

INTERROGATING PLANT IMMUNE SIGNALING PATHWAYS AND ELEVATED
TEMPERATURE SENSITIVITY IN *ARABIDOPSIS THALIANA*

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ABSTRACT

Elevated temperatures a few degrees above the average will occur more often in the coming decades, posing a unique threat to plant's survival that evolved for cooler temperatures. Temperature influences many elements of a plant's immune system, which has been increasingly under threat as pathogens expand their range. Previous research has highlighted that the plant defense hormone salicylic acid is disrupted at elevated temperature in numerous plant species in response to pathogens. Research in *Arabidopsis thaliana* has identified molecular mechanisms contributing towards compromised SA biosynthesis and signaling which enhances their susceptibility to the bacterial pathogen *Pseudomonas syringae* pathovar. tomato DC3000. Understanding how elevated temperature compromises SA biosynthesis and signaling will be key for developing solutions to enable plants to have robust temperature tolerance and survive pathogens.

In this dissertation, I will highlight how plants respond to elevated temperature and pathogen infection and examine the cross-section of this interaction through understanding plant stress hormones and how they interact with the immune system. Secondly, this thesis highlights research underpinning: 1). How elevated temperature interferes with upstream signaling elements of pathogen perception mechanisms that contribute towards SA biosynthesis and 2) How does supplementing exogenous SA in the form of benzothiadiazole (BTH) maintains plant immunity at elevated temperature despite the loss of canonical signaling like gene expression of *PATHOGENESIS RELATED1 (PR1)*.

This research is explored through the lens of the *Arabidopsis thaliana* –

Pseudomonas syringae pv. tomato DC3000 plant-pathosystem. This study revealed that Pattern Triggered Immunity (PTI), the upstream plant perception mechanism that perceives conserved epitopes of pathogens, is weakened by elevated temperature. By using flg22, a pathogen derived conserved peptide, I identified that $[Ca^{2+}]$ flux and signaling are compromised at elevated temperature, and several defense outputs appear compromised that depend on $[Ca^{2+}]$ signaling. Salicylic acid has putatively been linked to $[Ca^{2+}]$ -dynamics and this thesis investigates how temperature modulation of $[Ca^{2+}]$ would interfere with SA biosynthesis and signaling.

Furthermore, this study identified that the $[Ca^{2+}]$ -independent branch of immunity remains robust across the temperature range in this study. By investigating this temperature insensitive immune-signaling pathway, I revealed that their immune outputs can be enhanced at elevated temperature in response to BTH. This provides a novel framework for identifying how exogenous SA can prime a plant immune system in the absence of canonical signaling responses.

This dissertation is dedicated to my family, friends, and fellows who kept me on the path with unfaltering faith. You'll never know how much you helped.

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LIST OF ABBREVIATIONS

ABA	Abscisic Acid
BTH	Benzothiadiazole
[Ca ²⁺]	Calcium ions
CaM	Calmodulin
C(D)PK	Calcium Dependent Protein Kinase
cfu	colony forming units
CML	Calmodulin Like Protein
Col-0	<i>Arabidopsis thaliana</i> ecotype Columbia
ET	Ethylene
ETI	Effector Triggered Immunity
flg22	22 amino acids derived from bacteria flagella
JA	Jasmonic Acid
HR	Hypersensitive Response
LRR-RLK	Leucine Rich Repeat Receptor Like Kinase
NB	Nucleotide Binding
M(A)PK	Mitogen Activated Protein Kinase
OD	Optical Density
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
<i>Pst</i>	<i>Pseudomonas syringae</i> pathovar tomato
PTI	Pattern Triggered Immunity
qPCR	quantitative Polymerase Chain Reaction

RLCK	Receptor-Like Cytoplasmic Kinase
ROS	Reactive Oxygen Species
SA	Salicylic Acid
TF	Transcription Factor

CHAPTER 1 INTRODUCTION

1.1 The changing climate and its direct impact on plants

1.1.1 The influence of climate change on our agricultural systems

Anthropogenic-induced climate change poses a large-scale issue for the survival of plants, the basis of the food chain for terrestrial ecosystems and human civilization. The increased release of greenhouse gasses like CO₂ from fossil fuel use alongside shifts in land use and agricultural practices pose many severe issues for plant survival in the coming decades (Stern and Kaufmann, 2013). The results of these activities pose distinct and overlapping risks that threaten our agricultural systems and hurt plant health; elevated levels of CO₂, an increase in global temperature averages, and more frequent weather extremes, which ultimately drive changes in environmental factors like water availability and soil erosion (Romero *et al.*, 2022, Bibi and Rahman *et al.*, 2023). These environmental factors have many indirect emerging effects on the pressures to agriculture, such as increased distribution and emergence of plant pathogens and pests (Singh *et al.*, 2023, Franke *et al.*, 2022). By the end of the century, average temperatures are predicted to be 2.6-4.3°C higher, with temperatures rising even higher during the growing season (Domeisen *et al.*, 2023). Not only does the average shift under future climate models, the frequency and intensity of temperature swings, such as heat waves and cold snaps, will increase in frequency, challenging plants to a broader range of temperatures during key crop developmental periods like anthesis and grain filling stages (Bathiany *et al.*, 2018, Cheabu *et al.*, 2018).

The timing of these environmental and biotic stresses may compound, leading toward catastrophic crop losses. In the last 30 years, heat waves have been attributed to 10-20% losses in wheat production, and 8-12% losses in maize, with future

predictions looking more pessimistic. Under a climactic model predicting a 2.8°C – 3°C increase in average temperatures, there is an expected 25% loss in maize productivity, 15% yield reduction in rice, but modest increases in cool weather crops like wheat, which will see their production range expand significantly in high latitudes (Jägermeyr *et al.*, 2021, Cao *et al.*, 2022). Even with the range expanding for some of these crops, there are still new threats to crops in these novel growing regions. This is compounded by pathogen and pest pressures, which currently make up about 20-40% crop yield losses annually, roughly equaling \$220 billion in losses (IPCC report, 2021). This combination of warming temperature mixed with increased disease pressure from rapidly evolving pathogens presents a massive obstacle for maintaining high yields with a growing global population.

To mitigate these losses in the future under a less certain climate, it is paramount to understand how the environment and plants coordinate and respond to diseases in an environment-dependent manner. Research across recent decades has identified numerous molecular mechanisms underpinning how plants both perceive and respond to stressors in isolation, both abiotic and biotic. While these discoveries have greatly increased genetic resources to develop more productive and resilient crops, many of these discoveries have typically focused on binary plant-stress interactions. Understanding how temperature increases will impact a plant's ability to respond to heightened pathogen pressure will be one of the key determinants towards developing temperature-resilient tools to maintain high agricultural yields for a growing global population. This introduction will explore 3 main areas of focus 1). How plants respond to heightened temperature and how they cope with co-occurring abiotic stress, 2). How

the plant immune system perceives and responds to pathogens and pests and how hormones shape these responses and 3). How interactions between elevated temperatures influence interactions with plant defense and immunity.

1.1.2 Mechanisms plants utilize to respond to elevated temperature

As sessile organisms, plants must face and endure environmental stressors to successfully grow and reproduce. To respond to the environment, the plant must first perceive those changes and transduce that perception into a signal that enables the plant to alter its physiology through structural, chemical, and genetic mechanisms. The specific environmental stressors may have unique impacts on plant physiology, but many pathways employ converged strategies like hormone signaling, secondary messengers, or physiological structures to cope with stress. Nevertheless, plants must be able to first integrate these signals and respond accordingly to maximize their survival.

Plants have multifaceted methods to respond to temperature changes in the environment. While climate models predict increases in ambient temperature, this may also take the form of varying extremes in temperature. Either effect will perturb plant growth patterns in the environment and agronomic contexts. Plants respond to warming conditions 1-2°C above their optimum through a combination of mechanisms. One such mechanism integrates light signaling into temperature perception through plant phytochromes. Phytochromes are red and far-red light-sensitive proteins that change their conformations in response to the ratio of the light signals to induce light-dependent or shade-adapted responses (Quail 2002, Li *et al.*, 2011). Phytochromes adopt an active state under red light and are inactive under far-red light conditions, and this

conversion between active and inactive states is temperature dependent. As temperatures increase, phytochromes undergo a higher rate of conversion to the inactive state which allows plants to perceive slight variations in temperature and alter their growth and metabolism (Legris *et al.*, 2016, Jung *et al.*, 2016). Since phytochromes serve as an integrator of light and temperature, plant physiological changes associated with elevated temperature and plants grown in shade conditions are highly similar. This photomorphogenesis and thermogenesis is characterized by petiole and internode elongation, an upward angling of leaves to cool the plant or achieve more optimal light conditions, thinner leaf lamina, reduced chlorophyll content, greater stomata density, and shortened time to flowering (Quint *et al.*, 2018, Chen *et al.*, 2022). To induce these changes, phytochrome proteins physically interact with PHYTOCHROME INTERACTING FACTORS (PIFs). Active phytochromes repress nuclear localization of PIF and prevent their transcriptional activity, whereas inactive phytochromes at elevated temperature or under shade conditions disassociate from PIFs, allowing them to regulate shade and elevated temperature-controlled genes (Pfeiffer *et al.*, 2012). PIF4 and PIF7 play a predominant role in thermomorphogenesis whereas PIF1,3,4,5 and 7 play a major role in shade induced growth (Fiorucci *et al.*, 2019, Ciolfi *et al.*, 2013). PIFs positively regulate plant growth hormones auxin and gibberellin, alter signaling through the circadian clock, and the photosynthetic apparatus to give rise to the physiological changes to better tolerate heat and shade (Franklin *et al.*, 2011, Gray *et al.*, 1998, Sun *et al.*, 2013).

Plants can perceive $\sim 1^{\circ}\text{C}$ temperature changes and alter their genetic regulation via temperature kinetics of protein-protein interactions between transcription factors.

Recent research has identified that liquid-liquid phase separation (LLPS) serves as a novel mechanism for temperature perception to occur. Regions of high and low “solute density”, in this instance referring to organic molecules like DNA and peptides, can assemble and associate with varying affinities based on temperature-based condensation (Field *et al.*, 2023). LLPS was shown to regulate EARLY FLOWERING 3 (ELF3), a key regulator of the evening complex of the plant circadian clock, by regulating bio-condensates of this peptide with either its co-repressor or activator. ELF3 is negatively regulated by LUX ARRHYTHMO (LUX) and positively regulated by ELF4 to drive expression of evening clock genes like PIF4 (Nusinow *et al.*, 2011). In brief, ELF3 self-associates with the positive regulator ELF4 through ELF3’s prion-like domains which are temperature sensitive. Target genes of ELF3 display compromised expression at 30 – 35°C and overexpressing ELF4 removed temperature sensitivity. This model provides a mechanism through which transcriptional control is directly temperature dependent (Jung *et al.*, 2020). The use of prion-like domains to perceive temperature is intriguing and may be an emerging theme for perceiving small changes in temperature, where the environmental conditions alter the physical properties of transcriptional units, thereby physically gating specific genetic responses behind the appropriate stimulus.

While the average shift in temperatures will be higher in the coming decades, large fluxes in daily temperature will dramatically challenge plant survival in agricultural and economic contexts. Heat stress is defined by temperatures significantly above its optimal growing temperature and cause irreversible damage to plant growth (Wahid *et al.*, 2007). Extreme heat permeabilizes plant membranes which negatively impacts

photosynthesis by generating reactive oxygen species (ROS), resulting in a substantial decrease in photosynthesis (Sharkey 2005, Djanaguiraman *et al.*, 2018, Song *et al.*, 2014). High temperatures negatively effects plant reproductive capacity via multiple mechanisms. Heat stress results in earlier flowering through alteration of the circadian cycle of plant growth, leading to a mistiming of key reproductive events and reduced yield (Zinn *et al.*, 2010). Prolonged periods of heat result in enhanced flower abscission, drastically reducing plant yield from failed floral development (Monterroso and Wien 1990). Pollen germination is highly temperature sensitive across a range of plant species, resulting in failed fertilization events that lead to aborted fruits or non-viable pollen, ultimately reducing plant fecundity (Mesihovic *et al.*, 2016, Shenoda *et al.*, 2021). The primary means by which plants handle extreme heat is through the thermos-stabilizing heat-shock proteins (HSPs). HSPs like HSP70 and HSP90 are serve several roles under multiple biotic and abiotic stressors to stabilize the physical structure and function of proteins (Xu *et al.*, 2012 Haq *et al.*, 2019). HSPs are under transcriptional control by HEAT SHOCK FACTORS (HSFs), which bind to Heat shock elements (HSEs) in promoter regions of these and other chaperone proteins to maintain stabilization and mitigate oxidative damage to key proteins to maintain their function (Xu *et al.*, 2012, Guo *et al.*, 2016). Furthermore, plants generate osmoprotectant molecules like proline, glycine, and trehalose which help to mitigate oxidative radicals and enable the plant to conserve more water and prevent cellular damage (Sabagh *et al.*, 2021).

While plants experience their highest temperatures during diurnal phases, the timing of elevated temperature events is equally as important as the magnitude of temperature changes. Plants experience significantly greater heat stress during evening

hours, impacting carbon metabolism and transport, and water usage due to heightened evapotranspiration (Sadok *et al.*, 2020, Yang *et al.*, 2023). Nighttime temperatures have been rising faster than diurnal temperatures, imposing a unique challenge toward mitigating plant heat stress in the field (Cox *et al.*, 2020). Warm evenings limit nutrient availability due to the soil drying out faster, reducing soluble mineral uptake and root-shoot transport (Giri *et al.*, 2017). This evening heat also disrupts plant's circadian clocks, interfering with timing appropriate developmental phases of growth and reproduction (Laosuntisuk and Doherty 2022, Desai *et al.*, 2021).

1.2.3 Combinatorial crosstalk of heat with drought and elevated CO₂

Combinatorial environmental stressors will have compounding negative effects on many plant species, but these environmental effects will be felt unevenly in a species-specific, temporal, and geographic context (Zandalinas and Mittler 2022). Many landraces and cultivars of various agronomic species like maize, bean, and rice have been developed for specific growing conditions to optimize yield in diverse geographic regions (Macholdt *et al.*, 2016, Bai *et al.*, 2022, van Etten *et al.*, 2019). However, the tools and cultivars developed for our current agricultural system may not be suitable for the increasing number of stressors that will be placed on our developed varieties. Furthermore, our natural systems are endangered both due to biodiversity loss, coupled with changing climates, which limit the flexibility and recoverability of these systems if they reach an overwhelming number of environmental stressors (Carrier-Belleau *et al.*, 2021, Pascual *et al.*, 2022).

Ambient increases in CO₂ induces stomatal closure in the short term and decreases the total number of stomata under longer periods of development (Xu *et al.*,

2016). These changes in stomatal conductance negatively impact plant tolerance to heat, where the CO₂-induced closure of stomata competes with evapotranspiration-induced leaf-cooling (Hamilton *et al.*, 2008). This may in-turn influence RUBISCO activity and photorespiration, while also leading to enhanced oxidative damage, ultimately resulting in stunted growth and reduced yield. This has been seen in rice where increases in temperature exacerbates grain filling under elevated CO₂ conditions (Chaturvedi *et al.*, 2017) and is further exacerbated by modest increases in temperature in C3 plants (Hamilton *et al.* 2008). Longer temporal studies with modified CO₂ levels and more dynamic environmental systems revealed that elevated CO₂ does not benefit plant photosynthesis in the long term (Luenziger *et al.*, 2011). Furthermore, the enhanced photosynthetic capacity of elevated CO₂ is compromised by both warming temperature, and excessive moisture or under drought conditions.

Temperature and water availability is thought to be one of the greatest multi-factorial stresses influencing our ecosystems and agriculture. Warm air has greater water capacity and thermo-cooling capacity relative to dry air. At >32°C temperatures, the “heat-bulb” phenomenon has serious detrimental effects on plants and animals alike, where cooling adaptations are insufficient to cool the organism due to similar temperatures between the organism and the environment (Wosula *et al.*, 2015, Khalifa 2003). Plants reduce thermoresponsive factors for cooling under high humidity, minimizing Absciscic Acid (ABA) governed responses and heat-shock elements (Georgii *et al.*, 2017). However, under dry hot temperatures, evapotranspiration in soils is accelerated, leading to earlier onset of drought during heat waves. Earlier onset of warm weather during growing seasons may help plants tolerate dry conditions later due

to earlier emergence while water resources are still available (Fahad *et al.*, 2017). When planting is timed poorly in the growing season, along with drought or heat susceptibility in climate optimized cultivars, these putative breeding benefits can be lost as seen in wheat (Zhao *et al.*, 2022). Developing varieties of plants that can better tolerate heat and drought at specific stages of their development will be key to maintaining suitable yields despite greater disruptive conditions.

1.2 Plant immunity and defense hormone signaling

1.2.1 Pattern Triggered Immunity

To initiate defense against a microbial pathogen, the plant first perceives this threat by recognizing conserved patterns on the microbe, triggering the first branch of the plant immune system known as Pattern-Triggered Immunity (PTI) (Thomma *et al.*, 2011). PTI is activated through a series of chronological events known as a “cell-autonomous pathway” and initiates a series of interactions between proteins and secondary molecules to trigger a plant immune response against detrimental organisms, both bacterial and eukaryotic. The conserved molecules of microbes recognized by plants are known as pathogen-associated molecular patterns (PAMPs) (Zhang and Zhou, 2010). Some PAMPs are derived from microbial proteins such as the conserved 22-amino acid peptide derived from bacterial flagellin (flg22), or the conserved 18 amino acid peptide derived from bacterial elongation factor-Tu (elf18); while other PAMPs can be derived from components of the microbial cell wall, such as bacterial peptidoglycan or fungal chitin (Felix *et al.*, 2002, Kunze *et al.*, 2004, Wan *et al.*, 2008). Furthermore, plant cells can perceive biotic stressors indirectly by sensing cellular damage, such as the leakage of the amino acid glutamate, or via the proteolysis

of other signaling peptides to induce proper responses against the corresponding plant predator (Hou *et al.*, 2019).

PAMPs are perceived directly via a series of molecular receptors located along the outer membrane of the plant cell. These receptors are known as Pattern Recognition Receptors (PRRs), which come in the form of leucine-rich repeat receptor like kinases (LRR-RLK) and receptor-like proteins (RLP) (Tang *et al.*, 2017). Many of these receptors are composed of an extracellular facing binding domain with an internally facing kinase complex that can initiate the molecular cascade of events required to activate a cellular response to the stressor. The recognition of receptors is typically one-to-one and often requires receptor-co-receptor complex to initiate downstream responses. Some examples of these PAMP-PRR receptor pairings are flg22 is perceived by FLAGELLIN SENSING 2 (FLS2), elf18 via ELONGATION FACTOR THERMO-UNSTABLE RECEPTOR (EFR), proteinaceous plant elicitor 1 (AtPEP1) to the PEP1 RECEPTOR (PEPR1) (Chinchilla *et al.*, 2006, Zipfel *et al.*, 2006, Yamaguchi *et al.*, 2006). Other PRRs such as CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1), contains extracellular LysM motifs, which recognize fungal chitin and bacterial peptidoglycans (Gimenez-Ibanez *et al.*, 2009). Upon PAMP detection, the PRR interacts with a co-receptor, such as BRASSINOSTEROID INSENSITIVE 1 – ASSOCIATED RECEPTOR KINASE 1 (BAK1) or LysM-CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5) and undergo auto and transphosphorylation events between receptor and co-receptor (Chinchilla *et al.*, 2007, Roux *et al.*, 2011, Cao *et al.*, 2014). Shortly after PAMP-receptor association, the cytoplasmic receptor like kinases (RLCKs) like BOTRYTIS-INDUCED KINASE 1 (BIK1) are recruited and phosphorylated (Lu *et al.*,

2010). BIK1 is a key internode of propagating the immune signal, which leads to phosphorylation and activation of mitogen-activated protein kinases (MAPKs) to induce downstream transcriptional reprogramming and ultimately induce plant immunity (Meng and Zhang 2013). Additionally, BIK1 interacts with and phosphorylates RESPIRATORY BURST NADPH OXIDASE HOMOLOG D (RBOHD) and RBOHF to initiate production of ROS (Li *et al.*, 2014). It is believed that many of these PRRs and RLKs scaffold together to perceive other biotic and abiotic stressors, such as through FERONIA (FER) to form large receptor complexes that allow the plant to detect and rapidly respond to key stressors and provide an appropriate response (Duan *et al.*, 2022).

Upon activation of the PRR complexes, cytoplasmic concentrations of calcium $[Ca^{2+}]$ rise rapidly from external and internal sources (Pirayesh *et al.*, 2021). Several $[Ca^{2+}]$ -channels have been recognized to play a role in PTI and plant immunity, such as CYCLIC NUCLEOTIDE GATED CHANNEL 2 (CNGC2) and CNGC4 along with GLUTAMATE LIKE RECEPTOR 2.7, GLR 2.8 and GLR2.9 (Tian *et al.*, 2019, Bjornson *et al.*, 2021). This $[Ca^{2+}]$ is perceived by various calmodulin (CaM) and calmodulin like proteins (CMLs) which bind to and transduce the $[Ca^{2+}]$ signature to activate appropriate cellular processes. The exact CaMs and CMLs involved in PTI are only now being identified on a targeted basis but has remained difficult with reverse genetics approaches due to extreme overlap in similarity between these proteins (Zhu *et al.*, 2017, Lu *et al.*, 2018). However, it is likely that this high diversity allows for tissue specificity and timing of developmental cues as the plant grows. In addition to CaM, calcium can be perceived directly by Calcium dependent protein kinases (CDPKs), which go on to directly phosphorylate and activate other important immune regulators.

CDPK5 has been shown to directly phosphorylate RBOHD and induce ROS production (Dubiella *et al.*, 2013). CPK4, 5 6, and 11 to phosphorylate transcription factors regulating plant immunity (Boudsocq *et al.*, 2010, Dubiella *et al.*, 2013) (Figure 1).

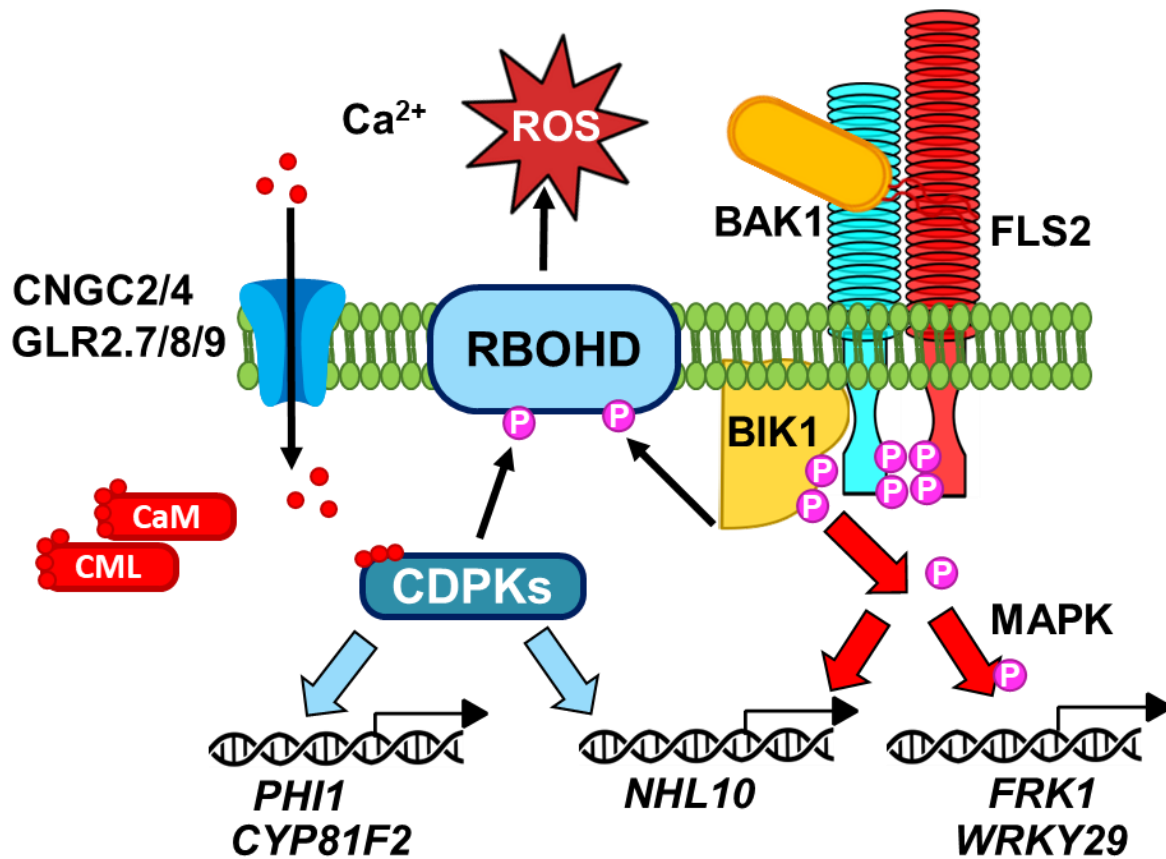


Figure 1. Basic framework for PTI signaling. PTI is initiated by ligand-specific plasma membrane-bound receptor-like kinases like FLAGELLIN SENSITIVE 2 (FLS2) and BRASSINOSTEROID INSENSITIVE-1 (BAK1) to perceive conserved molecular epitopes of pathogens like the bacterial flagella. This perception triggers a kinase cascade through the cytoplasmic kinase BOTRYTIS-INDUCED KINASE 1 (BIK1), which activates downstream gene expression and mediates ROS by phosphorylating NADPH/RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD). Furthermore, membrane bound calcium channels like CYCLIC NUCLEOTIDE GATED CHANNEL 2/4 (CNGC2/4) and GLUTAMATE RECEPTOR 2.7 (GLR2.7) are activated to flood the cytoplasm with [Ca²⁺]. These ions are perceived directly through calcium binding domains on various signaling proteins like CALCIUM DEPENDENT PROTEIN KINASE (CDPK) and interpreted through calmodulins (CaM) and calmodulin-like proteins (CML) to regulate immune responses like ROS and other overlapping downstream immune responses.

1.2.2 Effector Triggered Immunity

Pattern-triggered Immunity is often a target of attack by plant pathogens, where bacterial effector proteins are injected into the plant cell to target and disrupt various elements of PTI to minimize plant defense responses (Zhang *et al.*, 2022). In turn, plants have evolved a sophisticated detection methods for perceiving pathogen effector proteins to induce a stronger and more sustained branch of defense response, collectively known as Effector Triggered Immunity (ETI). This results in localized cell death, known as the hypersensitive response (HR), to minimize plant infection and reduce pathogen spreading. ETI is initiated by cytoplasm located nucleotide-binding leucine rich repeat receptors (NB-NLRs) which directly or indirectly perceive pathogen infection (Dangl and Jones, 2001). There are two types of NLRs, those containing a Toll/interleukin-1 domain, (TIR)-NLRs or a coiled-coil domain (CC-NLR). Upon activation of ETI, there is a strong sustained activation of the plant immune response, characterized by sustained levels of ROS and $[Ca^{2+}]$, strong MPK3/MPK6 activation, production of plant defense hormones, and inducing genes affiliated with plant immunity (Nguyen *et al.*, 2021).

It has recently been discovered that activating ETI through the NBS-LRR result in an assembly of a large heteromeric protein complex, such as those identified with CNL HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) (Wang *et al.*, 2019, Huang *et al.*, 2023). In brief, upon detection of the effector AvrAC from *Xanthomonas campestris* pv. *campestris*, the RESISTANCE RELATED KINASE 1 (RSK1) indirectly detects AvrAC activity and initiates a uridylation cascade through PBL2 to assemble large oligomeric pore-forming complexes known as a resistosome, similar to the inflammasome

complexes often observed in mammalian immune systems (Wang *et al.*, 2019). The resistosome associates with the plasma membrane and forms a negatively charged pore, putatively involved in the transfer of non-specific cation transfer into the plant cell, driving a sustained $[Ca^{2+}]$ flux that would result in ETI. While PTI has known $[Ca^{2+}]$ channels involved in activating short term immunity induction, the identity of ETI-specific channels that are not affiliated with the resistosome complex remain enigmatic.

PTI and ETI share similar downstream response characteristics, but ETI exhibits stronger and sustained outputs compared to PTI, such as ROS burst, MAPK activation, cytoplasmic $[Ca^{2+}]$ bursts, and transcriptional reprogramming for induced defense responses like antimicrobial biosynthetic genes, and the production of the plant hormones SA, JA, and ET which play a role in maintaining a robust response and gating the appropriate levels of defense signaling components (Li *et al.*, 2019). The interplay between PTI and ETI has begun to be united whereby ETI depends significantly on PTI activation. ETI activation by an inducible transgenic system expressing the avirulence gene AvrRpt2 in Arabidopsis (in the absence of PTI activation/bacteria) was absent. However, when presented with a PAMP or nonpathogenic bacteria, full ETI symptoms like HR and ROS returned (Ngou *et al.*, 2021). Additionally, the PTI-compromised triple Arabidopsis mutant *bak1/bkk1/cerk1* (*bbc*), which has mutations in three PRR co-receptors (Xin *et al.*, 2016) was significantly reduced in ROS, HR and ETI-controlled gene expression when using the AvrRpt2 inducible promoter system. These experiments show how ETI depends on functional PTI processes to achieve full immunity (Yuan *et al.*, 2021). While this is an intriguing discovery that unifies the two branches of plant immunity, the direct connection between these two pathways is still

being revealed.

1.2.3 Plant hormones are stress signal integrators to enable plant survival during infection.

Plant hormones are small molecules that serve as molecular messengers to integrate environmental and biotic stimuli into transcriptional reprogramming of plant cells to better optimize their physiology to aid survival. Plant hormones are involved in several elements of growth, development, reproduction, response to stress and disease, to name but a few of their functions. Additionally, many hormones have synergistic and antagonistic roles of plant physiology, such as the multifaceted cooperation between growth hormones and the antagonism viewed through the “growth-defense tradeoff” and immune signaling (Niels *et al.*, 2020, He *et al.*, 2022, Huot *et al.*, 2014).

Salicylic Acid (SA) is an important hormone that regulates a multitude of stress responses but has been primarily implicated in defense against biotrophic pathogens. Mechanisms used to enhance SA-responses in plants like the synthetic analog benzothiadiazole (BTH) or genetic approaches such as over activating the SA signaling pathway confer broad resistance to agricultural pests and pathogens (Görlach *et al.*, 1996, Oldroyd and Staskawicz, 1998, Cao and Li, 1998, Liang *et al.*, 2022). Additionally, SA biosynthesis is a hallmark of PTI and ETI activation but at different magnitudes and are partially responsible for differences in immune responses (Tsuda *et al.*, 2008, Gao *et al.*, 2013). Activating SA-dependent pathways results in an upregulation of PTI and ETI interdependent components like MPK3/6, RBOHD, and the FLS2-BAK1 receptor complex (Beckers *et al.*, 2009, Tateda *et al.*, 2014, Xu *et al.*, 2014). Due to the

multifaceted nature of SA in disease resistance, understanding how the SA pathway is affected by other hormone signaling pathways and the environment will be crucial for mitigating disease outbreaks and minimizing yield losses from pathogens.

SA is synthesized in the chloroplast in *Arabidopsis* via the isochorismate pathway. ISOCHORISMATE SYNTHASE 1 (ICS1) catalyzes the conversion of chorismate to isochorismate in the chloroplast and is then transported to the cytosol by the transporter ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) (Wildermuth *et al.*, 2001, Nawrath *et al.*, 2002). AvrPphB SUSCEPTIBLE 3 (PBS3) and ENHANCED *PSEUDOMONAS* SUSCEPTIBLE 1 (EPS1) then induce the conversion of isochorismate into salicylic acid in the cytoplasm (Torrens-Spence *et al.*, 2019). Salicylic acid can also be conjugated to various molecules, notably glucose, to form glycosylated-SA (SAG) to be transported to the vacuole or can be methylated by SALICYLATE 1-O-METHYLTRANSFERASE (BSMT1) to be released as gaseous Methyl-SA (George Thompson *et al.*, 2017, Attaran *et al.*, 2009).

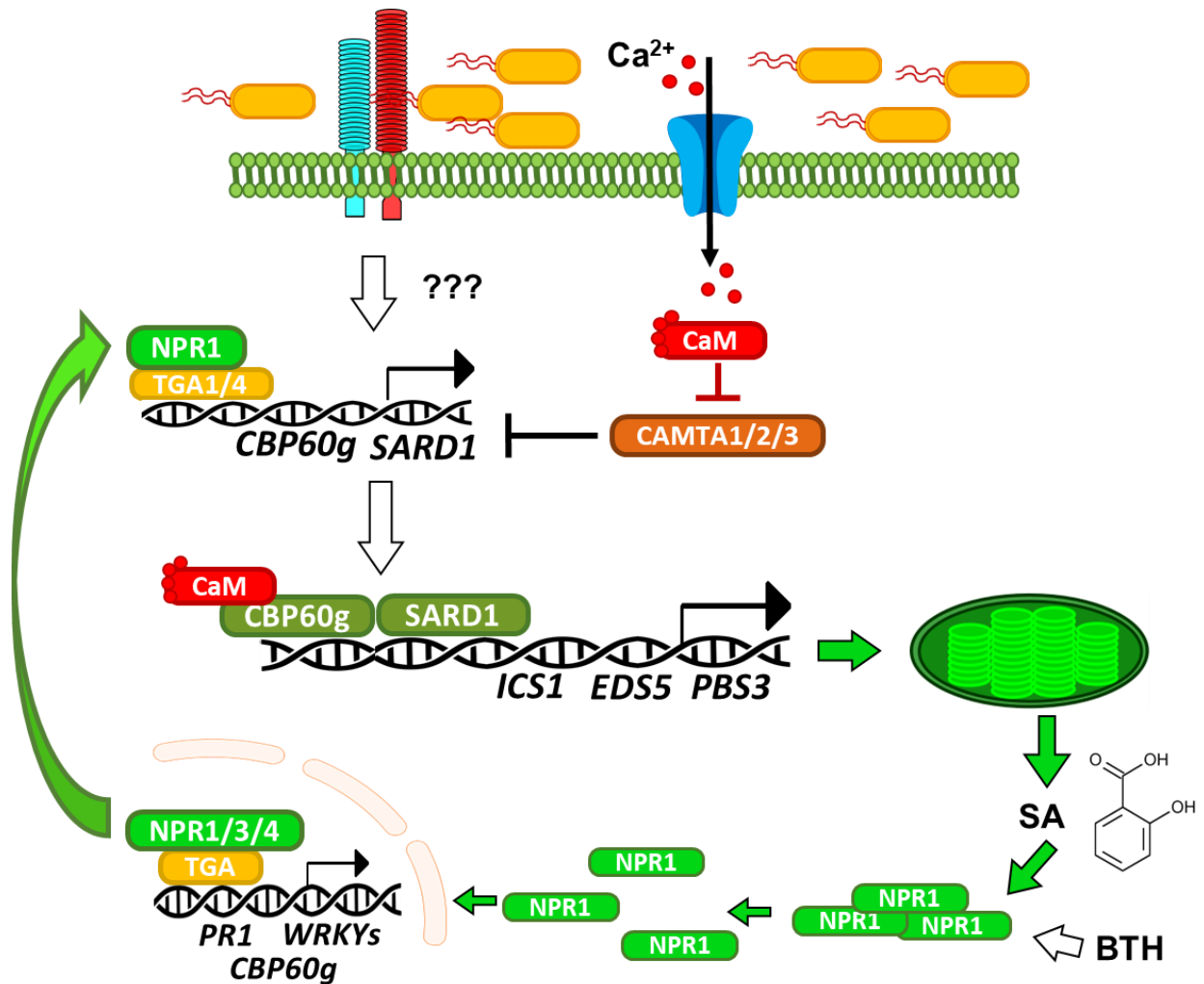


Figure 2. Overview of SA biosynthesis and signaling in *Arabidopsis*. The pathway from pathogen perception via PTI to initiating SA biosynthesis remains to be fully elucidated. After pathogen perception through PTI, the SA biosynthesis regulatory TFs CALMODULIN BINDING PROTEIN 60g (CBP60g) and SAR DEFICIENT 1 (SARD1) are induced. CBP60g and SARD1 are partially redundant TFs that depend on CaM binding and induce expression of the SA biosynthetic genes ISOCHORISMATE SYNTHASE 1 (ICS1), ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) and AvrPphB SUSCEPTIBLE 3 (PBS3) undergo the conversion of chorismate to salicylic acid through the chloroplast and cytoplasm. The release of SA is perceived by the master SA regulatory TF NONEXPRESSOR OF PR1 (NPR1), via oxidation of NPR1 oligomers which enables NPR1 to enter the nucleus. NPR1, along with NPR3 and 4 interact with TGA2/3/5/6 TFs to induce immune responsive genes like *PR1*. CBP60g and SARD1 expression is partially controlled by NPR1 and TGA1/4 TFs and facilitates an “amplification loop” of SA biosynthesis and signaling. CALMODULIN BINDING TRANSCRIPTIONAL ACTIVATOR (CAMTA) TFs negatively regulate CBP60g, SARD1 expression, inhibiting SA biosynthesis and downstream SA signaling elements under typical *Arabidopsis* growing conditions.

SA biosynthesis is under the control of two partially redundant transcription factors CALCIUM BINDING PROTEIN 60G (CBP60g) and SAR DEFICIENT 1 (SARD1), which bind to the promoters and positively regulate expression of ICS1, PBS3, and EPS1 (Torrens-Spence *et al.*, 2019). However, the transcriptional control of CBP60g and SARD1 is not entirely clear under pathogen infection. In the autoactive defense mutant *snc2-1D*, WRKY54 and WRKY70 positively regulate CBP60g/SARD1 (Chen *et al.*, 2021). The RLCKs PCRK1 and PCRK2 positively regulate CBP60g/SARD1 gene expression after pathogen infection (Kong *et al.*, 2016). Prior to perceiving a stimulus that induces SA biosynthesis, the negative regulators CALMODULIN BINDING TRANSCRIPTION ACTIVATOR 1 (CAMTA1), 2 and 3 inhibit CBP60g/SARD1 expression (Sun *et al.*, 2020). Upon pathogen infection and other abiotic stimuli like cold stress CAMTA1/2/3 relieve their repression of CBP60g/SARD1 and enable SA biosynthesis in response (Kim *et al.*, 2013). While it has been hypothesized that CAMTA negative regulation is dependent on $[Ca^{2+}]$ and calmodulin (CaM) binding to CAMTA is required to relieve negative regulation of SA biosynthesis, it appears that CAMTA's negative regulation is not dependent on CaM binding (Liu *et al.*, 2015, Kim *et al.*, 2017).

SA is perceived by the SA receptor and transcriptional activator NONEXPRESSOR OF PR1 (NPR1). NPR1 exists in an oligomeric form in the cytosol, but at the same time as SA biosynthesis occurs, a redox event induces monomerization of NPR1, which is subsequently transported into the nucleus (Mou *et al.*, 2003, Tada *et al.*, 2008). The other SA perceiving transcriptional regulators, NPR3 and NPR4 co-repress NPR1 and induce degradation under a low or significantly elevated SA

environment (Ding *et al.*, 2018). When SA is present, NPR3 and NPR4 relieve their repression of NPR1 and enables interactions with the TGA transcription factors. NPR1 interacts with TGA2/3/5/6 to drive expression of defense related genes, redox regulation genes, and marker genes like *PATHOGENESIS RELATED PROTEIN 1 (PR1)* (Zhou *et al.*, 2000 Zhang *et al.*, 2003, Fonseca *et al.*, 2022). Intriguingly, NPR1 also targets the promoters of CBP60g and SARD1 through interacting with TGA1/4, demonstrating that NPR1 participates in an amplification loop of SA biosynthesis (Sun *et al.*, 2018). Since CBP60g induce negative regulators of SA biosynthesis like NUDT6, the SA signaling pathway can self-regulate via induction of both positive and negative regulators to tailor the SA response (Sun *et al.*, 2018).

While SA plays a substantial role in plant defense against biotrophic pathogens, JA plays a major role directing plant responses to insects and synergistically acts with ethylene to defend against necrotrophic pathogens (Koo *et al.*, 2009, Lorenzo *et al.*, 2003). Jasmonate is synthesized in the chloroplast, deriving its biosynthesis from linoleic acid via the lipoxygenase (LOX) pathway (Wasternack and Feussner 2018). JA is synthesized rapidly after detecting cellular wounding or perception of insect elicitors like oral secretions (Tian *et al.*, 2013). JA is perceived by the JA F-Box receptor CORONATINE INSENSITIVE 1 (COI1). COI1 targets the 12 JAZ transcriptional repressors for polyubiquitination and subsequent degradation (Katsir *et al.*, 2008). JAZ transcriptional repressors constitutively repress MYC TFs transcription factors, so upon JA detection by COI1, JAZ degradation releases MYC TFs to modulate plant defense responses like biosynthesis of glucosinolate, an anti-herbivory response (Dombrecht *et al.*, 2007, Schweizer *et al.*, 2013).

The gaseous plant hormone ethylene has a myriad of responses in plants, most notably fruit ripening, but also in plant development, flooding tolerance, PTI development and defense against necrotrophic pathogens (Inqal *et al.*, 2017, Zipfel 2013, Zimmerli *et al.*, 2004). Ethylene is perceived by two-component-like protein receptors where homo and heterodimers interact and perceive ethylene using the proteins ETHYLENE RESPONSE SENSOR 1 (ESR1) or ETHYLENE INSENSITIVE 4 (EIN4), and ETHYLENE RESPONSE 1 (ETR1), (Hua *et al.*, 1998, Banno *et al.*, 2001). This initiates a signaling cascade to stabilize EIN2, a master transcriptional regulator of the ethylene response (Alonso *et al.*, 1992). EIN2 cleavage releases the C-terminus to induce activity of positive regulators of the ethylene signaling pathway EIN3 and EIL1, which induce the expression of ETHYLENE RESPONSE FACTOR (ERF) the transcription factors (Qiao *et al.*, 2012, Zhang *et al.*, 2017). The ERF family is large and diverse and regulate a multitude of genes involved in defense, development, and abiotic stress tolerance (Mizoi *et al.*, 2012). Furthermore, ethylene plays a substantial role in regulating PTI and ETI such as positively regulating expression of FLS2 (Mersmann *et al.*, 2010, Guan *et al.*, 2015). Ethylene also acts synergistically with other plant defense hormones like SA and JA to induce robust and sustained defense responses after PTI activation (Tsuda *et al.* 2009).

SA and JA both have synergistic and antagonistic functions in maintaining plant survival against biotic stressors. SA hyperaccumulators have enhanced defense against biotrophic pathogens, but lack proper defenses against herbivory, whereas plants lacking proper SA signaling display heightened resistance to insects (Spoel *et al.*, 2007, Kempel *et al.*, 2011). Furthermore, some pathogens hijack plant hormone signaling

pathways to make their hosts more susceptible. The bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 secretes coronatine, a JA-Ile mimic, activates JA responses and enhances the plant's susceptibility to bacterial infection (Cui *et al.*, 2005). However, SA and JA are required for a robust and efficient PTI and ETI network for maintaining high levels of receptors and signaling components (Hatsugai *et al.*, 2017, Tsuda *et al.*, 2009), demonstrating the complexity between hormone crosstalk being beneficial or antagonistic to tailored defense responses.

1.3 How does heat impacts plant-pathogen interactions

1.3.1 Temperature is a major regulator of plant disease and susceptibility

Historical perspectives and agricultural observations have long identified that outbreaks of problematic plant diseases coincide with specific environmental events, leading to a feast or famine future for the plant hosts and their agricultural caretakers, or towards the virulent diseases and pests plaguing plants. This has been coined as the “plant-disease triangle”, a summation of how the environment can affect the interaction between hosts and pathogens and is a cornerstone of plant pathology (McNew 1960, Scholthof 2007) (Figure 3). The Irish Potato Famine is a classic example of this paradigm in action, represented by a genetically uniform potato during an exceptionally wet and cool period in Ireland which led to crop failure from the virulent oomycete *Phytophthora infestans* (Engler and Werner, 2015). Since this period, a slew of other factors have been discovered that shape the interactions controlling plant disease and pathogen virulence, such as the microbial communities and the advent of modern chemical and technological interventions to aid in plant survival (Paasch *et al.*, 2023, Chappelka and Grulke 2015). With current climate projections predicting a warmer and

more variable climate, how this triangle will shift for each pathosystem will be largely dependent on host and pathogen-specific factors (Yang *et al.*, 2023). As the climate warms, agronomic regions will expand towards the poles, but so too will their pathogens, providing more complexity to range expansion in agriculture (King *et al.*, 2018, El-Sayed and Kamel, 2020). The environmental changes in the coming years will place significant pressure on the currently available crop cultivars and may also catalyze disease outbreaks akin to those in the past. By understanding how the environment and plant immunity interact on a molecular level, we will be able to generate climate resilient cultivars and strategies to mitigate losses from opportunistic plant diseases.

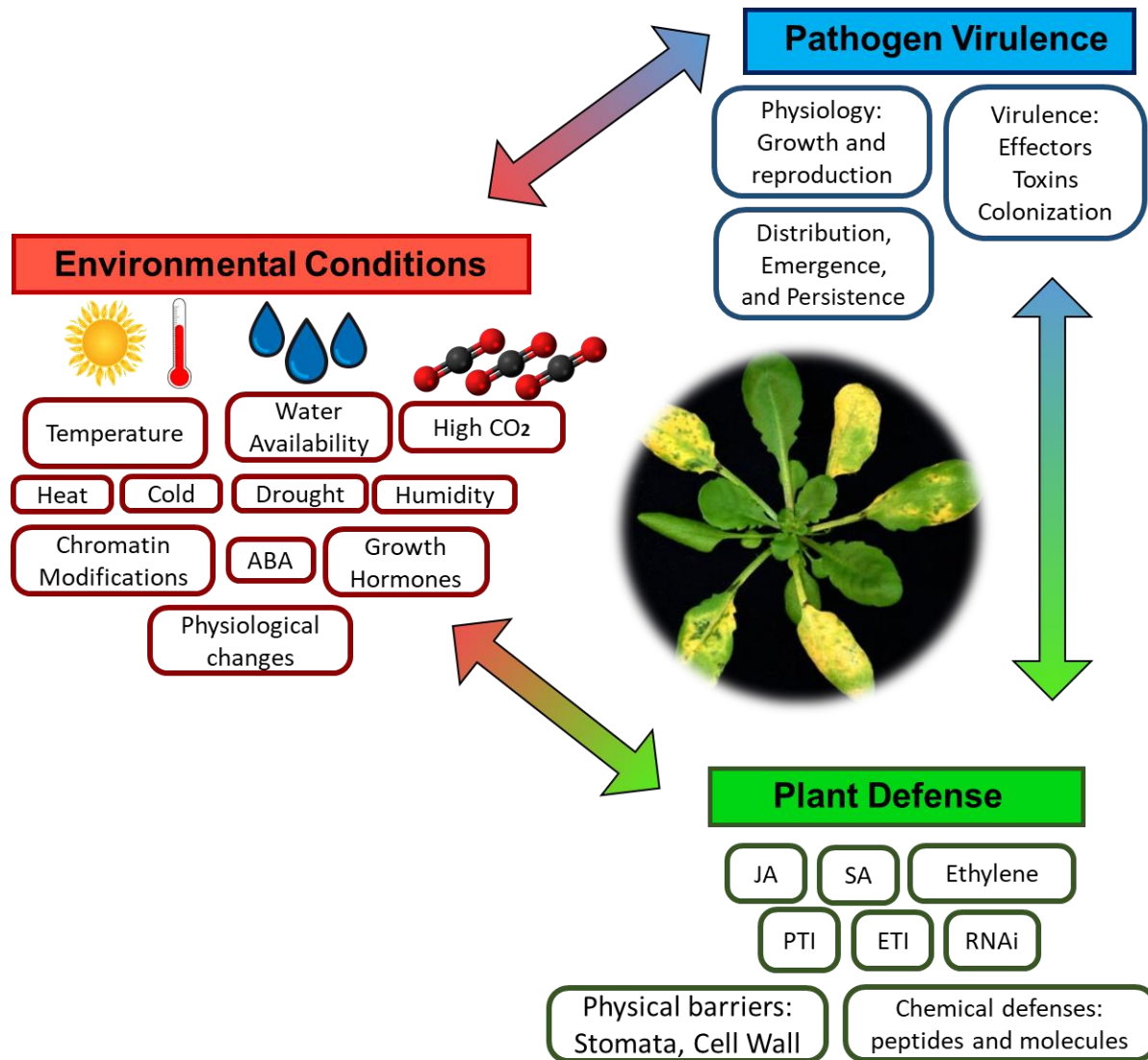


Figure 3. Environmental influence on plant disease. Environmental conditions shape interactions between host plants and their pests and pathogens. The environment can influence hosts and pathogens directly by modifying many elements of their growth, physiology, metabolism, and development. These changes can then indirectly affect how plants and pathogens interact while adjusting to the environment to determine whether disease emerges.

Temperature is a major factor driving plant disease outbreaks, as temperature influences many key elements of an organism's physiology. Between plant-pathogen interactions, there is an optimal temperature for both the host and pest that favor each respectively and subtle shifts in temperature dictate the outcome for each organism, ultimately determining the fate of the disease. The first documented research study exploring the impact of plant disease and temperature was examining the temperature specificity of *Fusarium graminearum* and immunity breakdown in wheat and corn (Dickson and Holbert, 1928). When wheat and corn were grown outside their optimal temperatures (4-12°C and 20-24°C respectively), the plants displayed enhanced disease symptoms. Within this study, temperatures above 12°C and below 20°C for wheat and corn promoted *F. graminearum* disease severity, demonstrating temperature specificity of host defense and pathogenicity. The increases in temperature and duration of the growing season will allow for greater pathogen population levels and persistence in the soil, exacerbating plant disease while also enabling more abiotic-tolerant pathogens to emerge and spread (Wu *et al.*, 2020). Thus, it is important to step ahead of the rapidly evolving pathogens to implement new solutions to develop more temperature-tolerant crops that are optimized for disease resistance.

At the genetic level, plant immunity breakdown at elevated temperature was first reported in tobacco *N* gene against *Tobacco Mosaic Virus* (TMV), where HR was lost above 28°C (Whitham *et al.* 1994). This breakdown of *N*-gene resistance correlated with a disruption in SA biosynthesis as TMV-induced SA and *PR-1* expression was lost at 32°C. Since then, numerous temperature sensitive elements of plant immunity have been identified. ETI exhibited by the R gene Xa21 against *Xanthomonas oryzae* in rice

functions at temperatures at and below 27°C but is completely absent at 31°C. Other R-genes like Xa3, Xa4, Xa5, and Xa10 also show a reduction in protein levels after being exposed to high temperatures under field-growing conditions, but the R-gene Xa7 showed enhanced resistance in plants growing at 35°C, demonstrating temperature specificity for different R-genes within the host plant (Webb *et al.*, 2010). This specificity was thought to select against pathogenic populations that demonstrate enhanced virulence under sub-optimal (hot) conditions for the pathogen, ultimately selecting for bacteria that maintain higher virulence at lower temperatures. This enhanced immunity observed in Xa7 at elevated temperature was associated with a downregulation of ABA signaling under pathogen infection, suggesting that high temperature induced ABA can be compromised after Xa7 activation (Cohen *et al.*, 2017). ABA suppressed SA-mediated defenses in the rice – *X. oryzae* pathosystem and has been observed in the model plant *Arabidopsis thaliana* to promote disease (Xu *et al.*, 2013, De Torres Zabala *et al.*, 2009). While these studies have identified correlations between ABA and temperature mediated resistance, the mechanism that dictates temperature sensitive immune outputs is not entirely clear. This tradeoff represents a delicate and sensitive act for balancing a crop's tolerance toward ABA-governed stressors like drought and heat with maintaining disease resistance.

Since ETI-based approaches provide targeted and strong immunity to specific pathogens in crop systems, there has been substantial research investigating ETI in the model plant *Arabidopsis thaliana* and how it is influenced by elevated temperature. Key symptoms of ETI, like HR, bacterial replication and expression of specific genes have been observed to be temperature sensitive in numerous studies examining *Pst.* DC3000

carrying AvrRpt2, AvrRpm1, AvrB, HopZ1, AvrPphB and AvrRps4 (Wang *et al.*, 2009, Cheng *et al.*, 2013, Menna *et al.*, 2015, Mang *et al.*, 2012). Notably, ETI displays differential sensitivity to various effectors at elevated temperature in an accession specific manner. For example, AvrHopZ1 and AvrRpt2 display temperature sensitivity at 27°C, where HR is lost but immunity is retained in the wild type *A. thaliana* Col-0, but both HR and ETI was compromised in the Tsu-1 and Wei-0 accessions (Menna *et al.* 2015). However, AvrRps4 and AvrRpm1 display weakening of immunity and loss of HR in Col-0, providing some discrepancies between ETI and elevated temperature (Wang *et al.* 2009, Cheng *et al.* 2013). These differences could be related to underlying signaling events that may separate and bifurcate HR symptoms from immunity, but also may vary due to differences in laboratory settings and methods of inoculation or temperature treatment.

A genetic dissection of *A. thaliana* immunity and elevated temperature using plant “lesion mimic” mutants displaying constitutive defense responses and cell death lesions has provided novel insights into the mechanisms underlying temperature sensitivity. Many autoimmune mutants display plant dwarfism cell-death lesions, and constitutively high expression levels of defense genes and elevated levels of SA. They also display restoration of growth and downregulation of defense responses when grown at elevated temperature. For instance, the *snc1-1* mutant shows temperature sensitivity but is reversed with a specific point mutation in the *snc1-3* mutant, indicating some elements of intact immunity at elevated temperature (Zhu *et al.*, 2010). The point mutation in *snc1-3* background also enables enhanced nuclear translocation of SNC1, demonstrating that nuclear exclusion may play a role in R-gene regulation at specific

temperatures. However, the role that SA plays in *snc1* dwarfism remains unclear, as *cbp60g/sard1* double mutants in the *snc1* background display enhanced defense responses and dwarfism, putatively since negative regulators of SA signaling are controlled by CBP60g and SARD1 and thus are unable to attenuate defense signaling (Sun *et al.*, 2018). Disrupting PTI via effectors is a common approach pathogens use to enhance the susceptibility of their host, so plants often induce ETI responses when PTI appears disrupted (O'Brien *et al.*, 2011). The double mutant *mkk1/mkk2*, *mpk4*, and *bir1-1* also display this temperature sensitive phenotype where cell death and plant dwarfism is reversed at 28°C (Zhang *et al.*, 2012, Gao *et al.*, 2010). However, not all constitutively active defense mutants display disrupted immunity at elevated temperatures. The *A. thaliana* mutant *zed1-D* was identified from an EMS screen as a dwarf mutant with high *PR1* expression at 28°C but not at 18°C. *zed1-D* is a gain of function autoimmune mutant that activates downstream ETI through ZAR1 and is dependent on SA, demonstrating complex regulation of immune activation, SA, and temperature (Wang *et al.*, 2017). How these point mutations confer temperature sensitivity to nuclear accumulation of R genes, along with temperature-sensitive immunity, remains to be revealed in a cohesive framework.

Elevated temperature promotes plant disease outbreaks indirectly by affecting insect vectors and arthropod pests, where temperature dictates key elements of the insect life cycle like emergence, persistence, and metabolism of herbivorous pests and disease-spreading insects (González-Tokman *et al.*, 2020). For example, *Arabidopsis thaliana* displays enhanced susceptibility to *Trichoplusia ni*, but did not correlate with enhanced JA biosynthesis and signaling. Elevated temperature enhanced *T. ni* feeding

and growth rates which facilitated them to overcome plant defenses (Havko *et al.*, 2020). Emergence of the Asian citrus psyllid, the primary vector for the *Candidatus liberibacter asiaticus* bacteria that causes citrus greening emerged faster under warming but not extreme heat conditions, suggesting citrus greening may spread earlier and further with warming temperatures (Antolinez *et al.*, 2022). The corn earworm *Helicoverpa zea* is highly sensitive to winter soil temperatures, which have warmed in northern latitudes, enabling this pest to persist at higher populations, enabling more problematic pest outbreaks (Lawton *et al.*, 2022). The ability for pathogens and their hosts to spread further and persist longer will challenge agricultural producers due to the heightened pressure placed on an extended growing season, possibly compromising improved yields from longer growing regimes.

1.3.2 The *Arabidopsis thaliana*- *Pst* DC3000 pathosystem and how elevated temperature influences plant immunity.

Arabidopsis thaliana is a model in the plant-molecular science sphere as it is highly fecund, goes from generation to generation in relatively short time spans, has a small nuclear genome, has many characterized mutants, exhibits a large geographic range with distinct populations, and is widely amenable to many experimental techniques in the lab (Koornneef and Meinke, 2010). The use of *Arabidopsis* in conjunction with the bacterial pathogen *Pseudomonas syringae* pv tomato DC3000 (*Pst*. DC3000) has enabled significant breakthroughs in our understanding of plant-microbe interactions and the genes and pathways involved in plant immunity and how pathogens evolve to target and mitigate plant defenses (Katagiri *et al.*, 2002). *Pseudomonas syringae* pv tomato DC3000 (*Pst*. DC3000) have evolved numerous mechanisms to

evade PTI perception. *Pst.* DC3000 contains a type III secretion system (T3SS) that enables delivery of bacterial effectors into the host plant cell to disrupt host immune processes, establishing a favorable environment for the pathogen (Alfano *et al.*, 1997). For example, the bacterial effector AvrPto targets and inhibits kinase activity of FLS2 and EFR, resulting in compromised PTI (Xiang *et al.*, 2008). A bacterial mutant that lacks a type III secretion system which is essential for delivering effector proteins into the host plant, *hrcC*⁻, is unable to cause disease, demonstrating the essential role bacterial effectors have in disarming plant immunity (Yuan and He 1996). The delivery of these effectors, combined with environmental conditions, dictate the virulence of *Pst.* DC3000 and the ability for host *Arabidopsis* plant to deploy sufficient resistance. Furthermore, the genetic resources available for the host and pathogen enable a deeper view of the molecular mechanisms that influence the host-pathogen dynamic.

The *A. thaliana* – *Pst.* DC3000 pathosystem has been used to understand the dual impact of temperature on both the host and pathogen to elucidate how optimal environmental conditions facilitate disease severity at a molecular level. Elevated temperature has been shown to enhance *Pst.* DC3000 replication and enhance disease symptoms in *A. thaliana*. (Wang *et al.*, 2009), but the underlying mechanisms have remained elusive. Elevated temperature enhances the virulence of *Pst.* DC3000 as there is higher effector translocation at 30°C than at 23°C. At the same time, high temperature negatively impacts plant immunity by compromising SA biosynthesis, which occurs independently of pathogen virulence. (Huot *et al.*, 2017). Furthermore, Benzothiadiazole (BTH), a synthetic analog of SA, enhances plant immunity at both 23°C and 30°C, which was surprising since canonical SA-responsive genes like *PR1*

were not induced at 30°C. This demonstrates an uncoupling of BTH/SA mediated protection through NPR1 and highlights intact elements of plant immunity that is SA-regulated at elevated temperature. How NPR1 is capable of inducing immunity at elevated temperatures represents an avenue of research to pursue to glean insight into temperature-resilient immunity.

Loss of SA biosynthesis at elevated temperature coincides with failure to induce transcription of *CBP60g* and *SARD1* after pathogen infection, indicating that regulation of these transcription factors is a key step to understand why SA biosynthesis is compromised. Our lab identified that overexpression of *CBP60g* was sufficient to restore SA biosynthesis at 28°C after *Pst*. DC3000 infection, further highlighting that *CBP60g* transcription is an essential element facilitating temperature sensitivity (Kim *et al.*, 2022).

We also found that *CBP60g* expression after BTH treatment at elevated temperature was compromised, which was associated with a reduction in GBPL3-dependent nuclear condensates that regulate gene expression at the *CBP60g* loci. This suggests that BTH can protect plants against *Pst* DC3000 infection without the expression of *CBP60g* module. Additionally, positive regulators of *CBP60g* expression like NPR1, TGA1 but not the condensate promoting GBPL3, along with RNA Polymerase II, and key mediator subunits (Kim *et al.*, 2022). This suggests BTH activates both *CBP60g*-regulated defense module, including SA biosynthesis, and another defense module that is controlled by NPR1 and TGA1 at normal temperature (Figure 2). It is intriguing that loss of the *CBP60g* module at 28°C does not affect BTH-mediated resistance against *Pst* DC3000, suggesting the existence of an NPR1/TGA1-

dependent temperature-resilient defense pathway. One possibility is that some immune signaling steps (e.g. PRR signaling) upstream of CBP60g is temperature sensitive but that BTH activates these early steps at 28°C to boost PTI, which is sufficient for resistance against *Pst*. DC3000 without the CBP60g module. As will be described in the next chapter, multiple results from my dissertation support this model.

While temperature sensitivity of ETI has been reported extensively, the effect of temperature on PTI has been explored only recently in relation to environmental conditions and there are no studies investigating temperature-immunity interactions through PTI. Initial research exploring PTI revealed a temperature sensitivity in the output of the defense genes *WRKY29* and *FRK1* after flg22 treatment, with the highest expression occurring between 23-32°C in protoplasts and young seedlings briefly treated with elevated temperature. Additionally, MPK3/6 phosphorylation occurred at relatively equal amounts between 23 and 28°C but lower at 16°C, leading researchers to conclude PTI exhibits enhanced activity at elevated temperatures (Cheng *et al.* 2013). This coincides with decreased expression of genes encoding coronatine biosynthesis and effector proteins in *Pst*. DC3000 *in vitro*, leading the authors to hypothesize that PTI would predominate plant immunity at warmer temperatures due to heightened bacterial replication and less induction of effector proteins (Ulrich *et al.*, 1995, Weingart *et al.*, 2004, Dijk *et al.*, 1999). However, more recent research found that effector translocation was enhanced at elevated temperature *in planta*, suggesting transcriptional regulation of effectors does not correlate with effector translocation (Huot *et al.*, 2017). Furthermore, heat shock decreases the protein levels of FLS2 and weakens ROS output of plants stimulated by flg22 or *Pst*. DC3000, suggesting that the

effects of temperature on PTI may be dependent on the nature of temperature pre-treatments (Janda *et al.*, 2019).

1.4 Aim of Research

Previous research in our lab has highlighted the dual influence of temperature on both pathogens and the host plant (Huot *et al.*, 2017). Key findings this study, and the subsequent study from (Kim *et al.*, 2022) suggested that temperature sensitivity of the expression of the CBP60g defense module, including salicylic acid biosynthesis under pathogen infection, is a key contributing factor promoting plant susceptibility to *Pst* DC3000 infection. However, plant immunity against *Pst* DC3000 included by BTH displays temperature insensitivity, despite the lack of the CBP60g defense module and suggests a temperature insensitive mechanism is still intact at elevated temperature. To broadly understand how the plant immune system is affected by temperature, I focused my dissertation research on PTI activation through flg22 to isolate host-specific processes that are temperature sensitive. The following chapters will highlight how elevated temperature impacts plant immunity through PTI and identify which elements of PTI may be impacted by temperature and which pathways remain resilient.

CHAPTER 2 PROBING TEMPERATURE SENSITIVE ELEMENTS OF PTI

2.1 Materials and Methods

Plant Growing Conditions

Arabidopsis thaliana plants were grown with potting soil of 2:1 “Arabidopsis mix” with additional perlite and covered in a standard fiberglass mesh for 4.5 days after sterilization and stratification. Soil was autoclaved at (121°C, 15PSI) 45 minutes prior to cooling and assembling pots to remove contaminants. Plants were grown on a 12/12 Light/Dark cycle at 23°C at $90 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$. Seeds were thinned after 7-10 days to isolate 4 plants per pot for experiments. Plants were supplemented with ½ strength Hoagland nutrient solutions (Arrhenius *et al.*, 1922) every two weeks.

All Arabidopsis plants were either the wild type (Col-0) or derived from the wild type background, T-DNA mutants were identified performing genotyping by PCR to identify homozygous individuals for inserts, which includes the *ics1-2* (Wildermuth *et al.*, 2001), *npr1-6*, (SAIL_708F09) *cbp60g/sard1* (Wang *et al.*, 2011) and *bak1-5/bkk1-1/cerk1* (Xin *et al.*, 2016), 35S::CPK5 (Dubiella *et al.*, 2013) and 35S::CBP60g (Qin *et al.*, 2018).

Temperature, Chemical, and UV-C treatment

After Arabidopsis plants reached 30 days of age, plants were transferred to experimental chambers set to 23°C or 28-30°C. Plants were kept at elevated temperature for 24 hours prior to chemical treatments, being moved 3 hours after the lights turned on. For experiments with flg22, stocks from a 10mM solution in DMSO were diluted to the noted concentrations (100nM – 1μM). Mock (0.1% DMSO) or flg22 were pressure infiltrated into marked adult leaves using blunt-ended syringe. Tissue was harvested as described at indicated or for the flg22-treated samples, subsequent

experiments for disease assays were initiated 24 hours after flg22 treatment. For treatment using SA or benzothiadiazole (BTH), plants were either sprayed with a mock solution containing 0.1% DMSO or 100 μ M BTH solution with 0.025% Silwet to sufficiently coat plants. Plants were returned to chambers and left to dry at their respective temperatures. For UV-C exposure, adult plants were moved to temperature specific growth chambers that were outfitted with UV lights set to 254nm (COOSPIDER 25W 110V). Plants were placed 30 cm below the UV lights and exposed to UV-C for 15 minutes, before the UV lights were turned off and kept at their respective temperatures for 24 hours prior to harvesting tissue for further analysis.

Disease Assays and flg22/BTH protection assays

Pst DC3000 was removed from a frozen glycerol stock and streaked out onto agar-based LM media (10.0g Bacto Tryptone, 6.0g, 1.5g K₂HPO₄, 0.6g NaCl, 0.4g MgSO₄ * 7*H₂O L⁻¹) containing Rifampacin (100mg L⁻¹, Cayman Chemical). Bacteria were incubated until individual colonies formed to be used for liquid culturing, and plates were kept at 4°C for up to 5 days week prior to assaying. One day prior to disease assay, individual colonies were picked using a sterile pipette and added to 10mL of LM media containing 1:100 dilution of Rifampicin. Bacteria were cultured overnight at 28°C and were assessed the following morning to ensure bacteria were in log-phase growth using a Spectrophotometer to measure optical density (OD) at an absorbance wavelength of 600nm (OD₆₀₀). 1mL of bacterial culture was spun down for 5 minutes @ 4000 rpm using a tabletop centrifuge and resuspended in 0.25mM MgCl₂. Bacterial suspensions were then remeasured to achieve a target OD₆₀₀ of 0.002 (~1 x 10⁶ cfu/mL⁻¹) in 50mL of 0.25 mM MgCl₂. Bacterial suspensions were plated on LM +

Rifampicin plates to confirm bacterial inoculum concentrations.

After plants had adjusted to the temperature chambers and/or any chemical treatments, bacterial inoculum was infiltrated into target leaves of interest using a blunt ended syringe until the leaf was thoroughly saturated. Plants were immediately returned to their respective test chambers to dry. Two days after bacterial inoculation (unless otherwise noted), individual leaves were removed from the plant and 4mm leaf discs were excised from infected tissue and placed in impact-resistant tubes containing zircon beads and 10mM MgCl₂ and macerated using a TissueLyzer (Qiagen) for 2 x 30s at 25 Hertz (Hz). Samples were serially diluted and 10mL of bacteria were plated onto LM + Rif agar plates to incubate overnight at 30°C. Colony forming units (CFUs) were counted using a microscope and the final reading of CFU/cm² was calculated using the formula: (CFU * Dilution factor)/(Volume diluted)/(Volume plated * leaf disc area).

ROS Elicitation Measurements

Three 4mm leaf discs were harvested from temperature pre-treated plants and placed with abaxial side facing downward in 200µL of sterile water in a white plexiglass 96-well plate, wrapped in tin foil, and were incubated at 23°C overnight to attenuate leaf wounding from excising discs. The following morning, water was removed and replaced using a 100µL eliciting solution containing 34 mg/mL luminol, 20µg/mL peroxidase, and 100nM flg22 using a multichannel pipette in dim light. Plates were quickly transferred to a Spectramax L microplate reader (Molecular Devices) and Relative light units (RLUs) were recorded over 60 minutes with the following settings: Total Photon Counts, absorbance – 0.5s, interval – 2 minutes.

Measuring cytoplasmic [Ca²⁺] flux

The method for calculating relative calcium flux was accomplished using a modified method from Tanaka et al. 2013. 35S::aequorin plants in Col-0 background were grown as described previously. 3 4mm leaf discs for technical replicates were harvested from 8 temperature-treated plants and placed in a 96-well microplate containing 100μL of 10mM CaCl₂ and 10mM coelenterazine and incubated overnight, wrapped in tinfoil at room temperature. The following morning, a Spectramax L microplate reader was set up with a 200μM flg22 and mock solution for treatment was prepared for plate-injection. Plates were set inside the machine for a few minutes to adjust. Spectramax L settings were set to: Inject 100μL, Absorbance – 470nm, absorbance – 0.2 seconds, interval 6 seconds.

Callose Staining and Quantification

Temperature acclimated plants were treated with a Mock solution containing 0.001% DMSO or 100μM flg22 and cleared with Ethanol overnight. Leaves devoid of chlorophyll were fixed with a 25% acetic acid, 75% ethanol solution for 2 hours, and subsequently washed with three 15 minute washes: a 75% ethanol, 50% ethanol, and 150mM K₂HPO₄ pH 9.5. Flg22-induced callose was visualized on an Olympus IX71 microscope with a 120-watt metal halide lamp and a DAPI filter (Semrock, excitation 377-50 nm and emission 447/60 nm). Images were taken and displayed at 10x resolution prior to callose quantification. Callose count was processed using ImageJ, where images were converted to 32-bit grayscale, where the threshold was adjusted so that leaf vasculature and cell walls were not visible. Callose area was determined by using the “analyze particles” tool and area was averaged across the samples. 8 leaves

were used from 8 different plants for two independent experimental replicates.

RNA extraction and qRT-PCR

After flg22 treatments, plants were harvested at the indicated time points for respective genes: 30 min – *CYP81F2*, 3 hours – *FRK1*, *WRKY29*, *NHL10*, 24 hours – *CBP60g*, *SARD1*, *ICS1*. *FLS2* and *MPK3* gene expression was measured 24 hours after temperature treatment. Tissue was harvested into impact-resistant screw-top cryotubes containing stainless steel beads and were flash frozen in liquid N₂ and moved to -80°C until processed for RNA extraction.

Frozen tissue was ground using a TissueLyzer for 2 x 30s at 30Hz and quickly re-frozen in liquid N₂ to minimize tissue degradation. RNA was extracted using RNEasy kits (Qiagen) using the manufacturers protocol with an on-column DNase1 (Qiagen) digestion for 1 hour prior to elution using 50µL of RNase-free water. Total RNA concentrations were measured and normalized to 50ng/µL on a NanoDrop 1000 spectrophotometer (Thermo Fisher). Using the Superscript IV VILO mastermix (Thermo Fisher), cDNA was synthesized according to the manufacturers recommended methods using 300ng of total RNA as the input. cDNA was diluted 1:30 with nuclease-free water following synthesis and stored at -20°C for short term storage or -80°C for long term storage.

To measure relative gene expression using qPCR, all reactions were performed using the SYBR Green master mix (Life Technologies), where reactions were performed with 2ng of input cDNA, 5µL of SYBR GREEN PCR master mix, 0.25 µL of each 10mM primer to be carried out in 10µL reactions. And 7500 Fast Real-Time PCR system (Applied Biosystems). Target gene expression was normalized to *PROTEIN*

PHOSPHATASE 2A SUBUNIT A3 (PP2A3) using the formula $2^{-\Delta CT}$, where ΔCT is defined by $CT_{\text{Gene of Interest}} - CT_{PP2A3}$. qPCR reactions for each treatment type were performed using 3 technical replicates from 4 plants (n=4).

Table 1. qPCR Primers Used in the study

Gene Name	Primer Name	Primer Sequence (5' → 3')
PP2A3	PP2AA3 – qRT_F	GGTTACAAGACAAGGTTCACTC
	PP2AA3 – qRT-R	CATTCAGGACCAAACCTCTTCAG
FRK1	FRK1 – qRT-F	CTTCCATCGAGGTACAAAGATGAC
	FRK1 – qRT-R	CAGTGCTCATGACAGTAGAAGC
NHL10	NHL10 – qRT-F1	TTCCTGTCCGTAACCCAAAC
	NHL10 – qRT-R1	CCCTCGTAGTAGGCATGAGC
WRKY29	WRKY29 – qRT-F	CTCCATACCCAAGGAGTTATTACAG
	WRKY29 – qRT-R	CGGGTTGGTAGTTCATGATTG
ICS1	ICS1 – qRT- F	ACTTACTAACCAGTCCGAAAGACGA
	ICS1 – qRT -R	ACAACAACCTCTGTACATATACCGT
CBP60g	CBP60g – qRT – F	AAGAAGAATTGTCCGAGAGGAG
	CBP60g – qRT – R	GGCGAGTTTATGAAGCACAG
SARD1	SARD1 – qRT – F	CCTCAACCAGCCCTACGTTA
	SARD1 – qRT -R	TAGTGGCTCGCAGCATATTG
FLS2	FLS2 – qRT_F2	ACTCTCCTCCAGGGGCTAAGGAT
	FLS2 – qRT-R2	AGCTAACAGCTCTCCAGGGATGG
BAK1	BAK1 – qRT_F	GTCAGAAAGTAGTGTCGCCA
	BAK1 – qRT-R	ACTTGTAGCGTCAGGACAGC
MPK3	MPK3 – qRT_F	TGACGTTTGACCCCAACAGA
	MPK3 – qRT-R	CTGTTCTCATCCAGAGGCTG
MPK6	MPK6 – qRT_F	CCGACAGTGCATCCTTTAGCT
	MPK6 – qRT-R	TGGGCCAATGCGTCTAAAAC

Protein Extraction and Immunoblot

One leaf of 4 plants was pooled for measuring basal levels of PTI protein components and flash frozen in Qiagen tubes containing stainless steel beads. For flg22-induced MPK3/6 phosphorylation, two leaves from two treated plants was harvested and pooled and quickly flash frozen in liquid N₂ at the indicated time points. Tissue was homogenized into a fine powder using a TissueLyzer (Qiagen) for 2 x 45 seconds at 30 Hz. 0.2 grams of ground tissue was then extracted using an extraction buffer containing 50mM Tris-HCl (pH 8.0), 150mM NaCl, 10% glycerol, 1% (v/v) Igepal, 0.5% (w/v) sodium deoxycholate, 1 EDTA-free protease inhibitor tablet (Roche) and held on ice for 15 minutes. Samples were cleared by centrifugation at 10000 rcf for 5 minutes and normalized on a Bradford Assay (Biorad). Plant extracts were loaded with a 5x loading buffer made of 10% (w/v) sodium dodecyl sulfate, 20% glycerol, 0.2 M Tris-HCL pH 6.8 and 0.05% (w/v) bromophenol blue and denatured using a thermocycler. The temperatures settings are: 37°C 20 minutes, 50°C for 15 minutes, 70°C for 8 minutes, 95°C for 5 minutes, and cooled to 21°C. 4-12% NuPage 4%-12% Bis-Tris gels (Thermo Fisher) were loaded with 10-15 µL of sample and ran for 100V (volts) for 3 hours. Proteins were transferred to a Polyvinylidene fluoride (PVDF) membrane at room temperature for 90 minutes at 20V. Blots were blocked in 3% milk, 2% Bovine serum albumin (BSA) with the exception of phosphor-p44/42 MAPK blots (5% BSA). Primary antibodies specific to *A. thaliana* (FLS2, Agrisera 1:5000), MPK3 and MPK6 (Sigma, 1:3333), p44/42 MAPK blots (Erk1/2, Thr202/Tyr204) (Cell Signaling, 1:10000), and Hemagglutinin (HA)-tagged CBP60g (Thermo-Fisher, 1:10,000) were blocked overnight at 4°C. Secondary antibodies (Horseradish peroxidase conjugated goat anti-rabbit)

were blotted following washes (Agrisera, 1:20,000). Proteins were visualized using a Chemi-Doc (Biorad) and detected using a HRP-Luminol substrate). Ponceau-S staining was performed after visualizing protein bands to confirm for equal loading.

Plant hormone extraction and quantification using Liquid Chromatography/Mass Spectrometry (LC/MS)

2-3 leaves (50-100 mg total) from temperature, chemical, and/or pathogen treated leaves were harvested, weighed, and flash frozen in 2mL cryogenic tubes containing 3 zircon beads. These tissues were harvested 48 hours after temperature treatment and 24 hours after the biologic treatment. Tissues were subsequently homogenized on the TissueLyzer (Qiagen) at 2 x 45s @ 30Hz and spun down briefly to collect the tissue at the bottom of the tubes. Tissues were then extracted in 0.5 mL of an extraction buffer containing 80% methanol, 0.1% formic acid, and 0.1 g/L of butylated hydroxytoluene, and 100nM of ABA-D₆ overnight at 4°C on a plate shaker. Samples were then spun down the following day, transferring the aqueous phase for column filtration using 0.2µm Ploytetrafluoroethylene (PTFE) membranes (Millipore) and transferred to autosampler vials for subsequent Mass Spectrometry Liquid Chromatography. 10µL of samples were injected and separated on an Ascentis Express fused-core C18 column, heated to 50°C on an Acquity ultra performance chromatography system (Waters Corporation). Samples were separated and analyzed using a gradient of 0.15% Formic Acid in water, and a 100% methanol solution and applied over 150 seconds using a flow rate of 0.4 mL/minute. Samples were separated from a ratio of 50:1 formic acid: methanol to 100% methanol over a linear gradient increase.

Selected Ion Mode (SIM) was performed in the negative ES channel for salicylic acid (SA: m/z 137>93) and salicylic acid-conjugated glucoside (SAG: m/z 299.1>137) and the internal ABA-D₆ standard (m/z 269.1>159.1) to observe transitions from de-protonated parent compounds to daughter ions were monitored on a Quattro Premier tandem mass spectrometer (Waters Corporation). Capillary voltage, cone voltage, and extractor voltage were set to 3500V, 25V, and 5V with the desolvation gas and cone gas (N₂) set to 50L/hour and 600/hour respectively. To determine hormone concentrations in respective extracts, peak area integration was performed relative to the internal standard (ABA-D₆) using QuanLynx v4.1 software (Waters Corporation). Analytes were quantified using a standard curve (1000nM – 7.8 nM) that were analyzed to the internal standard, using blank runs to prevent carry over for accurate measurements. Concentrations (nM) were converted to ng using the molecular weight of target hormones and extraction volume, which were normalized to the measured fresh weight harvested to give ng/FW as the final output. 4 plants were used for each biological replicate.

2.2 Results

2.2.1 Immunity provided by PTI is weakened by elevated temperature in a dose-dependent manner

Defense against the hemibiotrophic pathogen *Pst.* DC3000 is compromised at elevated temperature in *Arabidopsis thaliana* (*Arabidopsis*) and has been investigated by several groups to identify contributing factors that lead to compromised immunity (Wang et al. 2009, Cheng et al. 2013 Huot et al. 2017, Kim et al. 2022). Salicylic acid biosynthesis is compromised at elevated temperature, independent of enhanced virulence by *Pst* DC3000, suggesting that compromised SA biosynthesis is a host-specific phenomenon (Huot et al. 2017). Since enhanced pathogen virulence is observed at elevated temperature, the capacity to shut down immune signaling pathways is also likely enhanced, potentially confounding the mechanism through which temperature interferes with SA biosynthesis. To isolate and identify upstream signaling pathways that contribute towards SA biosynthesis, I decided to focus on PAMP-Triggered Immunity (PTI) since this pathway can be assessed without temperature influence on a virulent pathogen. It has been shown that PAMP application before *Pst.* DC3000 infection enhances plant defenses and prevents the pathogen from replicating sufficiently and causing disease in the host (Chinchilla et al. 2006). I treated *Arabidopsis* plants with flg22 and performed a protection assay in wild-type (Col-0) plants at normal (23°C) and elevated (28°C) temperatures. I observed flg22 protection was temperature sensitive at a low (100nM) concentration of flg22 but resilient at a high (1µM) dose of flg22 (Figure 4A). Flg22 protection is significantly weaker at lower concentrations, suggesting that some aspect(s) of PTI are compromised at 28°C that contribute towards

reduced protection and enhanced susceptibility. The 1 μ M dose was sufficient to restore plant immunity at 28°C, also suggesting that there are some element(s) of the plant immune system that can compensate to provide full immunity at elevated temperature. To further reinforce this paradigm, I infected temperature adjusted plants to *Pst* DC3000 Δ *hrcC*, a non-virulent mutant strain of pseudomonas that is unable to cause disease (He *et al.*, 2003). Bacterial populations at both temperatures were not significantly different, suggesting that some elements of PTI are sufficient to prevent infection and growth by non-pathogenic bacteria (Figure 4D). However, compromised PTI is insufficient to enable non-pathogenic bacteria to proliferate, suggesting other elements of microbial homeostasis are still intact at elevated temperatures (Xin *et al.*, 2016).

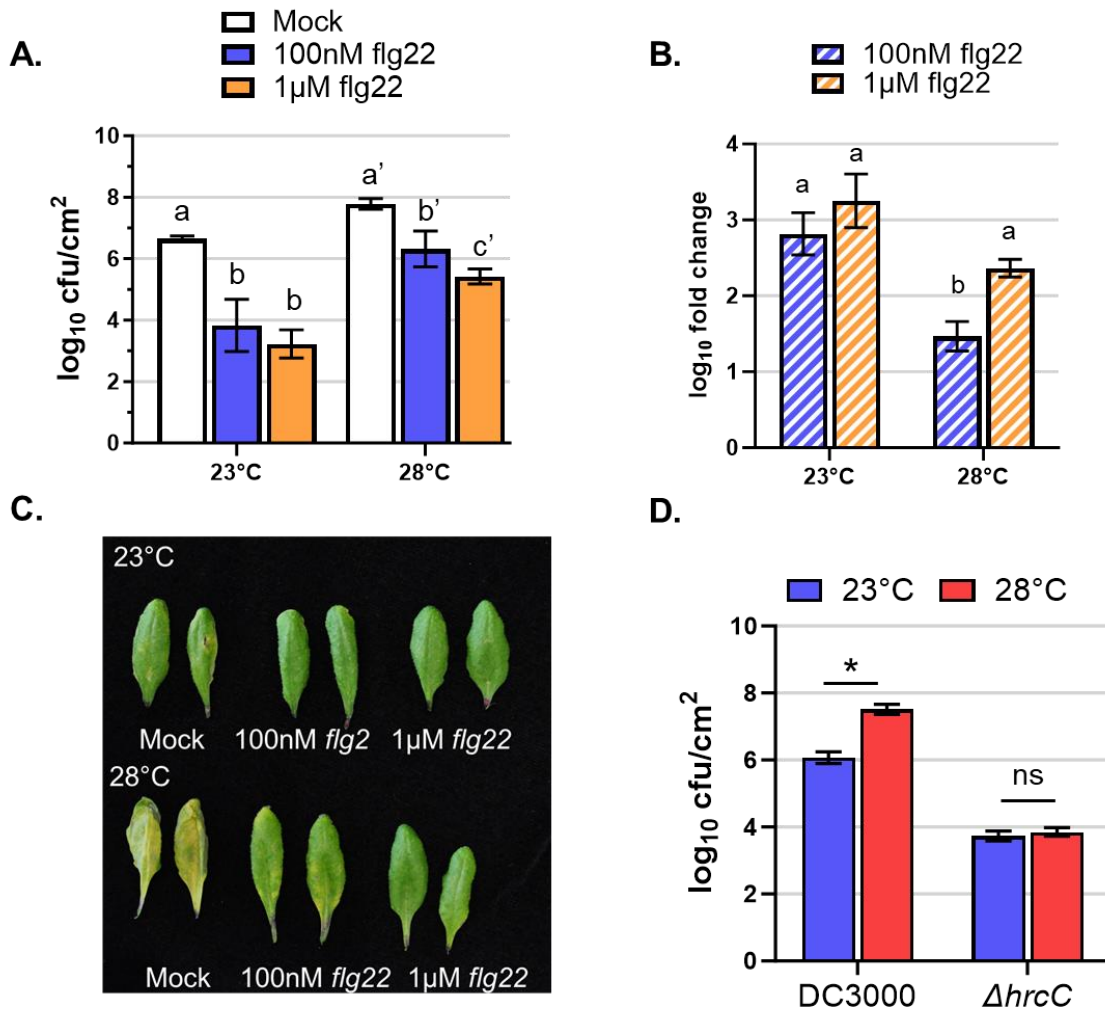


Figure 4. Elevated temperature compromises flg22-mediated protection in a dose-dependent manner. (A) Bacterial growth in plants adjusted to elevated temperature for one day were treated with indicated doses of flg22 with subsequent infection of Pst.DC3000. Bacterial populations were assessed 2 days after Pst. DC3000 infection (n = 4) (B) The degree of protection against DC3000 between mock and flg22 treated plants. (C) Visual symptoms of disease appear in flg22 treated plants at 28°C but not at 23°C. (D) Comparing bacterial populations in temperature treated plants between virulent Pst DC3000 with Pst Δ hrcC. These experiments are representative of three independent experimental replicates where error bars indicate standard errors of the mean (SEM). Asterisks indicate statistical significance based on a t-test (P < 0.05). Letters denote significance based on 2-Factor ANOVA with a Tukey HSD post hoc analysis (P < 0.05), where samples sharing letters were not statistically significantly different. Prime (') marks indicate two groups were analyzed in two different groups based on temperature

2.2.2 Elevated temperature compromises flg22-induced SA biosynthesis at elevated temperature

To further explore whether SA biosynthesis was compromised under flg22 induction, I harvested tissue from temperature-adjusted plants 24 hours after flg22 treatment. Expression of two key transcription factors genes for SA biosynthesis *CBP60g* and *SARD1*, was deficient at elevated temperature, as well as the key biosynthetic gene *ICS1* (Figure 5A). I did not observe any substantial accumulation of SA or its glycosylated form SAG at elevated temperature, even at high concentrations of flg22, suggesting that the enhanced immunity provided by higher levels of flg22 is not SA dependent. The loss of SA biosynthesis occurs independently of enhanced pathogen virulence at elevated temperatures as flg22 behaves similarly as *Pst*. DC3000, demonstrating temperature sensitive SA-biosynthesis is host-specific (Figure 5B). It has been shown previously that loss of SA biosynthesis in the SA biosynthetic mutant *ics1*, which lacks the first step of making SA by converting chorismate to isochorismate, exhibits weakened, flg22-induced immunity (Tsuda et al. 2009). Thus, if compromised SA biosynthesis is responsible for diminished flg22 protection (Figure 4), *ics1* should not display temperature sensitive flg22 protection. While *ics1* exhibits weaker flg22 protection at 23°C and 28°C, the protection appears somewhat temperature sensitive 28°C (Figure 5C-D). This suggests that there is an SA-independent mechanism also at play in the temperature sensitivity of flg22-induced immunity.

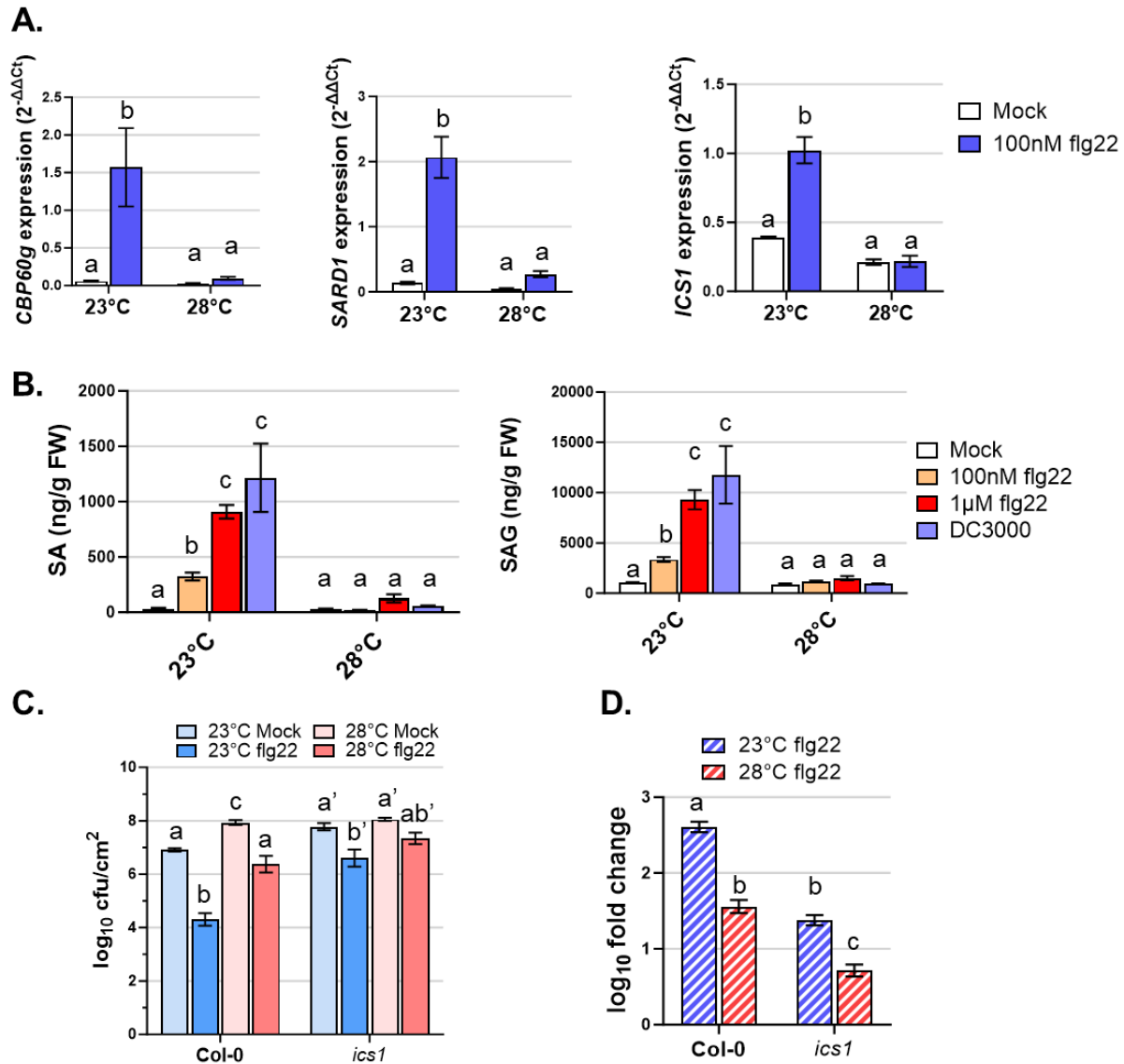


Figure 5. flg22 does not induce SA biosynthesis at elevated temperature and phenocopies SA-deficient mutants. (a). Expression levels of key regulatory SA biosynthetic genes *CBP60g*, *SARD1*, and *ICS1* 24 hours after 100nM flg22 treatment. Relative expression was determined using qPCR and normalized to expression of the gene *PP2A3*. (b). SA and SAG metabolites 24 hours after treatment were quantified using LCMS. Standard curves enabled transfer of nM concentration into ng, which was normalized to fresh weight. (c). Bacterial populations of *Pst.* DC3000 in Col-0 and *ics1* with flg22 pre-treatments. (d) change in flg22 protection between Col-0 and *ics1* at the indicated temperatures. For all experiments, n=4, error bars represent SEM and all experiments are representative of 3 independent experimental replicates. Letters indicate statistical significance based on 2-Way ANOVA ($p < 0.05$) with Tukey's HSD post-hoc test. The symbol (') denotes that two groups were analyzed in two groups based on genotype.

2.2.3 flg22-induced SA biosynthesis is not recovered by overexpressing the master transcriptional regulator CBP60g.

Previous research from our lab has identified that SA biosynthesis during *Pst.* DC3000 infection can be restored at elevated temperature by overexpressing CBP60g, a key transcription factor that governs SA biosynthetic genes (Wang *et al.*, 2011, Kim *et al.* 2022). To investigate whether 35S::CBP60g was also capable of restoring SA-biosynthesis induced by flg22, I performed a protection assay to assess temperature sensitivity to flg22. I found that flg22 protection was significantly weaker relative to the mock-treated plants at 28°C while showing robust protection at 23°C. The degree of protection relative to Col-0 appears relatively weaker due to 35S::CBP60g displaying enhanced resistance under mock conditions (Figure 6A), limiting the total duration of protection through the course of the assay (Figure 6B). Furthermore, unlike DC3000 which shows robust SA biosynthesis at both temperatures in 35S::CBP60g, flg22 does not restore SA biosynthesis in a temperature-insensitive manner (Figure 6C). While this is not surprising due to the enhanced susceptibility of flg22-treated plants at 28°C, this may suggest that flg22 induces SA in a relatively differentiated pattern relative to *Pst.* DC3000, especially since flg22-induced levels of SA are typically lower in magnitude compared to a pathogen in Col-0. Nevertheless, it is apparent that 35S::CBP60g is incapable of fully restoring flg22-induced SA biosynthesis and immunity in this system and suggests that a different pathway through PTI is inhibited by elevated temperature.

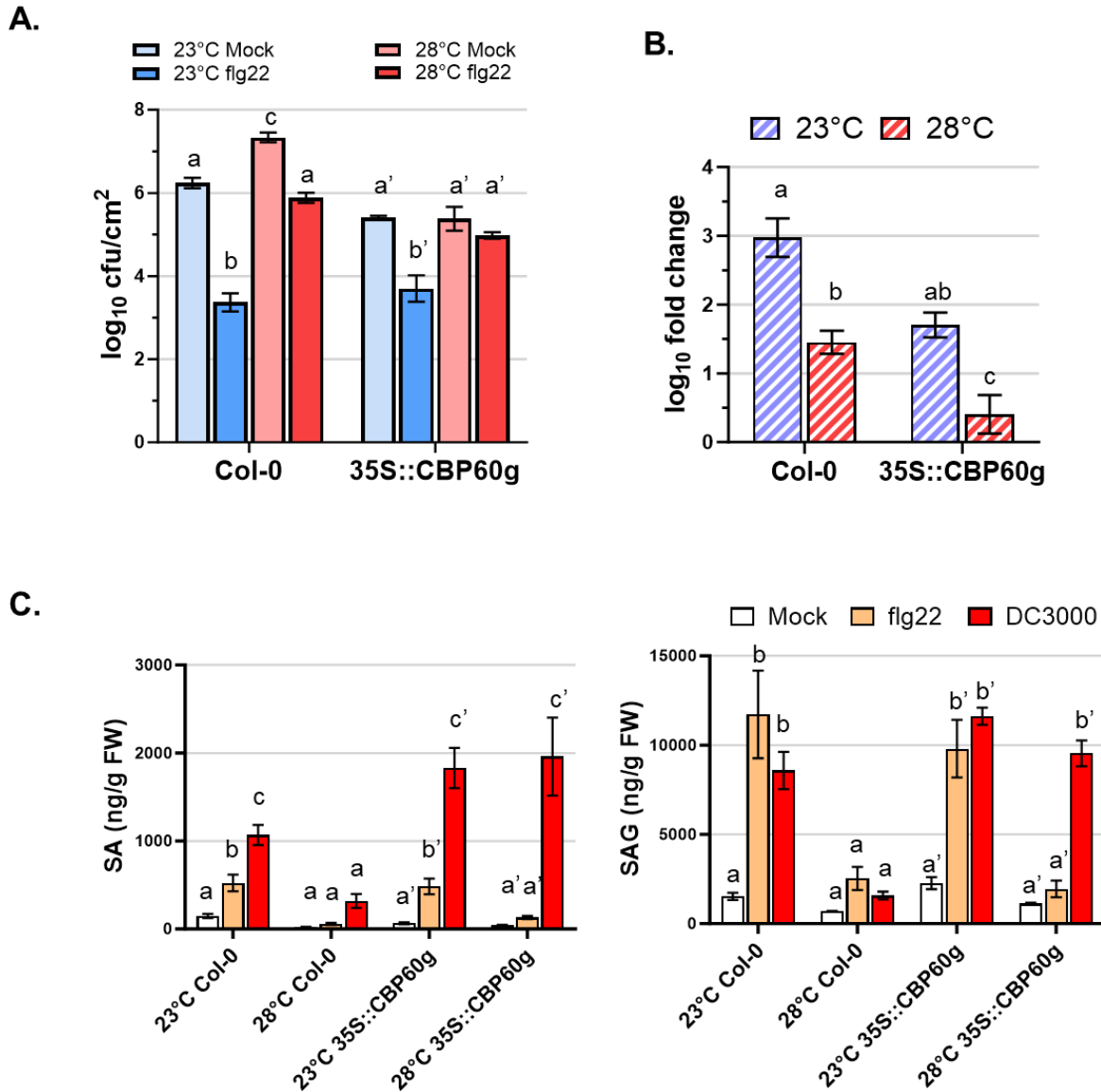


Figure 6. Overexpression of CBP60g is insufficient to restore flg22-induced SA biosynthesis at elevated temperature. (A) Bacterial populations of *Pst*. DC3000 in Col-0 and 35S::CBP60g with 100nM flg22 pre-treatments 2 days after infection. (B) Change in flg22 protection between Col-0 and 35S::CBP60g at indicated temperatures. Letters indicate significance determined via 2-Way ANOVA and Tukey's HSD post-hoc test ($P < 0.05$) (C) SA and SAG metabolites 24 hours after 100nM flg22 and *Pst* DC3000 treatment were quantified using LCMS. Standard curves of SA and SAG enabled transformation of nM into ng, which was normalized to fresh weight. For all experiments, $n=4$, error bars indicate SEM, and results are representative of 3 independent experimental replicates. Letters indicate significance determined via 2-Way ANOVA and Tukey's HSD post-hoc test ($P < 0.05$). The symbol (') denotes that two groups were analyzed in two groups based on genotype.

2.2.4 flg22-induced ROS and calcium are compromised at elevated temperature

My results so far suggest some aspects of PTI are compromised at elevated temperature due to disruptions in SA-dependent and SA-independent processes after flg22 treatment. To further examine signaling pathways upstream of SA biosynthesis, I investigated if early PTI-associated cellular responses are temperature sensitive that could contribute or correlate towards weakened flg22-protection. ROS production occurs as one of the first PTI responses observed after PAMP treatment and is a commonly observed response to assess PTI strength. Elevated temperature pretreatment of plants substantially lowered both the peak of ROS generation and the total ROS generated over time (Figure 7A-B).

ROS is generated by two mutually exclusive pathways that activate RBOHD function, the primary enzyme involved in apoplastic ROS generation through flg22. RBOHD is phosphorylated and activated by two independent phosphosites, via phosphorylation via BIK1 after flg22 recognition from the PRR complex (Kadota *et al.*, 2014), and a separate set of sites phosphorylated by CALCIUM DEPENDENT KINASE 5 (CDPK5), CDPK6, and CDPK11 (Dubiella *et al.*, 2013). Since CDPK function is directly tied to available cytoplasmic calcium bursts, I investigated calcium levels induced by flg22 exposure using the 35S::aequorin construct which enables *in-vivo* fluorescence to gauge cellular $[Ca^{2+}]$ bursts after flg22 treatment (Knight *et al.*, 1991). flg22 elicited $[Ca^{2+}]$ was significantly lower in plants adjusted to 28°C relative to plants grown at 23°C (Figure 7C-D). It is interesting to note that the basal levels of $[Ca^{2+}]$ appear slightly lower during the time course and the plants still respond to flg22, but to a significantly lower degree compared to plants grown at 23°C. Finally, to assess if the

ROS phenotype was dependent on loss of SA biosynthesis, I tested ROS elicitation between Col-0 and *ics1* at 23°C. I did not observe a noticeable difference between the two genotypes (Figure 7E-F). This suggests that the loss of ROS was independent of compromised SA biosynthesis.

ROS is tightly involved in callose deposition, where mutants deficient in RBOHD display compromised ROS in response to flg22. Based on the previous temperature-sensitive ROS phenotype, I hypothesized that callose deposition would be temperature-sensitive. Callose development is highly sensitive to ROS and *rboh*d mutants display significantly reduced callose in response to flg22 and pathogens (Luna *et al.*, 2011). I observed a modest reduction in callose deposited at 28°C relative to conventionally grown plants (Figure 8B). Furthermore, this phenotype was observed to be SA independent as well as Col-0 and *ics1* displayed similar levels of flg22-elicited callose, which corresponds to the ROS phenotype observed (Figure 8D).

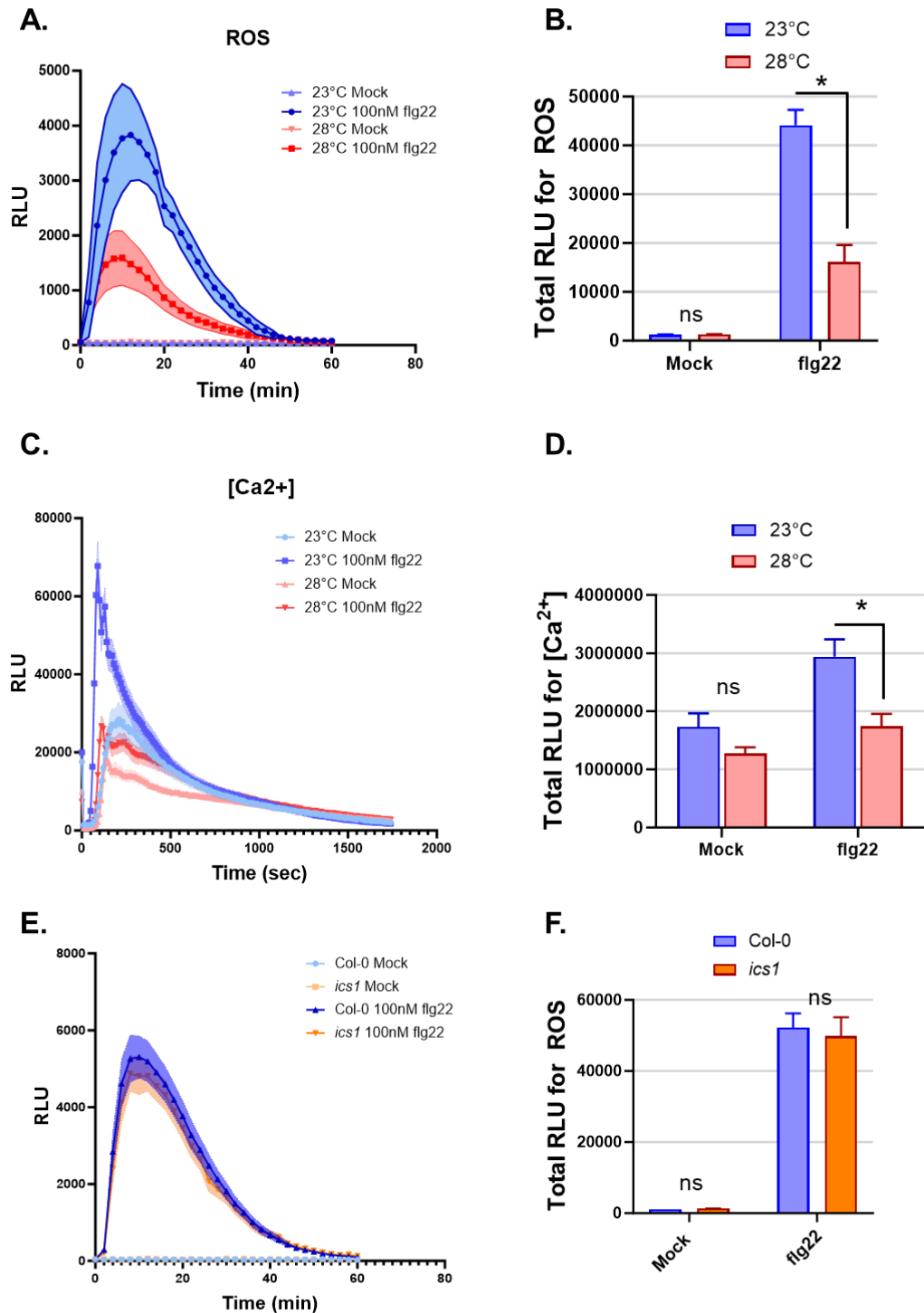


Figure 7. flg22-induced ROS and [Ca²⁺] flux is reduced at elevated temperature. (A) ROS production induced by 100nM flg22 in Col-0 plants that were temperature treated for 24 hours, excised, and placed in microplates overnight prior to measuring. Time points were recorded every 2 minutes over 24 hours to generate a temporal dynamic. Error

Figure 7 (cont' d)

range indicates a 95% confidence interval. (B) Total ROS elicited over 60 minutes ($n=8$, error bars indicate SEM). Asterisk indicates significance in a Student's t -test ($P < 0.05$). This experiment represents results from 3 separate experimental replicates. (C) 35S::aequorin plants were temperature adjusted for 24 hours and leaf discs excised and pretreated with coelenterazine overnight before flg22 stimulation. Time points were taken every 10 seconds after elicitation over 20 minutes. Error range indicates a 95% confidence interval. (d). Total RLU recorded under the 20-minute duration between various temperature treated plants. ($n=8$, error bars indicate SEM). Asterisks indicate statistical significance using Student's t -test ($P < 0.05$). This experiment represents results from 2 experimental replicates. (e) ROS elicited from Col-0 and *ics1* leaf discs from plants grown at 23°C after 100nM flg22 treatment in the same manner as (a). (f) Total ROS elicited over 60 minutes ($n=8$, error bars indicate SEM). Asterisk indicates significance for Student's t -test ($P < 0.05$). This experiment represents results from 2 independent experimental replicates.

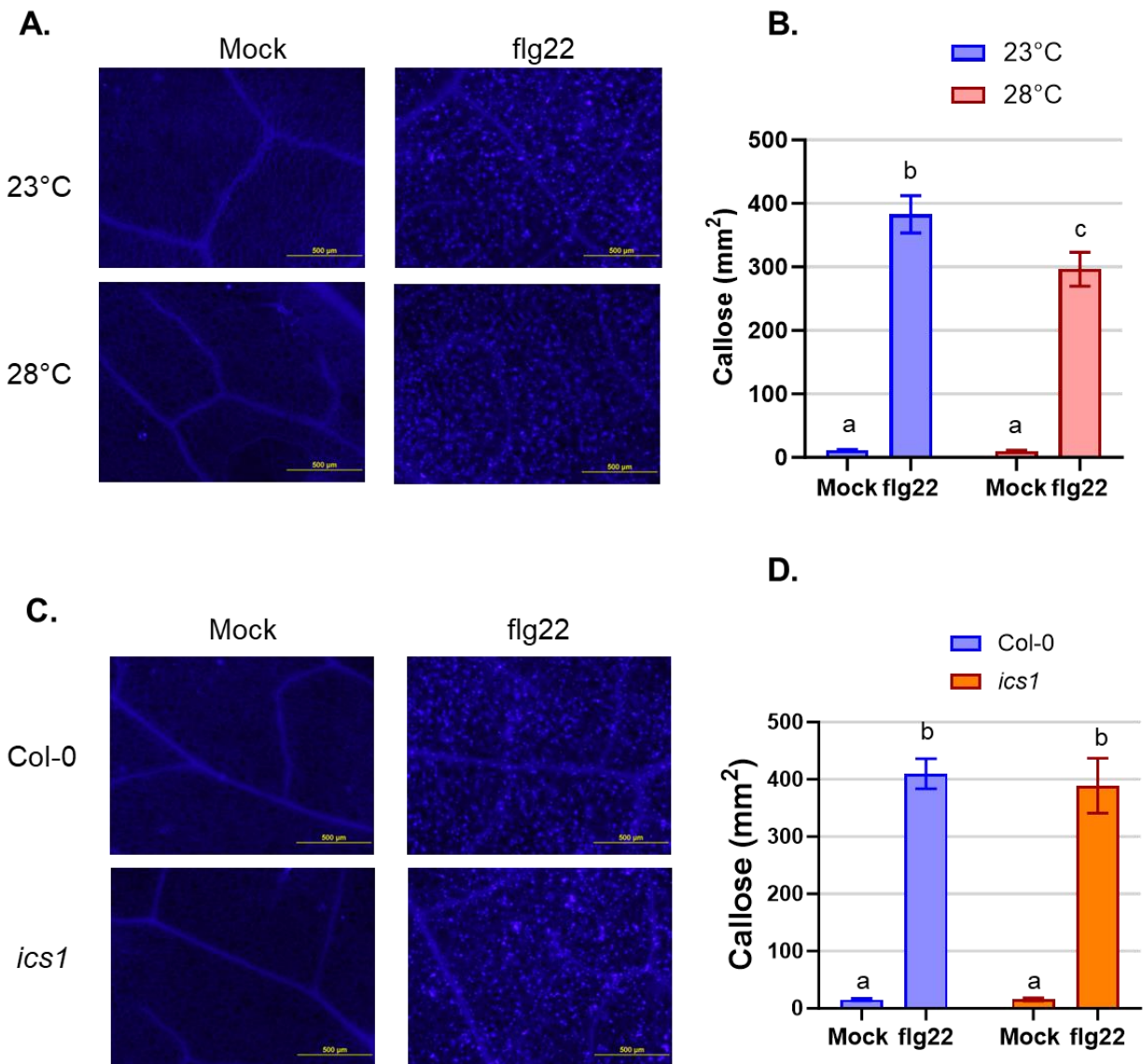


Figure 8. Elevated temperature reduces flg22-induced callose deposition in an SA-independent manner. (A)(C) Representative images of callose staining 24 hours after 100nM flg22 treatment of temperature-treated Col-0 plants and comparing Col-0 to *ics1* at 23°C. Scale bar = 100μm, images were taken with a DAPI filter (B)(D). Quantification of callose from temperature-treated plants (n=8, error bars indicate SEM). Letters denote statistical significance using 2-Way ANOVA and Tukey's HSD post-hoc test ($P < 0.05$). Data is a representation of 2 independent experimental replicates.

To explore how downstream elements of PTI are impacted by elevated temperature, I examined flg22-induced marker gene expression. There appears to be $[Ca^{2+}]$ /CDPK dependent and MAPK-dependent gene expression during PTI. Specifically, some downstream PTI marker genes are dependent primarily on either MAPK3/4/6, others on CDPK4/5/6/11, while some are dependent on both pathways (Boudsocq *et al.*, 2010). I observed MAPK-dependent gene expression of *FRK1* and *WRKY29* to be temperature insensitive (Figure 9A). *CYP81F2* and *PHI1* are two CDPK-dependent genes and are induced early on after flg22 treatment and their expression appears temperature sensitive at 28°C (Figure 9C) *NHL10*, which is a gene that is co-regulated by both MAPK and CDPK displays temperature sensitivity as well with reduced expression at 28°C (Figure 9B). Taken together, the expression pattern of these genes suggests that $[Ca^{2+}]$ -dependent gene expression during PTI is temperature sensitive, in line with the observed decrease in $[Ca^{2+}]$ flux at 28°C. These findings together suggest that calcium flux and signaling in response to flg22 is inhibited at elevated temperature.

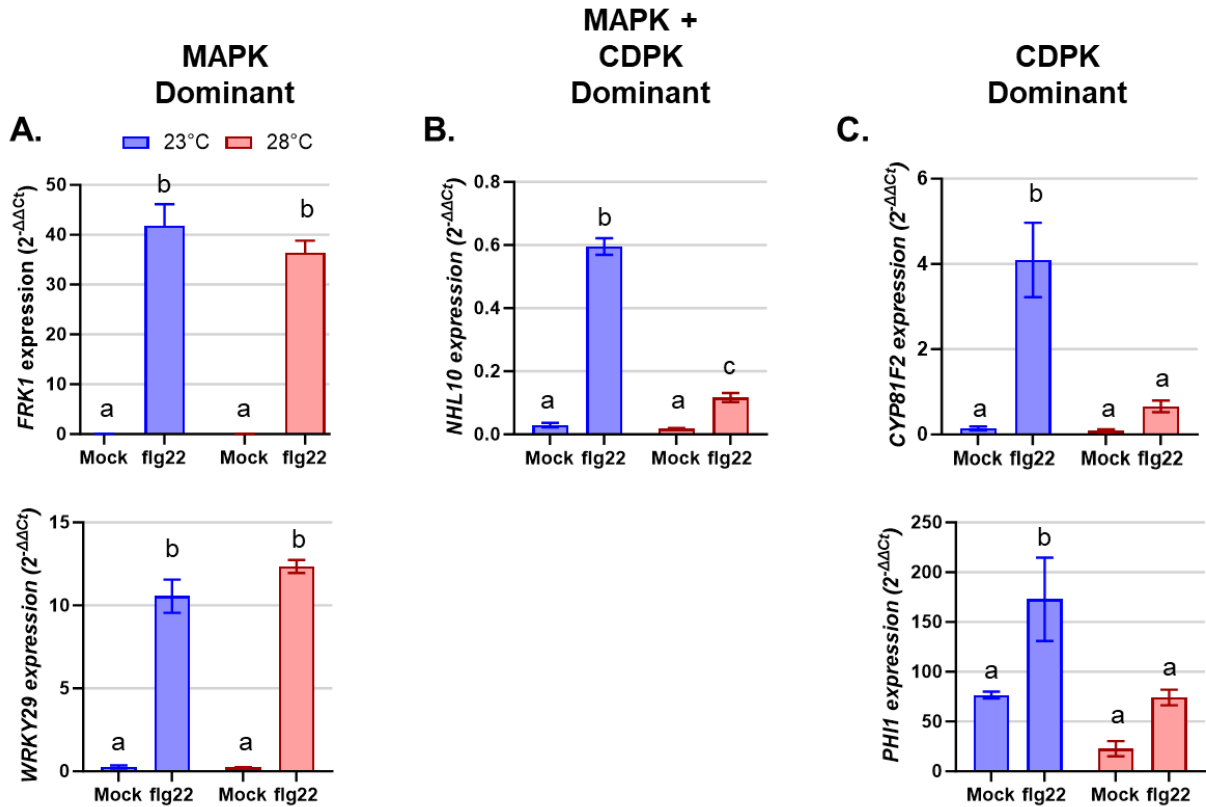


Figure 9. CDPK, but not MAPK-dependent gene expression is temperature sensitive. Marker genes were investigated in temperature treated plants exposed to 100nM flg22 (n=4). (A) The expression of *WRKY29* and *FRK1* is dependent on MAP kinases. Gene expression was taken 3 hours post flg22 treatment. (B). The gene *NHL10* is activated via both MAP kinases and CDP kinases and was assessed 3 hours after flg22 treatment. (C). The expression of *CYP81F2* and *PHI1* is dependent on predominantly CDPKs and were assessed 30 minutes after flg22 treatment. Expression of all genes presented was assessed using qPCR and were normalized to *PP2A3* expression. All experiments were performed with 3 independent experimental replicates (n=4, error bars indicate SEM). Letters denote statistical significance using 2-Way ANOVA and Tukey's HSD post-hoc test ($P < 0.05$).

2.2.5 Elevated temperature modulates the basal protein levels of PTI components

The dose response of flg22 protection and diminished outputs of PTI demonstrate there is a limitation on PTI signaling at elevated temperature. I therefore hypothesized that diminished outputs of PTI may be reflective of lower levels of PTI signaling components. To investigate the levels of PTI components associated with flg22-elicitation, I investigated whether the levels of PTI proteins are temperature sensitive. Indeed, plants that were pre-treated at 28°C displayed reduced levels of FLS2 and MPK3 proteins compared to those at 23°C (Figure 10A). BAK1 and MPK6 did not show differential protein levels between the two temperatures (Figure 10B). With respect to flg22-induced MAPK3/6 phosphorylation, it appears that elevated temperature negatively impacts the MPK3/6 phosphorylation status, intensity, and duration. However, the reduced basal levels of MPK3 would also be represented in phosphorylation status, where I observed a decrease in both. Interestingly, MPK3/6 phosphorylation status does not correspond perfectly with differences in downstream gene expression like *FRK1* (Figure 9A). There may be other temperature-insensitive components downstream of MPK3/6 phosphorylation that regulate *FRK1* expression.

I also examined the transcript levels of PTI components and found that the basal transcript levels of *FLS2*, *BAK1*, and *MPK3* were not significantly different at 23°C and 28°C (Figure 13A). This was somewhat surprising that there is a change in protein levels but no significant change in transcript levels. It appears that elevated temperature influences protein levels of some PTI components that may interfere with downstream immunity provided by flg22.

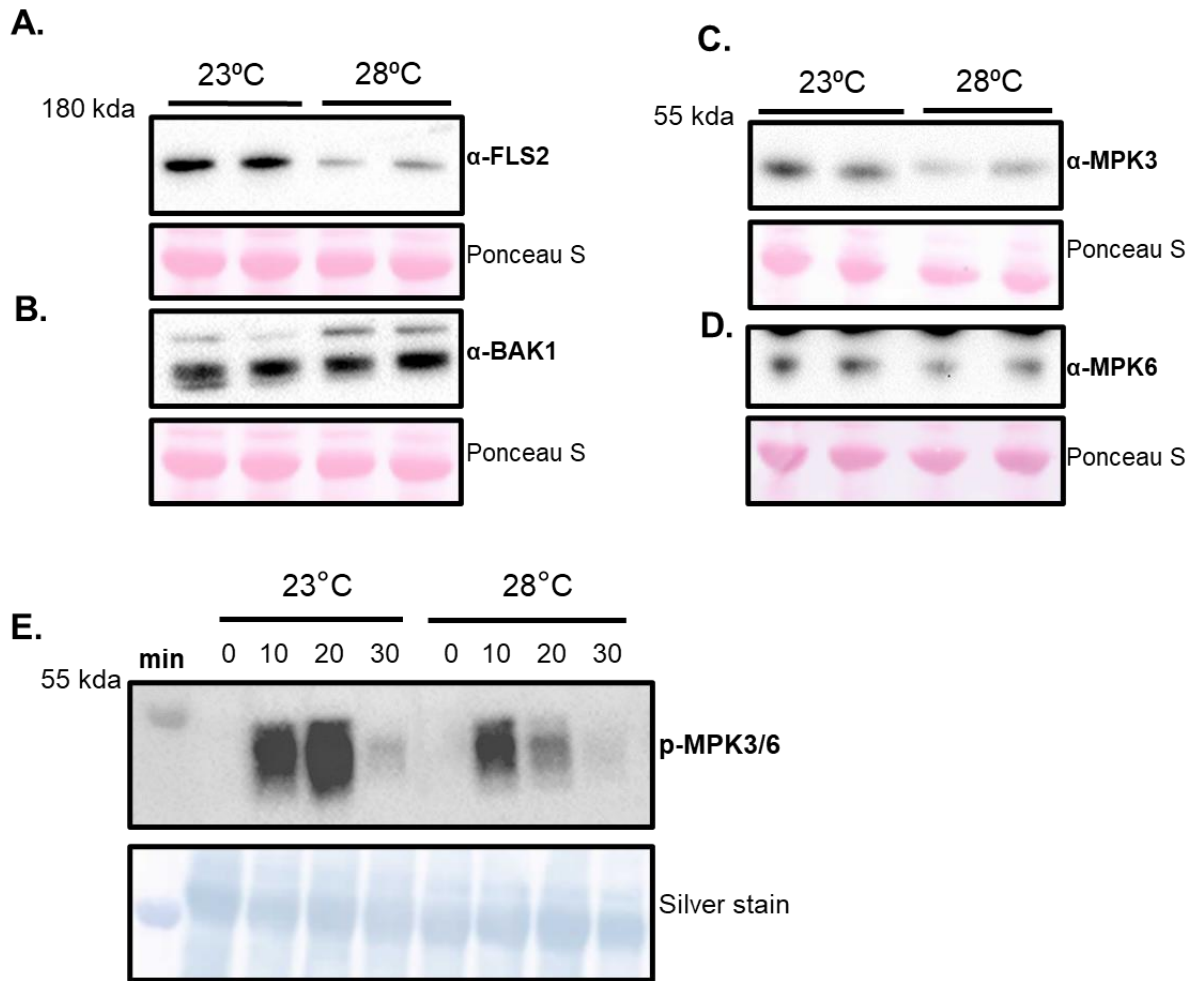


Figure 10. Elevated temperature reduces basal protein levels of some, but not all observed, PTI components. (A) Total FLS2 proteins, (B). Total BAK1 protein, (C). Total MPK3 and (D). Total MPK6 antibodies. Proteins were detected using HRP-conjugated antibodies that interact with antibodies specific to each protein assessed. Plant tissue was pooled from 4 independent plants that had been temperature treated for 24 hours. Each lane represents 2 different biological pools of samples. All experiments were replicated 2 independent times. (E). Phosphorylated MPK3/6 proteins detected using a p44/42-ERK antibody. 2 leaves were harvested at indicated time points after flg22 infiltration. Ponceau-S and Silver staining were provided to demonstrate equal loading of samples in wells.

2.2.6 Effect of overexpressing CPK5 on flg22-induced immunity at elevated temperature

Disruptions in early upstream calcium signaling suggest that this node of PTI is temperature sensitive. Therefore, I hypothesized that by overexpressing key signaling mediators of $[Ca^{2+}]$ signaling, the reduced $[Ca^{2+}]$ signal might restore $[Ca^{2+}]$ -dependent phenotypes at elevated temperature. I decided to investigate overexpression of CPK5 (35S::CPK5), which has previously been reported in the literature to display enhanced $[Ca^{2+}]$ phenotypes at normal temperature, such as enhanced immunity against bacterial pathogens, spontaneous lesions, elevated levels of SA, and heightened $[Ca^{2+}]$ -dependent gene expression (Dubiella *et al.*, 2013, Guerra *et al.*, 2020). I hypothesized that 35S::CPK5 plants would be capable of restoring flg22 protection at elevated temperatures by amplifying the calcium-dependent branch of immunity. Indeed, I found that flg22-protection assays displayed similar levels of protection at 23°C and 28°C in 35S::CPK5 plants (Figure 11A-B). With these promising results, 35S::CPK5 plants were further investigated to observe which elements of PTI were recovered at elevated temperature relative to Col-0. Since 35S::CPK5 has elevated levels of SA levels at 23°C, I investigated whether SA biosynthesis was recovered at 28°C. As previously reported, 35S::CPK5 plants have elevated levels of SA and SAG at 23°C regardless of treatment (Guerra *et al.*, 2020). However, the elevated levels and inducibility of SA were found to be temperature sensitive at 28°C (Figure 11C). To determine whether 35S::CPK5 plants were restored in immunity through the calcium-dependent processes such as ROS and $[Ca^{2+}]$ -dependent gene expression. I found that *FRK1* expression was not temperature sensitive in 35S::CPK5, as it is in Col-0, but *NHL10* and *CYP81F2*

expression was temperature sensitive at 28°C (Figure 12A). Furthermore, flg22-induced ROS was also temperature sensitive and did not differ from Col-0 (Figure 12B). This suggests that overexpressing CPK5 was not sufficient to restore temperature sensitive PTI-associated processes. It is likely that 35S::CPK5 has high basal levels of SA when grown at 23°C and that this large residual pool of SA likely remains bioactive at 28°C for inducing immunity. Presumably, while there might be reduced induced SA biosynthesis at elevated temperature, this remaining pool of elevated SA may contribute to the enhanced flg22 protection observed at 28°C in 35S::CPK5 plants.

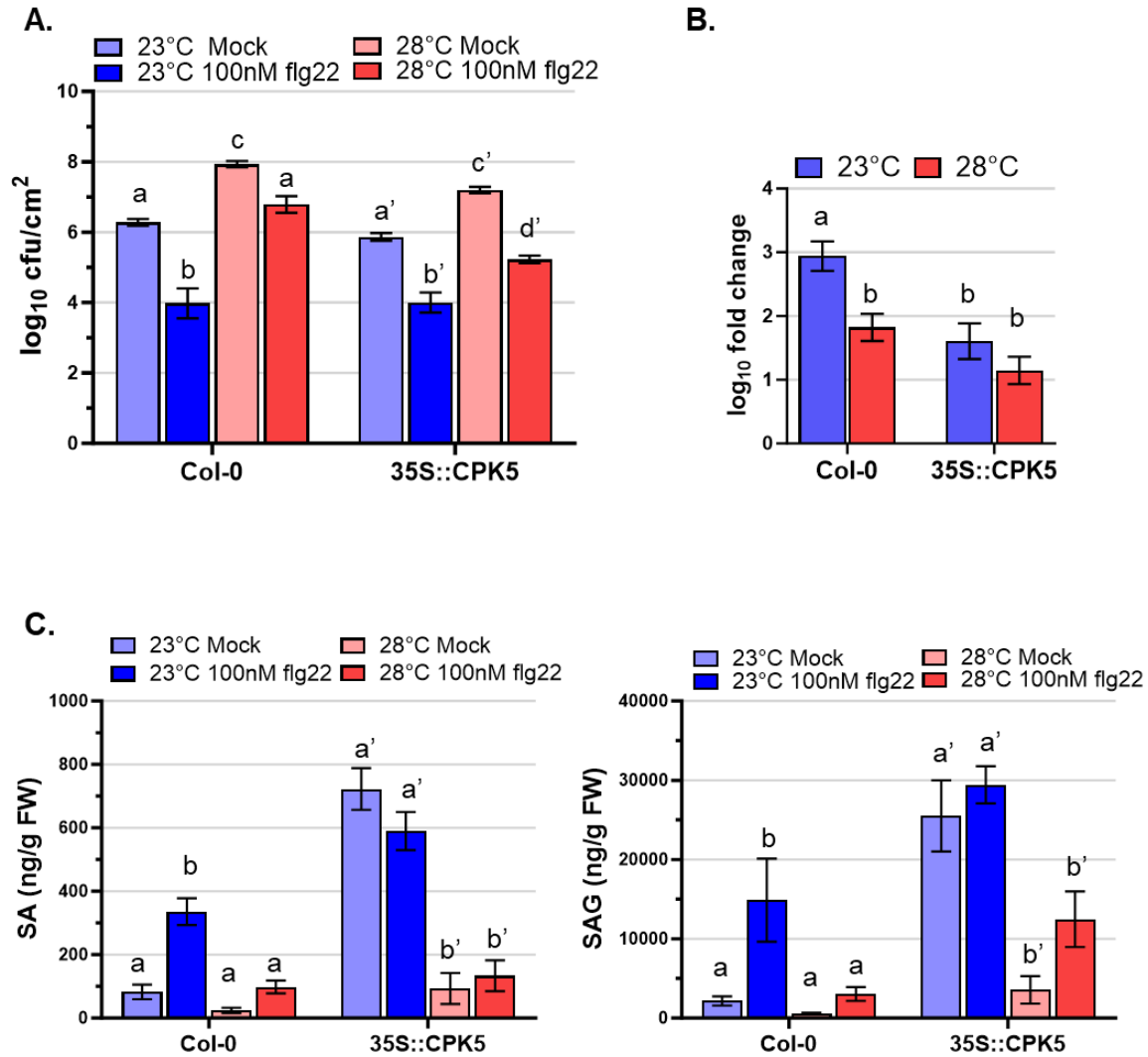


Figure 11. Overexpression of CPK5 restores flg22-based protection at elevated temperature but is temperature sensitive to SA biosynthesis in PTI signaling (A). Bacterial populations of DC3000 2 days after flg22 treatment of temperature acclimated Col-0 and 35S::CPK5 plants. (B) The degree of protection against DC3000 between mock and flg22 treated plants as visualized in a. (C) SA and SAG levels between Col-0 and 35S::CPK5 at 23 and 28°C 24 hours after flg22 treatment were assessed using LCMS. All experiments underwent 3 independent experimental replicates to determine representative results (n=4, error bars = SEM). Letters denote statistical differences between groups as determined by 2-Way ANOVA and Tukey's HSD post-hoc test ($P < 0.05$). The symbol (') denotes that two groups were analyzed in two groups based on genotype.

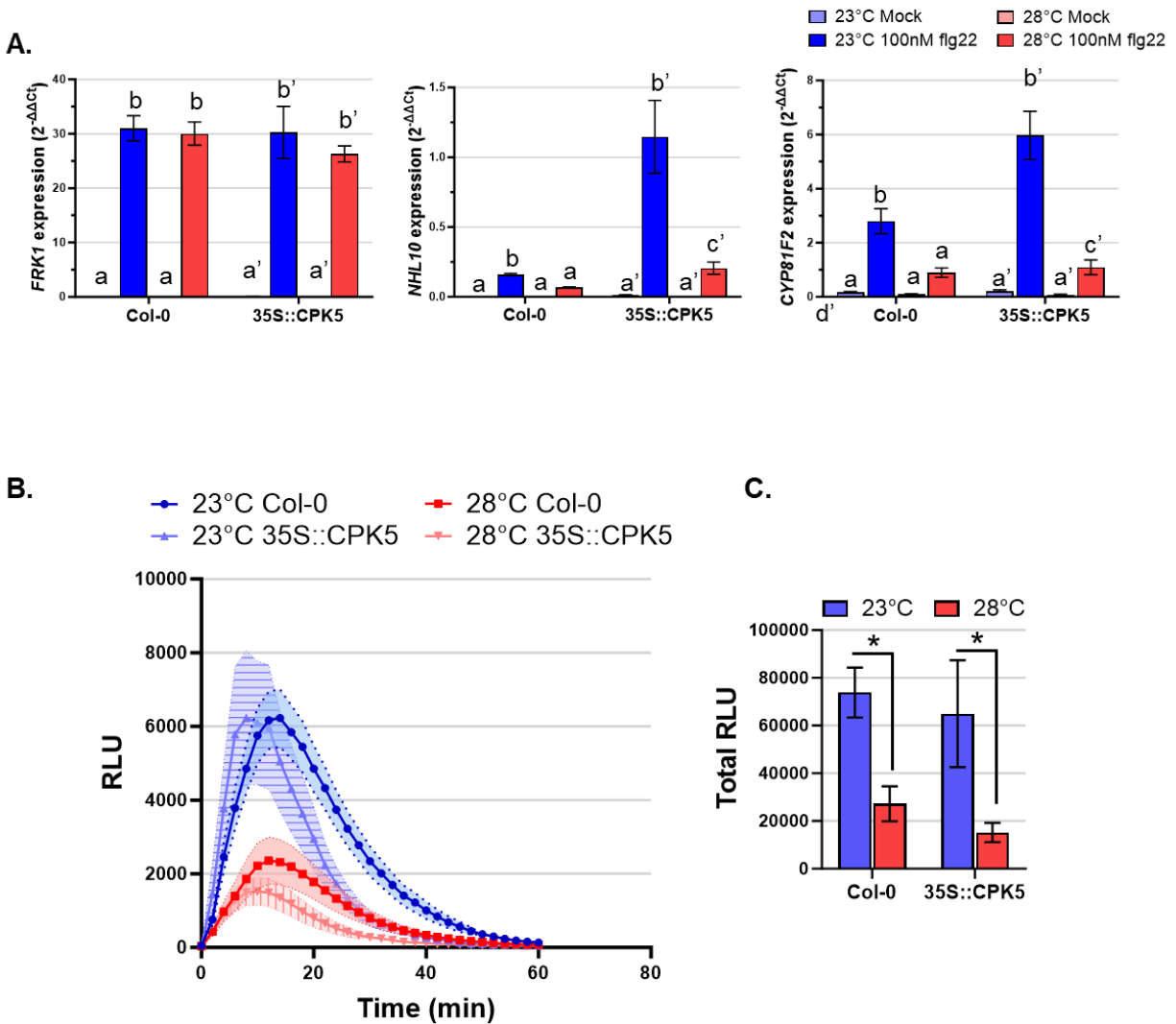


Figure 12. 35S::CPK5 plants display temperature sensitivity in flg22-induced gene expression and ROS. (A). Expression of *FLS2*, *NHL10*, and *CYP81F2* were determined in temperature acclimated 35S::CPK5 and Col-0 at 3 hours, 3 hours, and 30 minutes post flg22 treatment. Two independent experiments were used to determine a representative result ($n=4$, Error Bars = SEM). (b). Time course of flg22 induced ROS in temperature acclimated leaf discs from Col-0 and 35S::CPK5 ($n=8$) with 3 technical replicates. (c). Total RLU detected during the 60-minute time course. Statistical significance was determined using 2-Way ANOVA ($P < 0.05$) and letters denote statistical significance between sample type and treatment. The symbol (') denotes that two groups were analyzed in two groups based on genotype.

2.2.7 Induced SA signaling at elevated temperature enhances PTI outputs.

Previous research by our lab (Huot *et al.*, 2017) showed that forced SA signaling by BTH, a synthetic analog of SA, was sufficient to restore Arabidopsis immunity to *Pst.* DC3000 without rescuing SA signaling. The underlying mechanism behind this phenomenon has remained unclear. I explored gene clusters reported by Huot *et al.*, 2017 to investigate potential pathways that are responsible for temperature-insensitive immunity at 28°C. Go-Enrichment via previous DAVID analyses suggests that “Response to Pathogen” was highly represented in genes that were equally induced under both conditions. Investigating this cluster revealed many genes associated with PTI, such as *FLS2*, *BIK1*, and various PTI-associated kinases. I therefore hypothesized that BTH was inducing immunity at both temperatures by magnifying the PTI responses. SA has been shown to enhance the levels of PTI components in Arabidopsis, where both MAPK levels and phosphorylation status were elevated after BTH treatment, along with compromised BTH-induced immunity in the *mpk3* and *mpk6* null mutants against *Pst.* DC3000 (Beckers *et al.*, 2009). BTH also has been observed to induce *FLS2* and *BAK1* that localize at the cell membrane and enhance ROS generation in response to flg22 (Tateda *et al.*, 2014, Xu *et al.*, 2014). I hypothesized that the temperature-insensitive immunity observed in BTH treatment may be caused by the ability of BTH to enhance PTI outputs at both temperatures.

To confirm gene expression results from Huot *et al.*, 2017, I tested gene expression and protein levels of individual PTI components of Col-0 plants that were subjected to 23°C vs 28°C and subsequent BTH treatment. Expression of *FLS2*, *BAK1*, and *MPK3* was significantly induced at 28°C, but not to the same degree as observed at

23°C (Figure 13A). Further assessing protein levels from the same plants suggest that BTH enhanced the protein levels of FLS2 and MPK3 (Figure 13B). To determine if BTH was enhancing PTI outputs at 28°C, I performed a ROS assay on temperature and BTH pre-treated plants. I observed that BTH does enhance flg22-induced ROS at both temperatures, but the magnitude of induction is temperature sensitive. This suggests that BTH enhances the outputs of PTI, but whether that is responsible for full BTH-induced immunity observed at both temperatures is not clear.

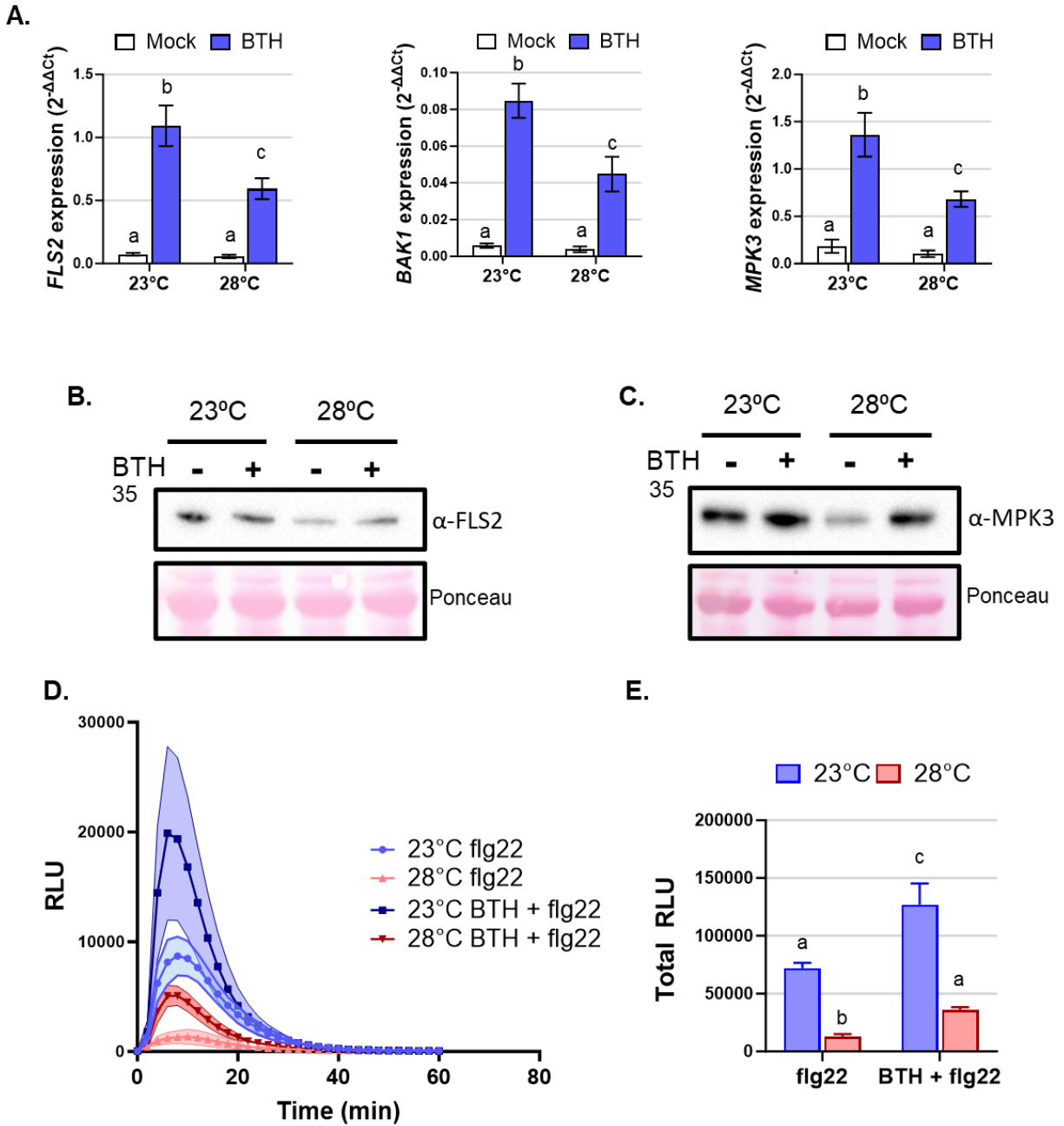


Figure 13. BTH enhances transcript and protein levels of some key PTI components and enhances PTI outputs at both temperatures. (A) Gene expression of *FLS2*, *BAK1*, and *MPK3* were assessed in Col-0 plants acclimated to elevated temperature 24 hours after to 100 μ M BTH treatment. Relative expression was determined using qPCR and normalized to expression of the gene *PP2A3*. These results are representative of two independent experimental replicates. Error bars indicate SEM and $n=4$. Different letters denote statistical significance between samples as determined using a 2-Way ANOVA using Tukey's post-hoc test ($P < 0.05$). (B) *FLS2* and (C) *MPK3* protein levels were observed in response to BTH treatment in temperature acclimated Col-0 plants. Proteins

Figure 13 (cont'd)

were visualized using secondary HRP-conjugated antibodies that recognize antibodies specific to FLS2 and MPK3 respectively. Ponceau S staining was included to show equal loading. Results are representative of three independent experimental replicates. (D). ROS generated in time lapse of temperature adjusted BTH treated Col-0 plants over 60 minutes. Shaded areas indicate a 95% confidence interval (n=8). (E). Total ROS elapsed from part (D) over the 60-minute period. Experiments were replicated 3 times to ensure reproducibility and results show the representative results. Letters indicate statistical significance ($P < 0.05$) using a 2-Way ANOVA and groups were assessed using Tukey's post hoc test.

While it has been explored previously that SA and BTH can enhance immunity by enhancing PTI, it has not been addressed whether PTI is required for SA-mediated defenses. Based on the enhanced PTI components and temperature-resilient immunity in BTH-treated plants at elevated temperature, I hypothesized that deficiencies in PTI result in a loss of BTH-induced protection. To test this hypothesis, I performed disease assays in the *bak1-5/bkk1-1/cerk1 (bbc)*, a mutant deficient in multiple PRR signaling pathways that utilize BAK1 and BKK1 as co-receptors to many PRR complexes, along with CERK1 which has been reported to perceive peptidoglycans and chitin (Xin *et al.*, 2016, Macho and Zipfel 2016). Using *npr1* as a control, which is deficient in BTH-induced immunity. I observed that *bbc* plants displayed substantially weaker BTH protection. To confirm that *bbc* plants are perceiving BTH similar to Col-0, I investigated *PR1* gene expression, which is a marker gene for SA signaling. While there is a modest decrease in *PR1* expression, it does not appear to be statistically significant, suggesting that *bbc* plants perceive BTH similarly to Col-0 plants. Overall, my results suggests that PTI is required for BTH-induced immunity and that BTH is capable of enhancing PTI responses at elevated temperatures.

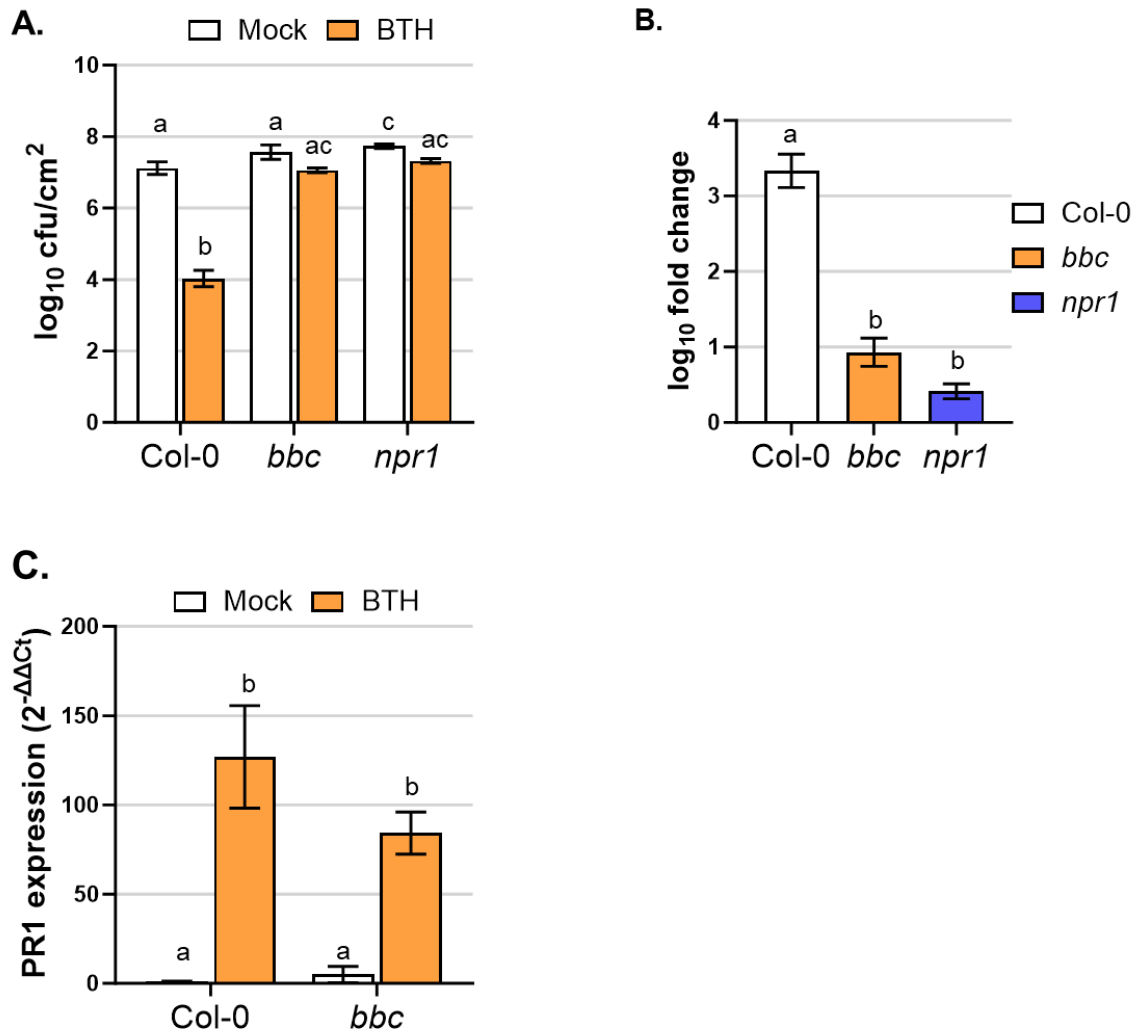


Figure 14. BTH protection is compromised in PTI deficient mutants. (A) Bacterial counts of *Pst* DC3000 in BTH-treated Col-0, *npr1*, and the PTI deficient mutant *bak1/bkk1/cerk1* (*bbc*) plants 2 days after infection. (n=4) 2 days after DC3000 (B) Change in bacterial levels demonstrated in (A). *npr1* is used as a control to show a complete defect in BTH-induced protection. Error bars indicate SEM and letters denote statistical significance determined via a 2-Way ANOVA with groupings determined by Tukey's post-hoc test ($P < 0.05$) Results from one experiment shown here are representative of three independent experimental replicates. (C) Gene expression of *PR1* 24 hours after BTH treatment in Col-0 and *bbc* plants (n=4, error bars represent SEM). Letters indicate statistical significance ($P < 0.05$) using a 2-Way ANOVA and groups were assessed using Tukey's post hoc test.

CHAPTER 3 DISCUSSION AND FUTURE DIRECTIONS

3.1 Discussion

3.1.1 Integrating Immunity into a broader understanding of temperature-PTI interactions.

This study provides a novel insight into how elevated temperature influences specific branches of PTI through an integrative approach to address how temperature influences immunity. Previous research had concluded that elevated temperature upregulates PTI signaling in *Arabidopsis* and suggested that there was a bifurcation in PTI and ETI that was temperature dependent where ETI was weaker at elevated temperature and PTI was enhanced (Cheng *et al.*, 2013). These experiments looked at the effects of a range of temperatures between 4°C and 32°C, on *FRK1* and *WRKY29* gene expression, and the phosphorylation status of MPK3/6 and BIK1. Between 23°C and 28°C, there was no significant difference in *FRK1* gene expression or MPK3/6 phosphorylation, while an upregulation of *WRKY29* was observed in protoplasts exposed to 28°C. These experiments were performed on 10-day old seedlings grown on plates and protoplasts from plate-grown young seedlings grown. These young plants and protoplasts were exposed to temperature treatments for 45 minutes prior to assaying. In contrast, my research highlights that flg22-induced immunity is partially suppressed in soil-grown adult plants with a 24-hour temperature treatment. The differential experimental conditions may underlie some of the discrepancies between our results. The importance of temperature treatment in the study of temperature-PTI interplay is further illustrated in research from Janda *et al.*, 2019. The researchers investigated how “heat shocks” between 32-42°C negatively influenced PTI in *A. thaliana* and found that 42°C exposure for 15-45 minutes drastically compromised flg22-

induced ROS and FLS2 expression and protein levels. These findings support my research converging on claims that PTI is sensitive to significantly warmer temperatures relative to typical temperatures *A. thaliana* experiences.

3.1.2 Temperature-Calcium interactions may serve as a convergent point of sensitivity in plant immunity.

Calcium has previously been hypothesized to regulate SA-dependent phenotypes in *Arabidopsis*. (Du *et al.*, 2009, Lenzioni *et al.*, 2018). There are several types of calcium sensing proteins that could provide links between temperature sensing and immunity (Figure 15).

The six *Arabidopsis* CAMTA transcription factors were the first proteins that linked the calcium response to SA regulation in the context of cold-temperature tolerance, providing a pre-established link between temperature and SA-biosynthesis (Pooviah *et al.*, 2009, Kim *et al.*, 2013, Du *et al.*, 2009). Within the normal temperature range of *Arabidopsis*, CAMTA TFs appear to repress expression of SA-related genes, like *PR1* and *CBP60g* as the *camta1*, 2, and 3 mutants display smaller stature, heightened basal SA levels and constitutive *PR1* expression and higher-order *camta* mutants display enhanced SA-dependent phenotypes observed in the single order mutants. CAMTA TFs have a high affinity for $[Ca^{2+}]$ -bound CaM which binds to the IQ domain in the C-terminus and influences CAMTA's nucleotide-binding domain on the N-terminus. Mutations in the IQ domains influence the nucleotide-binding ability to repress transcription of SA biosynthetic genes (Kim *et al.*, 2017). CaM binding is associated with release from transcriptional repression, suggesting that $[Ca^{2+}]$ -spiking in response to pathogen infection would release CAMTA TFs from repressing genes like *ICS1* and

represent part of a mechanism to allow expression of SA-related genes in response to pathogen infection (Reddy *et al.*, 2011). Furthermore, chilling temperatures induce intracellular calcium spiking, which is thought to be perceived via CaM, CML, and CAM-binding transcription factors like CAMTA (Eckardt 2009). Exposure to 4°C induces SA biosynthesis and freezing tolerance in part by the release from SA-regulatory sequences and degradation of CAMTA TFs, where plants appear dwarfed due to high SA levels. Growing *camta1/3* double mutants at 25°C-27°C reverses the growth inhibition observed at 19-20°C and reduces the intracellular SA levels (Pooviah *et al.*, 2009). The *camta2/3* mutants have high residual SA levels that may confer enhanced immunity when the plants are adjusted to elevated temperature, but SA biosynthesis is unresponsive at 30°C. While CAMTAs serve as negative regulators of SA biosynthesis, their removal is insufficient to drive *ICS1* expression at 30°C after pathogen infection (Huot *et al.*, 2017). This suggests that removing CAMTA repression at elevated temperatures is insufficient to drive SA biosynthesis, suggesting other transcriptional regulators may not be present or functional to drive *ICS1* gene expression at elevated temperature.

While CAMTA serves as a transcriptional regulator that links [Ca²⁺] to negatively repressing SA-related responses, CBP60g demonstrates a positive regulator of plant immunity that links [Ca²⁺]-signaling to SA biosynthesis. CBP60g has a calmodulin-binding domain at the N-terminus, which is required for its transcriptional activity (Wang *et al.*, 2009, Wang *et al.*, 2011). 35S::CBP60g does not display constitutively high expression of *ICS1* at 23°C and requires an immune elicitor like *Pst.* DC3000 to induce CBP60g function and *ICS1* expression. This immune elicitor acts at the level of CBP60g

since 35S::ICS1 plants display constitutively high levels of SA and are significantly dwarfed, indicating that the pathogen-derived signal does not influence ICS1 function (Kim *et al.*, 2022). Beyond CBP60g, there are other classes of calmodulin-binding TFs that have been implicated in regulating SA biosynthesis. CBP60b was identified as a positive regulator of SA since *cbp60b* plants display reduced levels of SA after pathogen challenge (Li *et al.*, 2021). CBP60a likely acts as a negative regulator of SA biosynthesis as *cbp60a* null mutants display heightened basal and induced levels of SA after pathogen challenge and are more resistant to biotrophic pathogens (Truman *et al.*, 2013).

CPK5 also appears as a positive regulator of SA biosynthesis and signaling as CPK5 overexpression displays constitutively active SA-dependent responses like dwarfism, spontaneous cell death, and constitutively high levels of SA (Guerra *et al.*, 2020). CPK5 has a very low K_m (100nM) towards $[Ca^{2+}]$, which allows this kinase to rapidly respond to rapid and early influx of $[Ca^{2+}]$ and initiate early immune phenotypes like RBOHD phosphorylation to induce ROS. Furthermore, CPK5 is capable of phosphorylating and activating WRKY28, a TF known to positively regulate expression of *ICS1* and *PBS3* via promoter binding (Van Verk *et al.*, 2011, Gao *et al.*, 2013). This provides a direct link between $[Ca^{2+}]$ and SA biosynthesis where CPK5 activation by $[Ca^{2+}]$ induces SA biosynthesis. It would be interesting to see if CPK5-dependent phosphorylation of upstream PTI responses on RBOHD and downstream WRKY28 phosphorylation are affected by elevated temperature, providing a mechanistic link between PTI and SA biosynthesis through calcium.

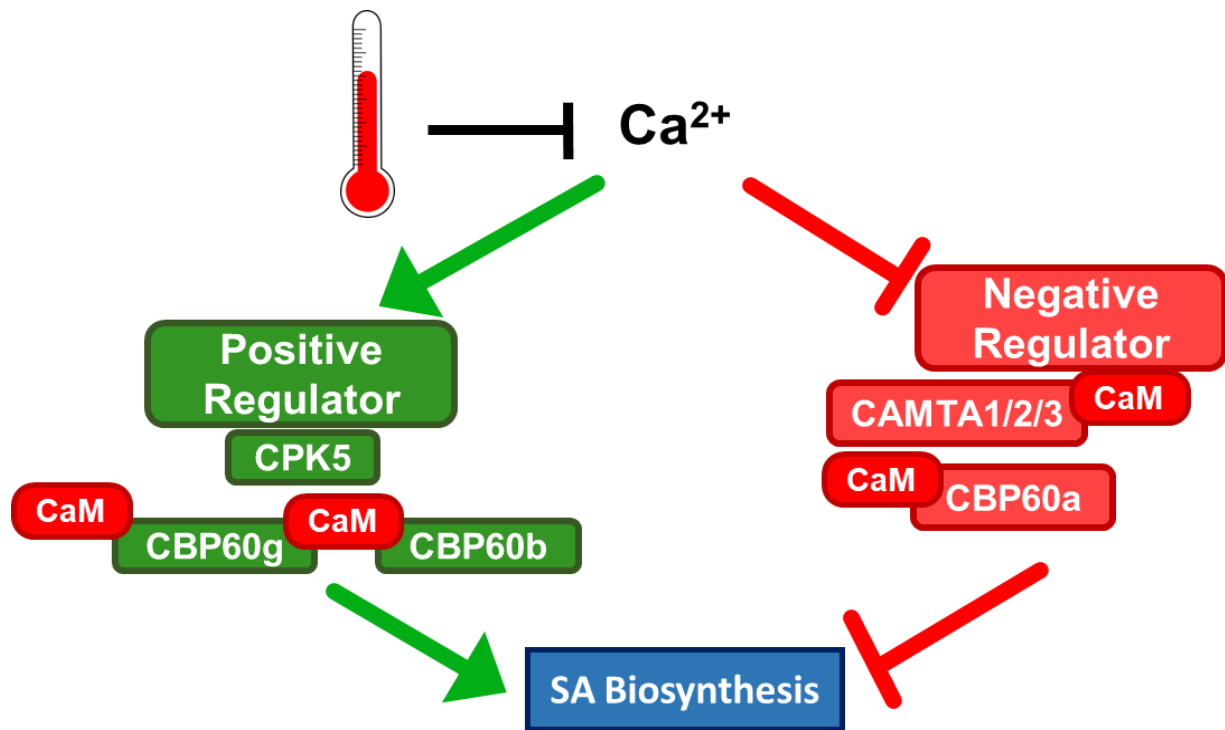


Figure 15. Calcium putatively serves as a regulator of SA biosynthesis. Calcium serves as a secondary messenger that influences SA biosynthesis and signaling. Many positive regulators like CPK5 and CBP60g require calcium to induce transcriptional reprogramming that drives SA biosynthesis. Furthermore, negative regulators like CAMTA and CBP60a negatively influence SA-responses like biosynthesis. These $[\text{Ca}^{2+}]$ transients may be separated in time and space that determine the order of events that dictate the activity of SA biosynthesis to maximize defense without overwhelming the cell.

35S::CBP60g and 35S::CPK5 both lacked flg22-induced SA biosynthesis at elevated temperature, suggesting that merely overexpressing a $[Ca^{2+}]$ -dependent point of sensitivity is insufficient to restore SA biosynthesis in response to flg22. This study identifies that cellular basal $[Ca^{2+}]$ levels are significantly lower at 28°C and 23°C. Since both proteins are gated by CaM/ $[Ca^{2+}]$, their functions would still be limited at 28°C as the cellular levels of $[Ca^{2+}]$ must reach a higher concentration to activate these proteins. Loss of flg22-induced SA biosynthesis in 35S::CBP60g may be due to calcium-dependent negative regulators like CAMTA being unable to release their transcriptional repression of SA biosynthetic genes.

Previous research from our lab identified that $\Delta hrcC$ was capable of inducing SA biosynthesis in the 35S::CBP60g background at elevated temperature, but not in Col-0 (Kim et al. 2022). This further highlights the differential dynamics between eliciting with a singular high concentration PAMP, versus a multitude of PAMPs on the microbe that persists within the plant, which may continually provide immune stimulation that is significant to activate CBP60g for *ICS1* expression. Interestingly, $\Delta hrcC$ displayed a temporal induction of SA, where SA levels were significantly higher at 12 hours post-infection but was significantly attenuated, yet temperature insensitive, at 24 hours (Kim et al., 2022). This temporal difference may explain the different kinetics of flg22 vs. microbial elicitation of SA-biosynthesis. I investigated protein levels of HA-tagged CBP60g in response to flg22 and *Pst*. DC3000 at early and late time points (Zhang et al., 2010). CBP60g protein levels were significantly induced in response to flg22 at 4 hours at both temperatures but protein levels were significantly attenuated at 24 hours at 28°C but not 23°C. Gene expression 6 hours after flg22 treatment revealed that this

expression also appears to be transient (Figure A1), correlating closely to the protein data. The transient nature of this induction was surprising, since no SA biosynthesis was detected after 24 hours of treatment. Our previous research identified that CBP60g is regulated in part by the NPR1-TGA1/4 complex, which is non-functional at elevated temperature. The null mutants *npr1* and *tga1/4* show reduced (but not absent) SA levels in response to pathogens (Sun *et al.*, 2018). It has been shown that NPR1 modulates expression of SA biosynthetic genes via a positive feedback loop through NPR1 to tightly control immune outputs (Li *et al.*, 2018). The absence of a functional NPR1-TGA complex at 28°C suggests there must be a secondary mechanism of inducing CBP60g at early time points that is amplified through the NPR1 signaling pathway. I hypothesize there may be a temporal and temperature-sensitive bifurcation in CBP60g induction, where early induction of CBP60g would induce low levels of SA, which would amplify CBP60g expression through NPR1. Understanding the temporal and temperature sensitivity of the CBP60g promoter and the TFs that regulate its expression will be key for connecting how PRRs directly activate SA biosynthesis and may identify new targets that regulate plant immunity.

Negative regulation of PTI by elevated temperature provides new evidence suggesting how ETI is also perturbed at elevated temperature in *Arabidopsis*. Many components of PTI and ETI are shared, such as RBOHD, BIK1, MPK3/6, [Ca²⁺] Channels, and phytohormones like SA, JA, and ethylene (Kadota *et al.*, 2014, Yuan *et al.*, 2021, Ngou *et al.*, 2021). ETI has been observed to be temperature-sensitive depending on the pathogen effector and host NLR combinations. For example, the hypersensitive response (HR), which manifests as spontaneous cell death to limit

pathogen growth, is temperature sensitive in *Arabidopsis* when elicited by AvrHopZ1a and AvrRpt2. Additionally, natural variation in *Arabidopsis* identified natural accessions that maintained immunity at 27°C (Menna *et al.*, 2015). AvrRpt2, AvrRpm1, and AvrRps4 all demonstrate compromised HR and resistance at 28°C which is not due to reduced levels of RIN4 or RPS2 which are key NLRs for perceiving pathogen-derived effector proteins (Wang *et al.*, 2009, Cheng *et al.*, 2013). The linkage between components of PTI and ETI would support the hypothesis that plant immune systems are modulated by the environment via $[Ca^{2+}]$. Since HR, ROS, and $[Ca^{2+}]$ are intimately linked for plant immunity phenotypes, it would be interesting to see if temperature interactions with HR correspond to changes in the $[Ca^{2+}]$. These findings are reinforced by the study showing that CDPK1/2/4/5/6 and 11 are involved in positive regulation of HR, further highlighting how $[Ca^{2+}]$ perturbations induced by the environment would impact the entire plant immune system (Gao *et al.*, 2013). Many crop varieties developed disease resistance through extensive breeding approaches to isolate NLR genes which confer immunity to specific plant diseases via ETI. Underpinning how the environment modulates both PTI and ETI will be paramount for maintaining disease resistance as global temperatures shift.

Temperature perception through $[Ca^{2+}]$ -signaling is a repeating theme across biological taxa, as mammals perceive heat directly through the mechanosensitive $[Ca^{2+}]$ -ion channel TRPV1 (Liu *et al.*, 2003). Exposure to temperature extremes has been shown to stimulate $[Ca^{2+}]$ transients in the cytoplasm in response to heat shocks or near-freezing temperatures in plants (Plieth *et al.*, 1999, Saidi *et al.*, 2009). Adapting to changing temperatures, known as thermotolerance, seems to require the plant $[Ca^{2+}]$ -

ion channel CNGC2/4, which forms a heteromeric complex in the plasma membrane to form a complete pore (Finka *et al.*, 2012). CNGC2/4 have been investigated as known regulators of PTI. Null mutations in *cngc2* confer enhanced resistance but a loss of cell death (Tian *et al.*, 2019, Clough *et al.*, 2000, Chan *et al.*, 2003). CNGC2/4 may serve as a nexus point that integrates both environmental and biotic signals to mount the appropriate response. The lowered basal $[Ca^{2+}]$ levels observed in this thesis, along with Hilleary *et al.*, 2020, suggest that there is some mechanism that decreases the availability of $[Ca^{2+}]$. If temperature alters the efficiency of these channels either through direct temperature-channel kinetics, changes to the plasma membrane composition, or through post-translational modifications, then such a study would provide how these $[Ca^{2+}]$ channels integrate both abiotic and biotic stress.

In summary, $[Ca^{2+}]$ has emerged as an important regulator of SA biosynthesis where both negative and positive regulators work in a tightly controlled manner to rapidly respond to a changing environment (Figure 15). However, within the context of this research, it is difficult to assess what are the causative mechanism(s) underlying the reduced basal and flg22-induced $[Ca^{2+}]$. Research from Hilleary *et al.*, 2020 underscores that directly manipulating cytoplasmic $[Ca^{2+}]$ influences SA biosynthesis. The *aca4/11* mutant which lacks tonoplast-localized extracellular facing calcium pumps exhibits elevated cytoplasmic $[Ca^{2+}]$ and constitutively high SA levels. $[Ca^{2+}]$ and SA-related phenotypes are suppressed at 28°C, including the basal level of $[Ca^{2+}]$, suggesting that inflow of $[Ca^{2+}]$ is inhibited by warmer temperatures. Thus, the lowered basal $[Ca^{2+}]$ at elevated temperature may serve to decrease the sensitivity of CaM and

[Ca²⁺]-dependent proteins, limiting their activity at elevated temperatures.

3.1.3 Elevated temperature negatively impacts basal PTI levels

The observed decrease in basal PTI components at elevated temperature partially explains why flg22-mediated protection is dose-dependent, as higher doses of flg22 may compensate for the reduced levels of FLS2 and associated signaling components. FLS2 protein levels can be regulated via E3-ubiquitination along with phosphorylation of the co-receptor BAK1, which drives the degradation of the PRR receptor complex (Lu *et al.*, 2011, Yan *et al.*, 2012). A previous finding that FLS2 protein levels and localization are quickly modified to negatively impact PTI after exposure to heat shocks suggests that these changes happen rapidly, and that post-translational modifications impact PTI in a temperature-dependent manner (Janda *et al.*, 2019). Furthermore, PTI is regulated by the phytohormones SA, JA, and ethylene, which have been explored in the context of basal resistance in *Arabidopsis* in our lab (Tsuda *et al.* 2009, Huot *et al.* 2017). CBP60g targets and promotes the transcription of many PTI components in addition to SA biosynthesis, like MPK3, MKK1, BAK1, and FLS2 (Sun *et al.*, 2018), suggesting that SA biosynthesis is also correlated with maintaining appropriate levels of PTI components. However, in the *ics1* mutant, FLS2 protein levels remain unaffected, suggesting this decrease in PTI components is not directly caused by SA levels directly (Tateda *et al.*, 2014). Experiments investigating how PTI protein levels in the *cbp60g/sard1* are affected would help reveal if these TFs play a role in maintaining basal levels of PTI proteins.

While JA and Ethylene show upregulation at elevated temperatures, it is also unclear whether these hormone pathways govern the temperature-sensitive PTI protein

levels as well (Clarke *et al.*, 2009, Huot *et al.*, Havko *et al.*, 2020). Heightened JA responses by exogenous JA negatively influence flg22-ROS and other PTI phenotypes, suggesting that elevated temperature's influence on JA signaling may contribute towards reduced PTI levels (Ishiga *et al.*, 2009). However, ethylene is a positive regulator of PTI components as mutants deficient in ethylene have reduced FLS2 levels and weaker flg22 protection (Boutrot *et al.*, 2010). It would be interesting to investigate whether these two pathways influence the flg22-phenotypes at elevated temperature to further understand how PTI levels are modulated in response to the environment.

3.1.4 Temperature interferes with canonical SA signaling but primes PTI outputs through a novel mechanism

How exogenous BTH or high endogenous levels of SA prime PTI at elevated temperature is perplexing, considering that the NPR1/TGA/CBP60g module is temperature sensitive in response to BTH (Kim *et al.*, 2022). Since BTH protection is NPR1 dependent, the observed increases in PTI components are likely through an intact NPR1 signaling module that drives this phenotype, but not those affiliated with regulating SA-specific responses like *CBP60g* and *PR1* expression. It would be important to identify what targets of NPR1 are responsible for primed PTI outputs. The dependency of PTI for SA/BTH-induced immunity has been explored in the context of MAPK activation, where *mpk3* and *mpk6* mutants display weakened BTH-induced protection (Beckers *et al.*, 2009). Exogenous BTH enhances ROS and PRRs to exert flg22-dependent phenotypes and is NPR1 (Tateda *et al.*, 2014). This research provides evidence that through the analysis of the *bbc* mutant, PTI not only contributes towards BTH-induced immunity, PTI needs to remain functional for BTH to induce immunity. The

recent discovery that ETI is dependent on functional PTI further underscores that modifications to PTI intensity will determine the functionality of the entire plant immune system and the outcome of plant-pathogen interactions in *Arabidopsis* (Ngou *et al.*, 2021, Yuan *et al.*, 2022). Identifying the targets of NPR1 that are involved in potentiating PTI would identify compensatory mechanisms that enhance disease resistance in an environment-independent context. From this research, I have generated a model through which elevated temperature negatively impacts PTI by compromising $[Ca^{2+}]$ -signaling. However, plants have a temperature-insensitive branch of immunity, which is modulated by SA, that is capable of inducing resistance against pathogenic bacteria (Figure 16).

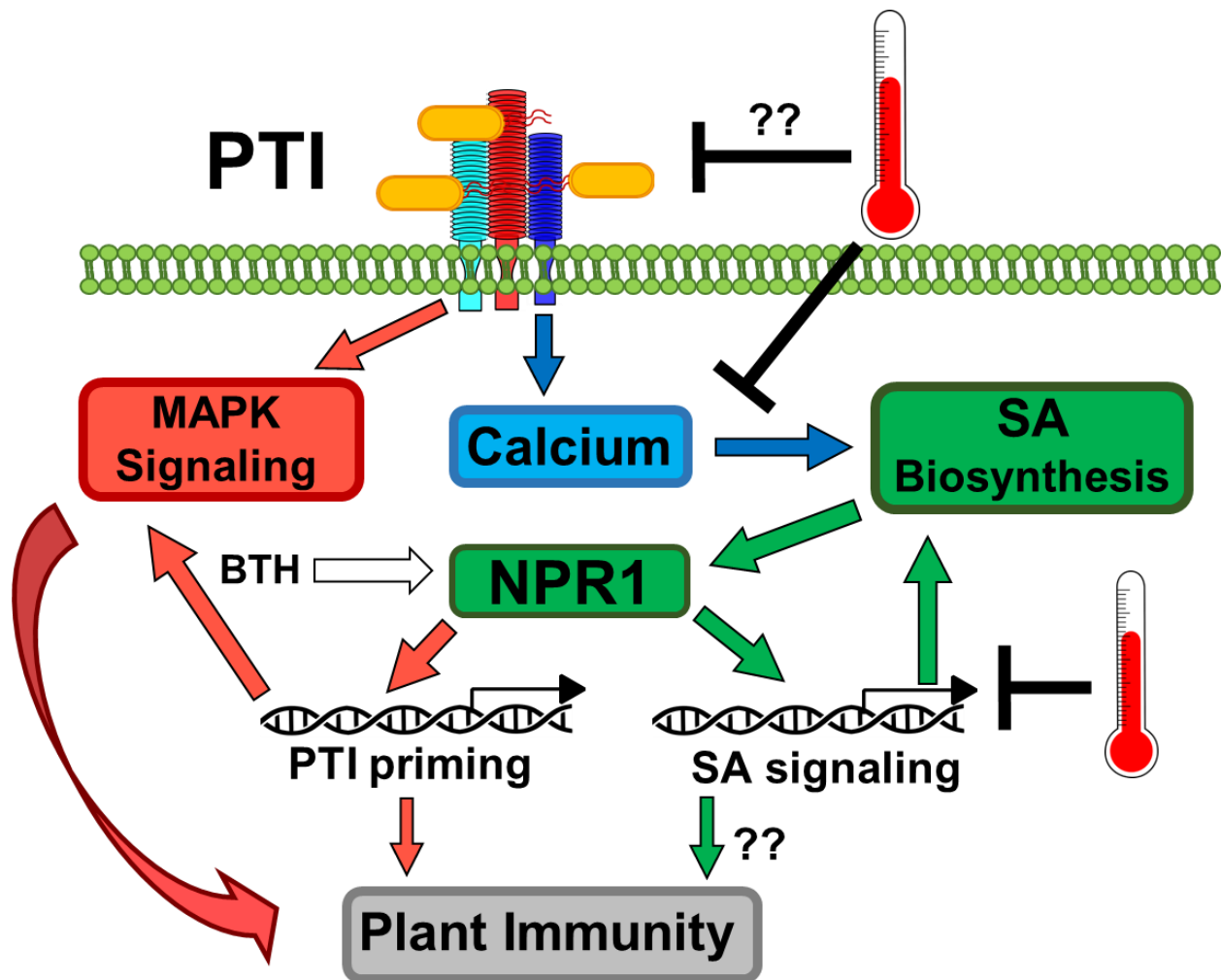


Figure 16. A framework for how elevated temperature interferes with PTI. Elevated temperature negatively influences the calcium branch of PTI while the MAPK branch of immunity remains intact downstream of the PRR complex. This negative influence on $[Ca^{2+}]$ -dependent processes may overlap with SA biosynthesis that ultimately inhibits overall immunity at elevated temperatures. Elevated temperature may negatively regulate PRR complexes, either through RBOHD, receptor levels or localization. Meanwhile, high basal SA levels along with exogenous BTH induce immunity by enhancing elements of $[Ca^{2+}]$ -independent responses. Non-canonical NPR1-dependent signaling in response to SA analog BTH remains intact at elevated temperature which induces immunity despite loss of the SA marker gene expression and disruption to the SA amplification loop.

3.2 Future Directions

This research serves to integrate temperature into plant immunity through the lens of flg22 and PTI under highly controlled environmental conditions to provide a foundation for exploring environment by temperature interactions. Three main research questions directly emerge from the findings of this study: (1). Through what mechanism does elevated temperature influence $[Ca^{2+}]$ dynamics in response to flg22? (2) How widely conserved is the temperature sensitivity of $[Ca^{2+}]$ dynamics in response to biotic stress? (3). How does BTH influence PTI to enhance immunity at elevated temperature despite loss of canonical signaling?

3.2.1 Pathways towards understanding elevated temperature – calcium signaling interactions for flg22.

The observed decrease in FLS2 protein in this study, along with previous research demonstrating FLS2 expression, total protein levels, and PM localization is compromised after heat shock suggest that there is PRR instability at high temperatures (Janda *et al.*, 2019). It would be interesting to study the dynamics of the FLS2 protein, whether it is undergoing degradation via endocytosis or whether the kinetics via flg22 affect the receptor complex. By experimenting on 35S::FLS2-GFP, one could observe if the decrease in flg22 protection and PTI outputs are still temperature sensitive. If overexpressing FLS2 is insufficient to restore these responses, it suggests two different outcomes: that FLS2 may undergo post-translational regulation such as membrane trafficking that minimizes flg22 outputs, or that there are other temperature sensitive elements associated with PRR signaling that are independent of FLS2 protein levels. If the limiting factor that decreases the calcium signal is receptor levels at the membrane,

then overexpressing FLS2 would reveal whether the decrease in FLS2 proteins contribute to the compromised calcium phenotype. Additionally, FLS2 might undergo altered plasma membrane trafficking at higher temperatures, potentially interfering with outputs of PTI and counter overexpression to restore $[Ca^{2+}]$ -signals and immunity. Regardless, FLS2 appears to undergo some modifications in response to temperature and researching the dynamics of this, along with other members of the PRR signaling complex may reveal how PTI signaling and $[Ca^{2+}]$ -dependent phenotypes might be impacted.

If PRRs are not the site through which temperature compromises PTI, there are a few identified $[Ca^{2+}]$ -channels known to directly be involved in PTI, serving as an initial area to explore how elevated temperature may interfere with their function. CNGC2 and CNGC4 have been previously reported to aid in heat shock survival in moss and *A. thaliana*, suggesting that these calcium channels are involved in temperature perception (Finka *et al.*, 2012). The dimer formation of CNGC2/4 is also involved in $[Ca^{2+}]$ -signaling in response to PTI activation (Tian *et al.*, 2019). CNGC2/4 are directly phosphorylated by BIK1 to induce channel opening, providing a link between BIK1-activation and $[Ca^{2+}]$ -flux. Furthermore, since BIK1 induces ROS by phosphorylating RBOHD and activates downstream MAPK signaling, BIK1-might serve as a temperature sensitive node of PTI since BIK1 can initiate $[Ca^{2+}]$ -dependent and $[Ca^{2+}]$ -independent PTI pathways. Thus, it would be important to examine RBOHD and CNGC2/4 phosphorylation by BIK1 at different temperatures *in vivo*. This would resolve whether CNGC2/4 play a role in temperature sensitive calcium fluxes, as well as if ROS sensitivity at elevated temperature is due to disruptions in BIK1, CPK5 signaling, or directly on RBOHD itself.

While CNGC2/4 may serve to integrate a temperature-calcium interaction proposed in this study may not fully explain the temperature sensitivity of PTI. Null mutants of *cngc2* and *cngc4* null mutants display slightly attenuated but mostly intact PTI phenotypes in soil-grown plants, likely due to other $[Ca^{2+}]$ channels involved in PTI signaling. Additionally, how temperature may directly influence CNGC2/4 channel opening is not well understood, making it difficult to observe if there is a PTI-independent effect on these channels at different temperatures. Another $[Ca^{2+}]$ -channel that can be explored for temperature regulation is OSCA1.3, a plasma membrane localized calcium channel that regulates stomatal closure in response to PAMP perception (Thor *et al.*, 2020). OSCA1.3 is rapidly phosphorylated by BIK1, serving as another direct target of the PRR signaling complex. Since elevated temperature promotes leaf cooling via stomatal opening and evapotranspiration, it would be interesting to explore how plants balance thermal homeostasis with preventing pathogen entry. This would also provide a direct model to demonstrate how $[Ca^{2+}]$ signaling derived from higher temperature versus PTI-induced $[Ca^{2+}]$ -flux impact on stomatal aperture and thus tolerance to elevated temperature.

Reverse genetics may help to better elucidate $[Ca^{2+}]$ -homeostasis via previously identified ion channels, as there are likely other unidentified factors that regulate $[Ca^{2+}]$ homeostasis and fluxes at elevated temperature. A forward genetic screening that utilizes a high-affinity genetically encoded $[Ca^{2+}]$ -biosensor line such as R-GECO or GCaMP6 would accelerate our understanding of the factors that influence the $[Ca^{2+}]$ levels (Waila *et al.*, 2018). There were two fundamental differences in $[Ca^{2+}]$ phenotypes between the temperatures, a decrease in the magnitude in response to

flg22, and a lowering of the basal level of $[Ca^{2+}]$ in an undisturbed leaf. It is currently unclear if these two phenotypes are related, but the inducibility of $[Ca^{2+}]$ -transients are still intact at elevated temperature, suggesting that lowering cellular concentration of $[Ca^{2+}]$ might be rate limiting for $[Ca^{2+}]$ -dependent proteins. Many of the CDPKs within the same clade display significantly different affinities (K_m) to $[Ca^{2+}]$, suggesting that the decrease in basal levels may disrupt some of their function since the total $[Ca^{2+}]$ -signal is lower (Delormel and Boudsocq 2019). Screening mutagenized plants at 28°C with enhanced basal $[Ca^{2+}]$ concentrations at elevated temperature could identify if a negative regulator of $[Ca^{2+}]$ dynamics is responsible for this temperature sensitive phenotype. Elevated levels of cellular $[Ca^{2+}]$ often have dwarfed phenotypes and lesions on older leaves, so visual screening may aid in matching the cellular phenotype to an organismal phenotype. There is the possibility that the minimized $[Ca^{2+}]$ signatures are dependent on the loss of a positive regulator of $[Ca^{2+}]$, which would require a modification towards the screening approach. If there were a mutant available that was known to have constitutive $[Ca^{2+}]$ signaling at elevated temperature, mutagenizing this population to identify plants that restore temperature sensitive $[Ca^{2+}]$ would identify a positive regulator. As of now, no known mutant exists, which would make this approach impractical. Nevertheless, identifying novel components of pathogen-inducible $[Ca^{2+}]$ homeostasis that are responsive to the external environment would be essential for understanding how plants integrate environmental and biotically derived signals and respond accordingly.

3.2.2 Investigating conservation of $[Ca^{2+}]$ signaling and SA biosynthesis in other biotic and abiotic signaling pathways.

$[Ca^{2+}]$ flux occurs in response to numerous PAMPs like elf18 and chitosan, which elicit similar yet distinct $[Ca^{2+}]$ kinetics through EFR-BKK1 and CERK1 (Ranf *et al.*, 2011). Similar PRR complexes like those including PEPR1 and DORN1 can also respond to cellular damage to induce plant defense, like the peptide pep1 and extracellular ATP (Krol *et al.*, 2010, Chen *et al.*, 2017). Many of these PAMPs have conserved co-receptors like the PRR co-receptor BAK1, and BKK1, but there is substantial diversity in downstream RLCKs that may denote the specificity of responses to the specific elicitor (Rao *et al.*, 2018). However, PRR activation via PAMP elicitors often converge downstream with conserved factors like MPK3/4/6 phosphorylation and $[Ca^{2+}]$ -dependent processes (Galletti *et al.*, 2011, Eschen-Lippold *et al.*, 2012). It would be interesting to identify if these PAMP and DAMP elicitors display temperature-sensitive outputs, and if these alterations track with both $[Ca^{2+}]$ dynamics and immunity. Conservation or variation in $[Ca^{2+}]$ responses between these elicitors may provide insight into which $[Ca^{2+}]$ -channels and/or PRR complexes display temperature sensitivity, enabling a broader insight into how conserved this temperature-induced downregulation of $[Ca^{2+}]$ -dynamics is manifest through other defense elicitors. Furthermore, physical wounding enables rapid $[Ca^{2+}]$ spiking, which has been linked to downstream JA responses and attenuating $[Ca^{2+}]$ signals corresponds to a decrease in JA-related responses (Sun *et al.*, 2006) Since elevated temperature enhances JA responses in *A. thaliana* (Havko *et al.*, 2020), it would suggest that $[Ca^{2+}]$ responses that link wounding to JA responses might be temperature insensitive, if not enhanced at

elevated temperature. The Glutamate-Like Receptor $[Ca^{2+}]$ 3.3/3.6 (GLR3.3/3.6) channels are involved in JA induction after mechanical wounding and are important for inducing JA responses in systemic tissue (Mousavi *et al.*, 2013). CBL37 has been linked to positive regulation of JA-biosynthesis, further linking biotically induced $[Ca^{2+}]$ to defense regulation (Scholz *et al.*, 2014). GLRs and other $[Ca^{2+}]$ -channels may be a point of research to investigate linkages between $[Ca^{2+}]$ -signaling, JA-induction, and temperature sensitivity.

While this study explored biotic activation of $[Ca^{2+}]$ transients, there are several abiotic stimuli which also induce $[Ca^{2+}]$ transients that allow plants to respond to their environment. Drought stress is often a co-occurring phenomenon with elevated temperatures and poses a substantial threat to our agricultural systems. Thus, understanding how these two plant stressors co-interact at the molecular level may enable new discoveries to enhance plant productivity. Drought is perceived by changes in water potential in the roots which induce $[Ca^{2+}]$ transients via the $[Ca^{2+}]$ -channel OSCA1 (Yuan *et al.*, 2014). These $[Ca^{2+}]$ -transients are perceived and integrated into a signaling cascade involving CDPKs, CaM and CML (Wilikins *et al.*, 2016). These $[Ca^{2+}]$ -dependent proteins then in turn induce ABA in local and systemic tissues to survive water depletion via stomatal closure and physiological changes to prevent water loss (Zhang *et al.*, 2020). While drought stimulates stomatal closure, elevated temperature favors leaf cooling via stomatal opening, demonstrating an antagonistic relationship between controlling leaf temperature while minimizing water loss (Reynolds-Henne *et al.*, 2010). It would be interesting to see how elevated temperature may modulate these drought-induced $[Ca^{2+}]$ -transients and $[Ca^{2+}]$ -dependent

responses, as they are essential for regulating the stomata and ultimately, plant survival to heat and drought stress.

While this research has explored how SA induction is perturbed under biotic stimuli, little research has explored how abiotic stimuli influence SA biosynthesis and how temperature influences these inductions. UV-radiation is a constant stress plants face when exposed to the natural environment. UV-light damages the photosynthetic apparatus and produces damaging ROS species that oxidize, damage, and misfold proteins, along with inducing genomic mutations, resulting in a rise in DNA repair processes and SA biosynthesis (Gill *et al.*, 2020). One method plants use to survive UV stress is by inducing anti-oxidative pathways to scavenge ROS species, often utilizing glutathione, along with an upregulation of protectant processes SA signaling to mitigate ROS damage and scavenge harmful radicals (Saleem *et al.*, 2021). I investigated if SA biosynthesis was temperature sensitive under UV-C elicitation and was surprised to find temperature insensitivity between 23°C and 28°C when elicited by UV. Furthermore, this pathway was dependent on CBP60g and SARD1, demonstrating pathogen and UV-elicited ROS converge on the same SA biosynthetic module (Figure A2). While it is uncertain what the direct pathway is that induces SA-biosynthesis after UV stress, it is not conferred by the UV-sensing UVR8 pathway as *uvr8* mutants display enhanced *PR1* expression after UV-B radiation, suggesting UV is inducing SA in response to a different stimulus (Kliebenstein *et al.*, 2002). However, this highlights that temperature may not directly interfere with the physical associations with phase-separation driving transcription at the CBP60g loci, unlike the other temperature sensitive transcriptional regulation observed with ELF3 and CDK8-GBPL3 interactions (Jung *et al.*, 2020, Kim *et*

al., 2022). While these results suggest gaps in our understanding of SA regulation, they suggest other temperature-resilient mechanisms of inducing SA-biosynthetic regulators like CBP60g which may be developed for more robust plant immunity in dynamic environments.

3.2.3 Strategies to identify mechanisms of BTH protection by enhancing PTI.

How BTH is capable of inducing plant immunity at elevated temperature through modifying PTI is a largely unexplored area of research. The temperature-induced schism between the canonical signaling pathway including CBP60g/SARD1/PR1 and for genes involved in enhancing PTI highlights unknown branching elements of NPR1 signaling that are responsible for inducing plant defenses. In this sense, temperature serves as a useful probe to interrogate which targets of NPR1 are transcriptionally active or inactive under different environmental conditions. Previous reports suggested CBP60g was responsible for PTI enhancement as CBP60g binds to the promoters of BAK1, BKK1, and MPK3 to positively regulate gene expression (Sun *et al.* 2018). However, BTH induction of CBP60g is temperature sensitive, suggesting that NPR1-CBP60g regulation is likely not the culprit of PTI enhancement, suggesting a novel element of NPR1-PTI regulation that remains to be discovered.

One way to identify if NPR1 targets are responsible for priming PTI at elevated temperature would be to couple ChIP-seq with RNA-seq analyses after BTH treatment to identify which genes are directly targeted by NPR1 and which cis-promoter element(s) NPR1 binds to influences transcription. This approach would physically identify targets of NPR1 that are influenced by its binding to the promoter. There are pitfalls to this approach, primarily that the presence or absence of NPR1 may not reveal

other transcriptional regulators important for driving expression. Research previously performed by our group identified that transcriptional activation at the CBP60g loci is related to reduced BTH-induced nuclear condensates along with a loss of recruiting transcriptional regulators like MED6, MED16, and GBPL3. While NPR1 could be detected at the CBP60g promoter, the loss of GBPL3 was associated with a reduction in nuclear condensates that drive CBP60g transcription at 28°C. Elevated temperature impairs recruitment of GBPL3 at some NPR1-dependent targets like CBP60g, but not at NPR1, demonstrating that GBPL3 binding to the promoter does not indicate transcriptional activity, and that other factors like the mediator subunits MED16 and MED6 might also dictate transcription. This suggests that there might be post-translational regulation of NPR1 or other transcriptional units that facilitate target gene transcription. This could be explored further with either exploring the proteomic state of NPR1 by examining modifications to NPR1, such as phosphorylation or SUMOylation at normal and at elevated temperatures. These modifications may be detected using a NPR1 Co-immunoprecipitation and coupled with mass spectrometry approach to detect if there are any temperature sensitive modifications to NPR1. It has been shown that the phosphorylation status and SUMOylation of NPR1 positively impacts its transcriptional regulation, but as of now, no known proteins involved in modifying NPR1 play a role in temperature sensitive expression of *PR1*, nor do they significantly impact disease susceptibility at elevated temperature (Wang *et al.*, 2006, Weigel *et al.*, 2001, Spoel *et al.*, 2009, Kim *et al.*, 2022). One potential challenge is that NPR1 is present at target genes that are temperature-sensitive, modifications that positively and negatively influence transcription would be pooled with co-IP samples, making it difficult to assess

how these modifications influence activity. A quantitative proteomic approach to compare relative changes in NPR1 modifications would reveal relative changes in the ratio of protein modifications observed between 23°C and 28°C. Another approach to assess how NPR1 is inducing temperature insensitive genes associated with inducing immunity via PTI priming would be to conduct a protein-protein interaction atlas of NPR1 to identify proteins that interact in a temperature specific manner. If the post-translational modifications influence protein-protein interactions, this approach will facilitate identifying which proteins are important for inducing NPR1-dependent gene expression independent of understanding the underlying modifications.

Uncovering the targets of NPR1 that are responsible for PTI priming will be highly valuable for enhancing the plant immune systems in numerous regards. BTH protection being temperature-insensitive highlights that there are still fully intact elements of the plant immune systems at elevated temperature, or at least there are other elements of the plant immune network that can compensate for vulnerabilities in other pathways. Furthermore, since the linkage between PTI and ETI has become significantly clearer in recent years where ETI highly depends on functional elements of PTI to induce immunity (Yuan *et al.*, 2021, Ngou *et al.*, 2021). Understanding NPR1's role to regulate PTI and likely ETI through SA will be essential for fortifying plant immunity in the face of new and diverse plant pathogens. Since many new cultivars being developed for disease resistance focus on NLR mediated immunity, understanding how PTI is regulated by NPR1 will only serve to develop more efficacious strategies to develop disease resistance in the face of a changing climate.

3.2.4 Exploring temperature - salicylic acid regulation with alterations to growing conditions.

There are many avenues to further investigate the effect of temperature on plant immunity in *A. thaliana* at a fundamental level. Since plants often develop in a constantly shifting environment, the static temperature change between 23°C to 28°C used in this and other studies is relatively arbitrary for understanding how temperature perturbs *Arabidopsis*. Temperature changes are relative, and *A. thaliana* exhibits robust diversity in a wide range of environmental conditions across the globe and has expanded its range widely after the last glacial maximum (Beck *et al.*, 2007). Many accessions, including Col-0, exhibit an annual lifestyle, emerging shortly after the spring snowmelt, whereas others exhibit a biennial lifestyle, remaining dormant during cold winter conditions until warmer spring weather signals to induce reproduction (Krämer 2015). The temperatures *A. thaliana* experiences during their natural life cycle often are significantly colder than those observed in this study during their lifetime, but daily temperature oscillations can be within 5°C of change. It would be interesting to investigate whether relative temperature shifts, rather than the static 23°C – 28°C transition have similar impacts on plant immunity. One could test this by growing *A. thaliana* at a lower temperature (15-20°C) and increasing the temperature by ~5°C to see if similar temperature sensitive immune outputs are observed. Furthermore, growing *A. thaliana* under dynamic temperature ranges prior to inducing elevated temperature treatments may also influence the severity of immunity phenotypes. This would reveal whether temperature sensitive immunity is dictated by absolute temperature or relative shifts in temperature.

Surface temperature is correlated to sunlight duration, suggesting that day-length may interact with the timing of temperature sensitive immunity during the plant's life cycle (Craufurd and Wheeler 2009). Day length tightly controls *A. thaliana* transitions from vegetative growth to reproduction and foliar plant immunity is attenuated during the reproductive stage. (Cecchini *et al.*, 2002, Griebel and Zeier 2008). Furthermore, salicylic acid serves as a positive regulator of flowering in *A. thaliana*, highlighting a link between immunity, survival, and reproduction in the face of biotic and abiotic stress (Glander *et al.*, 2018). Future research to investigate the link between timing of heat during the development of *A. thaliana* and its relation to day-length may reveal whether the temperature sensitivity of SA-biosynthesis and PTI is developmentally gated. Experiments modifying the day length during vegetative growth at elevated temperature may influence temperature-modulation of immunity outcomes. These studies would broadly inform how temperature impacts the immune system at different stages of plant development and may identify crucial periods where and when pathogen outbreaks can be at their most severe.

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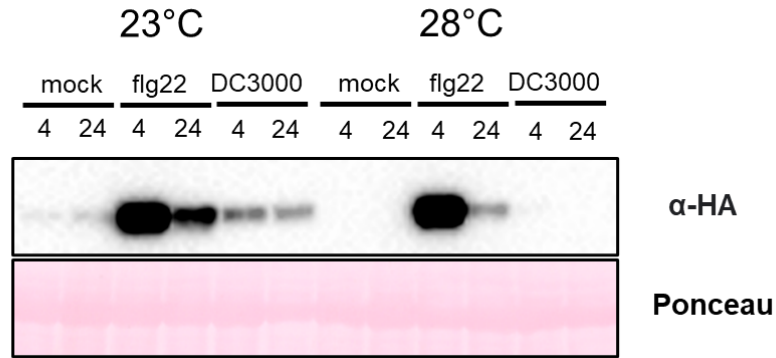
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APPENDIX

APPENDIX A: SUPPLEMENTAL EXPERIMENTS

A.



B.

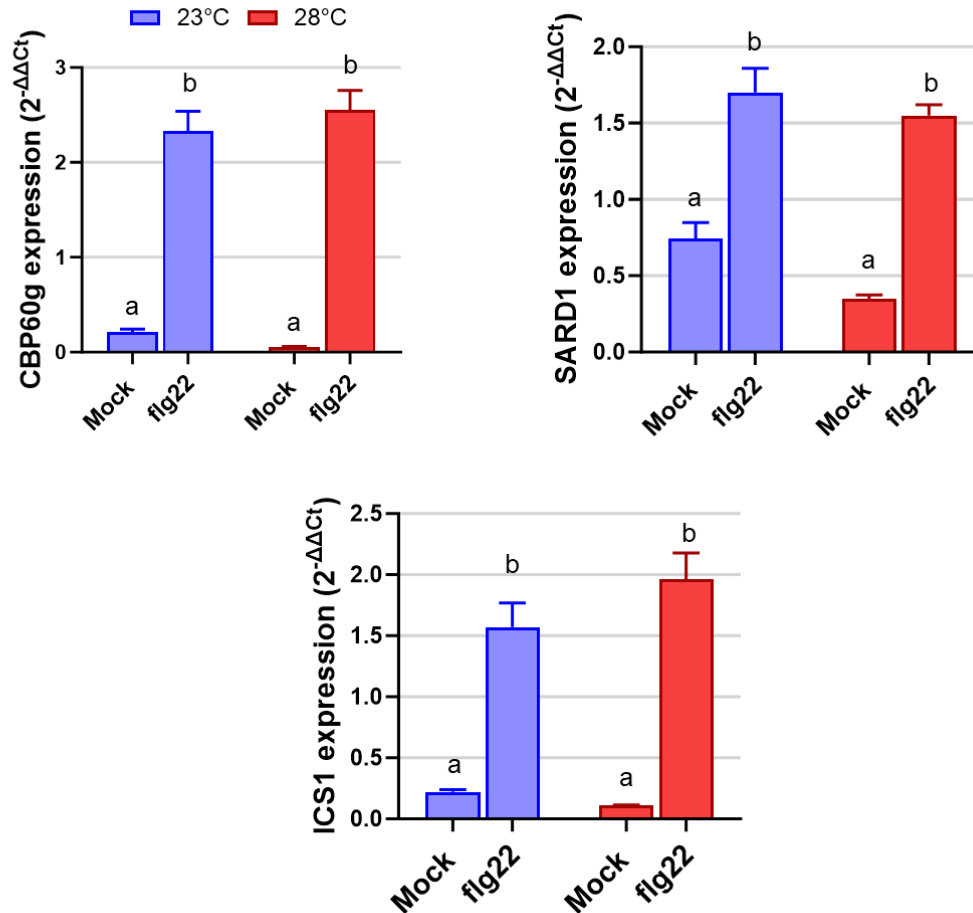


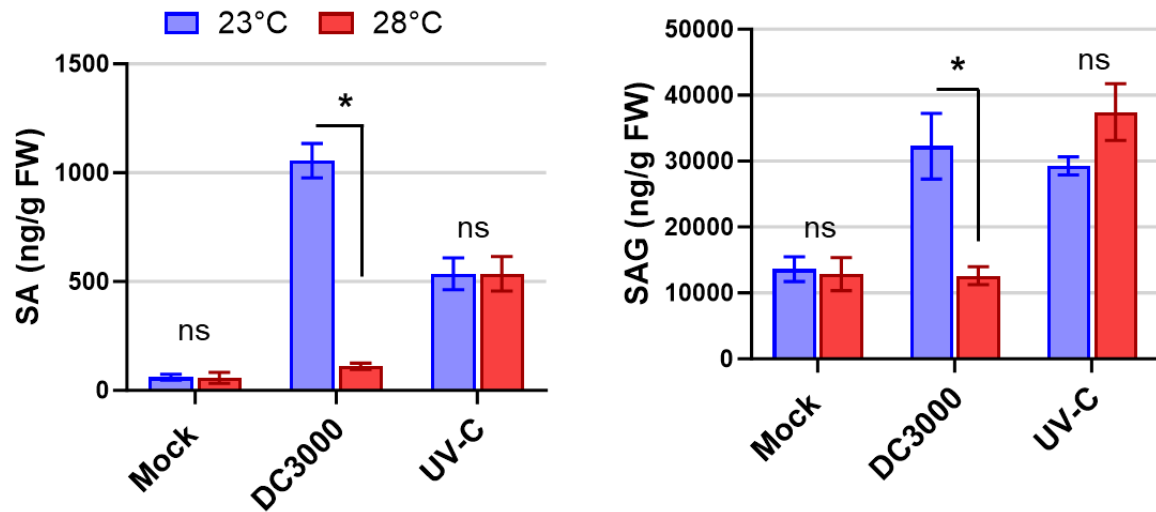
Figure A1. flg22 induces CBP60g protein levels and gene expression at early time points after flg22 treatment at 28°C.

(a) Transgenic CBP60g-HA plants were exposed to their respective temperature

Figure A1 (cont'd)

treatments and then treated with 100nM flg22 or DC3000. Tissue samples were harvested 6 and 24 hours after flg22 treatment and were probed to assess CBP60g protein levels via SDS-PAGE/Western Blot. Each lane represents 4 plants pooled together for protein extraction. Equal loading of the wells for SDS-Page was assessed using Ponceau-S staining. Experimental results are representative of 3 experimental replicates. (b). Expression of CBP60g and SARD1 was assessed 6 hours after 100nM flg22 time points. Relative expression was determined using qPCR and normalized to expression of the gene *PP2A3* (n=4, Error bars indicate SEM). Letters indicate statistical significance ($P < 0.05$) using a 2-Way ANOVA and groups were assessed using Tukey's post hoc test.

A.



B.

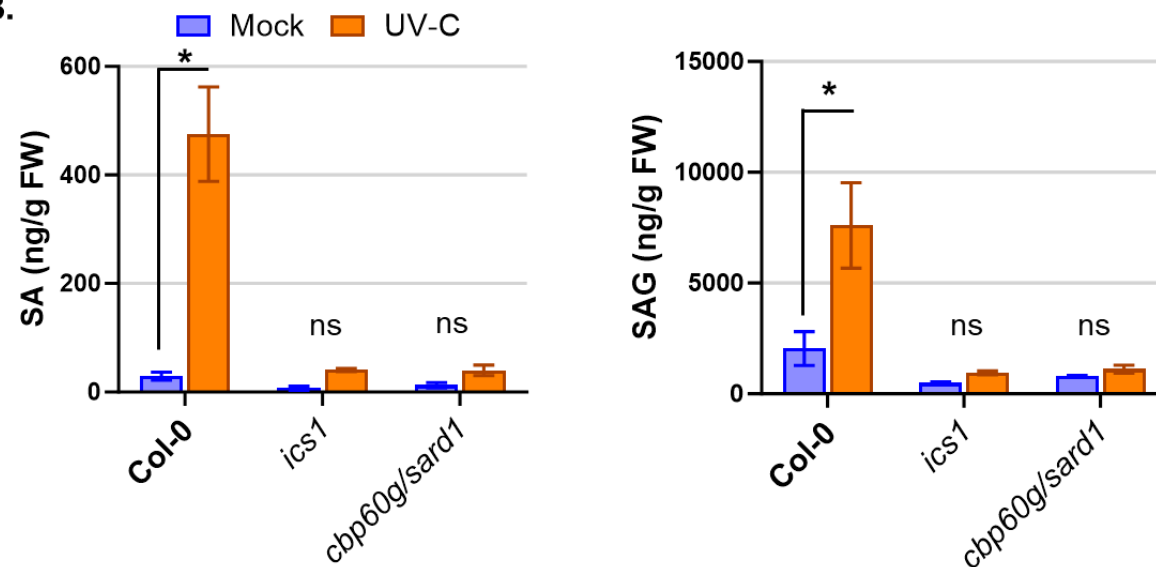


Figure A2. UV-induced SA biosynthesis is not temperature sensitive between 23°C and 28°C (a). SA-levels collected from plants 24 hours after a 15-minute exposure to UV-C (254nm), along with the negative control flg22 at 28°C (n=4, Error Bars represent SEM). Asterisks indicate significance and ns for no significance for Student's t-test ($P < 0.05$). The results are representative of 3 biological replicates. (b). UV-C induced SA biosynthesis was assessed in Col-0 and the *cbp60g/sard1* double mutant which is unable to induce SA biosynthesis in response to pathogens. (n=4, Error Bars represent SEM). Asterisks indicate significance and ns for no significance for Student's t-test ($P < 0.05$).