td>
<td>489.5</td>
<td>1</td>
<td>489.5</td>
<td>F (1, 44) = 1.490</td>
<td>P=0.2057</td>
</tr>
<tr>
<td>Residual</td>
<td>14455</td>
<td>44</td>
<td>328.5</td>
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</tr>
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</table>

**SARD1 (0.02 Pst DC3000)**

<table>
<thead>
<tr>
<th>ANOVA table</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.1035</td>
<td>1</td>
<td>0.1035</td>
<td>F (1, 44) = 4.342</td>
<td>P=0.0454</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.1459</td>
<td>1</td>
<td>0.1459</td>
<td>F (1, 44) = 5.979</td>
<td>P=0.0180</td>
</tr>
<tr>
<td>Pathogen</td>
<td>1.076</td>
<td>1</td>
<td>1.076</td>
<td>F (1, 44) = 44.10</td>
<td>P=0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>1.073</td>
<td>44</td>
<td>0.02439</td>
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</tr>
</tbody>
</table>

**CBP60g (0.02 Pst DC3000/AvrRpt2)**

<table>
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<tr>
<th>ANOVA table</th>
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<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>744.7</td>
<td>1</td>
<td>744.7</td>
<td>F (1, 44) = 1.202</td>
<td>P=0.2673</td>
</tr>
<tr>
<td>Temperature</td>
<td>3400</td>
<td>1</td>
<td>3400</td>
<td>F (1, 44) = 5.805</td>
<td>P=0.0166</td>
</tr>
<tr>
<td>Pathogen</td>
<td>886.7</td>
<td>1</td>
<td>886.7</td>
<td>F (1, 44) = 1.503</td>
<td>P=0.2267</td>
</tr>
<tr>
<td>Residual</td>
<td>25956</td>
<td>44</td>
<td>589.9</td>
<td></td>
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</tr>
</tbody>
</table>

**SARD1 (0.02 Pst DC3000/AvrRpt2)**

<table>
<thead>
<tr>
<th>ANOVA table</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.01570</td>
<td>1</td>
<td>0.01570</td>
<td>F (1, 44) = 0.4260</td>
<td>P=0.5174</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.07928</td>
<td>1</td>
<td>0.07928</td>
<td>F (1, 44) = 2.162</td>
<td>P=0.1465</td>
</tr>
<tr>
<td>Pathogen</td>
<td>0.2125</td>
<td>1</td>
<td>0.2125</td>
<td>F (1, 44) = 5.708</td>
<td>P=0.0200</td>
</tr>
<tr>
<td>Residual</td>
<td>1.621</td>
<td>44</td>
<td>0.03605</td>
<td></td>
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