DISSERTATION

CYTOKININ-MEDIATED PROCESSES PROMOTE HEAT-INDUCED DISEASE SUSCEPTIBILITY OF PLANTS TO BACTERIAL PATHOGENS

Submitted by

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ABSTRACT

CYTOKININ-MEDIATED PROCESSES PROMOTE HEAT-INDUCED DISEASE SUSCEPTIBILITY OF PLANTS TO BACTERIAL PATHOGENS

As global human populations continue to grow and temperatures are expected to rise, the pressure to increase food productivity and develop more stress-resistant crop varieties intensifies. Increased temperatures, a consequence anticipated as a result of global climate change, is expected to have an overall negative impact on crop productivity and agricultural systems. When exposed to non-optimal, high temperature conditions plant defense responses to pathogen attack are attenuated, leading to a process referred to here as heat-induced disease susceptibility. The plant growth hormone cytokinin is known to regulate responses to both biotic and abiotic pressures, making it an ideal target to study heat-induced disease susceptibility. The overarching goal of this dissertation was to understand the role of cytokinin in heat-induced disease susceptibility, to identify novel strategies to combat this process and design new ways to teach future generations about the impact of climate change on agricultural systems and science policy.

First, I identified that a plant lacking a functional cytokinin signaling pathway, *ahk2,3* mutated on the cytokinin signaling receptors AHK2 and AHK3, was less susceptible at elevated temperatures to the bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). My results show that *ahk2,3* plants are less susceptible under high temperature conditions with *Pst* DC3000 populations proliferating at a lower rate compared to wild-type plants overtime, suggesting that heat-induced susceptibility is partially dependent on cytokinin-

signaling. Our results show that differences in susceptibility under elevated temperatures of *ahk2,3* and wild-type plants is not attributed to an increase in defense responses, but rather by a possible change in the availability of nutrients for *Pst* DC3000. Together the data reveals that under high temperature conditions cytokinin promotes late-physiological processes, centered around primary metabolism, that are contributing to increased pathogen proliferation. These results led to the identification of cytokinin-regulated genes that could be utilized for breeding efforts to obtain loss-of-heat induced disease susceptibility that could be translated to crop species.

Second, I identified that another member of the Brassicaceae family, *Brassica napus*, also exhibited heat-induced disease susceptibility to the bacterial pathogen, *P. syringae* pv. *maculicola* (*Psm* ES4326). Gene expression analysis confirms that similar to Arabidopsis, *B. napus* plants increase cytokinin signaling in response to high temperature stress. To further address if cytokinin was important for heat-induced disease susceptibility of *B. napus*, I utilized a chemical approach. *B. napus* plants were sprayed with the cytokinin-signaling antagonist, PI-55, prior to inoculation and results show that a single application of PI-55 led to a loss of susceptibility under heat to *Psm* ES4326. Additionally, this application of PI-55 did not lead to any adverse vegetative growth parameters, suggesting a potential novel chemical approach to combat heat-induced disease susceptibility in Brassicaceae crops.

Lastly, I constructed a new approach to teach future generations about the impact of climate change on plant diseases in agricultural systems. “Plant Diseases and Climate Change” is an active learning activity designed to give college students experience in synthesizing information and developing a solution, in the context of plant pathology. This exercise uses the issue of heat-induced susceptibility of rice in the Philippines to improve student understanding of

the interactions between abiotic and biotic factors affecting global food security. By using an international agricultural pathosystem, I aim to inform students how environmental pressures can impact economically important plant systems, the role scientists and experts play in policy making to preserve food security, and the importance of agriculture on a global scale.

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DEDICATION:

This dissertation is dedicated to my sister Erin Olivia Shigenaga (1994-2009).

I love you and miss you every day.

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Chapter 1

Introduction and Scope of Dissertation

1.1 Plant Hormones in Plant-Pathogen Interactions

In both agricultural and natural settings plants are constantly exposed to a diverse set of stimuli. From varying levels and quality of light, to varying levels of water availability, soil salinity, temperature, and interactions with other organisms, plants must constantly adapt to a changing environment. This adaptation requires a remarkable level of not only signal perception, but also of signal integration, a capacity that, in plants, is modulated by plant hormones.

Plant hormones are major regulators of plant development and responses to the environment. These small organic molecules are perceived by their cognate receptors in target plant cells, leading to the activation of signaling pathways and hormone-regulated transcriptional responses, culminating in changes in plant cell physiology. Hormonal crosstalk, or the integration of different hormonal pathways, is essential for plant fitness. Recent protein interactome approaches have revealed that the interaction amongst individual plant hormonal networks is more extensive than previously thought, underscoring the importance of hormonal crosstalk to plant life (Altman et al., 2020). Correspondingly, the lines separating the specific contributions of individual plant hormones to plant physiology are now blurred, with various hormones contributing to numerous physiological functions; to the point that the classical definition of growth (auxin, cytokinins, gibberellins, brassinosteroids, strigolactones) and stress/defense hormones (salicylic acid, jasmonic acid, ethylene, abscisic acid) no longer applies (Shigenaga et al., 2016).

The importance of plant hormones to plant physiology can also be appreciated by the importance of these molecules for plant-interacting organisms. This has been particularly well studied in the interactions of plants and plant pathogens. During invasion of the host, as well as during host colonization, plant pathogens use effectors (secreted proteins, metabolites or nucleic acids of pathogen origin) to manipulate host cells and create conditions that are favorable for pathogen growth and multiplication. Such manipulations include not only suppression of defense responses, but also changes in plant metabolism to feed the growing number of pathogens that start to multiply in the infected plant tissue, leading to plant susceptibility, and to what is manifested as plant disease. Many of these effectors are known to target, directly or indirectly, plant hormone metabolism and signaling, highlighting the importance of plant hormones for plant defense and susceptibility to pathogens (Kazan and Lyons, 2014; also curated in www.planthormones.org).

In the next sections, I describe how one specific class of plant hormones, cytokinins (CK), are important for defense responses and also for disease susceptibility, and how CK dependent processes could be used for loss-of-susceptibility approaches to provide durable resistance against pathogens.

1.2 Cytokinins in Plant-Pathogen Interactions: A Double-Edged Sword

While the classification of plant hormone functions no longer applies, historically, the plant hormones salicylic acid (SA) and jasmonic acid (JA) have been associated with defense responses to pathogens. SA is mostly known for its role promoting resistance against biotrophic pathogens, or those that obtain their nutrients from living plant cells. JA, in conjunction with the plant hormone ethylene, is mostly associated with resistance against necrotrophic pathogens, or

those that kill plant host cells to acquire nutrients for growth (reviewed in Shigenaga et al., 2016). These two signaling pathways are mostly antagonistic to each other: when the SA pathway is up-regulated in response to a biotrophic pathogens the JA pathway tends to be suppressed; and when the JA pathway is activated in response to an attack by a necrotrophic pathogen, the SA pathway is suppressed (Spoel et al., 2007). Along with negative effects on plant growth, this antagonistic crosstalk is a major hurdle in the engineering of resistant plants by overexpression of either the SA or JA pathways, as resistance tradeoffs in one of the pathways occur when the converse pathway is activated (reviewed in Shigenaga et al., 2017).

Although the plant hormone CK is broadly known as a hormone involved in the regulation of plant growth, a role for this hormone in plant-pathogen interactions has recently been established (reviewed in Albrecht and Argueso, 2017). Earlier studies, mostly on crop species, showed that exogenous application of the hormone most often led to decreased pathogen growth, especially when CK was applied in high concentrations (micromolar) (Argueso et al., 2012; Babosha, 2009). Conversely, exogenous applications of low levels (nanomolar) of CK to plants sometimes led to increased susceptibility to pathogens (Argueso et al., 2012; Babosha, 2009). The fact that application of exogenous CK to plants led to varying outcomes of disease, sometimes leading to decreased pathogen growth and sometimes to decreased susceptibility, prevented the elucidation of a defining role for CKs in plant-pathogen interactions.

1.3 Cytokinins in Defense Responses Against Pathogens

It was only with studies using the model plant *Arabidopsis thaliana* (hereafter, Arabidopsis) that a clearer picture of the role of CKs in plant-pathogen interactions became apparent, through the use of mutants impaired in CK signaling and metabolism. Using transgenic

plants with increased levels of CK by overexpression of *ISOPENTENYL TRANSFERASES* (*IPTs*), which encode CK biosynthesis enzymes, researchers showed that endogenous accumulation of CK could phenocopy the results of exogenous application of micromolar levels of CK to plants, leading to plants with decreased susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Choi et al., 2010). Moreover, these results could not only be expanded to other pathogens, such as the biotrophic oomycete *Hyaloperonospora arabidopsidis (Hpa)*, but the effect of exogenous CK was shown to be really dependent on plant physiological functions rather than a direct role of CK on the pathogen, as CK receptor mutants failed to produce the same response (Argueso et al., 2012).

Similar observations of a positive role of CK in defense responses have now been extended to other plant species, including crops (Grosskinsky et al., 2011; Zou et al., 2020; Shigenaga et al., unpublished), and shown to be effective against pathogens of different lifestyles, such as vascular pathogens (Alonso-Diaz et al., 2021), necrotrophic pathogens (Alonso-Diaz et al., 2021; Gupta et al., 2020), viruses (Zou et al., 2020) and even nematodes (Shanks et al., 2016). The phenotype of decreased susceptibility to pathogens by exogenous application of high levels of CK is accompanied by several plant defense responses, such as increased defense gene expression, production of reactive oxygen species (ROS), changes in stomatal immunity, as well as increased production of antimicrobial compounds such as phytoalexins (Argueso et al., 2012; Arnaud et al., 2017; Choi et al., 2010; Grosskinsky et al., 2011; Shanks et al., 2016). Of interest, CK does not directly activate these responses; rather, it seems to trigger physiological conditions that prime plants and potentiate defense, as the activation of defense responses only happens after pathogen detection (Argueso et al., 2012).

As is commonly the case in plant hormones, crosstalk between different plant hormonal pathways is also involved in the action of CKs in plant immunity. Using mutants in the biosynthesis and perception of SA, Arabidopsis plants were shown to require SA for the function of CKs in plant defenses. Arabidopsis *eds16* mutants, which have a mutation in the gene encoding the SA biosynthetic enzyme ISOCHORISMATE SYNTHASE 1 (ICS1), fail to display decreased disease susceptibility to the biotrophic oomycete *Hpa* after treatment with high levels of CK (Argueso et al., 2012). Similarly, mutations on the gene encoding the master regulator of SA signaling, the transcriptional activator NON-EXPRESSER OF PR-1 (NPR1), also lead to a failure in displaying decreased susceptibility to the hemi-biotrophic bacterial pathogen *Pst* DC3000 after CK application (Choi et al., 2010). Therefore, CKs function in the amplification of SA-dependent defense responses, upon recognition of biotrophic pathogens.

Recently, a function for CKs in immunity against necrotrophic pathogens was also uncovered. Using tomato as a host, researchers showed that application of micromolar amounts of CKs can also have a protective effect against the necrotrophic fungal pathogen *Botrytis cinerea* (Gupta et al., 2020). Using a tomato mutant impaired in JA signaling, it was determined that proper JA signaling is needed for this response (Gupta et al., 2020). Similarly to what happens with responses to biotrophic pathogens, amplification of defense responses to necrotrophic pathogens by CKs is activated only upon pathogen infection, and include the expression of the JA defense marker gene *PLANT DEFENSIN* (*PDF1.2*), as well as production of ROS, both of which are much stronger in pathogen-infected CK-treated plants than in pathogen-infected mock control plants (Gupta et al., 2020).

Taken together, the current role of CKs in immunity against pathogens can be described as that of a priming agent. Priming is an activated state where plants become able to employ

stronger and faster defense responses, resulting in enhanced pathogen protection (Conrath et al., 2015). The mechanisms involved in defense priming are currently unknown, but its activation, before pathogen infection, has minimal negative effect on the plant energy status, and provides a high level of protection with considerably low fitness costs (Conrath et al., 2015). What is interesting about this priming effect of CKs is that it can activate the two main different pathways known to contribute to defense: The SA pathway regulating defense against necrotrophic pathogens and the JA pathway that regulates resistance against necrotrophic pathogens. The specific pathway activated depends on the type of pathogen being recognized.

The mechanisms by which CKs mediate this priming effect are still unclear but are beginning to be elucidated. The activation of crosstalk between CK and SA was shown to be mediated by the physical interaction between the CK-regulated transcription factor ARR2 and the SA transcription factor TGA3, which together with NPR1 bind to the promoters of SA-regulated genes to activate their expression (Choi et al., 2010). Using Arabidopsis mutants in the various steps of the CK signaling pathway, it was demonstrated that the phosphorelay signaling that is initiated by binding of CK to its receptors is needed for the priming effect (Argueso et al., 2012). Mutation of specific phosphorylation residues of proteins involved in the CK signaling pathway, known as two-component elements, abolished priming by CKs. Conversely, mutations that create a phospho-mimic effect in these same residues led to further activation of priming (Argueso et al., 2012). Finally, priming by CK seems to lead to an increase in the levels of cell surface plant immune receptors, that recognize Pathogen-Associated Molecular Patterns (PAMPs) to initiate PAMP-Triggered Immunity (PTI) (Gupta et al., 2020). Given the fact that priming by CK requires pathogen recognition, and that its ensuing specific activation of the JA

or SA pathways also depends on the type of pathogen being recognized, a link between CK priming, PAMP cell surface receptors and PTI amplification seems likely.

1.4 Cytokinins in Disease Susceptibility: Manipulation of Host Nutrient Allocation

As mentioned above, CKs can also have an effect in increasing the susceptibility of plants to pathogen attack. This effect is most commonly seen when lower concentrations of CKs are applied to plants (Babosha et al., 2009; Argueso et al., 2012). Such a physiological role for CKs in promoting conditions that increase plant susceptibility would signify that CK-regulated processes could be exploited by pathogens for their manipulation of host cells. Interestingly, this is exactly the case. Several plant-pathogenic organisms can produce CKs, or can induce its production *in planta*, or manipulate CK signaling in plant cells (reviewed in Spallek et al., 2018). This is also the case of some species of plant beneficial microbes, whose effects of enhanced plant growth and protection from disease were found to be CK-mediated (Grosskinsky et al., 2016).

Among the plant pathogens that can produce CKs to generate conditions for pathogen growth and multiplication, the most classic example is the bacterial pathogen *Agrobacterium tumefaciens*. During its pathogenic process, *Agrobacterium* cells insert the CK biosynthesis gene *trans-zeatin synthesizing* (*tzs)* into the plant genome, leading to CK biosynthesis in plant cells, increased levels of cell division and the formation of plant galls (Hwang et al., 2010). The obligate biotroph *Plasmodiophora brassicae,* another root gall-forming plant pathogen, causes clubroot disease in cruciferous plants and its genome contains genes encoding two CK biosynthesis enzymes, which contribute to pathogenesis (Malinowski et al., 2016). The cyst nematode *Heterodera schachtii*, which infects Arabidopsis, is able to produce and secrete CKs

(Siddique et al., 2015). RNAi-based silencing of the nematode gene encoding a CK biosynthetic enzyme (*HsIPT*) was shown to decrease the size of nematode-induced syncitia and to lead to decreased nematode size overall (Siddique et al., 2015), thus indicating that production of CK is part of *H. schachtii* virulence strategy. Many other examples exist, including parasitic plants, which utilize haustoria-like appendices to invade plant cells, and can induce the biosynthesis of CK in plant cells, which help in haustorium formation and pathogenesis (Spallek et al. 2017).

In addition to CK synthesis, either directly by pathogens or by plants after pathogen induction, pathogens can also use effectors to change CK signaling in plant cells. This is the case of *Pst* DC3000, which cannot produce CKs, but whose effector HopQ1 was shown to have an enzymatic activity similar to the CK activating enzyme LOG1 (Hann et al., 2014). HopQ1 is important for *Pst* DC3000 virulence in Arabidopsis, and its mode of action in plant cells includes the increase in the levels of active CK in plants, and interestingly, also a decrease in the levels of the PAMP cell surface receptor FLAGELLIN SENSITIVE 2 (FLS2), thus linking levels of PAMP cell surface receptors and CK function in disease susceptibility. Of note, HopQ1 is conserved across many bacterial genera, including in *Xanthomonads* and *Ralstoniales*, thus suggesting that these bacteria may also manipulate CK signaling in plants through their HopQ1 orthologues (Hann et al., 2014).

The examples cited above suggest that CKs can function as virulence factors, increasing plant susceptibility to pathogens. However, the mechanisms by which CKs mediate plant susceptibility are not fully known. Of all the physiological roles associated with CKs, promotion of cell division and regulation of source-sink relationships are the most like to be involved with pathogen susceptibility. Many of the pathogens that produce CKs as a mechanism of virulence also induce the formation of structures associated with increases in cell division, such as galls,

knots, tumors and nodules. In addition to accommodating the pathogens physically, these structures also assist in the metabolic needs of plant pathogens, as their increased rates of cell division usually result in diversion of host metabolism to these structures to allow for plant cell growth, thus functioning as sinks for metabolites and nutrients. Infection by several CK-producing, gall-forming pathogens alters carbohydrate metabolism in the host, resulting in increased sugar and starch content at the site of infection (Brodmann et al., 2002, Evans and Scholes 1995, Williams et al., 1968).

An effect of CK in providing suitable metabolic conditions for pathogen growth and multiplication during infection is also seen with pathogens that do not induce cell division. Green islands are small areas of live, green leaf tissue surrounded by yellow, senescing tissue, normally seen in plants infected with biotrophic fungi (Bushnell, 1967). Green islands are known to have increased CK content within the green areas (López-Carbonell et al., 1998). These green regions also display increased rates of photosynthesis in comparison to the surrounding senescing tissue (Walters et al., 2008), as well as increased levels of sugars and starch (Angra and Mandahar, 1991; Angra-Sharma and Mandahar, 1993; Raggi, 1974, 1976), therefore suggesting a role for CKs in maintaining the local sites of photosynthesis, and associated nutrient content, to help pathogens thrive. However, pathogens that do not form green islands can also use CK to manipulate nutrient allocation during infection. For example, the genome of the rice blast fungus *Magnaporthe oryzae* encodes a CK biosynthetic gene, *CKS1* (Chanclud et al., 2016). *M. oryzae* mutants in *CKS1* have reduced virulence and are impaired in their ability to multiply *in planta,* but not *in vitro*, implicating pathogen nutrition through CK-regulated host processes in virulence (Chanclud et al., 2016). Further, *M. oryzae* successful infection is associated with the allocation

of sugars and amino acids to the sites of pathogen growth (Chanclud et al., 2016), thus suggesting a function for CK in acting to change source-sink relationships in this process.

1.5 Cytokinin-Regulated Processes as a Source of Susceptibility Genes to be Targeted for Loss-of-Susceptibility (LOS)-mediated Resistance

The examples mentioned above indicate that during infection, CKs may play a key role in creating and maintaining infection sites as sinks tissues (McIntyre et al., 2021). Thus, CK-regulated processes may be essential for pathogen nutrition, and therefore genes involved in the regulation of some of these CK-regulated processes could be considered Susceptibility (*S*) genes. Susceptibility genes are genes from the plant that are required for pathogen growth in host cells. One of the best examples of *S* genes are the rice *SWEET* genes, encoding sugar transporters involved in the regulation of source-sink relationships in plants, through the transport of sugars out of mesophyll cells into the apoplast (Chen et al., 2010). Rice *SWEET* genes are targeted by the Transcription Activator-Like (TAL) effectors of the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*. Activation of *SWEET*s by TALs changes the accumulation of sugars at sites of infection, to accommodate pathogen nutritional needs (Chen et al., 2010).

Interestingly, natural or induced mutations in genes encoding SWEET transporters leads to resistance to *Xanthomonas oryzae* pv. *oryzae* and other *Xanthomonas* species (Oliva et al., 2019). This resistance is not mediated by activation of defense of responses, but by the loss of an *S* gene, required for the pathogen growth in host cells, in this case the *SWEET* genes required for pathogen feeding. Differently from resistance mediated by Resistance (*R*) genes, which activates plant immunity upon recognition of pathogen effector activity, resistance mediated by loss-of-

susceptibility is durable, because it is based on host processes that are often essential for plant cells and does not depend on the recognition of pathogen effectors that are constantly mutating.

Thus, identification of *S* genes can be used for strategies of creating durable resistance through loss-of-susceptibility, by mutation of *S* genes through gene editing, or the identification of natural alleles providing loss-of-function. Given the importance of CKs in mediating host metabolic processes and mechanisms of nutrient allocation that are important for pathogen feeding, I therefore hypothesize that CK-mediate processes could be used in loss-of-susceptibility approaches to engineer durable resistance against pathogens.

1.6 Scope of Dissertation

The overarching goal of this dissertation was to understand the role the plant hormone CK plays in heat-induced disease susceptibility of plants to bacterial pathogens, to identify novel approaches to improve crop protection under elevated temperatures, and use the topic of heat-induced disease susceptibility to teach future generations about the negative impact climate change may have on agricultural systems.

In Chapter 2, I address the role of CK in heat-induced disease susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. *tomato* DC3000 through a genetic approach. Various genetic tools such as a CK signaling mutant *ahk2,3*, mutated on the genes encoding CK signaling receptors, were used to elucidate the role of CK in this process. Plants lacking CK signaling were less susceptible under elevated temperature, indicating that heat-induced susceptibility of Arabidopsis is a CK-mediated process, likely associated with regulation of primary metabolism and important for pathogen feeding.

In Chapter 3, I address the role of CK in heat-induced disease susceptibility of *Brassica napu*s to *P. syringae* pv. *maculicola*. To elucidate the role of CK in this process I utilized a chemical approach by exogenously applying the CK signaling antagonist PI-55 to plants prior to inoculation. Results indicate that inhibiting CK signaling leads to loss of heat-induced disease susceptibility of *B. napus*, indicating that this could be a useful chemical approach to combat heat-induced disease susceptibility in crop plants.

In Chapter 4, I created an active learning lesson plan utilizing the concept of heat-induced disease susceptibility of plants to teach students the negative impacts of climate change on agricultural systems, in the context of plant pathology. Through a hands-on game, students learn about the importance of agriculture on a global scale, as well as the importance of scientists and experts in agricultural practices and policy making.

Finally, in Chapter 5 I present my main conclusions about this work, as well as my insights on how the knowledge presented here may be used in the future to combat disease and to teach the public and next generation of scientists about the importance of plant pathology, science and agriculture, in a changing word.

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Chapter 2

Cytokinin-mediated processes promote heat-induced disease susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. *tomato* DC3000[1]

2.1 Summary

Under increased temperatures, such as those predicted as a result of global climate change, plant defense responses are attenuated leading to a process referred to as heat-induced disease susceptibility (HIS). The plant growth hormone cytokinin (CK) is known to regulate responses to both biotic and abiotic pressures. To address the role of CK in HIS of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), we compared how wild-type Col-0 plants and a CK-signaling receptor mutant (*ahk2,3* mutated on *ARABIDOPSIS HISTIDINE KINASE 2* and *3*) respond to *Pst* inoculation under different temperatures conditions, normal (22°C) and high (28°C). Host susceptibility levels and pathogen fitness were initially determined by *Pst* populations *in planta*. Results show that, when exposed to higher temperatures, *ahk2,3* plants are less susceptible than Col-0 plants, with *Pst* populations proliferating at a lower rate compared to Col-0 overtime, suggesting that HIS is partially dependent on CK signaling. Additionally, plants with constitutive CK signaling exhibit increased susceptibility at 28°C supporting that CK signaling is needed for HIS to occur.

Hormone quantification showed that exposure to 28°C led to an increase in CK precursor content in Col-0 plants and a synthetic CK reporter line confirmed that high temperature increased CK signaling. Furthermore, DEX-inducible CK biosynthesis lines confirmed that CK

[1] This chapter contains preliminary data for a future publication with the following authors: Alexandra M. Shigenaga, Sarah M. Johns, Carolina Cassano Monte Bello, Mirella Farinelli-Ortiz, Yumiko Takebayashi, Marcelo Figueiredo, Hitoshi Sakakibara, Scott Nissen, Camila Caldana, Daniel R. Bush, & Cristiana T. Argueso.

content is both necessary and sufficient for HIS to occur in Arabidopsis. Interestingly, when addressing how high temperature affects defense responses results indicate that both Col-0 and *ahk2,3* plants show a decrease in defense responses or show a down-regulation of defense response genes, suggesting that the loss-of-susceptibility of *ahk2,3* at 28ºC is not attributed to more robust defenses. Metabolomics and autoradiography results show that CK promotes HIS of Arabidopsis through changes in primary metabolism and nutrient availability for *Pst* DC3000. Together the data reveals that under high temperature conditions, CK promotes late-physiological conditions, centered around primary metabolic processes, that may be contributing to pathogen proliferation.

2.2 Introduction

Plant diseases, pests, and insects are some of the leading causes of decreased crop yields and accounting for approximately 20% of total crop loss (Strange and Scott, 2005; Oerke, 2006). Additionally, adverse abiotic stresses, including increased temperatures, have a devastating effect on agricultural systems and are anticipated to account for up to 50% of crop loss (Wang et al., 2003; Zhao et al., 2017). Although individual stresses can negatively impact crop yield, this effect can be exacerbated in the presence of two or more stressors (Chakraborty and Newton, 2011). It has become evident that plants become more vulnerable to biotic pressures, after exposure to environmental stress (Atkinson et al., 2013; Prasch and Sonnewald, 2013). Understanding how plants respond to simultaneous stresses is important not only for epidemiological considerations, but can also be the basis of increasing breeding efforts to improve crop resilience.

Plants become more susceptible to disease when exposed to elevated temperatures, resulting in a process referred to here as heat-induced disease susceptibility (HIS) (Cohen and

Leach, 2020). HIS has been demonstrated in various plant-pathogen interactions and although HIS has been identified more with fungal (Madgwick et al., 2011; Mikkelsen et al., 2015; Onaga et al., 2017) and viral pathogens (Moury et al., 1998; Király et al., 2008), it has also been documented for bacterial pathogens (Velásquez et al., 2018). For example, in rice, exposure to elevated temperatures has been shown to increase susceptibility to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Webb et al., 2010). Previous studies have also shown that wild-type Arabidopsis plants become more susceptible to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) under high temperature conditions (Wang et al., 2009; Cheng et al., 2013; Menna et al., 2015; Huot et al., 2017). However, the underlying mechanism that drives HIS of Arabidopsis to *Pst* DC3000 is currently unknown.

The hemi-biotrophic pathogen *Pst* DC3000 has been described as a model plant pathogen and requires two important mechanisms to cause disease (Xin and He, 2013). These virulence mechanisms include the type III secretion system (T3SS), to translocate various effector proteins into plant cells to suppress immune responses (Xin and He, 2013), and the hormone-mimic phytotoxin coronatine (COR), which facilitates re-opening of stomata for bacterial entry into the apoplast (Melotto et al., 2006; Melotto et al., 2008). The T3SS of *Pst* DC3000 is induced by various host signals and can deploy up to 28 T3S effectors to cause disease. T3S effectors act to suppress defense responses, as well as target cellular processes such as photosynthesis, metabolism, and hormone signaling to manipulate host physiology for the pathogen's benefit (Cunnac et al., 2009; Cunnac et al., 2011; Xin and He, 2013; Hann et al., 2014; Turner et al., 2020).

HIS of Arabidopsis to *Pst* DC3000 can be attributed to decreased defense responses and/or increased pathogen virulence. Moderately high temperature conditions (6-8 ºC above

normal temperatures) have been shown to decrease the abundance of immune receptors (Janda et al., 2019), and lead to the down-regulation of defense related genes (Huot et al., 2017), and impairment of effector-triggered immunity (Cheng et al., 2013; Menna et al., 2015). Prior to 2017, increased susceptibility of Arabidopsis under heat stress was linked solely to decreased defense responses (Wang et al., 2009), as there was evidence from previous *in vitro* and protoplast studies to support that high temperature negatively impacted the T3SS (Smirnova et al., 2001; Cheng et al., 2013). However, recently studies have shown that under elevated temperature *Pst* DC3000 increases translocation of effector proteins, and that increased proliferation *in planta* requires an intact T3SS (Huot et al., 2017).

In addition, HIS of Arabidopsis to *Pst* DC3000 is also due to compromised defense responses dependent on the plant hormone salicylic acid (SA) (Huot et al., 2017). SA is a classic defense hormone, important for activating defense responses to (hemi)biotrophic pathogens, such as *Pst* DC3000 (Ding and Ding, 2020). However, SA does not act alone in activating defense responses (Shigenaga and Argueso, 2016). The plant growth hormone, cytokinin (CK) well-known for promoting cell division, nutrient remobilization, and inhibiting senescence (Hwang and Sakakibara, 2006; Choi and Hwang, 2007; Werner et al., 2008; Mok, 2019; Wang et al., 2019), has been shown to act synergistically with SA to promote defense responses (Choi et al., 2010; Argueso et al., 2012; Jiang et al., 2013; Arnaud et al., 2017). *Pst* DC3000 has a T3S effector that acts like a CK biosynthetic enzyme to produce CKs (Hann et al., 2014), a virulence mechanism utilized by other pathogens as well (Chanclud et al., 2016). Additionally, increase in CK content has been linked to being important in nutrient allocation during plant pathogen interactions by regulating source-sink relationships (reviewed in McIntyre et al., 2021),

In Arabidopsis, CK perception and signaling is carried out through a two-component system, similar to those in bacterial and fungal systems (Schaller et al., 2008; Argueso et al., 2012). Arabidopsis contains three CK histidine kinase signaling receptors, ARABIDOPSIS HISTIDINE KINASE (AHK2, 3 and 4). The *AHK2* and *AHK3* genes are expressed mostly in shoot tissues, whereas *AHK4* is mainly expressed in roots (Riefler et al., 2006). After CK is recognized by these receptors, it initiates a phosphorelay activating the ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER (AHP1-6) proteins (Hutchison et al., 2006). This leads to a phosphorylation of the ARABIDOPSIS RESPONSE REGULATORS (ARRs), which are categorized in two major groups: Type-B and Type-A. Type-B ARRs are known as positive regulators and contain a DNA-binding domain, and function as transcription factors (Argyros et al., 2008; Zubo et al., 2017). Type-A ARRs are negative regulators, lack a DNA-binding domain and act to inhibit CK signaling to prevent overactivation of the signaling pathway (To et al., 2004; To et al., 2007). In addition to the Type-B ARRs, the CK response factors (CRFs) are another class of transcription factors that mediate CK responses (Rashotte et al., 2006). Studies have shown that the Type-B ARR protein ARR2 promotes SA-mediated plant defense responses by binding the SA-regulated transcription factor TGA3, which is necessary to activate NON-EXPRESSER OF PR GENES 1 (NPR1)-dependent immune responses (Choi et al., 2010). Other studies have shown that, even though CK promotes SA-mediated defense responses, SA actually inhibits CK-mediated defense processes (Argueso et al., 2012).

CK has been shown to regulate responses to both biotic and abiotic pressures, including drought, salinity, cold and heat stress (Argueso et al., 2009; O'Brien and Benková, 2013; Zwack and Rashotte, 2015; Cortleven et al., 2019). Increases in CK content leads to plants more tolerant to these environmental stresses (Xu et al., 2010; Peleg et al., 2011; Reguera et al., 2013; Skalák

et al., 2016). In relation to plant responses to heat stress, CK has been hypothesized to directly participate in heat signaling due to overlap of temperature and CK signaling at the transcriptomic level (Černý et al., 2014) and given that increases in temperature result in an increase in biologically active forms of CK (Dobrá et al., 2015; Skalák et al., 2016). Increased endogenous levels of CK through the use of a transgenic line containing an inducible CK biosynthesis gene (*ISOPENTENYLTRANSFERASE* or *IPT*) demonstrated that plants are more well adapted to heat-stress with increased CK content (Skalák et al., 2016). Inhibition of CK-degradation enzymes (CK oxidase/dehydrogenases or CKXs) increases CK content and also leads to faster recovery of plants from heat stress (Prerostova et al., 2020). However, a decrease in CK content in response to increased temperature has also been observed (Todorova et al., 2005). While it is clear that that CK is important for thermo-responsiveness and heat signaling in plants (Černý et al., 2014; Skalák et al., 2016; Prerostova et al., 2020), it is yet to be determined if CK is important for plant responses during HIS.

To understand how plants become more susceptible under high temperature conditions, we investigated this process in the Arabidopsis-*Pst* DC3000 pathosystem. To address the role of CK in this process we compared Col-0 plants to a CK receptor mutant (*ahk2,3*). Our results show that *ahk2,3* plants are less susceptible to *Pst* DC3000 under heat stress, indicating a role for CK-signaling in promoting HIS. Hormone quantification and a CK synthetic reporter line confirmed that plants exposed to 28ºC show an increase in CK biosynthesis and signaling. Utilization of DEX-inducible CK biosynthesis lines further confirmed that CK-mediated processes are the basis of HIS. Although *ahk2,3* plants are less susceptible, our data indicates this is not due to increased defense responses but rather is associated with changes in nutrient availability for *Pst* DC3000. These results suggest that under high temperature CK promotes physiological

conditions that contribute to pathogen proliferation. Thus, we propose a model where CK is promoting HIS through a late physiological process that is dependent on primary metabolic processes. These CK-dependent genes may be considered high temperature susceptibility genes, as they are necessary for enhanced multiplication of pathogens at high temperatures. As such, they may form the basis of future engineering efforts of advanced crops with enhanced and durable resistance to pathogens at high temperatures, through approaches of genome editing to achieve loss-of-susceptibility.

2.3 Methods

Plant Materials and Growth Conditions:

Arabidopsis thaliana ecotype Columbia (Col-0, wild-type) and transgenic seeds in Col-0 background were stratified for 2-4 days at 4ºC before being placed on soil. All lines used for experiments were homozygous. Lines used in the study include: *ahk2-7 ahk3-2 (ahk2,3)* and the over-expressor Type-B ARR10 line, *35S*::ARR10 (Zubo et al., 2017); *pTCSn::GFP* (pTCSn) (Zürcher et al., 2013); dexamethasone (DEX)-inducible lines *proCaMV35S*>GR>*ipt* expressing *ipt* from *Agrobacterium* (*DEX::IPT*) (Craft et al., 2005) and *proCaMV35S*>GR>*HvCKX2* expressing *CKX2* from *Hordeum vulgare* (*DEX::CKX2*) (Černý et al., 2013). All plants were soil grown in a Conviron growth chamber (Model# ATC60) at Colorado State University Plant Growth Facility, unless stated otherwise. Plants were grown in Pro-mix HP Mycorrhizae soil for 5-7 weeks under a 10:14 hour day:night light regime at 160 ± 20 μmol $m^{-2}s^{-1}$ at 22ºC, 65% relative humidity (RH) in the day and 20ºC, 55% RH at night.

Temperature Treatments:

For all increased temperature assays, plants were moved to a growth chamber (Percival Model# PGC-15) set for 10:14 hour day:night light regime at 160 ± 20 μmol $m^{-2}s^{-1}$ at 28ºC, 65% RH in day and 55% RH at night. 5-7 week-old plants were used for all experiments, except for hypocotyl elongation, microscopy experiments, and flood inoculation assays.

Disease Assays:

Control plants remained at 22ºC for the entire experiment, while heat-treated plants were moved to the 28ºC chamber 24 hours before pathogen inoculation and remained there for the entirety of the experiment. On the day of inoculation plants were watered in the morning. Arabidopsis leaves were infiltrated with a bacterial suspension as described by (Tornero and Dangl, 2002) with noted changes. *Pseudomonas syringae* pv. *tomato* DC3000 EV (*Pst* DC3000) was streaked on King's B (KB) Media supplemented with Rifampicin (Rif, 50mg/mL) and Kanamycin (Kan, 50mg/mL) and incubated for 48 hours at 28ºC. 24 hours before inoculation a lawn plate of *Pst* DC3000 was streaked onto a new $KB_{rif,kan}$ plate and incubated at 28ºC. On the day of inoculation, the bacteria were resuspended in 10mM $MgCl_2$ for a bacterial concentration of $1x10^6$ CFU/mL (equivalent to OD_{600}=0.0002). Plants were inoculated by leaf infiltration with a needless syringe, four fully developed leaves per plant. After inoculation plants were covered with a lightly sprayed dome for 24 hours post inoculation (hpi), which was then cracked and removed 48 hpi. The amount of *in planta* bacteria was quantified 1 hpi (day 0) and 3 days post inoculation (dpi). Leaf discs were pooled for one sample, four samples were collected for each genotype/treatment at each time point. Leaf discs were ground in 10mM $MgCl_2$ and serial dilutions of ground tissue were used to determine the CFU per cm^2 of leaf disc tissue. Day 0

dilutions were plated on $KB_{rif,kan}$ plates and day 3 dilutions were plates on $KB_{rif,chx}$ (cycloheximide) plates, both were incubated at 28ºC for 24 hours before counting.

For dip inoculations, the bacterial solution was prepared at an OD_{600} of 0.05 in a 10mM $MgCl_2$ solution with 0.025% Silwett. Two-week-old plants were grown on soil covered with mesh before being dipped into bacterial solution. The amount of *in planta* bacteria was quantified 1 hpi and 3 dpi. Day 0 dilutions were plated on $KB_{rif,kan}$ plates and day 3 dilutions were plates on $KB_{rif,chx}$ plates, both were incubated at 28ºC for 24 hours before counting.

For flood inoculations the bacterial solution was at an OD_{600} of 0.1 (5×10^7 CFU/mL) in a 40ml solution of sterile distilled water containing 0.025% Silwett. Two-week-old seedlings, grown on 1X Murashige Skoog (MS) vertical plates before being flooded with bacterial solution for 2 minutes (Ishiga et al., 2011). After flood inoculation plates were placed back in the appropriate chamber and 2 dpi plants were imaged (see *Fluorescence Microscopy* section below).

***In planta* Bacterial Growth Curve:**

Disease assay conditions were followed. Control plants remained at 22ºC for experiment, while heat treated plants were moved to 28ºC chamber 24 hours before inoculation and remained there for the experiment. *Pst* DC3000 was grown and set to OD_{600} of 0.0002 ($1x10^6$ CFU/mL). Plants were inoculated with a needless syringe, as described above. Leaf discs were collected and pooled for each sample for day 0 through day 3. Leaf discs were ground in 10mM $MgCl_2$ and serial dilutions were plated to determine bacterial populations in planta. Days 0-1 were plated on $KB_{rif,kan}$ plates and days 2-3 were plated on $KB_{rif,chx}$ plates, then stored at 28ºC overnight.

***In vitro* Bacterial Growth Curve**:

A single colony overnight culture of *Pst* DC3000 was grown in $KB_{rif,kan}$ liquid culture at 28ºC, 225 rpm. The overnight culture was diluted to an OD_{600} of 0.05 in Liquid Media (LM, from Hanahan, 1983), which was separated into three flasks. Three flasks were used for each temperature (22ºC or 28ºC), shaking at 225rpm. *Pst* DC3000 populations at each time point were assessed by measuring the OD_{600} with a spectrophotometer (Bio-Rad SmartSpec 3000).

Cytokinin Quantification:

Frozen tissue, over 100 mg fresh weight (FW), was crushed to a fine powder using a TissueLyser (Qiagen, Hilden, Germany) with a zirconia bead (diameter, 5 mm). Extraction and determination of CKs from fresh tissue were performed as described previously by ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (AQUITY UPLC System/XEVO-TQS; Waters, Bedford, MA, USA) with an ODS column (AQUITY UPLC HSS T3, 1.8 μm 2.1 × 100 mm) (Kojima et al., 2009). The measured CK molecular species were tZ, tZ-7-*N*-glucoside (tZ7G), tZ-9-*N*-glucoside (tZ9G), tZ-*O*-glucoside (tZOG), tZR, tZR-*O*-glucoside (tZROG), tZRPs, tZRPs-*O*-glucoside (tZRPsOG), iP, iP-7-*N*-glucoside (iP7G), iP-9-*N*-glucoside (iP9G), iP-riboside (iPR) and iP-ribotides (iPRPs).

Fluorescence Microscopy:

Col-0 or transgenic *pTCSn::GFP* plants were grown on 1X MS vertical plates for 10 days. Roots were examined with a Leica DM5500B at 10X magnification using a UV emission filter to view the GFP signal. Col-0 were used as a control for absence of fluorescence. Images were composited and contrast adjusted to visualize the GFP signal in the root tissue. For each

treatment, 6 individual plants were imaged, and at least two biological replicates were completed.

Stomatal Aperture Assays:

Stomata aperture was measured according to (Melotto et al., 2006) with the following changes. Plants were exposed to light 100 mE/m^2/s for 3 hours to allow stomata to open fully. Epidermal peels of fully expanded leaves from Col-0 and *ahk2,3* plants were taken 24 hours after temperature treatment. Epidermal peels from 3 different plants per genotype were placed in MES buffer (25 mM MES-KOH [pH 6.15] and 10mM KCl) or MES buffer with 5uM flg22 peptide (GenScript Catalog No. RP19986, flagellin22 sequence: QRLSTGSRINSAKDDAAGLQIA). Epidermal peels were then placed on glass slides with the buffer they were previously incubated in. Images were taken within 20 minutes using a light microscope (Nikon Eclipse 50i) with an attachable camera (Nikon DS-Fi1). Stomata aperture was measured in the NIKON NIS Elements program. Experiment was repeated a minimum of three times.

Hypocotyl Elongation Assay:

Seeds were sown on 1X MS media horizontal plates and stratified at 4°C prior to being moved to the appropriate plant growth chamber. Seedings were grown under short day conditions, 120-130umE/m^2/s, in either the normal temperature (22°C) or high temperature plant growth chambers (28°C) for 5 days. At 5 days after germination (dag) Col-0 and *ahk2,3* plants were carefully pushed onto MS medium and plates were scanned for hypocotyl measurements. Hypocotyl length was measured using ImageJ Software (Version 1.51). Experiment was done in triplicate with 25 seedlings per genotype and treatment.

RNA Extraction and qRT-PCR:

Total RNA was extracted using RNeasy Plant kit (QIAGEN), following manufacturer's instructions. Quality and integrity of RNA was assessed by A_{260}/A_{280} and A_{260}/A_{230} ratios. RNA samples of good quality underwent DNAse Treatment (TURBO DNase-Free) and were checked for absence of genomic DNA by qRT-PCR using primers for AT5G66770 (For 5'-GGTTTGGTTTGGTTATCGCCAGGA-3', Rev 5'-TGGCTTCATCTCTTTGGCCTGGA-3'). cDNA was synthesized with Qscript (QuantaBio) and checked for full cDNA amplification through qRT-PCR using primers for *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE GAPDH* (AT1G13320). Primers used were: *GAPDH-1* (For 5'-TAGATCGCTCGGAACTTGGAAA-3', Rev 5'-CCTCACCAAAACTCAAATCACTCC-3'); *GAPDH-3* (For 5'-AACTAGGACGGATCTGGTGCCT-3', Rev 5'-GCTATCCGA ACTTCTGCCTCATTAT-3'), and *GAPDH-5* (For 5'-AAATTTAAC GTGGCCAAAATGATGC-3', Rev 5'-GTTCTCCACAACCGCTTGGT-3'). qRT-PCR reactions were performed with PerfeCTa SYBR Green (QuantaBio) on a CFX Connect Real-Time System (BioRad). cDNAs with Ct/Cq differences between each GAPDH primer of less than 1.5 were considered fully extended and of good quality. AT4G05320 *UBIQUITIN10* (*UBQ10)* was used as housekeeping gene in all reactions (For 5'-CGTTAAGACGTTGACTGGGAAAACT-3', Rev 5'-GCTTTCACGTTATCAATGGTGTCA-3'). Gene specific primers used are listed in **Table 2-1**. At least three biological replicates of each experiment were obtained.

RNA-Seq Whole Transcriptomics Analysis:

RNA quality and integrity were assessed using a TapeStation 2200 (Agilent) with High Sensitivity RNA materials (Agilent Screentape 5067-5579, Agilent Sample Buffer 5067-5580).

An RNA gel was also run to confirm RNA quality before being sent for sequencing. Paired-end, 150bp reads were sequenced by Novogene (Sacramento, CA, USA) at a depth of 40 million reads. Novogene used an Illumina based library construction kit (NEB Next Ultra 2). Illumina adapters were trimmed from paired-end reads using the CLC Genomics Workbench (20.0.3). Following adapter trimming, quality filtering was performed on CLC Genomics Workbench (20.0.3) followed by RNA-Seq and differential expression analysis. For RNA-Seq analysis Illumina reads were aligned with the *Arabidopsis thaliana* reference genome (TAIR10) with the set parameters: genome annotated with genes and transcripts, one reference sequence per transcript. Differential expression gene (DEG) analyses were conducted in the CLC Genomics Workbench (20.0.3), parameters for DEGs include False Discovery Rate (FDR, adjusted p-value) less than 0.05 and a log fold-change of 1.5, equal or greater than for up-regulated genes and equal of less than for down-regulated genes. Gene Ontology (GO) Term Enrichment for Plants from TAIR (https://www.arabidopsis.org/tools/go_term_enrichment.jsp) was used to identify statistical over- and under-represented GO biological processes. Metabolic pathways were analyzed using the MAPMAN software (Thimm et al., 2004). Heatmaps were generated in R (v3.2.2) using pheatmaps R package (Kolde, 2012). Promoter analysis was done with the BAR tool Promomer (http://bar.utoronto.ca/ntools/cgi-bin/BAR_Promomer.cgi) (Toufighi et al., 2005). The Type-B ARR binding motif 5'-(A/G)GAT(T/C)-3' was utilized for promoter analysis, set to measure the number of binding sites 1000bp upstream from the promoter region (TAIR10_upstream_1000). For the GO Term Enrichment and MAPMAN analyses only DEGs identified based on the above parameters were used.

Autoradiography:

Source leaves were marked based on (Farmer et al., 2013). Two 1uL droplets of ^{14}C-sucrose (American Radiolabeling Chemicals, Inc.) at a working concentration of 0.1uCi/10uL in a 0.25% Tween 20 solution were applied to the marked source leaf at the beginning of the light cycle. At timepoints mentioned in **Figure 2-16** legend, the applied leaf was cut off and "washed" in 5mL of 10% methanol and 1% NIS leaf wash solution. The leaf wash solution was then mixed with 10mL of the scintillation solution (Ecoscint^{TM} XR). The leaf wash and scintillation solution were then placed in a TRI-CARB 2300TR Liquid Scintillation Counter to measure how much of the applied ^{14}C-sucrose was absorbed by the leaf. Plant tissue for phosphorimaging was allowed to dry in an herbarium press at room temperature for 7-10 days before exposure to Phosphor Screen film. All samples for phosphorimaging were exposed to the film for 2 days before imaging. All phosphorimages were taken on the Typhoon Trio Imager (GE Healthcare) at a resolution of 50 microns.

Starch Staining:

Plant tissue was collected at the end of day. Leaf tissue was either mock-inoculated (10mM $MgCl_2$) or inoculated with *Pst* DC3000, by infiltration. Treated leaf tissue was collected 40 hpi and placed in a fixation solution (5% Formic Acid, 80% Ethanol, 15% Water), boiled at 80ºC for 10 minutes and then cooled for 1 minute. Fixation solution was removed, and the leaf tissue boiled in ethanol at 80ºC for 5 minutes to remove excess chlorophyll. Ethanol was removed and Lugol's solution (Sigma Aldrich) was added for 3 minutes and then tissue was rinsed with sterile water. Samples were boiled at 80ºC in water to fix the Lugol's staining for 15

minutes before being placed at 4ºC for 15 minutes. Samples were then placed in 50% glycerol for imaging.

Metabolite Extraction and GC-TOF MS Data Analysis:

Arabidopsis leaf tissue was collected at the beginning of the light period. Primary metabolites were extracted from 4.2 mg of lyophilized Arabidopsis leaf material (n = 3 technical replicates) according to da Silva et al., (2021). Metabolite extraction was done using methyl-tert-butyl-ether extraction (MTBE) buffer (Giavalisco et al., 2011). After concentrating polar fractions from the MTBE extractions, the polar fractions were derivatized with N-methyl-N-trimethylsilyltrifluoroacetamide and then analyzed by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) analysis using an Agilent 7890N GC system coupled with a Pegasus HT and Leco TOF-MS (Lisec et al., 2006). TargetSearch was used for peak detection, retention time alignment with fatty acid methyl esters, and mass spec comparison with reference libraries (Cuadros-Inostroza et al., 2009). Additionally, metabolite identification was manually supervised and a standard curve was used for absolute sugar quantification. For each sample, metabolites were quantified based on the peak intensity for a selected mass, normalized to the fresh weight (FW) and total ion count, then $\log_2$ transformed (da Silva et al., 2021). Data normalization and statistical analysis were performed in R version 3.2.2 and RStudio version 1.1.463. Pairwise comparisons of metabolites between the Col-0 temperature mock controls and *ahk2,3* samples were calculated using Student's t-test (p-value ≤ 0.05). Heatmaps were generated in RStudio version 1.1.463 using pheatmaps R package (Kolde, 2012).

Absolute starch quantification was also done according to da Silva et al., (2021). Following the MTBE extraction, the insoluble material was solubilized at 95°C in 0.1 M NaOH,

then neutralized, and digested enzymatically overnight. The released glucose after the overnight enzymatic digestion was then used to determine absolute starch content (μmol/g FW) of the samples spectrophotometrically by measuring the reduction of $NADP^+$ to NADPH (Hendriks et al., 2003). The statistical analysis described above for primary metabolites was used for starch content.

Statistical Analysis:

A minimum of three biological replicates were done for each assay, with at minimum three technical replicates for each genotype and treatment, unless stated otherwise. Sample sizes for each experiment are noted in figure legends. For experiments with two comparisons a two-WAY ANOVA was used and for one comparison a Student's t-test was used to evaluate statistical significance. Significance for these tests was based on a p-value ≤ 0.05.

2.4 Results

Elevated temperatures lead to increased disease susceptibility, in a CK-dependent manner.

Heat waves, or periods of moderately elevated temperature fluctuations 5-8ºC above optimal conditions, are anticipated to be a side effect of climate change and negatively impact plant systems (De Boeck et al., 2010; Bita and Gerats, 2013; Huot et al., 2017). Exposure to these moderately increased temperatures, even if for a short period, has been shown to decrease resistance of Arabidopsis to *Pst* DC3000, resulting in increased pathogen proliferation (Wang et al., 2009; Cheng et al., 2013; Huot et al., 2017; Li et al., 2020), a process known as heat-induced disease susceptibility (HIS). Given that CKs are plant hormones with a function in abiotic stress tolerance, and to understand the mechanisms underlying HIS, we tested whether an Arabidopsis

CK-receptor mutant (*ahk2,3;* harboring mutations on *ARABIDOPSIS HISTIDINE KINASE 2* and *ARABIDOPSIS HISTIDINE KINASE 3*) had comparable responses to wild type Arabidopsis plants (Col-0) in relation to HIS. Col-0 and *ahk2,3* plants were grown in a plant growth chamber under short days and normal temperature (22ºC) conditions. At 6 weeks of age half of the Col-0 and *ahk2,3* plants were moved to a plant growth chamber with similar settings, but with the temperature set to 28ºC, while the control plants remained at 22ºC. All plants, at both temperature regimens, were inoculated with *Pst* DC3000 by syringe infiltration 24 hours after placement in the appropriate chamber (**Figure 2-1A**), and then kept at their respective chambers for 3 more days. Bacterial populations were determined 1 hour post inoculation (hpi) and again 3 days post inoculation (dpi), in both genotypes tested. Our results confirm previous work (Wang et al., 2009; Cheng et al., 2013; Huot et al., 2017; Li et al., 2020) showing that wild-type Col-0 plants are more susceptible after exposure to 28ºC (**Figure 2-1B**), by an average of 7 fold in relation to wild type plants kept at the normal growth temperature of 22ºC. Plants of the CK receptor mutant *ahk2,3* allowed for increased *Pst* DC3000 proliferation at 22ºC in comparison to wild type plants under the same temperature, confirming previous findings that CK signaling is important for defense against *Pst* DC3000 in an SA-dependent manner (Choi et al., 2010). However, *ahk2,3* plants were less susceptible to *Pst* DC3000 at 28ºC, indicating that CK signaling is promoting host susceptibility at 28ºC in Col-0 plants (**Figure 2-1B**). To confirm that CK signaling is important for HIS, we used a CK hypersensitive line, *35S::ARR10*, over-expressing the transcription factor *ARR10*, a positive regulator of CK transcriptional responses (Zubo et al., 2017). Our results show that constitutive activation of CK signaling led to increased susceptibility at 28ºC, resulting in HIS levels greater than that seen in Col-0 (**Figure 2-1C**). In **Figure 2-1C**, HIS of Col-0 exhibits an increase in 0.85 Log CFU/cm^2 from 22ºC to 28ºC,

whereas HIS of *35S::ARR10* shows an increase of 1.2 Log CFU/cm^2, supporting that CK signaling is important for this physiological process.

Using liquid culture assays, we confirmed that *Pst* DC3000 does not proliferate as well *in vitro* at 28ºC compared to 22ºC, suggesting that the increased susceptibility of Col-0 to *Pst* DC3000 is a plant-dependent process (**Figure 2-2A**). Therefore, to understand the role of CK signaling in HIS we conducted an *in planta Pst* DC3000 growth curve over a three-day period. When comparing *ahk2,3* to Col-0 at 28ºC, *Pst* DC3000 proliferates at a slower rate between 24 hpi and 48 hpi in *ahk2,3* plants (**Figure 2-2B**). These results indicate that CK is likely promoting HIS of Col-0 to *Pst* DC3000 through a late physiological process, as the difference in bacterial proliferation is seen after ~40 hpi. Taken together these results indicate that CK signaling is important for increased susceptibility of *Pst* DC3000 *in planta* at 28ºC.

Increase in temperature leads to an increase in CK biosynthesis and signaling.

As CK plays an important role in SA-mediated defense responses against *Pst* DC3000 (Choi et al., 2010; Argueso et al., 2012), we wanted to evaluate how CK levels and downstream signaling were impacted by heat and pathogen stress. Previous studies have confirmed that SA content is negatively impacted by elevated temperature (Huot et al., 2017), but the effect of heat on CK remains unclear (Dobrá et al., 2015; Skalák et al., 2016; Prerostova et al., 2020). To address how high temperature affects CK content, we measured the CK content of Col-0 plants grown at 22ºC and 28ºC using GC-MS (Kojima et al., 2009). Col-0 plants were grown at 22ºC, then moved to 28ºC 48-hours prior to tissue collection, to ensure that we captured the timepoint in which we observe a difference in bacterial proliferation between Col-0 wild-type and *ahk2,3* plants, as seen in the *in planta* growth curve. We observed an increase in the iP-type, N^6-(Δ^2-

isopentenyl)-adenine, and tZ-type, *trans*-zeatin, CK precursors in response to 28ºC (**Figure 2-3A**). tZ and iP are the two most abundant forms of CK in plants (Osugi and Sakakibara, 2015). Although, the bioactive forms of CK, tZ and iP, did not show a significant difference in content between normal and high temperature conditions (**Figure 2-3A), a**n accumulation of CK precursors has been noted to generally result in increased CK activity (Kiba et al., 2019). The concentrations of all measured CK metabolites are outlined in **Table 2-2**.

As a result of increased CK precursor biosynthesis, we anticipated an increase in CK signaling. To address if CK signaling increased in response to 28ºC, we examined the response of the CK reporter line *pTCSn::GFP* to exposure to 28ºC. The *pTCSn::GFP* is a synthetic CK reporter line that shows fluorescence in Type-B ARR responding cells, and display strong signal on root tips and stomata guard cells (Zürcher et al., 2013). *pTCSn::GFP* plants were grown on vertical plates containing 1X Murashige-Skoog (MS) media in a growth chamber at 22ºC. Ten days after germination (dag) plates were exposed to elevated temperature (28ºC) or maintained at the control temperature (22ºC) and imaged at early (5 hours) and late (40 and 60 hours) timepoints on a fluorescence microscope. Non-transgenic wild type Col-0 plants were used as a negative fluorescence control (data not shown). Our results show that plants exposed to short-term (5 hour exposure) or long-term (40 or 60 hour exposure) high temperature conditions resulted in increased fluorescence in CK-responding cells, compared to plants grown at 22ºC (**Figure 2-3B**). The peak of *pTCSn::GFP* signal occurred around 40h after heat exposure, coinciding with the time point in which Col-0 plants show increased susceptibility under HIS (**Figure 2-3B)**.

To further confirm the increase in CK signaling at 28ºC, as a result of increased CK precursor accumulation, the expression of CK signaling components was measured through gene

expression analyses using qRT-PCR and RNA-Seq for global gene expression analysis, described in more detail below. The schematic labeled in **Figure 1A** was utilized for the RNA-Seq experiment, with Col-0 and *ahk2,3* plants grown at 22ºC for five- to six-weeks, and 24 hours prior to inoculation heat treated plants were moved to a 28ºC chamber. Control plants remained at 22ºC and heat-treated plant remained at 28ºC, for the remainder of the experiment. Leaves were syringe inoculated with either a mock solution (10mM $MgCl_2$), or with *Pst* DC3000 (OD_{600}= 0.0002). Tissue was collected approximately 40 hpi for RNA extraction, followed by library preparation and RNA sequencing using Illumina technology. Tissue for qRT-PCR was collected under the same experimental conditions as RNA-Seq. RNA-seq data analysis for differentially expressed genes (DEGs) was performed with CLC Genomics Workbench (20.0.3) (see *Methods*). Data other than CK signaling components will be discussed later in this chapter. As expected, the CK-regulated Type-A ARRs *ARR5* and *ARR7* showed an increase in expression after exposure to 28ºC, as determined by qRT-PCR (**Figure 2-4**). Additionally, our RNA-Seq data showed an upregulation of many CK signaling components (both Type-A and Type-B ARRs), including CRFs and the CK biosynthetic gene *IPT3* in response to elevated temperature. Further, Col-0 plants exposed to 28ºC showed a decrease in the expression of genes encoding CK degradation enzyme genes (*CKX1*, *CKX2*, and *CKX4*) compared to Col-0 exposed to 22ºC (**Figure 2-4**). Together these data further support that Col-0 plants exposed to 28ºC result in increased CK biosynthesis and CK signaling.

To further elucidate the role of CK in HIS, we monitored levels of *pTCSn::GFP* signal after infection with *Pst* DC3000, under normal (22ºC) and high (28ºC) temperature regimens. *pTCSn::GFP* plants were grown on vertical plates containing 1X MS media as in **Figure 2-3B**. Ten-day-old plants on plates were exposed to heat stress (28ºC) or maintained at the control

temperature (22ºC), and 24 hours later inoculated with *Pst DC3000* using a flood inoculation protocol (Ishiga et al., 2011), followed by imaging at 40 hpi. In comparison to *pTCSn::GFP* plants maintained at 22ºC, *pTCSn::GFP* exposed to 28ºC showed a strong signal (**Figure 2-5A**). However, inoculation with *Pst* DC3000, either at 22ºC or 28ºC, led to a decrease of the *pTCSn::GFP* signal in CK-responding cells, especially at 28ºC (**Figure 2-5A**). Additionally, we assessed the effect of combined temperature and *Pst* DC3000 stresses on CK signaling by measuring the expression levels of the CK signaling gene *ARR7* on Col-0 plants by qRT-PCR using a similar experimental design, at early (5 hpi) and late (40 hpi) timepoints (**Figure 2-5B**). The expression of *ARR7* was upregulated under high temperature and *Pst* DC3000 at 5 hpi, in comparison to plants at 22ºC (**Figure 2-5B**). This early up-regulation was followed by a decline in *ARR7* levels at 40 hpi. A reduction in CK-regulated gene expression and signaling in response to infection with several types of pathogens has been documented before, although only at normal (22ºC) temperatures (Argueso et al., 2012; Hann et al., 2014). Together these data suggest that there is an early activation of CK signaling in response to heat stress, that later lowers in response to *Pst* DC3000.

CK-regulated processes are the basis for HIS.

To address the importance of CK content and test whether CK-mediated processes are necessary for HIS of Arabidopsis to *Pst* DC3000, we compared HIS phenotypes of Col-0 to transgenic plants expressing the dexamethasone-inducible constructs *CaMV35S* > GR > *HvCKX2* (*DEX::CKX2*) and *CaMV35S* > GR > *ipt* (*DEX::IPT*), encoding a CK degradation enzyme or a CK biosynthesis enzyme, respectively (Skalák et al., 2016). Use of these DEX-inducible lines allowed us to address how decreased and increased CK content impacts HIS. Six-

week-old *DEX::CKX2* and *DEX::IPT* homozygous lines, as well as Col-0 wild type plants, were grown in a plant growth chamber under short days and normal temperature conditions (22ºC). Plants were sprayed with 20uM dexamethasone (DEX) prior to being moved to the 28ºC chamber, or maintained at the normal temperature chamber, 24 hours before inoculation with *Pst* DC3000 by syringe infiltration. HIS was assessed by measurement of bacterial populations at 3 dpi. Wild type Col-0 plants showed increased bacterial populations under high temperature, demonstrating that the experimental conditions led to normal HIS development (**Figure 2-6**). The disease phenotypes of the DEX-inducible lines at 22ºC were as expected, considering the role of CK in defense against *Pst* DC3000 (Choi et al., 2010): the DEX-induced *CKX2* line, with decreased CK content, was more susceptible to *Pst* DC3000 at 22ºC, and the DEX-induced *ipt* line, with increased CK content, was more resistant (**Figure 2-6**). Results of the DEX-inducible lines showed that CK content is both necessary and sufficient for HIS to occur: when comparing *Pst* DC3000 proliferation in the DEX-*CKX2* line, there is no difference in susceptibility between 22ºC and 28ºC indicating that HIS is lost in the absence of CK (**Figure 2-6**). However, when comparing *Pst* DC3000 proliferation in the DEX-inducible *ipt* line, in which there is an increase in CK content, plants are more resistant at 22ºC and are highly susceptible at 28ºC (**Figure 2-6**). In **Figure 2-6**, HIS of Col-0 plants shows a 1.1 Log CFU/cm^2 increase, whereas HIS of *DEX::IPT* shows an increase of 1.3 Log CFU/cm^2 further supporting that increase in CK content alone is sufficient for HIS to occur. These results indicate a direct correlation between CK content and susceptibility of plants to *Pst* DC3000 at 28ºC. These disease phenotypes for the DEX-inducible lines coincide with the gene expression signatures of Col-0 plants exposed to 28ºC, with *CKX* genes being down-regulated and *IPT* genes up-regulated (**Figure 2-4**).

CK-regulated physiological processes are activated under elevated temperature.

To further address the importance of CK in HIS, we assessed whether Col-0 and *ahk2,3* plants respond similarly to elevated temperature. Additionally, we evaluated if CK-mediated physiological processes are impacted under high temperature conditions. In response to elevated temperatures, *Arabidopsis* plants will exhibit various physiological changes including accelerated flowering, hypocotyl elongation, and early senescence (Larkindale et al., 2005; Jespersen et al., 2016; Kazan and Lyons, 2016; Kim et al., 2020), the latter two have both been linked to CK (Osborne, 1962; Dyer and Osborne, 1971; Cary et al., 1995; Zwack and Rashotte, 2015).

Growth of Arabidopsis plants in high temperature conditions triggers early flowering (Balasubramanian et al., 2006). Col-0 wild type plants grown under short day and high temperature conditions (28ºC) flowered at approximately 4 weeks post germination (**Figure 2-7A**), which is approximately 6 weeks earlier than the normal flowering time of Col-0 plants at 22ºC under short day conditions (Sharma et al., 2016). *ahk2,3* plants also had an accelerated flowering time, flowering at approximately 5 weeks post germination (**Figure 2-7B**). Previous studies have also shown that CK is involved in heat-induced hypocotyl elongation (Richmond and Lang, 1957; Černý et al., 2014). Col-0 plants grown at 28ºC displayed hypocotyl elongation, when compared to 22ºC, but *ahk2,3* showed a less pronounced response (**Figure 2-7C**). Together, these results show that CK-dependent physiological processes are activated during heat stress, and that impairment of CK signaling in *ahk2,3* prevents these physiological processes from fully occurring.

Down-regulation of defense responses contribute to increased susceptibility of Col-0 at 28ºC, but these are not the only factors.

Previous studies have shown that defense responses of Col-0 plants are down-regulated under high temperature conditions (Huot et al., 2017; Janda et al., 2019). We therefore addressed whether the loss of HIS observed in the CK signaling mutant *ahk2,3* was a result of increased defense responses in this genetic background (**Figure 2-8A**). Stomatal immunity is an essential component of plant defense responses and stomatal function is directly associated with abiotic stress regulation (Melotto et al., 2006). Further, stomatal immunity can be induced by CK application, via an SA-dependent pathway (Arnaud et al., 2017). Epidermal peels of Col-0 and *ahk2,3* plants were placed in MES buffer with or without 5μM of the flagellin peptide flg22, known to activate Pathogen-Associated Molecular Pattern (PAMP)-triggered immunity and result in stomatal closure (Melotto et al., 2006; Zhang et al., 2008; Arnaud et al., 2017). Epidermal peels were exposed to 22ºC or 28ºC, and stomatal aperture measured (**Figure 2-8B**). Our results confirm that Col-0 displays stomatal closure in response to flg22 at 22ºC, however this response is lost in *ahk2,3* plants (**Figure 2-8A**). This confirms previous reports of CK signaling being important for stomatal immunity (Arnaud et al., 2017). However, at 28ºC Col-0 stomatal response was impaired, as the stomata remained more open after exposure to flg22 (**Figure 2-8A**). Interestingly, our results show that this response is also CK-dependent, as impairment of stomatal immunity at 28ºC is lost in *ahk2,3* plants (**Figure 2-8A**). To determine the contribution of stomatal immunity to HIS, we performed a HIS assay using plants inoculated with *Pst* DC3000 by dip inoculation, in which bacterial cells enter the apoplast naturally through the stomata. Our results show that HIS by dip inoculation is not as pronounced in Col-0 plants, further suggesting that stomatal immunity is not fully impaired under heat stress (**Figure 2-8C**).

Similar to our results by infiltration inoculation (**Figure 2-1A**), at 22ºC *ahk2,3* plants showed increased susceptibility to *Pst* DC3000 in comparison to Col-0 plants, supporting previous findings (Choi et al., 2010; Arnaud et al., 2017), and improved stomatal immunity under heat stress (**Figure 2-8A**). Given the similarity in susceptibility phenotypes of *ahk2,3* plants regardless of method of inoculation (**Figure 2-1A**; **Figure 2-8C**), we can conclude that stomatal immunity is not likely contributing to the overall loss of HIS in this genetic background.

To investigate other defense responses, we analyzed the pattern of expression of genes associated with defense responses against pathogens. Previous studies have confirmed that the expression patterns of such genes are down-regulated in response to elevated temperature (Huot et al., 2017). To address this question, we analyzed gene expression changes under heat stress and whether there is an increase in defense response genes in *ahk2,3* plants that lack HIS at 28ºC. When comparing tissue from Col-0 and *ahk2,3* plants at 22ºC and 28ºC inoculated with *Pst* DC3000 or a mock control by qRT-PCR, we found that the defense response genes *PR-1* and *WRKY18* were down-regulated in all heat-exposed plants, regardless of genotype (**Figure 2-9A**). RNA-Seq analysis also showed that plants exposed to 28ºC show a general down-regulation of defense response genes (**Figure 2-9B**). Gene Ontology (GO) analyses of the RNA-Seq results of Col-0 and *ahk2,3* plants at 28°C showed that both genotypes exhibited down-regulation of the biological processes Defense response (GO:0006952), Response to bacterium (GO:0009617), and Systemic acquired resistance (GO: 0009627) (**Figure 2-9C**), and of the top 15 GO biological processes down-regulated by 28ºC in Col-0 and *ahk2,3*, the majority were associated with defense responses (**Figure 2-9C**). Together these results indicate that *ahk2,3* displays suppression of defense responses by heat stress that is comparable to that of Col-0 wild type

plants under similar conditions, thus suggesting that physiological processes other than defense are promoting loss of HIS of *ahk2,3* plants at 28ºC.

Whole transcriptome RNA-Seq analysis reveals potential genes of interest associated with HIS.

To address what processes CK may be impacting to promote HIS, we conducted an RNA-Seq experiment to identify underlying processes. The RNA-Seq experiment comparisons are listed in **Figure 2-10A**, with the *ahk2,3* comparisons shaded in yellow. The most important comparisons for identifying genes of interest for HIS are comparison 3 and comparison 5 (**Figure 2-10A**). All samples were compared to Col-0 22ºC $MgCl_2$ as a baseline and the number of differentially expressed genes in each group is listed in **Figure 2-10B**. When comparing each transcriptional profile of each RNA-Seq sample in **Figure 2-10A**, the differentially expressed genes clustered based on temperature and pathogen stress (**Figure 2-11**). The heat map of hierarchical clustering shows that samples first separate based on temperature treatment, then separated based on *Pst* DC3000 inoculation (**Figure 2-11**).

Analysis of genes differentially expressed in Col-0 28ºC *Pst* and *ahk2,3* 28ºC *Pst* showed that the most upregulated GO Biological Processes included Jasmonic acid (JA) biosynthesis (GO:0009695) and JA metabolic processes (GO:0009694) (**Figure 2-12A**). Further investigation of these data showed that various JA-biosynthetic and signaling genes were up-regulated in both genotypes at 28ºC after inoculation with *Pst* (**Figure 2-12B**). Together these results suggest that JA is likely not contributing to HIS in Col-0 plants or lack of HIS in *ahk2,3* plants, supporting previous findings (Huot et al., 2017), and indicating that another process is responsible for the underlying mechanism of HIS.

To narrow down the list of differentially expressed genes identified from the RNA-Seq analysis that could be contributing to CK-dependent processes associated with HIS, we compared the differentially expressed Col-0 28ºC *Pst* and *ahk2,3* 28ºC *Pst* genes to a list of Type-B ARR10 transcriptional targets, identified by ChIP-Seq (Zubo et al., 2017). After identifying Type-B ARR10 candidate genes in each list, we overlapped these to identify unique candidates based on genotype background (**Figure 2-13A**). Differentially expressed genes that were specific for Col-0 28ºC *Pst* or *ahk2,3* 28ºC *Pst* were divided into up-regulated and down-regulated genes. To identify potential genes to target for decreased susceptibility, we focused on genes unique to Col-0 28ºC *Pst* and performed a promoter analysis to identify those with Type-B ARR binding motifs, indicative of transcriptional regulation by CK. The promoters of these genes were searched 1000bp upstream from their transcriptional start site for Type-B ARR binding motifs (5'-(A/G)GAT(T/C)-3'; based on (Zubo et al., 2017), using Promomer (Toufighi et al., 2005) (**Figure 2-13B**). A total of 34 genes were identified as potential Type-B ARR targets involved in promoting CK-dependent HIS in Col-0 plants (**Figure 2-13A**, **C**). Of these genes, a subset was selected based on physiological functions, including the CK Response Factors (CRFs), Ethylene Response Factors (ERFs), nitrate transporters, and polyamine oxidases (**Table 2-3**). We then took a loss-of-function approach to determine whether these genes contributed to HIS of Col-0 plants. T-DNA or EMS-generated mutants in each of these genes were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio). Plants were genotyped to identify homozygous mutants, and seeds harvested. Mutants were then assessed for HIS, using Col-0 and *ahk2,3* plants as HIS positive and negative controls, respectively. Preliminary data suggested that the mutants tested did not show any clear changes on HIS phenotypes (data not shown), but these results were complicated by poor soil conditions at the

time of assays, and therefore should be repeated. Further investigation will be necessary to properly determine the contribution of these genes to HIS.

Loss of HIS of *ahk2,3* plants is linked to decreased nutrient availability for *Pst* DC3000.

In parallel to our loss-of-function approach, we also took a targeted physiological approach to determine the mechanisms responsible for loss of HIS in *ahk2,3* plants. CK is a plant growth hormone that has long been implicated in the regulation of source-sink relationships and reallocation of nutrients (Roitsch and Ehneß, 2000). Studies have shown that an increase in endogenous CKs lead to changes in source-sink modifications that can lead to improved abiotic and biotic stresses (Werner et al., 2008; Peleg et al., 2011). Thus, we hypothesized that decreased susceptibility of *ahk2,3* plants at 28ºC might be due to decreased nutrient availability for *Pst* DC3000, leading to physiological conditions unable to support the increased bacterial populations allowed to grow at 28ºC. To address if changes in nutrient availability could be contributing to changes in susceptibility between Col-0 and *ahk2,3* plants at 28ºC, and therefore contributing to HIS, we analyzed our transcriptomics RNA-Seq data with MAPMAN (Thimm et al., 2004), to identify any trends in metabolic pathways based on gene expression. Interestingly, genes involved in sucrose degradation and trehalose biosynthesis, located under minor CHO/Carbohydrates, were highly up-regulated in Col-0 28ºC plants inoculated with *Pst* DC3000, whereas genes associated with starch degradation and light reactions were highly down-regulated (**Figure 2-14A**). However, the MAPMAN analysis for *ahk2,3* plants at 28ºC inoculated with *Pst* DC3000 showed a down-regulation of genes associated with starch biosynthesis and light reactions, with starch degradation being up-regulated, as well as biosynthesis of the minor sugars raffinose and trehalose (**Figure 2-14B**). Down-regulation of

genes associated with light reactions and photosynthesis has been well documented and is expected in response to *Pst* DC3000 inoculation (Bonfig et al., 2006; Nomura et al., 2012). However, up-regulation of sucrose biosynthesis and down-regulation of starch biosynthesis in Col-0 28ºC plants suggest that these plants may have more sucrose available for *Pst* DC3000. Whereas, the metabolic expression pattern of *ahk2,3* plants at 28ºC inoculated with *Pst* suggests less nutrient availability for the pathogen.

Our RNA-Seq data indicated that sucrose biosynthesis may be the determining factor for differences in host susceptibility between Col-0 and *ahk2,3* plants at 28ºC, and thus we wanted to address if changes in source and sink vegetative tissue could be contributing to CK-mediated HIS. We hypothesized that if sucrose is important for this biological process, we should see a difference in HIS between source and sink tissues. We therefore conducted HIS disease assays with leaves presumed to function either as source or sinks, in both Col-0 and *ahk2,3* plants (**Figure 2-15A**). Previous studies used $^{14}CO_2$ labeling to track carbon availability in source and sink vegetative tissues (Kölling et al., 2013; Kölling et al., 2015). Based on these studies and the well-documented role of CK in source-sink relationships (McIntyre et al., 2021) we wanted to address if source or sink leaf tissue would impact HIS. Source leaves were selected as fully developed leaves and around leaf number 8 (Farmer et al., 2013), whereas sink leaves were selected as the youngest leaves that were large enough for inoculation and around leaf number 16 (Farmer et al., 2013). Individual plants were used either for source or sink leaves. Four leaves of the same type were inoculated per plant, and we aimed to inoculate leaves with vascular connections, which meant leaf numbers n ± 5 and n ± 8 (Farmer et al., 2013). Results show that source or sink leaf type impacts HIS and is dependent on CK signaling (**Figure 2-15B**). More specifically, under normal temperature conditions sink leaves are less susceptible in Col-0 when

compared to source leaves, and this is CK-dependent as it is lost in *ahk2,3* plants (**Figure 2-15B**). Under high temperature conditions, source and sink leaves of Col-0 plants are equally susceptible, and this shift in susceptibility compared to 22ºC is CK-dependent as *ahk2,3* plants exhibit a decrease in susceptibility of source leaves (**Figure 2-15B**). Together these results suggest a role for source-sink relationships, potentially through nutrient availability, that is contributing to CK-mediated HIS.

To assess if sucrose translocation changes under high temperature conditions, we utilized an *in planta* ^{14}C-sucrose transport assay. First, we wanted to confirm that the source leaves and sink leaves from the above experiment were actually correctly identified. We hypothesized that after applying ^{14}C-sucrose to source leaves the sucrose would translocate to the sink leaves, which should be visualized by phosphoimaging. Results show that application of ^{14}C-sucrose to a single source leave will travel to sink tissue, either roots or young leaves (**Figure 2-16A**). Imaging at various time points (30 minutes to 6 hours post ^{14}C-sucrose application) showed that *ahk2,3* plants translocate sucrose at a faster rate than Col-0 plants, with plants at 22ºC translocating more than plants at 28ºC (**Figure 2-16A**). ^{14}C-sucrose transport in Col-0 plants was much slower compared to *ahk2,3* plants, with the only real difference showing between the 3- and 6-hour post ^{14}C-sucrose application time-points (**Figure 2-16A**), with plants at 28ºC translocating slightly more ^{14}C-sucrose. However, infiltration with *Pst* DC3000 or mock solutions led to no visible difference in ^{14}C-sucrose transport 6-hours post application (**Figure 2-16B**), suggesting that sucrose translocation equilibrium may have been disrupted by infiltration.

Pathogen-produced CKs can promote starch accumulation in leaves (Erickson et al., 2014), thus we wanted to address if starch content could be contributing to HIS. Col-0 and *ahk2,3* plants were exposed to 22ºC or 28ºC, infiltrated with a $MgCl_2$ control or *Pst* DC3000,

then stained for starch using a Lugol's iodine solution. Results from this assay show that Col-0 plants have a breakdown of starch in areas inoculated with *Pst* DC3000 at 22ºC, but this is lost at 28ºC (**Figure 2-17A**). Whereas, *ahk2,3* plants do not exhibit much starch accumulation regardless of temperature or pathogen stress (**Figure 2-17A**). This suggests that starch accumulation is a CK-dependent plant process and that breakdown of starch at normal temperature conditions is important for *Pst* DC3000 virulence. Lack of starch breakdown in Col-0 plants at 28ºC suggests that there are enough carbon sources available that *Pst* DC3000 doesn't need to breakdown starch for survival.

To confirm the starch staining results, we quantified important primary metabolites in starch biosynthesis and degradation. The experimental design was carried out based on **Figure 2-1A**. Leaf tissue from plants infiltrated with the $MgCl_2$ control or *Pst* DC3000 was collected 40 hpi and used for metabolomics analysis of primary metabolomics through a GC-MS approach (Caldana et al., 2013). The results show that Col-0 plants have an overall increase in starch content compared to *ahk2,3* plants, with Col-0 plants at 28ºC having significantly more starch content than all other genotypes and treatments (**Figure 2-17B**). These metabolomics results support our starch staining (**Figure 2-17A**), further confirming that plants lacking CK signaling are unable to produce and store starch appropriately. Additionally, only Col-0 plants at 22ºC inoculated with *Pst* DC3000 show a significant increase in maltose content (**Figure 2-17B**). Maltose is the main breakdown metabolite of starch (Niittyla, 2004; Weise et al., 2004), thus only Col-0 plants at 22ºC inoculated with *Pst* DC3000 should exhibit maltose accumulation based on starch staining results (**Figure 2-17A**). Together these absolute quantification results for starch and maltose support our starch staining results (**Figure 2-17A**).

Additionally, relative abundance metabolomics results indicate that Col-0 plants have a down-regulation of trehalose content, compared to *ahk2,3* plants (**Figure 2-18A**). Trehalose is disaccharide that is important for starch biosynthesis and has been linked to sugar utilization, as well as maintaining overall plant metabolic status (Kolbe et al., 2005; Lunn et al., 2014). An increase of trehalose in *ahk2,3* plants indicates that starch metabolism is mis-regulated (Wingler et al., 2000), supporting the MAPMAN and starch staining results. When comparing the relative abundance of sugars in Col-0 and *ahk2,3* plants at 28ºC inoculated with *Pst*, *ahk2,3* plants have overall less sugar content than Col-0 (**Figure 2-18A**).

When looking at the relative abundance of fructose, although *ahk2,3* 28ºC *Pst* samples shows a significant increase (p-value < 0.05) of this metabolite, all other tested genotypes and treatments also exhibit high levels of relative fructose. Especially when comparing Col-0 and *ahk2,3* plants at 28ºC, with or without *Pst*, both genotypes exhibit high relative abundance of fructose (**Figure 2-18A**). Although these results suggest that the difference in susceptibility is not due to fructose content, a sugar important for induction of T3SS (Stauber et al., 2012), comparing relative abundance of other important sugars show that *ahk2,3* plants inoculated with *Pst* DC3000 at 28ºC have a significant decrease in various sugar abundance including: sucrose, fucose, xylose, cellobiose, maltose, and raffinose (**Figure 2-18B**). These results suggest an overall decrease in available carbon sources for *Pst* DC3000 in the *ahk2,3* background under elevated temperature. Additionally, absolute sucrose accumulation results show that Col-0 plants have higher sucrose content compared to *ahk2,3* plants regardless of temperature of pathogen treatment (**Figure 2-18B**). However, it is important to note that when comparing sucrose accumulation of Col-0 and *ahk2,3* plants at 28ºC, Col-0 plants have more sucrose content, suggesting this could be contributing to increased susceptibility.

2.5 Discussion

Because a plant lacking CK signaling was less susceptible to *Pst* DC3000 under high temperature conditions (**Figure 2-1A**), it was of interest to understand how CK could be involved in regulating HIS responses. First, we investigated how CK biosynthesis and signaling were affected by high temperature conditions. Although the role of CK in heat stress responses is currently unclear (Todorova et al., 2005; Dobrá et al., 2015; Skalák et al., 2019; Prerostova et al., 2020), our results indicate that CK biosynthesis and signaling increases in response to elevated temperatures (**Figure 2-3A, B**). To understand if CK is important for HIS, we utilized DEX-inducible CK biosynthesis lines, which showed that CK content was not only necessary but sufficient for HIS to occur (**Figure 2-6**). Here we provide evidence that CK mediates plant response to *Pst* DC3000 at high temperatures through alterations of nutrient allocation.

After exposure to high temperature stress, either short or long term, *Arabidopsis thaliana* defense responses are disrupted and *Pst* DC3000 virulence is increased *in planta* (Wang et al., 2009; Cheng et al., 2013; Huot et al., 2017; Janda et al., 2019). Huot et al., (2017) elegantly showed that increases in temperature lead to a compromised SA-pathway, including down-regulation of SA-biosynthesis gene *ICS1* and SA-mediated defense genes such as *PR-1*. In addition to down-regulated SA-mediated defenses, this study showed an increase in effector translocation in response to high temperature (Huot et al., 2017). Additionally, studies have shown that other defense processes are also negatively impacted by high temperature, such as decrease in abundance of the immune receptor FLS2 (Flagellin Sensing 2) (Janda et al., 2019). Although down-regulation of SA-mediated responses has been well-documented to be involved in HIS of Arabidopsis to *Pst* DC3000, the underlying mechanism driving this physiological process is still unclear. Considering that CK acts synergistically with SA to increase resistance

against *Pst* DC3000, we reasoned that if high temperature impacts SA then the CK response would also be affected (Choi et al., 2010; Argueso et al., 2012). To provide insights into the CK-mediated physiological factors contributing to increased biotic susceptibility in Arabidopsis at elevated temperature, we utilized a temperature-exposure treatment and *Pst* DC3000 inoculation outlined in **Figure 2-1A**.

CK-Related Processes are Necessary for Heat Induced Susceptibility.

We found that plants deficient in CK signaling were less susceptible to *Pst* DC3000 than wild-type Col-0 plants at elevated temperature conditions (**Figure 2-1B**), whereas the mutant was more susceptible at 22°C, which was previously characterized by (Choi et al., 2010). This was in contrast to plants overexpressing CK signaling, which were less susceptible to *Pst* DC3000 at 22°C and showed an increase in susceptibility at 28°C (**Figure 2-1A**). Together these results indicate that CK signaling is promoting *Pst* DC3000 proliferation in Col-0 plants at 28°C. When comparing *Pst* DC3000 proliferation in Col-0 and *ahk2,3* plants at 28°C, bacterial growth plateaus in *ahk2,3* plants after 48 hours (**Figure 2-2B**). Consistent *Pst* DC3000 proliferation in both Col-0 and *ahk2,3* plants until 48 hours suggests that a late, CK-mediated process, is responsible for a difference in host susceptibility under high temperatures.

Additionally, DEX-inducible CK biosynthesis lines further confirmed that CK-mediated processes are both necessary and sufficient for HIS to occur (**Figure 2-6**). The DEX-inducible lines used in this study include a *DEX::CKX2* line, which encodes a CK oxidase/dehydrogenase that degrades CK, and a *DEX::IPT* line, which encodes the CK biosynthesis isopentenyl transferase enzyme. Results from the *DEX::CKX2* line show that CK content is necessary for HIS because breakdown of CK content leads to no difference in host susceptibility at 22°C and

28°C **(Figure 2-6**). Whereas, the *DEX::IPT* line shows that CK content is sufficient for HIS to occur, because with a constitutive increase in CK biosynthesis there is an increase in susceptibility from 22°C to 28°C (**Figure 2-6**). Together these results highlight the importance of CK in HIS and support previous work that increase in CK content will lead to increased resistance (Choi et al., 2010; Grosskinsky et al., 2011), whereas decreased CK content will lead to increased susceptibility (Choi et al., 2010).

Additionally, our results indicate that increased *Pst* DC3000 proliferation at 28°C is specific to a plant related process, given that *Pst* DC3000 does not proliferate more at 28°C in liquid culture (**Figure 2-2A**). These results are further supported by the *in planta* growth curve assay, indicating that the increase *Pst* DC3000 proliferation at 28°C is CK dependent. While there are contrasting results for how *Pst* DC3000 grows *in vitro* at normal versus high temperature conditions (Wang et al., 2009; Huot et al., 2017), currently the consensus from the literature is that HIS is a plant-dependent process. Thus, our results further support the consensus that HIS is plant-dependent and indicates that CK may drive HIS responses.

Increased defense responses are not contributing to loss of HIS in *ahk2,3*.

Considering various studies have cited that the down-regulation of defense responses is associated with increased susceptibility of Arabidopsis to *Pst* DC3000, we hypothesized that loss of susceptibility at 28°C in *ahk2,3* plants could be attributed to increased defense responses. One of the main virulence strategies of *Pst* DC3000 is to utilize a JA-hormone mimic, coronatine, to re-open closed stomata (Brooks et al., 2005; Melotto et al., 2006; Melotto et al., 2008). Additionally, CK has been shown to inhibit abscisic acid mediated stomatal closure to abiotic stress, such as heat stress (Tanaka, 2006). Recently, CK has been linked to being involved in SA-

mediated stomatal immunity in response to flg22 and *Pst* DC3000 (Arnaud et al., 2017). To address if stomatal immunity was impacted in HIS, and if CK was important, we measured stomatal aperture of Col-0 and *ahk2,3* plants exposed to 22°C or 28°C in the presence of flg22 (**Figure 2-8A, B**). Results support that at normal temperature conditions, Col-0 plants exhibit stomatal immunity to flg22 and this process is CK-dependent since stomatal immunity is lost in *ahk2,3* (**Figure 2-8A**). These results support the findings of Arnaud et al., (2017) and reaffirm CK is important for stomatal immunity under normal temperature conditions. However, under high temperature conditions stomatal immunity is lost in Col-0 plants (**Figure 2-8A**). This response is expected because plants exposed to high temperature will exhibit more open stomata as a cooling mechanism (Kostaki et al., 2020). However, inhibition of stomatal immunity at 28°C is CK dependent because under elevated temperature *ahk2,3* plants are able to innate stomatal immunity in response to flg22 (**Figure 2-8A**). Based on infiltration (**Figure 2-1B**) and dip (**Figure 2-8C**) inoculations exhibiting similar HIS disease phenotypes, we conclude that stomatal immunity is not a major contributing factor to loss of HIS in the *ahk2,3* background.

Various studies have shown that defense gene expression is negatively impacted by elevated temperatures (reviewed in Zarattini et al., 2021). Thus, we analyzed qRT-PCR and RNA-Seq data to recognize any patterns in defense gene expression between Col-0 and *ahk2,3* plants. The qRT-PCR data shows that the defense response genes *PR-1* and *WRKY18* are down-regulated in all 28°C exposed plants (**Figure 2-9A**), with the *PR-1* results reflecting Huot et al., (2017) findings. The RNA-Seq data shows down-regulation of representative defense response genes that are in several samples; and an overall trend in which plants exposed to 28°C do not increase expression of these defense response genes (**Figure 2-9B**). Comparing the GO Enrichment Analysis of Biological Processes in Col-0 28°C and *ahk2,3* 28°C treated plants

showed that the top 15 down-regulated processes are mainly associated with defense responses (**Figure 2-9C**). These results support that both Col-0 and *ahk2,3* plants have down-regulated defense responses in response to high temperature conditions, supporting previous studies (Huot et al., 2017; Janda et al., 2019; Leng et al., 2021; Zarattini et al., 2021). Thus, although the CK signaling mutant is less susceptible at 28°C than Col-0 plants, our results indicate that this is not due to increased defense responses, but rather that other processes are inhibiting *Pst* DC3000 proliferation in the *ahk2,3* background under elevated temperature conditions.

CK alters carbon availability under elevated temperature, impacting pathogen proliferation *in-planta*.

CK is also important for nutrient allocation (Roitsch and Ehneß, 2000; Werner et al., 2008), sugar (Kiba et al., 2019) and various abiotic stress responses (Peleg et al., 2011; Reguera et al., 2013) making it an ideal candidate to study HIS. Various studies have shown that over-expression of the CK biosynthesis gene *IPT* will increase sink strength of tissue or increase resilience to certain abiotic stresses, such as drought (Werner et al., 2008; Peleg et al., 2011; Reguera et al., 2013). Additionally, CK has been shown to be important for changes in source-sink relationships in plant-pathogen interactions (Lara et al., 2004) and targeting of CK-mediated processes has been shown to be an important virulence factor for various pathogens (Hann et al., 2014; Chanclud et al., 2016).

Carbon availability is a major factor in plant (Graf et al., 2010; Caldana et al., 2013) and pathogen growth (Zhang et al., 2016; Naseem et al., 2017; Huai et al., 2020), and considering CK's role in nutrient allocation and source-sink relationships it is probable that CK is stimulating a primary metabolic pathway to promote HIS (Roitsch and Ehneß, 2000; Werner et al., 2008).

Source-sink disease assays indicate that changes in carbon availability, based on leaf development stage (i.e., source or sink), impacts HIS, in a CK-dependent manner (**Figure 2-15B**). These results suggest that the late physiological CK-mediated process contributing to HIS is likely associated with sucrose, the major carbon compound exported from source to sink tissues (Lemoine, 2000; Durand et al., 2018). Interestingly, recent studies have shown that an increase in photosynthetically generated sugars, such as sucrose and glucose, will trigger CK biosynthesis (Kushwah and Laxmi, 2014; Kiba et al., 2019). Together our CK quantification (**Figure 2-3A**) and primary metabolomics data (**Figure 2-17B**, **Figure 2-18**), support that Col-0 plants at 28°C exhibit not only an increase in CK content and signaling, but are also rich in carbon availability in the form of starch and sucrose. Whereas, the *ahk2,3* CK signaling deficient plants have significantly less carbon availability in the form of starch (**Figure 2-17A, B**) and sucrose accumulation (**Figure 2-18B**), as well as lower sugar abundance levels overall (**Figure 2-11A**). In comparison to previous work, our metabolomics analysis reflects similar metabolic trends of Col-0 plants under normal temperature conditions, such as slight increase in trehalose and sucrose after infection with *Pst* DC3000 (**Figure 2-15B**) (Ward et al., 2010). Based on these results, we propose a model where CK biosynthesis may be increasing under elevated temperature, in response to increased sucrose content, to promote HIS through a late physiological process that is dependent on primary metabolism.

Studies have shown that *Pst* DC3000 delivery of T3SS effectors is impacted by apoplastic sugar availability (Yamada et al., 2016) and of the three major sugars in plants, fructose is the most important for activation of T3S of effectors (Stauber et al., 2012). However, our results indicate elevated levels of fructose in all genotypes and treatments in relation to 22°C mock inoculated samples (**Figure 2-18A**). Previous studies saw an increase in *Pst* DC3000

effector delivery under elevated temperatures (Huot et al., 2017). Together with an increase in relative fructose levels and effector translocation, this may indicate that T3S effectors are contributing to CK-mediated HIS, rather than just a failure of the host in supporting the pathogen metabolically. *Pst* DC3000 has been shown to utilize a T3S effector HopQ1, which acts a CK biosynthesis enzyme, to synthesize CKs as a virulence mechanism (Hann et al., 2014). Other studies have shown that pathogens, such as *Magnaporthe oryzae*, will produce CKs around the infection site leading to an to increase sugar and amino acid content necessary for infection (Chanclud et al., 2016). Thus, it's probable that T3S effectors, such as HopQ1, may be important for CK-mediated HIS.

Additionally, plant defense processes are energy intensive and have been linked to primary metabolic processes (Bolton, 2009; Rojas et al., 2014). Studies have shown that plants may run into an energy imbalance when attempting to activate defense and maintain growth (Scheideler et al., 2002), this is likely exacerbated by high temperature stress as well. Thus, the lower nutrient availability of *ahk2,3* plants at 28ºC may be contributing to loss of HIS in one of two, non-mutually exclusive ways: (1) The lack of apoplastic sugars available in *ahk2,3* are leading to decreased virulence of *Pst* DC3000 or (2) the lack of nutrient availability is due to *ahk2,3* response to elevated temperature and response to pathogen attack (i.e., loss of nutrients overtime to energy intensive processes). Overall, *ahk2,3* plants have lower carbon availability compared to Col-0 plants, which may be contributing to decreased susceptibility of *ahk2,3* plants to *Pst* DC3000 at 28ºC. Indicating that primary metabolic processes are contributing to HIS of Col-0 to *Pst* DC3000, in a CK-dependent manner. These results suggest that at 28°C CK signaling promotes starch and sucrose biosynthesis in wild-type plants, therefore plants lacking CK signaling would be poor hosts for *Pst* DC3000 under high temperature conditions.

As a consequence of climate change, global temperatures will continue to rise. This increase in environmental temperatures will put agricultural crop systems in an increasingly vulnerable state against pathogen attack (Velásquez et al., 2018). In this study, we used a variety of physiological and genetic approaches to characterize what factors are contributing to HIS of Col-0 to the bacterial pathogen *Pst* DC3000 in a CK-dependent manner. Our results support previous studies that carbon availability and nutrient allocation play a fundamental role in bacterial proliferation (Stauber et al., 2012; Naseem et al., 2017) and host susceptibility (van Schie and Takken, 2014a). More importantly, these changes in nutrient allocation are impacted by high temperature and driven by CK signaling, suggesting this sugar accumulation may be the mechanism driving CK-mediated HIS.

Analyzing differences in *ahk2,3* and Col-0 susceptibility under heat stress could lead to potential "loss-of-susceptibility" genes that could alleviate the pressure of increased temperatures on plant-pathogen interactions. For example, based on our results, genes associated with starch (**Table 2-4**: *BAM*, *PGMP*) and trehalose (**Table 2-4**: *TPS*, *TPP*) biosynthesis are down-regulated under high temperature conditions in *ahk2,3* plants. Thus, targeting these genes through gene editing may reveal that inhibiting their function will lead to decreased host susceptibility under conditions of increased ambient temperatures. The targeting of primary metabolic processes by pathogens is not a new discovery (Huai et al., 2019; Huai et al., 2020; Lacrampe et al., 2021; Luo et al., 2021) and various studies have shown that targeting these susceptibility genes through a genetic approach, to achieve loss-of-susceptibility, is a durable approach to achieve broad-spectrum resistance to pathogens (Pavan et al., 2010; van Schie and Takken, 2014b; Makinen, 2020; Thomazella et al., 2021).

The proposed susceptibility genes in **Table 2-4** are associated with important primary metabolic processes needed for optimal plant growth, and their use in a loss-of-susceptibility approach may lead to costs in plat yield. Therefore engineering of these genes for expression under a tissue-specific (Li et al., 2012) or inducible promoters (Leng et al., 2021) could allow for only brief activation for loss-of-susceptibility, to avoid a fitness costs. Recent studies have successfully utilized a heat-inducible promoter to activate SA-mediated defense genes *EDS16* and *PAD4* while overcoming the growth-defense tradeoff associated with constitutive defense (Leng et al., 2021). Overall, our results highlight the value of CK-based genetic approaches to improve crop protection under increased temperatures and provides the basis for future directions for plant engineering in a world with climate change.

2-6 Figures:

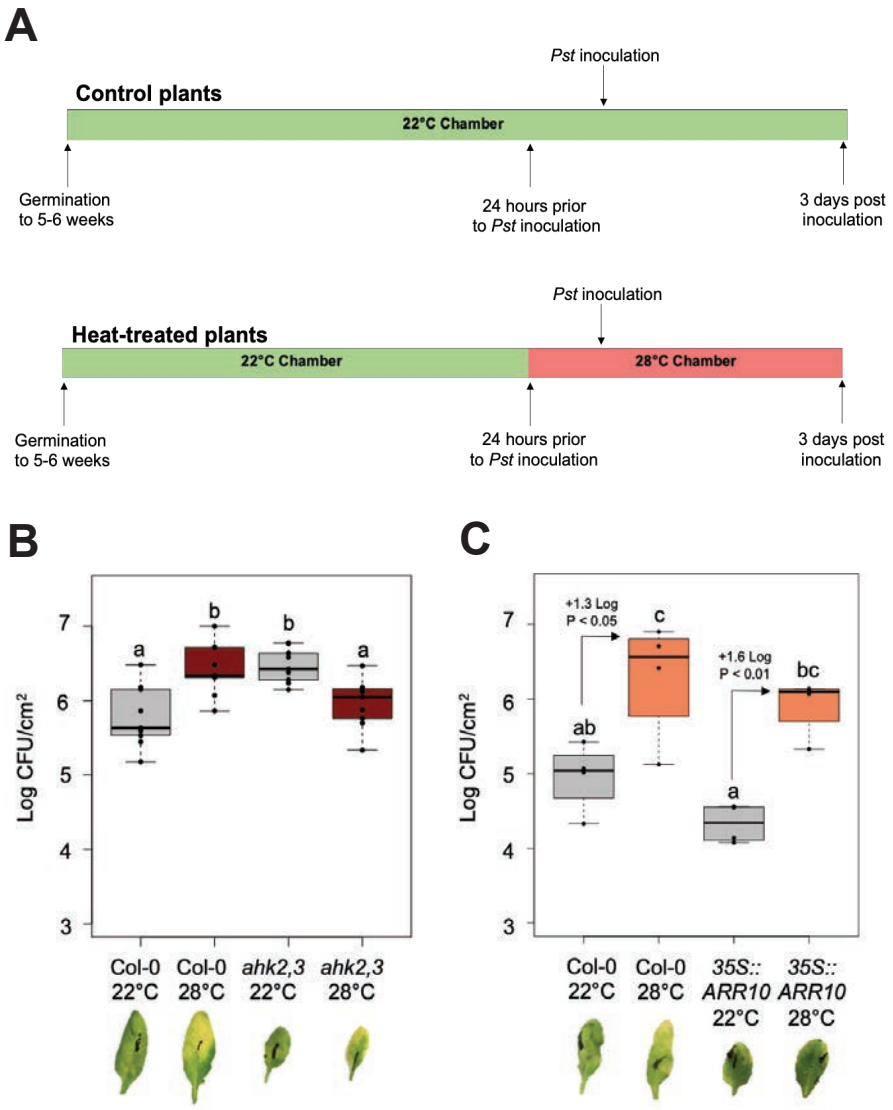


Figure 2-1: CK-mediated processes promote HIS of Arabidopsis to *Pst* DC3000.
A) Experimental design schematic for HIS disease assays. Plants are grown under normal temperature conditions until approximately 5 to 6 weeks. 24 hours prior to inoculation with *Pst* DC3000, control plants remained at 22ºC and heat-treated plants were moved to a 28ºC growth chamber. Control plants remained at 22ºC for the remainder of the experiment, 3 days post inoculation, and heat-treated plants remained at 28ºC. **B)** Bacterial growth in heat-treated Col-0 and *ahk2,3* (CK signaling mutant) plants three days post infiltration inoculation with *Pst* DC3000 at 1 x10^5 CFU/mL. n=8 pooled experiments, with 4 technical replicates per genotype and treatment. Error bars represent standard error. Samples with different letters indicate significant differences in host susceptibility as determined by a Two-Way ANOVA, Tukey HSD post-hoc analysis (p-value ≤ 0.05). **C)** Bacterial growth in heat-stressed Col-0 and *35S::ARR10* (CK-hypersensitive line signaling mutant) plants three days post infiltration inoculation with *Pst* DC3000 at 1 x10^5 CFU/mL. Data presented is a representative, with 4 technical replicates per genotype and treatment. Experiment conducted at least 3 times with similar results. Error bars represent standard error. Samples with different letters indicate significant differences in host

susceptibility as determined by a Two-Way ANOVA, Tukey HSD post-hoc analysis (p-value ≤ 0.05). Arrows indicate genotypes that exhibit increase in host susceptibility between 22°C and 28°C, with the increase in Log CFU/cm^2 noted and significant difference determined by a Two-Way ANOVA, Tukey HSD post-hoc analysis (p-value ≤ 0.05).

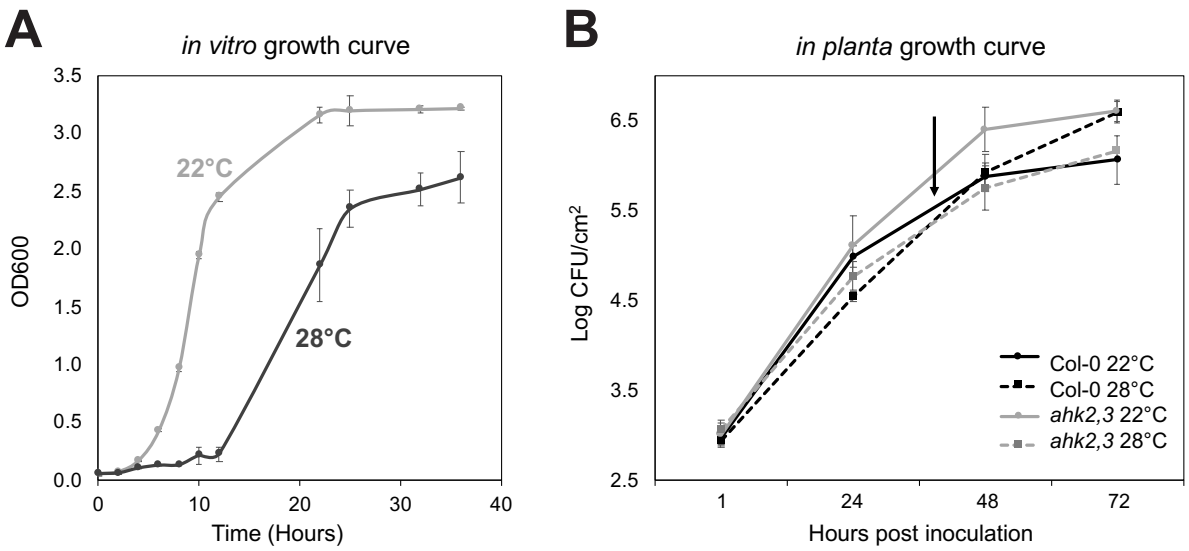


Figure 2-2: Temperature effects of *Pst* DC3000 proliferation overtime in liquid media and in *Arabidopsis thaliana* plants. A) Growth curve of *Pst* DC3000 in liquid media at 22°C and 28°C, 225 rpm for a 36-hour period. Error bars represent standard error, n=3 per timepoint. **B)** Growth curve of *Pst* DC3000 in Col-0 and *ahk2,3* (CK signaling mutant) over a three-day period post infiltration inoculation (1 x10^5 CFU/mL), n=4 plants per genotype and treatment. The arrow indicates the time-point, ~40 hours post inoculation, where *Pst* DC3000 proliferation continues in Col-0 plants at 28°C and begins to slow in *ahk2,3* plants at 28°C. A Two-Way ANOVA, Tukey HSD post-hoc analysis (p-value ≤ 0.05) was used to determine significant differences between samples, only *ahk2,3* 22°C is significantly different compared to Col-0 22°C at all time points. Each growth curve experiment was repeated at least three times with similar results. Error bars represent standard error.

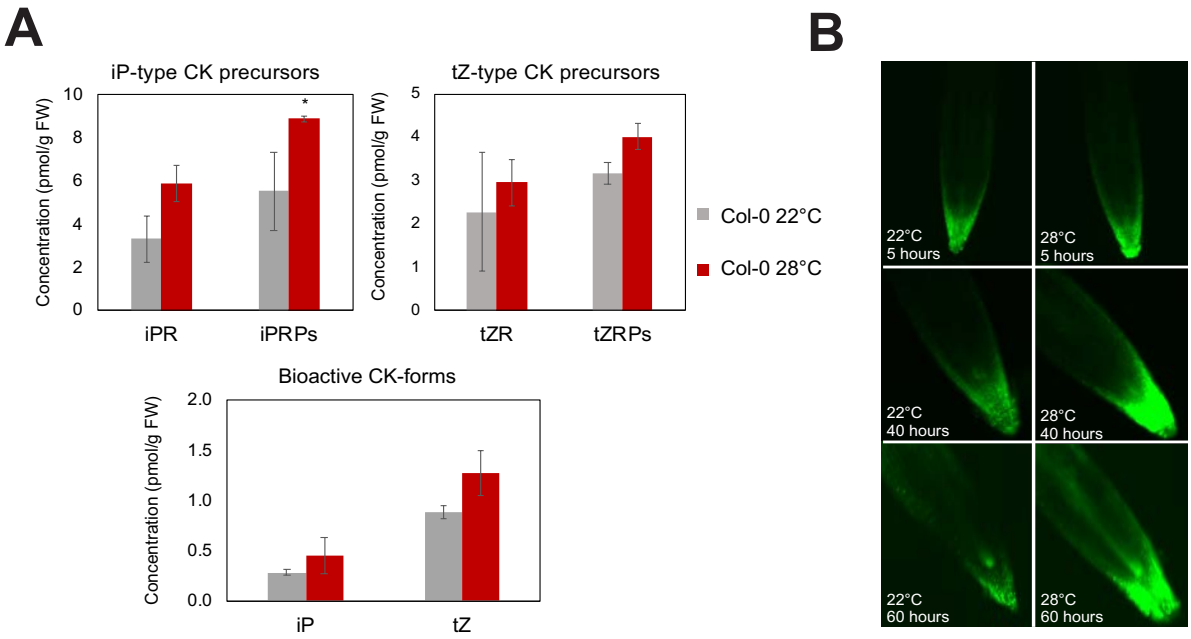


Figure 2-3: CK-biosynthesis and signaling increases in response to high temperature in Col-0. A. Changes in CK levels in Col-0 plants at 22°C and 28°C. iP-type precursor levels (top left), tZ-type precursor levels (top right), and bioactive CK forms iP and tZ levels shown as pmol/g FW. Six-week-old plants were grown at 22°C, then were exposed to 28°C for 48 hours prior to tissue collection (n=4). Samples with asterisks indicate significant difference from Col-0 22°C based on Student's t-test (p-value ≤ 0.05). Error bars represent standard error (n=4) for two experimental replicates. The concentrations of all measured CK metabolites are outlined in Table 2-2. **B.** A synthetic CK signaling reporter lines, *pTCSn::GFP*, shows where CK signaling is increased (n=6). Vertical root plates were exposed to heat stress and imaged following early (5 hours, top) and late (40 and 60 hours, middle and bottom) timepoints. Fluorescent microscopy experiments were repeated at least two times with similar results.

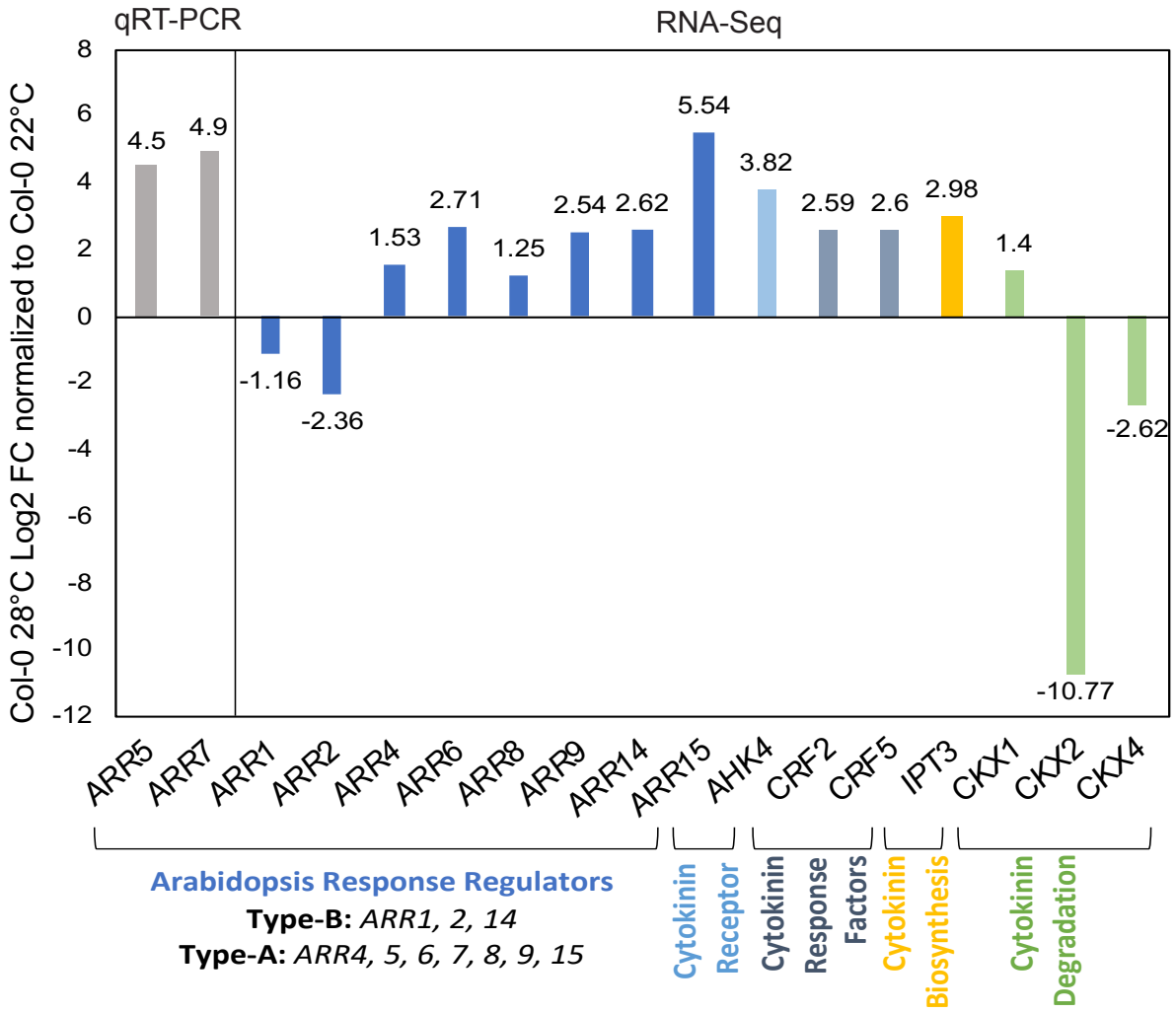


Figure 2-4: Expression of CK signaling components increases under high temperature conditions in Col-0 plants. CK signaling components increase in response to high temperature conditions, 28°C, shown by qRT-PCR and RNA-Seq Expression Analyses. Tissue for qRT-PCR gene expression analyses and RNA-Seq transcriptomics were collected at 40 hours post stress. Gene expression experiments were conducted at least two times with similar results. Col-0 28°C $MgCl_2$ qRT-PCR and RNA-Seq results are normalized to Col-0 22°C $MgCl_2$.

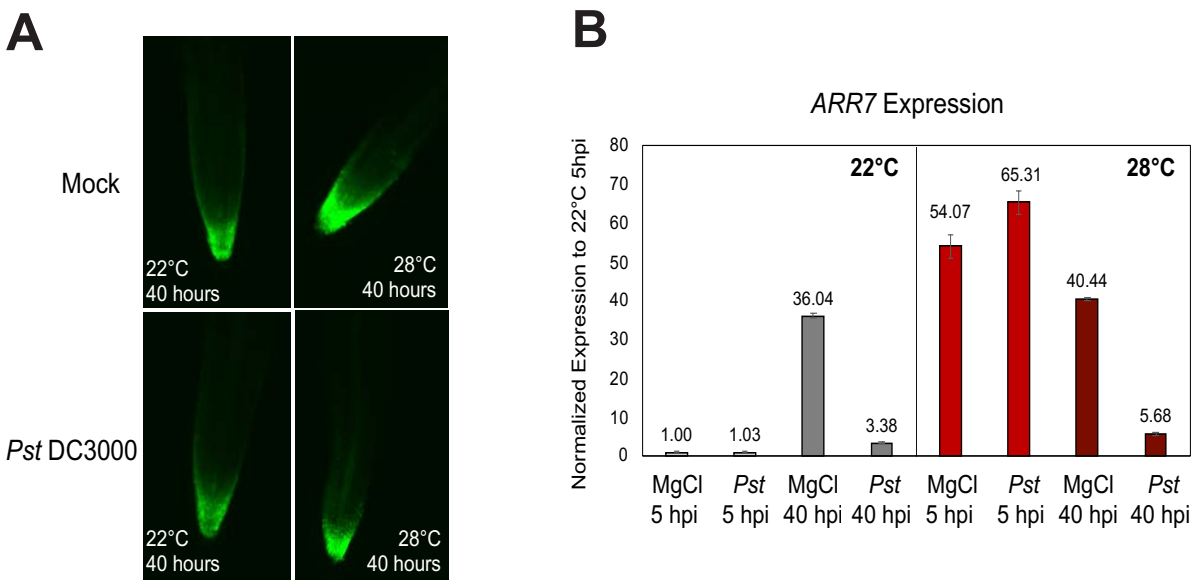


Figure 2-5: CK-signaling does not increase in the presence of *Pst* DC3000 at 22°C or 28°C. **A.** A synthetic CK-signaling reporter lines, *pTCSn::GFP*, shows where CK signaling is increased after 40 hours exposure to each temperature. Seedlings were grown on vertical root plates at 22°C. High temperature plates were exposed to heat stress 24 hours prior to flood inoculation with *Pst* DC3000 (OD_{600} = 0.1). Following inoculation plates were placed back at appropriate chamber until 40 hpi. Under high temperature conditions, 28°C, the signal is stronger than at 22°C and with *Pst* DC3000 inoculation. The experiment was repeated at least two times with similar results. **B.** Gene expression of CK signaling gene *ARR7* in response to temperature and *Pst* DC3000 stress. Left panel are plants exposed to 22ºC and right panel are plants exposed to 28ºC. Tissue was collected at either 5 hpi or 40 hpi of Col-0 plants. Expression levels are normalized to 22ºC $MgCl_2$ 5 hpi and standard error bars represent standard error (n=3), experiment was repeated at least two times with similar results.

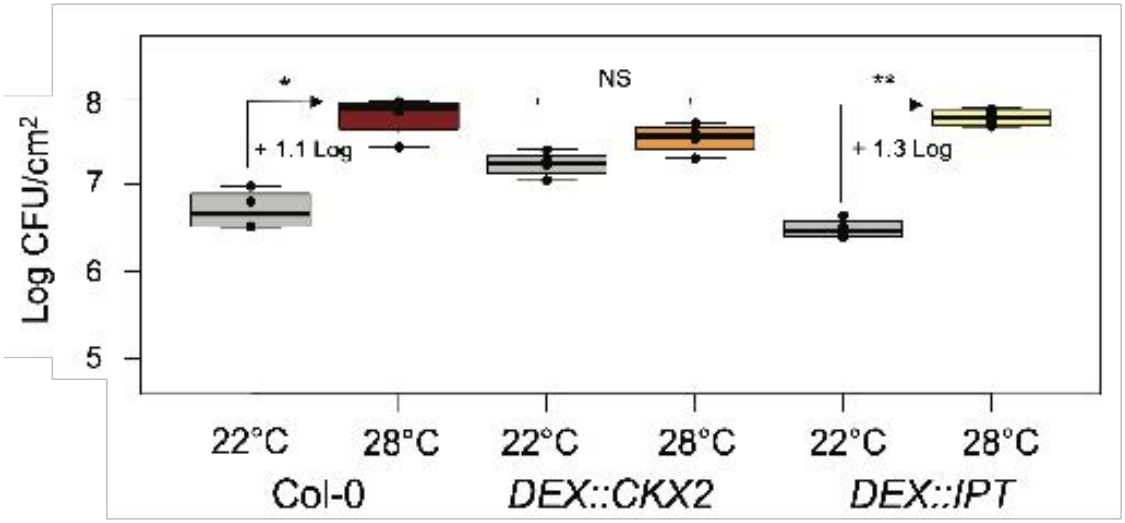


Figure 2-6: CK-regulated processes are the basis for HIS, CK content is both necessary and sufficient for HIS to occur. Bacterial growth in heat-stressed Col-0, *DEX::CKX2*, and *DEX::IPT* plants three days post infiltration inoculation with *Pst* DC3000 at 1 x10^5 CFU/ml. Error bars represent standard error (n=4). Plants were treated with DEX prior to being moved to 28°C (i.e., 24 hours prior to inoculation). Arrows indicate genotypes that exhibit increase in host susceptibility between 22°C and 28°C, with the increase in Log CFU/cm^2 noted. Samples with asterisks indicate significant differences in host susceptibility as determined by a Two-Way ANOVA, Tukey HSD post-hoc analysis (p-value ≤ 0.05), NS indicates no significant difference. Data presented is a representative, experiment conducted at least 3 times with similar results.

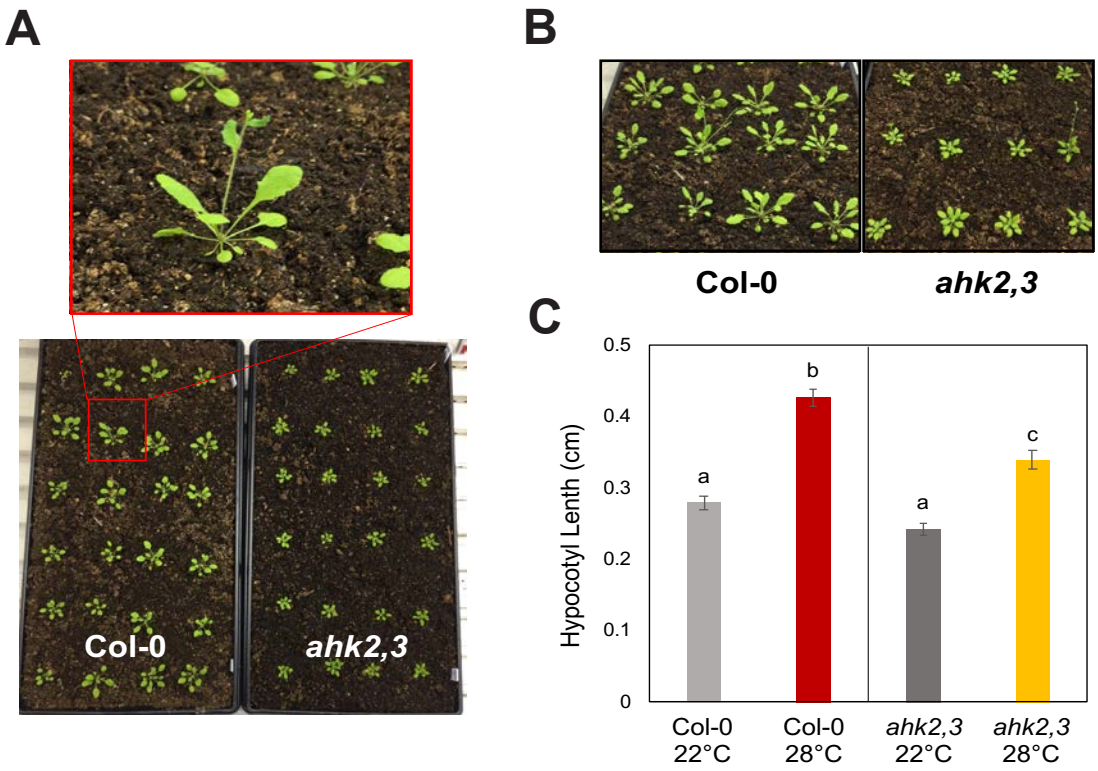


Figure 2-7: CK-regulated physiological processes are activated under elevated temperature. A. Comparison of Col-0 and *ahk2,3* growth phenotypes 4 weeks post germination under high temperature conditions. Col-0 and *ahk2,3* plants soil grown at 28°C exhibit accelerated growth phenotypes, with Col-0 bolting and flowering at 4 weeks (red square). **B.** Comparison of Col-0 and *ahk2,3* growth phenotypes 5 weeks post germination under high temperature conditions. *ahk2,3* plants exhibit early bolting and flowering at 28ºC, but are delayed compared to Col-0 plants. **C.** Hypocotyl length of Col-0 and *ahk2,3* plants after exposure to 22°C and 28°C. Seedlings were grown on horizontal MS plates and hypocotyl measurements were taken 5 days post germination. Experiment was repeated at least two times with similar results. Error bars represent standard error (n=25). Samples with different letters indicate significant differences in host susceptibility as determined by a Two-Way ANOVA, Tukey HSD post-hoc analysis (p-value ≤ 0.05).

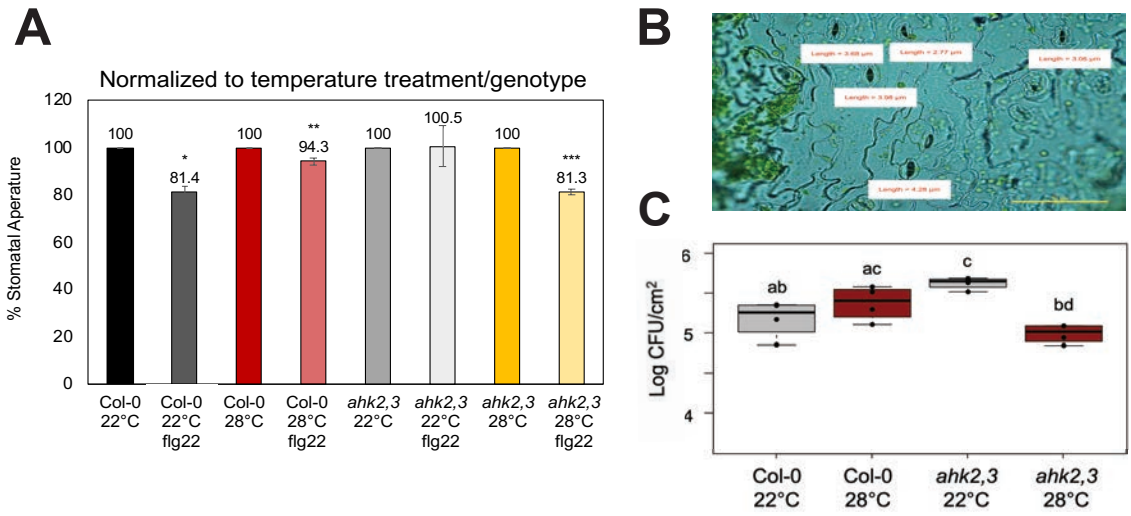


Figure 2-8: Decreased susceptibility of *ahk2,3* plants at 28°C is not attributed to increased defense responses. A. The role of stomata in CK-mediated heat induced susceptibility. Stomatal aperture in epidermal peels of Col-0 and *ahk2,3* plants exposed to water (darker color bars) or flg22 (lighter color bars). Results are shown as mean (n = 60 stomata, at least) normalized to temperature control. Samples with asterisks note significant different compared to normalized control based on Two-Way ANOVA (p-value ≤ 0.05). Experiment was repeated at least three times with similar results. **B.** Image representing how stomatal aperture was measured to assess the impact of temperature and CK signaling on the process. **C.** Dip inoculation of Col-0 and *ahk2,3* plants exhibit loss of HIS in *ahk2,3* but HIS of Col-0 is not as prominent. Error bars represent standard error (n=4), data shown is a representative but at least three experiments were done with similar results. Samples with different letters indicate significant differences in host susceptibility as determined by a Two-Way ANOVA, Tukey HSD post-hoc analysis (p-value ≤ 0.05).

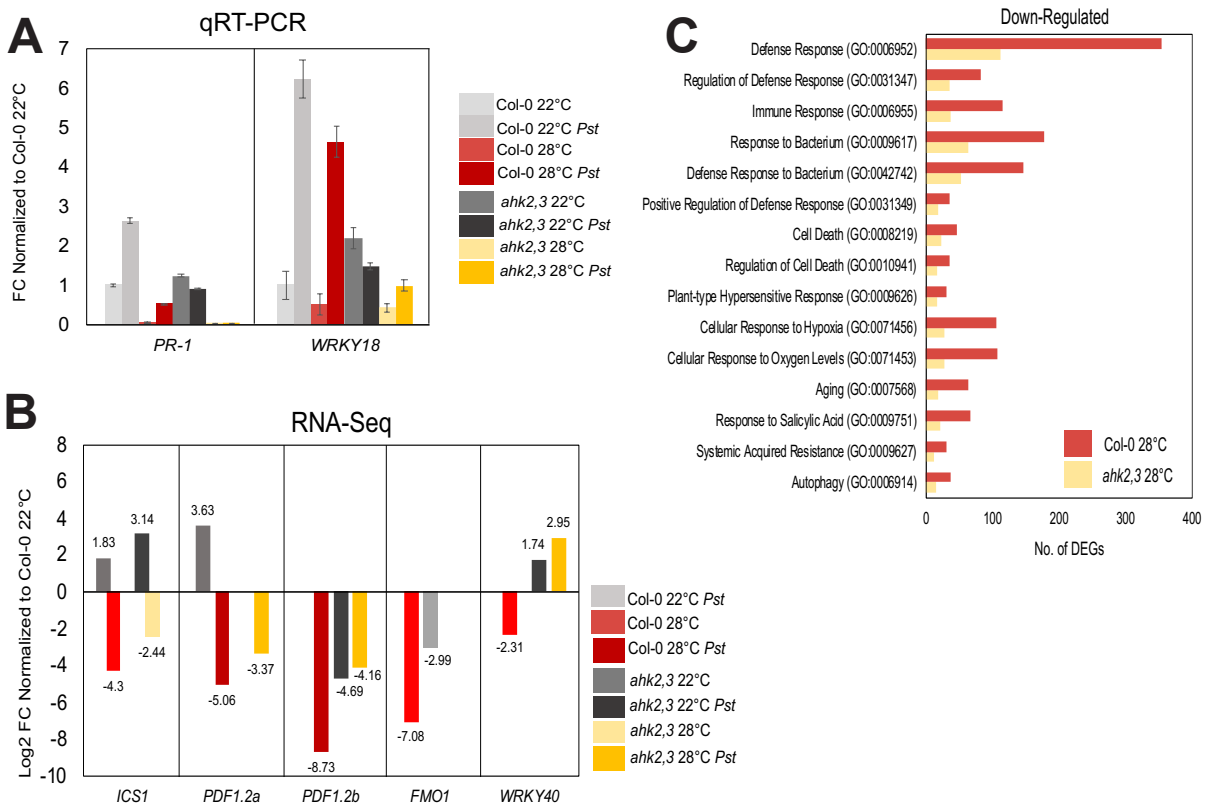


Figure 2-9: Col-0 and *ahk2,3* exhibit a decrease in defense response related genes at 28ºC. **A.** Defense response gene expression is impaired under heat-stress as determined by qRT-PCR. Col-0 and *ahk2,3* plants were treated with a mock solution ($MgCl_2$) or *Pst* DC3000 at 22°C or 28°C, tissue was collected at 40 hpi. The error bars represent standard error (n=4). Gene expression analyses by qRT-PCR was repeated at least three times with similar results. **B.** Defense response gene expression is impaired under heat-stress as determined by RNA-Seq Analysis. **C.** Heat stress alone leads to down-regulation of various defense response pathways as determined by RNA-Seq Analysis. Gene-Ontology (GO from TAIR) results based on the grouping of genes belonging to similar GO: Biological Processes. All genes in each GO: Biological Process were differentially expressed based on log fold change (±1.5 threshold) and FDR p-values (≤ 0.05) of two biological replicates. Colors indicate the represented GO: Biological Processes seen in the top right.

A

Group:	Comparison:	Genes Differentially Regulated by:
1	Col-0 22°C $MgCl_2$ **vs.** Col-0 28°C $MgCl_2$	High temperature
2	Col-0 22°C $MgCl_2$ **vs.** Col-0 22°C *Pst*	*Pst* DC3000
3	Col-0 22°C $MgCl_2$ **vs.** Col-0 28°C *Pst*	*HIS*
4	Col-0 22°C $MgCl_2$ **vs.** *ahk2,3* 22°C $MgCl_2$	Cytokinin signaling genes
5	Col-0 22°C $MgCl_2$ **vs.** *ahk2,3* 22°C *Pst*	*Pst* DC3000, cytokinin-independent
6	Col-0 22°C $MgCl_2$ **vs.** *ahk2,3* 28°C $MgCl_2$	High temperature, cytokinin
7	Col-0 22°C $MgCl_2$ **vs.** *ahk2,3* 28°C *Pst*	HIS, cytokinin-independent

Shaded boxes indicate *ahk2,3* RNA-Seq comparisons

B

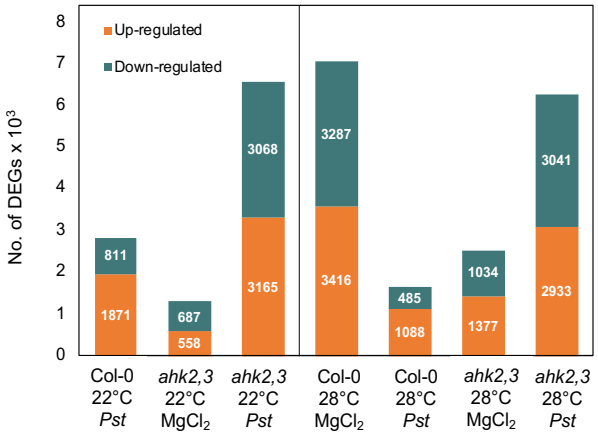


Figure 2-10: Experimental comparisons and number of total differentially expressed genes for RNA-Seq samples. A. Experimental design described in Figure2-1A was utilized for the RNA-Seq experiment. This table outlines the various comparisons and what processes are regulating those genes of interest. There are eight main comparisons, the ones shaded in yellow indicate *ahk2,3* comparisons. Comparison groups number 3 and 7 are the most important as these are associated with HIS. **B.** The number of differentially expressed genes up-regulated (orange) and down-regulated (teal) in each sample. All samples were compared to Col-0 22ºC $MgCl_2$.

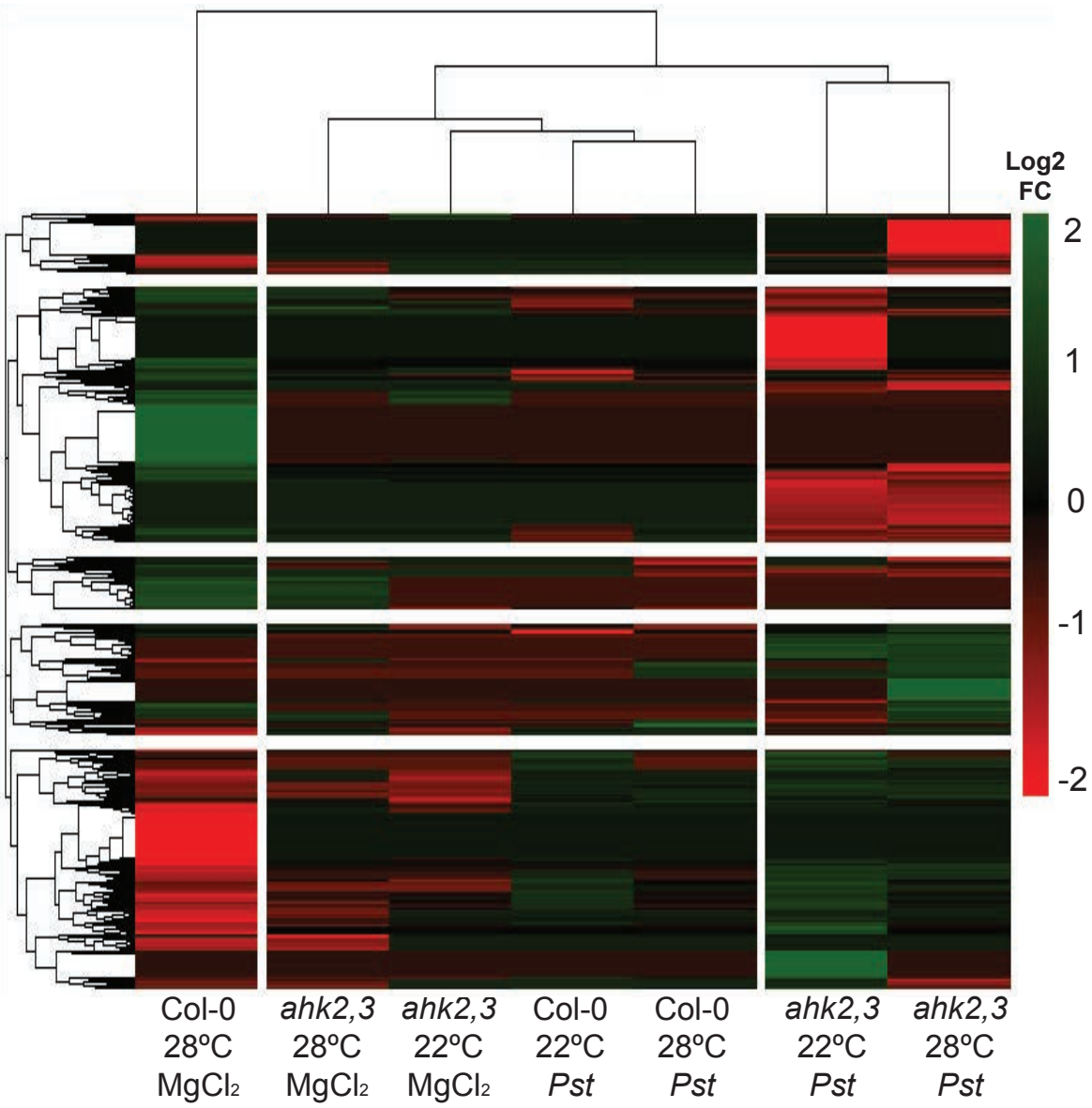


Figure 2-11: Hierarchical clustering of differentially expression in Col-0 and *ahk2,3* under temperature and pathogen stress. Heatmap was constructed using normalized Log2 fold change (FC) values to Col-0 22ºC $MgCl_2$ for significant differentially expressed genes (FDR p-value <0.05; Log2 FC of at least 1.5 for upregulated; Log2 FC of at least -1.5 for down-regulated). High/upregulated FC is represented by green, low/down-regulated FC is represented by red. Heatmap was created with R-package pheatmap.

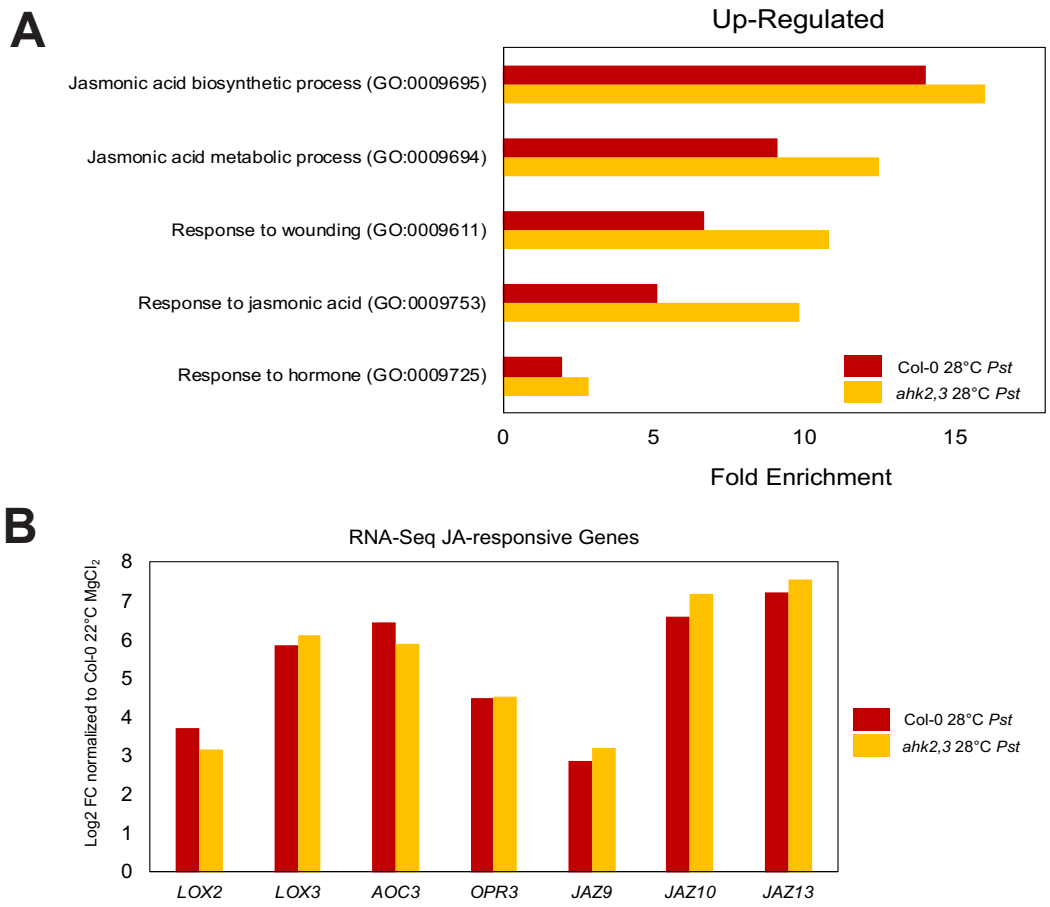
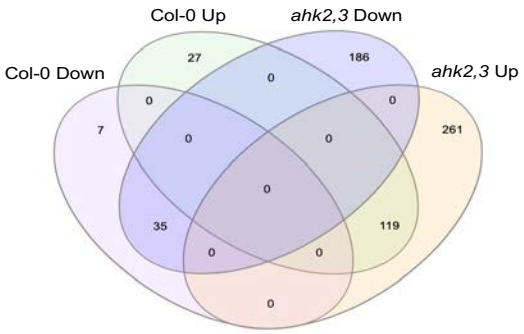


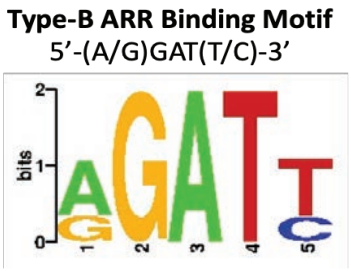
Figure 2-12: Increase in JA-mediated responses is not contributing to increase in host susceptibility. A. RNA-Seq results indicate the main GO Biological processes that are up-regulated in Col-0 28ºC *Pst* and also upregulated in *ahk2,3* 28ºC *Pst* and are associated with JA responses. Stacked bar chart represents fold enrichment of each GO Biological Process Term (observed vs expected). GO Enrichment analysis was done through TAIR, only differentially expressed genes were used for analysis. **B.** Representative JA-mediated genes up-regulated in Col-0 28ºC *Pst* and *ahk2,3* 28ºC *Pst* samples to support the GO Enrichment analysis.

A



DEGs that overlap with conserved type-B ARR candidate genes
Col-0 & *ahk2,3* 28°C *Pst* samples

B



C

Gene ID	Gene Name	Description	Log_2 FC	# of AGAT Binding Motifs
AT3G56410	AT3G56410	hypothetical protein (DUF3133)	1.22	9
AT5G13190	GILP	GSH-induced LITAF domain protein	1.24	5
AT3G61630	CRF6	cytokinin response factor 6	1.4	7
AT3G29575	AFP3	ABI five binding protein 3	1.47	4
AT5G65920	AT5G65920	ARM repeat superfamily protein	1.47	6
AT2G35940	BLH1	BEL1-like homeodomain 1	1.56	5
AT3G19030	AT3G19030	transcription initiation factor TFIID subunit 1b-like protein	1.63	5
AT4G16860	RPP4	Disease resistance protein (TIR-NBS-LRR class) family	1.68	7
AT5G59220	HAI1	PP2C protein (Clade A protein phosphatases type 2C)	1.72	8
AT1G21100	IGMT1	O-methyltransferase family protein	1.89	8
AT5G04310	AT5G04310	Pectin lyase-like superfamily protein	2.02	8
AT2G34070	TBL37	TRICHOME BIREFRINGENCE-LIKE 37	2.06	5
AT1G77760	NIA1	nitrate reductase 1	2.15	0
AT3G07340	AT3G07340	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	2.32	4
AT1G21110	IGMT3	O-methyltransferase family protein	2.48	3
AT1G14780	AT1G14780	MAC/Perforin domain-containing protein	2.54	3
AT5G36925	AT5G36925	hypothetical protein	2.57	5
AT4G21390	B120	S-locus lectin protein kinase family protein	2.75	6
AT5G13700	PAO1	polyamine oxidase 1	2.97	4
AT2G37430	ZAT11	C2H2 and C2HC zinc fingers superfamily protein	3	5
AT1G74930	ORA47	Integrase-type DNA-binding superfamily protein	3.35	3
AT1G70140	FH8	formin 8	3.51	1
AT4G39980	DHS1	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 1	3.6	6
AT5G37490	AT5G37490	ARM repeat superfamily protein	4.55	4
AT2G32510	MAPKKK17	mitogen-activated protein kinase kinase kinase 17	4.74	8
AT3G23250	MYB15	myb domain protein 15	5.01	3
AT4G17490	ERF6	ethylene responsive element binding factor 6	5.35	4
AT4G39403	PLS	polari	-4.69	1
AT5G25240	AT5G25240	stress induced protein	-3.85	2
AT5G19190	AT5G19190	hypothetical protein	-3.44	7
AT5G28840	GME	GDP-D-mannose 3',5'-epimerase	-1.77	4
AT3G61150	HDG1	homeodomain GLABROUS 1	-1.75	4
AT1G08880	H2AXA	Histone superfamily protein	-1.48	6
AT2G39450	MTP11	Cation efflux family protein	-1.32	3

Figure 2-13: Narrowing genes associated with HIS of Col-0 to *Pst* DC3000 based on Type-B ARR binding motifs. A. RNA-Seq differentially expressed genes of Col-0 28ºC *Pst* and *ahk2,3* 28ºC *Pst* samples, that overlapped with Type-B ARR candidates. Genes were selected based on overlap based on targets identified in (Zubo et al., 2017). **B.** The Type-B ARR binding motif was used for the promoter analysis. **C.** List of genes from Col-0 28ºC *Pst* that are Type-B ARR candidates and the number of Type-B ARR binding sites 1000bp above the promoter region. These genes were identified as potential CK-induced genes that are important for HIS.

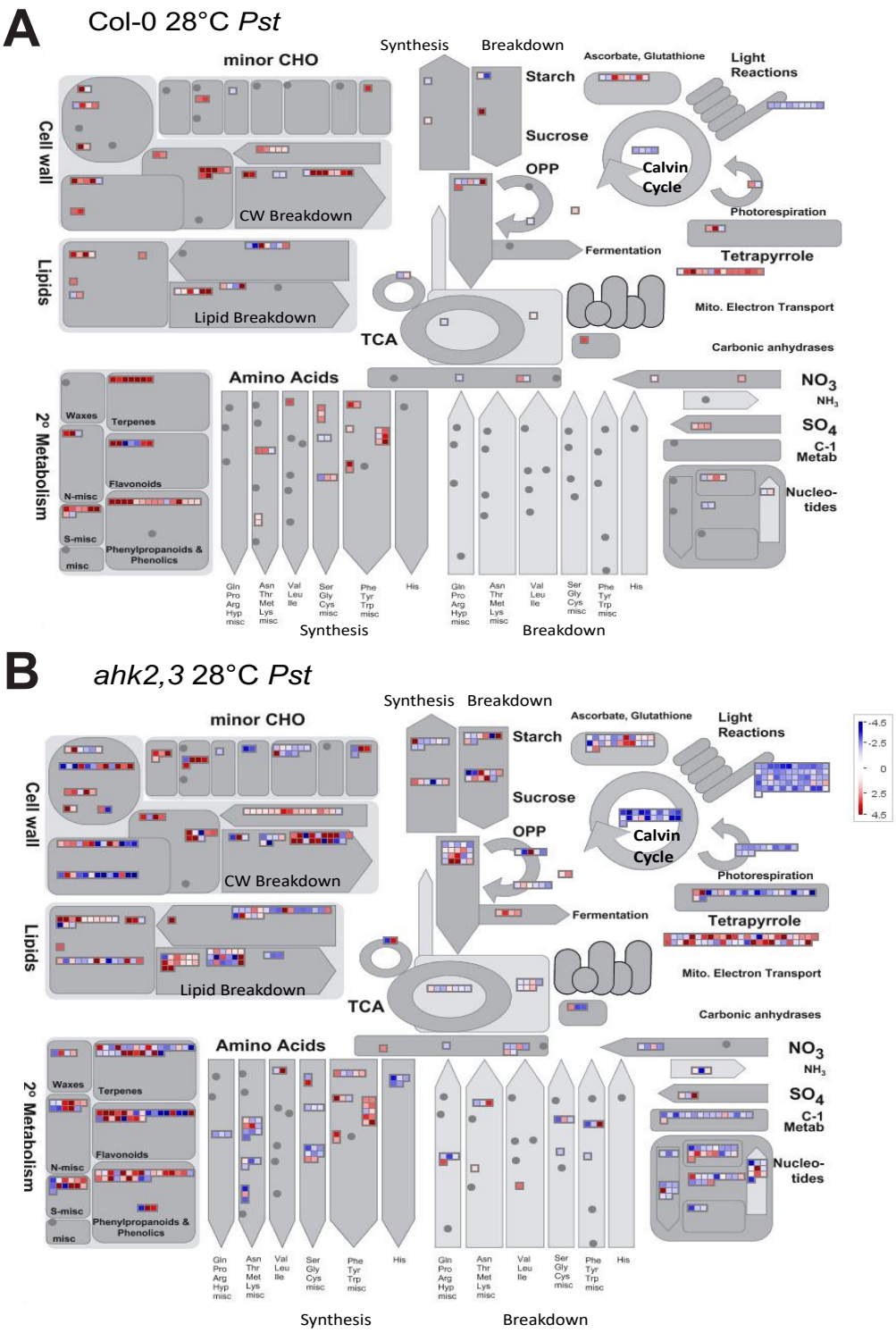


Figure 2-14: Metabolism changes in Col-0 28°C *Pst* and *ahk2,3* 28°C *Pst*. MAPMAN was used to observe metabolic changes in heat stressed and *Pst* DC3000 inoculated Col-0 (**A**) and *ahk2,3* (**B**) plants at approximately 40 hpi. The average fold change of two biological replicates is presented as illustrated in the fold change colors in the top right of each image (blue, repressed; red, induced).

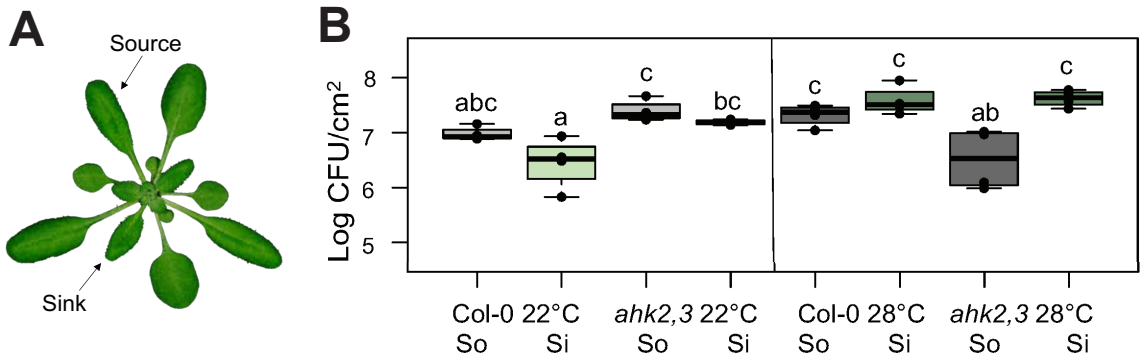


Figure 2-15: Developmental source and sink leaf type impacts HIS in a CK signaling dependent manner. A. Figure representation of a five-week-old Arabidopsis plant with the source (So) and sink (Si) leaf types labeled. For this experiment, there were four technical replicates for each leaf type per genotype and temperature treatment. Four leaves of each leaf type were also used for inoculation. These leaf types were based on previous studies identifying So and Si leaves of fully developed Arabidopsis plants (Kölling et al., 2013; Kölling et al., 2015). Arabidopsis photo from Flickr. **B.** Leaf type changes host susceptibility to *Pst* DC3000 and this is dependent on CK. Plants were inoculated with *Pst* DC3000 and tissue was collected after a three-day period post infiltration inoculation (1 x10^5 CFU/mL), data shown is a representative experiment. Experiment was repeated at least three times with similar results. Error bars represent standard error (n=4). The line in panel B is separating the 22ºC exposed plants and the 28ºC exposed plants.

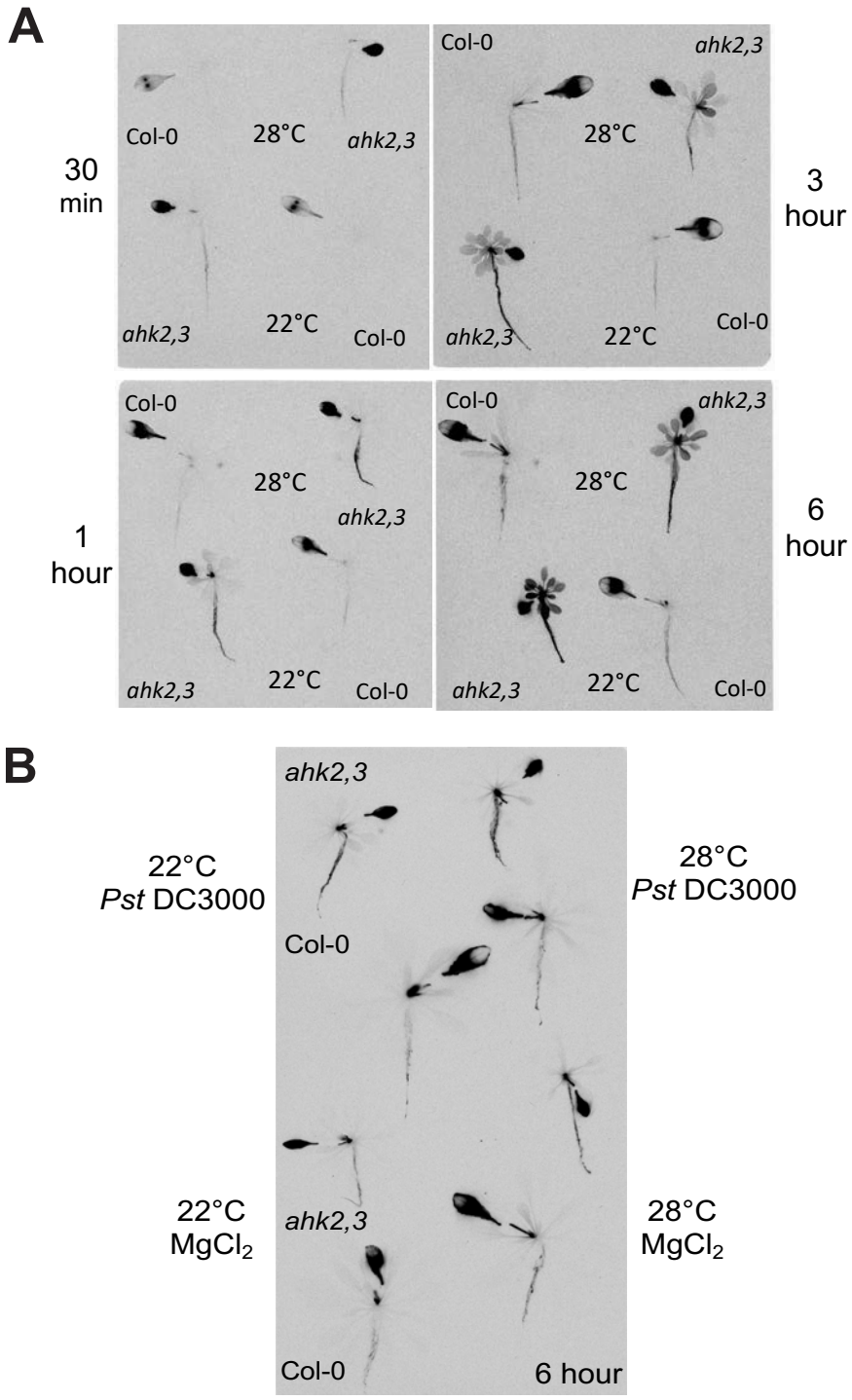


Figure 2-16: Sucrose movement through plants varies based on temperature and CK-signaling, but shows no difference in response to *Pst* DC3000 inoculation. A. C-14 sucrose translocation 30 minutes, 1 hour, 3 hours, and 6 hours post application. *ahk2,3* plants at 22ºC remobilize C-14 sucrose faster than Col-0 plants. Heat stress inhibits/decreases mobilization in *ahk2,3* plants, but shows a slight increase in Col-0 plants. **B.** A source leaf was infiltrated with either $MgCl_2$ or *Pst* DC3000. A source leaf connected by parastichies was then labeled with C-14 sucrose and images were taken at 6 hours post application. Sucrose travels similarly throughout

Col-0 and *ahk2,3* plants regardless of temperature or pathogen treatment. Each phosphorimage shows a representative plant for each genotype and treatment. C-14 sucrose was applied at the beginning of the light cycle and tissue was collected at each timepoint mentioned after C-14 sucrose application.

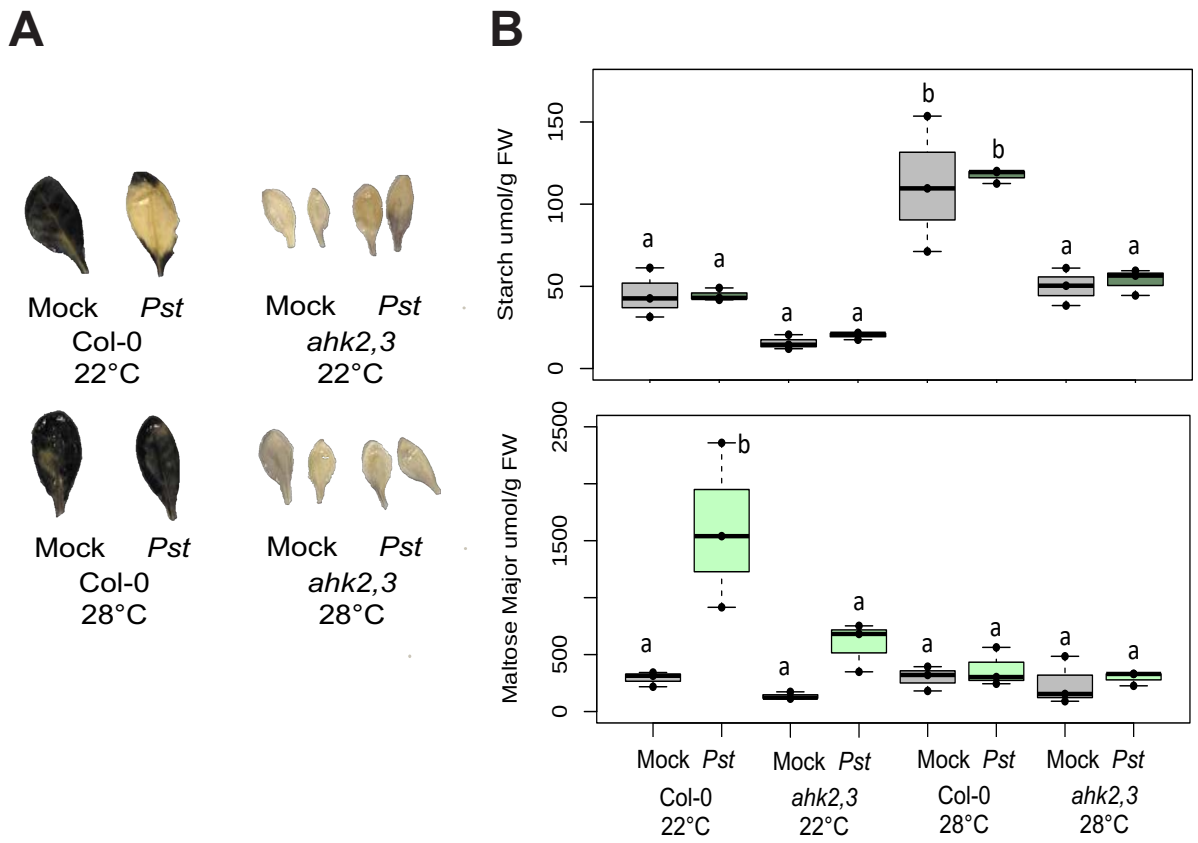


Figure 2-17: *ahk2,3* plants have lower carbon availability for *Pst* DC3000, compared to Col-0 plants. A. Lugol iodine staining of Col-0 and *ahk2,3* leaves infiltrated with a mock solution (10mM $MgCl_2$) or *Pst* DC3000 at 40 hpi. Plant tissue for lugol iodine staining was collected at the end of the light period. Black coloration indicates starch staining, yellow indicates lack of starch staining. **B.** Absolute quantification of starch (**top**) and maltose major (**bottom**). Starch and maltose major levels shown as µmol/g FW. Plant tissue for starch and maltose quantification was collected at the beginning of the light period. Error bars represent standard error (n=3). Samples with different letters indicate significant differences in host susceptibility as determined by a Two-Way ANOVA, Tukey HSD post-hoc analysis (p-value ≤ 0.05).

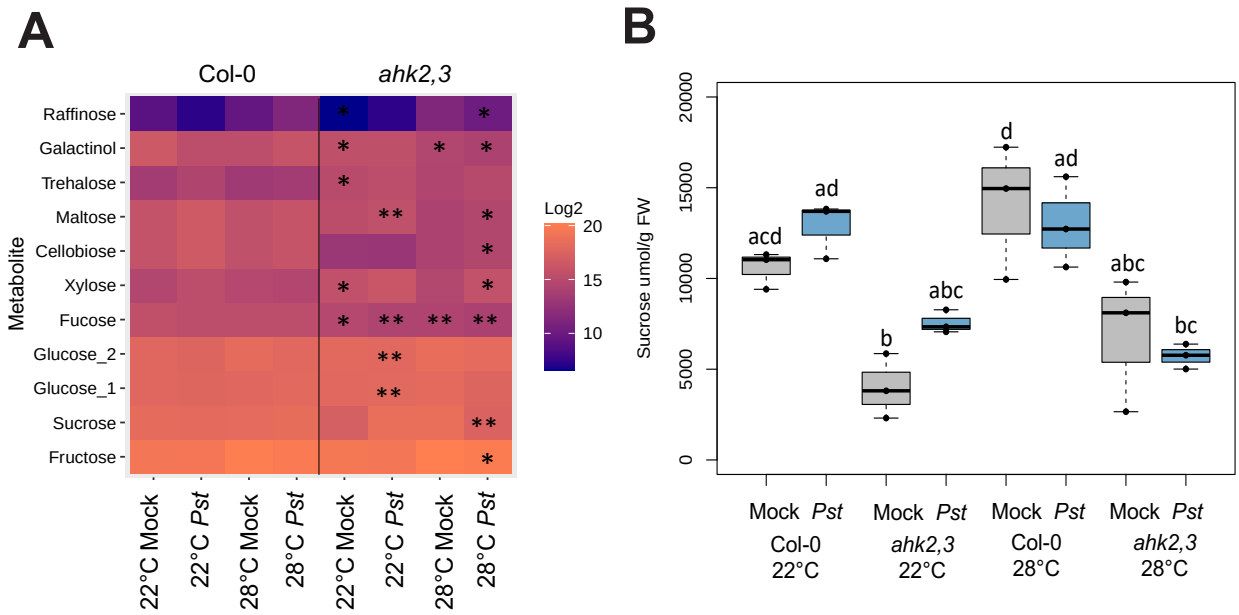


Figure 2-18: *ahk2,3* plants have lower sugar levels, especially under heat stress and combined heat-pathogen stress when compared to Col-0 plants. A. Heatmap of relative abundance levels of all sugars measured show a separation in major and minor sugars based on heat, genotype, and pathogen treatment. Data represents the average of biological replicates (n=2, with 3 plants per genotype and treatment), normalized log2 transformed values of relative abundance. Asterisks denote significant differences between metabolites in *ahk2,3* plants from the appropriate control, Col-0 22ºC Mock and Col-0 28ºC Mock, as determined by Student's t-test: *p<0.05, **p<0.01. **B.** Absolute quantification of sucrose. Sucrose levels shown as µmol/g FW. Error bars represent standard error (n=3). Samples with different letters indicate significant differences in host susceptibility as determined by a Two-Way ANOVA, Tukey HSD post-hoc analysis (p-value ≤ 0.05).

2-7: Tables

Table 2-1: Gene Specific Primers used for qRT-PCR Gene Expression Analysis.

Gene ID	Gene Name	Forward (5' → 3')	Reverse (5' → 3')
AT3G48100	*ARABIDOPSIS RESPONSE REGULATOR 5, ARR5*	TCTGAAGATTAA TTTGATAATGACGG	TCACAGGCTTCA ATAAGAAATCTTCA
AT1G19050	*ARABIDOPSIS RESPONSE REGULATOR 7, ARR7*	ACTGTAGAGAGT GGAACTAGGGCT	AGTCCTGGCATT GAGTAATCCGTC
AT2G14610	*PATHOGENESIS-RELATED PROTEIN 1, PR-1*	ACACGTGCAATG GAGTTTGTGGTC	TACACCTCACTT TGGCACATCCGA
AT4G31800	*WRKY DNA-BINDING PROTEIN 18, WRKY18*	TGGGTCAAGCAC AGTGACTTTGGA	GCAGCAGCAAGA GCAGCTGTAAAT

Table 2-2: Cytokinin concentration in leaf tissue of wild-type Col-0 plants exposed to normal (22°C) and high (28°C) temperature conditions. Data represents two pooled experiments, with n=4 per temperature treatment.

Temperature	Cytokinin Metabolite (pmol/g FW ± SD)					
	tZ	**tZR**	**tZRPs**	**cZ**	**cZR**	**cZRPs**
22°C	0.88 ± 0.52	2.28 ± 1.95	3.17 ± 0.75	0.71 ± 0.12	8.42 ± 3.41	4.99 ± 0.28
28°C	1.27 ± 0.65	2.96 ± 0.34	4.02 ± 0.41	0.76 ± 0.31	9.32 ± 4.45	2.98 ± 0.22 *
	DZ	**DZR**	**DZRPs**	**iP**	**iPR**	**iPRPs**
22°C	N.D.	0.27 ± 0.14	0.1 ± 0.04	0.28 ± 0.09	3.32 ± 3.03	5.51 ± 2.54
28°C	N.D.	0.32 ± 0.09	N.D.	0.45 ± 0.19	5.90 ± 2.37	8.85 ± 0.21 *
	tZ7G	**tZ9G**	**tZOG**	**cZOG**	**tZROG**	**cZROG**
22°C	42.05 ± 1.08	24.14 ± 1.05	1.83 ± 0.26	1.67 ± 0.11	0.63 ± 0.12	2.72 ± 0.24
28°C	51.68 ± 0.51 *	22.30 ± 1.07	3.00 ± 0.23 *	2.39 ± 0.19 *	0.65 ± 0.04	2.80 ± 0.02
	tZRPsOG	**cZRPsOG**	**DZ9G**	**iP7G**	**iP9G**	
22°C	0.17 ± 0.04	0.22 ± 0.03	0.08 ± 0.02	79.43 ± 2.38	1.48 ± 0.10	
28°C	0.14 ± 0.003	0.17 ± 0.003 *	0.06 ± 0.01 *	86.67 ± 1.68	1.72 ± 0.10 *	

*, statistically significant differences between 28°C and 22°C exposed plants ($p < 0.05$; Student's t-test).

tZ, trans-zeatin; tZR ,tZ riboside; tZRPs, tZ ribotides; cZ, cis-zeatin; cZR, cZ riboside; cZRPs, cZ ribotides; DZ, dihydrozeatin; DZR, DZ riboside; DZRPs, DZ ribotide; iP, N6-(Δ2-isopentenyl)adenine; iPR, iP riboside; iPRPs, iP ribotides; tZ7G, tZ-7-N-glucoside; tZ9G, tZ-9-N-glucoside; tZOG, tZ-O-glucoside; cZOG, cZ-O-glucoside; tZROG, tZR-O-glucoside; cZROG, cZR-O-glucoside; DZ9G, DZ-9-N-glucoside; iP7G, iP-7-N-glucoside; iP9G, iP-9-N-glucoside; tZ-type CK precursors, sum of tZR and tZRPs; iP-type CK precursors, sum of iPR and iPRPs; inactivated tZ-type, sum of tZ7G, tZ9G, tZOG, tZROG and tZRPsOG; inactivated iP-type, sum of iP7G and iP9G; N.D., not detected.

Table 2-3: List of potential Loss of Susceptibility candidate genes identified from Type-B ARR binding motif analysis.

Gene ID	Gene Name	Gene Description	ABRC Line	D.E.G in:
AT3G61630	*CRF6*	CRF6 encodes one of the six cytokinin response factors		Col-0 28°C *Pst*
AT4G39980	*DHS1*	Encodes a 2-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, which catalyzes the first committed step in aromatic amino acid biosynthesis	SALK_008842	Col-0 28°C *Pst*
AT4G17490	*ERF6*	Encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ATERF-6)	SALK_087356C	Col-0 28°C *Pst*
AT1G69870	*NPF2.13*	Encodes a low affinity nitrate transporter NRT1.7. Responsible for source-to-sink remobilization of nitrate	SALK_022429	Col-0 28°C *Pst*
AT1G59740	*NPF4.3*	Major facilitator superfamily protein	SALK_131109	Col-0 28°C *Pst*
AT5G13700	*PAO1*	Encodes a protein with polyamine oxidase activity	SALK_013026	Col-0 28°C *Pst*
AT2G43020	*PAO2*	Encodes a polyamine oxidase	SAIL_439_C04 / CS874173	Col-0 28°C *Pst*
AT1G65840	*PAO4*	Encodes a peroxisomal polyamine oxidase, involved in the back-conversion polyamine degradation pathway	SALK_020782	Col-0 28°C *Pst*

Table 2-4: List of potential Loss-of-Susceptibility candidate genes identified from metabolomics analysis and starch staining assays.

Gene ID	Gene Name	Gene Description	ABRC Line	D.E.G in:
AT4G17770	*TPS5*	Encodes an enzyme putatively involved in trehalose biosynthesis	SALK_007952	*ahk2,3* 28°C
AT1G68020	*TPS6*	Encodes an enzyme putatively involved in trehalose biosynthesis	SALK_031944	*ahk2,3* 28°C
AT1G70290	*TPS8*	Encodes an enzyme putatively involved in trehalose biosynthesis	SALK_203675C	*ahk2,3* 28°C
AT1G23870	*TPS9*	Encodes an enzyme putatively involved in trehalose biosynthesis	SALK_086992C	*ahk2,3* 28°C
AT1G60140	*TPS10_1*	Encodes an enzyme putatively involved in trehalose biosynthesis	SALK_029104	*ahk2,3* 28°C
AT2G18700	*TPS11*	Encodes an enzyme putatively involved in trehalose biosynthesis	CS456820	*ahk2,3* 28°C
AT4G22590	*TPPG*	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein	SALK_016673C	*ahk2,3* 28°C *Pst*
AT1G35910	*TPPD*	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein	SALK_013114C	*ahk2,3* 28°C *Pst*
AT4G12430	*TPPF*	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein	SALK_005461	*ahk2,3* 28°C *Pst*
AT3G20040	*ATHXK4*	Hexokinase	SALK_096977C	*ahk2,3* 22°C *Pst*
AT2G19860	*HXK2*	Encodes a protein with hexokinase activity	SALK_080584C	*ahk2,3* 22°C *Pst*
AT4G00490	*BAM2_2*	Encodes a chloroplast beta-amylase	CS859579	*ahk2,3* 28°C Mock and *Pst*
AT4G17090	*BAM3_1*	Encodes a beta-amylase targeted to the chloroplast	SALK_041214C	*ahk2,3* 28°C Mock and *Pst*
AT5G55700	*BAM4*	In vitro assay indicates no beta-amylase activity of BAM4. However mutation in BAM4 impairs starch breakdown. BAM4 may play a regulatory role	SALK_037355C	*ahk2,3* 28°C Mock and *Pst*
AT4G15210	*BAM5*	Cytosolic beta-amylase expressed in rosette leaves and inducible by	SALK_014698	*ahk2,3* 28°C Mock and *Pst*
AT2G32290	*BAM6*	Beta-amylase 6	SALK_023637	*ahk2,3* 28°C Mock and *Pst*
AT5G18670	*BAM9*	Putative beta-amylase BMY3	CS860019	*ahk2,3* 28°C Mock and *Pst*
AT5G51820	*PGMP*	Encodes a plastid isoform of the enzyme phosphoglucomutase involved in controlling photosynthetic carbon flow	CS210	*ahk2,3* 28°C *Pst*
AT5G51820	*PGMP*	Encodes a plastid isoform of the enzyme phosphoglucomutase involved in controlling photosynthetic carbon flow	SALK_016383C	*ahk2,3* 28°C Pst

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Chapter 3

Brassica napus displays heat-induced disease susceptibility to *Pseudomonas syringae* pv. *maculicola* in a cytokinin-dependent manner[2]

3.1 Summary

Heat-induced disease susceptibility (HIS) occurs when plants are exposed to unfavorable, high temperature growth conditions causing increased pathogen proliferation and decreased crop yields. However, without a clear understanding of how plants become more vulnerable to disease pressures at elevated temperatures, new effective strategies cannot be implemented in our agricultural systems. *Brassica napus* is an important worldwide oilseed crop that is anticipated to be highly vulnerable to increased temperatures, a side effect anticipated from global climate change. Thus, the question remained if *B. napus* plants would be more susceptible to pathogen infection under high temperature conditions. Previous work in *Arabidopsis thaliana*, a member of the Brassicaceae family, has shown HIS to the bacterial pathogen *Pst* DC3000, thus we decided to test if *B. napus* exhibited HIS to *P. syringae* pv *maculicola* (*Psm* ES4326). Results indicate that *B. napus* exposed to high temperature conditions are more susceptible to *Psm* ES436 and this process is dependent on prior nitrogen fertilization.

HIS of Arabidopsis has been linked to the plant growth hormone cytokinin (CK), thus we aimed to address whether CK would be important for HIS of *B. napus* to *Psm* ES4326. Gene expression analysis show that *B. napus* plants exposed to high temperature conditions, with or without *Psm* ES4326, show an increase in expression of a CK biosynthesis gene suggesting an increase in CK content and signaling. Additionally, *B. napus* plants exhibit CK-mediated

[2] This chapter contains preliminary data for a future publication with the following authors: Alexandra M. Shigenaga, Grace A. Johnston, Cristiana T. Argueso.

physiological responses, such as root length inhibition, similar to Arabidopsis under normal and high temperature conditions. Due to lack of genetic tools in *B. napus*, we utilized a chemical approach to assess if CK was important for HIS. The CK signaling antagonist, PI-55, was exogenously applied to plants 48 hours prior to inoculation and results show that a single application of PI-55 led to a loss of susceptibility at 28ºC to *Psm* ES4326. Additionally, this application of PI-55 did not lead to any adverse vegetative growth parameters. Results from this work will allow us to understand how CK influences HIS and provide a novel chemical approach to combat this process in Brassicaceae crop.

3.2 Introduction

Plants, being sessile creatures, are subject to a variety of environmental stressors in the field, ranging from various combinations of abiotic and biotic pressures (Gull et al., 2019). As a consequence of global climate change, increased temperatures are anticipated to be a serious limitation on plant growth and crop productivity (Hedhly et al., 2009; Bita and Gerats, 2013; Zhao et al., 2017; Wang et al., 2020). Heat stress for crop plants is described as an atypically high temperature that causes irreversible damage to the plant and negatively effects production (Teixeira et al., 2013; Kawasaki and Uchida, 2016). High temperature stress is predicted to have an especially adverse effect on various cool-season agricultural crops, including *Brassicaceae* crop species such as *Brassica napus*, or rapeseed (Hatfield and Prueger, 2015). *B. napus* is one of the most important oilseed crops worldwide (Friedt et al., 2018) and is highly sensitive to increased temperature stress, especially during the flowering and seed filling stage (Gan et al., 2004; Aksouh-Harradj et al., 2006; Huang et al., 2019). Since *B. napus* is an oilseed crop, the effect of heat stress on reproductive tissues has been widely studied (Angadi et al., 2000; Rahaman et al., 2018; Chen et al., 2021; Mácová et al., 2021). However, there has been far less

studies focusing on how increased temperatures might affect vegetative tissues (Koscielny et al., 2018) or response to biotic pressures (Yang et al., 2021).

Heat-induced disease susceptibility (HIS) is a phenomenon that occurs when plants are exposed to high temperature conditions and become more susceptible to pathogen stress (Cohen and Leach, 2020). This phenomenon has been documented in several crop species such as rice, *Oryza sativa* (Webb et al., 2010; Onaga et al., 2017), barley, *Hordeum vulgare* (Barna et al., 2014; Mikkelsen et al., 2015), and tomato, *Solanum lycopersicum* (Zacheo et al., 1995; Anfoka et al., 2016). Studies in *O. sativa* have shown that under high temperature conditions some resistance *R* genes are more effective, such as the *R* gene *Xa7*, which recognizes the bacterial pathogen *Xanthomonas oryzae pv. oryzae* (*Xoo*) (Webb et al., 2010; Cohen et al., 2017). This increase in heat-induced *R* gene effectiveness has been linked to the abiotic stress hormone abscisic acid (ABA) (Cohen et al., 2017). However, rice plants not expressing the *R* gene *Xa7*, or any *R* gene, exhibit HIS to the bacterial pathogen *Xoo* (Webb et al., 2010). Additionally, other studies have shown that *O. sativa* plants also exhibit HIS to the rice blast fungal pathogen *Magnaporthe oryzae* (Onaga et al., 2017). This increase of host susceptibility to *M. oryzae* was attributed to not only a decrease in host defense responses, but an increase in the expression of fungal effector genes *in planta* (Onaga et al., 2017). Studies in barley have also shown that after exposure to high temperature plants become more susceptible to the hemi-biotrophic fungal pathogen causing spot blotch disease, *Bipolaris sorokiniana* (Mikkelsen et al., 2015*)*. However, there are contrasting results about the impact of high temperature on barley susceptibility to the biotrophic powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei*. Recent studies show that exposure to elevated temperatures increase susceptibility (Barna et al., 2014), but other studies show that high temperature needs to be associated with increases in CO_2 for there to be an

increase in susceptibility to powdery mildew (Mikkelsen et al., 2015). Furthermore, studies in *S. lycopersiucum* show that after exposure to heat stress plants become more susceptible to nematode (Zacheo et al., 1995) and viral infections (Anfoka et al., 2016). Lastly, preliminary data has shown that Arabidopsis, a member of the *Brassicaceae* family, exhibits HIS to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Wang et al., 2009; Cheng et al., 2013; Huot et al., 2017), in a manner that is dependent on the plant hormone cytokinin (CK) (Shigenaga et al., Chapter 2).

CKs are plant hormones whose physiological functions are normally associated with plant growth. First discovered for their role in cell division (hence their name, from cytokinesis), this class of plant hormones is now known to regulate many other physiological functions, including responses to biotic and abiotic stresses (Argueso et al., 2009; Cortleven et al., 2019). CKs are adenine-derived molecules and can be classified by the presence of an isoprenoid or an aromatic chain at the N^6 position of their adenine moieties (Mok and Mok, 2001). In Arabidopsis and other plant species CK signaling is mediated by two-component element proteins, analogous to the two-component signaling system used by bacteria and fungi to perceive and respond to environmental stimuli (Kieber and Schaller, 2018). In the canonical form of this signaling system in plants, CKs are perceived by CHASE domain-containing Histidine Kinase (HK) receptors, located on the endoplasmic reticulum membrane and/or plasma membrane. Binding of CK to the CHASE domain leads to autophosphorylation of the HK receptors, leading to conformational changes and the initiation of a phospho-relay pathway involving downstream players; namely the Histidine-Phosphotransfer proteins (HPs) and Response Regulators (RRs), eventually culminating in the transcriptional activation of CK-regulated genes. CKs positively affect several aspects of plant growth, including plant nutrient assimilation and source-sink relationships, with

important consequences to yield (McIntyre et al., 2021). For this reason, this plant hormone is a frequent target of plant breeding programs, including those involving *Brassica* species.

B. napus and other Brassicaceae crops are susceptible to the bacterial leaf spot pathogen *P. syringae* pv. *maculicola (Psm)*. *Psm* was first identified on cauliflower, where it was described to prefer cool, wet conditions and to enter the host via the stomata (McCulloch, 1911). Since then *Psm* has been identified as an important bacterial disease of crucifers worldwide, especially in areas that provide optimal environmental conditions (Wechter et al., 2007; Takikawa and Takahashi, 2014; Zhao et al., 2017). *Psm* has been described as a heterogenous pathovar of *P. syringae* as it is closely related to *Pst* DC3000 in genetic makeup, nutritional requirements, and virulence strategies (Dong et al., 1991; Hendson et al., 1992; Cuppels and Ainsworth, 1995; Yan et al., 2008). Both bacterial strains produce the phytotoxin coronatine as a main virulence strategy to combat stomatal immunity (Wiebe, 1993; Cuppels and Ainsworth, 1995; Zheng et al., 2012). *Psm* has since been reported to be pathogenic on a variety of crucifers, including leafy *Brassica* cultivars (Zhao et al., 2000; Keinath et al., 2006; Takikawa and Takahashi, 2014). However, similar to *Pst* DC3000, *Psm* can also infect tomato (Wiebe, 1993). Common symptoms of bacterial leaf spot caused by *Psm* include small, brown necrotic spots with chlorotic halos and necrotic, water soaked, or chlorotic lesions (Zhao et al., 2000). As the infection progresses, the lesions continue to grow, causing extensive leaf damage and reducing plant quality. However, whether *B. napus* exhibits HIS to *Psm*, and whether this process is CK-dependent, is currently unknown.

Here we addressed whether *B. napus* was more susceptible to *Psm* under higher temperatures (i.e., affected by HIS), using the *Psm* strain ES4326 (*Psm* ES4326). To assess the role of CK in HIS, and given the amphidiploid nature of *B. napus* and the lack of extensive

genetic tools in this species (Neik et al., 2017), we utilized a chemical approach by using a CK signaling chemical antagonist. The CK signaling antagonist 6-(2-hydroxy-3-methylbenzylamino)purine, known as PI-55 (Spíchal et al., 2009), is structurally similar to the bioactive CK *trans*-zeatin, and the synthetic CK benzyl adenine (BA). In Arabidopsis, PI-55 has been shown to act as a CK signaling antagonist by competitively binding to the Arabidopsis CK signaling receptor AHK4, completely inhibiting *trans*-zeatin from binding to AHK4 (Spíchal et al., 2009). PI-55 can also bind Arabidopsis CK signaling receptors AHK2 and AHK3, but with weaker activity. In addition to being shown to act as a CK signaling antagonist in Arabidopsis, PI-55 has also been successfully used to inhibit CK signaling in *B. napus* (Guo et al., 2017) and tomato (Costa et al., 2021).

Recent studies in *B. napus* have identified five CK Histidine Kinase (CHK) receptors that have the CHASE-containing His kinase domains similar to the Arabidopsis HK receptors (Kuderová et al., 2015). Phylogenetic analyses have shown that the five BnCHK receptors are most closely related to the AHK2 and AHK3 receptors of Arabidopsis (Kuderová et al., 2015). Similar to Arabidopsis, CK is perceived by the CHKs in *B. napus*, activating a two-component phosphorelay signaling pathway to initiate CK-dependent processes. Thus, the fact that CK signaling is conserved between Arabidopsis and *B. napus* indicates that PI-55 could be used as a CK signaling inhibitor to elucidate the role of CK processes in *B. napus*.

In this study, we show that under elevated temperature conditions *B. napus* plants are more susceptible to *Psm* ES4326 infection and this process is dependent on prior fertilization. Interestingly, *B. napus* plants respond to exogenous CK application in a similar way to Arabidopsis plants and exhibit previously identified heat stress phenotypes. Utilizing the CK signaling antagonist, PI-55, we show that CK signaling is important for HIS of *B. napus* to occur.

Lastly, application of PI-55 to *B. napus* plants did not result in any developmental and/or yield costs suggesting that it could be a viable chemical approach for decreased bacterial disease outbreaks under increased temperature conditions. Understanding the role CK plays in HIS of *B. napus* to *Psm* ES4326 will be useful for creating strategies for increased crop resilience to pathogen stress under high temperature conditions.

3.3 Methods

Plant Materials and Growth Conditions:

Brassica napus (SKU: 0163A, Seed Savers Exchange) seeds were placed directly on pre-wet soil following package instructions (½ inch deep in the soil), with no domes required for germination. All plants were soil grown in Percival growth chamber (Model# PGC-15) at Colorado State University, unless stated otherwise. Plants were grown in Pro-mix HP Mycorrhizae soil for four to five weeks under 10:14 hour day:night light regime at 160 ± 20 μmol $m^{-2}s^{-1}$ at day parameters of 22ºC, 65% relative humidity (RH) and night parameters at 20ºC, 55% RH. Plants were fertilized with Miracle-Gro® Water Soluble All Purpose Plant Food (3:1:2 NPK ratio) at ½ tsp in 4L water 5-7 days prior to disease assays.

Temperature Treatments:

For all heat stress experiments, plants were moved to a high temperature growth chamber (Percival Model# PGC-15) set at 28ºC for 10:14 hour day:night light regime, and 65/55 %RH. Four to five-week-old plants were used for all experiments, except for root plates assays (*see below*).

CK-Inhibitor (PI-55) Application:

PI-55 (255.28 MW) CK-inhibitor was synthesized and provided by Lukas Spichal, Palacký University. PI-55 was dissolved in DMSO at a stock concentration of 50mM and stored at -20°C. For pathogen experiments a working concentration of 100uM PI-55 and a vehicle control solution of 0.1% DMSO were used. 0.002% Silwett was used as a surfactant for all working solutions. DMSO and 100uM PI-55 solutions were applied using an atomized sprayer (Preval, Inc.). The entire aerial part of plants was sprayed with until run-off. Plants were sprayed just prior to being placed in the appropriate growth chamber (i.e., 24 hours prior to inoculation with *Psm*).

Disease Assays:

Control plants remained at 22ºC for the entire experiment, while heat treated plants were moved to 28ºC chamber 24 hours before inoculation with pathogen and remained there for the entirety of the experiment. On the day of inoculation plants were watered in the morning. Assessment of bacterial growth in *B. napus* was done by infiltration inoculation. *Psm* ES4326, provided by Marc Nishimura at Colorado State University, was streaked on KB media plates supplemented with Rifampicin (Rif, 50mg/mL) and Cycloheximide (Chx, 100mg/mL) and incubated for 48 hours at 28ºC. 24 hours before inoculation a lawn plate of *Psm* ES4326 was streaked onto a new $KB_{rif,chx}$ plate and incubated at 28ºC. On the day of inoculation, the bacteria were resuspended in 10mM $MgCl_2$ for a concentration of $1x10^5$ CFU/mL (equivalent to OD_{600}=0.0002). Plants were inoculated by leaf infiltration with a needless syringe, 1 fully developed leaf (no. 2 or 3) per plant and 4 plants per treatment. The amount of *in planta* bacteria was quantified 1 hour post inoculation (hpi, day of inoculation) and 4 days post inoculation (dpi).

Leaf discs were pooled for one sample, four samples were collected for each treatment at each time point. Leaf discs were ground in 10mM $MgCl_2$ and serial dilutions of ground tissue were used to determine the number of CFU per cm^2 of leaf disc tissue. All dilutions were plated on $KB_{rif,chx}$ and were incubated at 28ºC before counting CFU.

***In planta* Bacterial Growth Curve:**

Disease assay conditions mentioned above were followed, with the noted changes below. Control plants remained at 22ºC for experiment, while heat treated plants were moved to 28ºC chamber 24 hours before inoculation and remained there for the entire experiment. *Psm ES4326* was grown and set to OD_{600} of 0.0002 ($1x10^5$ CFU/mL). Plants were inoculated with a needless syringe, as described above. Leaf discs were collected and pooled for each sample for Day 0 through Day 3. Leaf discs were ground in 10mM $MgCl_2$ and serial dilutions were plated to determine bacterial populations *in planta*. Days 0-3 were plated on $KB_{rif,chx}$ plates and incubated at 28ºC.

Primary Root Elongation Assay for CK Sensitivity:

B. napus seeds were grown on vertical plates containing 1X Murashige-Skoog (MS) media (Phytotech Labs) with 1% sucrose and 0.8% bactoagar (USBiological Life Sciences, A0930). MS plates were supplemented with 100nM of the synthetic CK benzyladenine (BA, Sigma Aldrich) or 0.1% DMSO vehicle control. *B. napus* seeds were liquid sterilized in a sterile hood prior to plating by incubation for 10 minutes in a 30% bleach solution, followed by 5 rinses in sterile water. Plates were moved to 4ºC for 4 days to synchronize germination, and then moved to the appropriate plant growth chambers. Germination for each seedling was marked.

Plates were scanned 4 days post germination (dpg) and primary root growth was measured using Image J (Version 1.51).

RNA Extraction and qRT-PCR:

Total RNA was extracted using RNeasy Plant kit (QIAGEN), following manufacturer's instructions. Quality and integrity of RNA assessed by A_{260}/A_{280} and A_{260}/A_{230} ratios. RNA samples of good quality underwent DNAse Treatment (TURBO DNase-Free), and cDNA was synthesized using Qscript (QuantaBio). qRT-PCR reactions were performed with PerfeCTa SYBR Green (QuantaBio) on CFX Connect Real-Time System (BioRad). *BnActin*, BnaC02g00690D, (F: 5′-ATCACCATCGGAGCTGAG-3′; R: 5′-GAAGCATTTCCTGTGGACG-3' (Kagale et al., 2006) was used as a housekeeping gene for all reactions. Gene specific primers are listed in Table 3.1. At least two biological replicates of each experiment were obtained, with three technical replicates for each treatment.

Vegetative Growth Experiment:

Four- to five-week-old *B. napus* plants were measured for vegetative growth parameters: height, fresh weight, dry weight, and hypocotyl length, 5 days post application of DMSO or 100uM PI-55. Fresh weight was measured with a Sartorius scale (Model ENTRIS822-1S). Height, fresh weight, and hypocotyl length were all measured at the end of the experiment. Following fresh weight measurements, the entire shoot tissue was placed in a paper bag and placed in an oven at 65C to dry for 5 days. Dry weight was measured with Ohaus Scout® scale (Model SPX222). Growth experiments were replicated at least two times.

Statistical Analysis:

A minimum of two biological replicates were done for each assay, with at minimum three technical replicates for each chemical treatment and inoculation, unless stated otherwise. Sample sizes for each experiment are noted in figure legends. For experiments with two comparisons a two-WAY ANOVA was used and for those with one comparison a Student's t-test was used to evaluate statistical significance. Significance for these tests was based on a p-value ≤ 0.05.

3.4 Results

Elevated Temperatures Lead to Increased Disease Susceptibility

Previous studies have shown that various plant species become more susceptible to pathogens after exposure to transient high temperature conditions, or heat waves (Reviewed in Velásquez et al., 2018; Desaint et al., 2021). However, to date, there have been no studies testing the impact of elevated temperatures on *B. napus* susceptibility to the virulent pathogen *Psm* ES4326. To test the effect of heat stress on *B. napus* susceptibility, plants were moved to a heat chamber set at 28°C, 24 hours prior to inoculation. Plants exposed to 28°C resulted in higher *Psm* ES4326 population counts at 4 days after inoculation, as well as an increase in disease symptoms (**Figure 3-1**). These results indicate that *B. napus* plants exhibit HIS to *Psm* ES3426.

To further investigate the differences in *Psm* ES4326 growth in *B. napus* plants at 22ºC and 28ºC, we conducted an *in planta* growth curve assay to determine bacterial multiplication and host susceptibility during a three-day period. Results show that the difference in *in planta* *Psm* ES4326 proliferation at 22ºC and 28ºC happens early, within a few hours of inoculation (**Figure 3-2**). These results suggest that increased proliferation of *Psm* ES4326 *in planta* at 28ºC is likely due to decreased plant defense responses, since the difference in susceptibility is early.

Studies in Arabidopsis show that heat stress has a negative effect on defense responses (Huot et al., 2017; Janda et al., 2019), more specifically these studies showed that a decrease in immune receptor *FLS2* (*FLAGELLIN SENSING 2*) accumulation and down-regulation of defense response genes such as *PR-1* (*PATHOGENESIS RELATED 1*). Thus, given the fact that Arabidopsis and *B. napus* are phylogenetically close (Parkin et al., 2005) it is probable that *B. napus* may down-regulate genes encoding defense-related genes in response to elevated temperature conditions.

Interestingly, in the course of our experiments we discovered that HIS of *B. napus* to *Psm* ES4326 requires prior nitrogen-phosphate-potassium (NPK) fertilization. Nitrogen, phosphate and potassium are essential nutrients for plant growth and physiology. We noticed that *B. napus* plants not fertilized prior to infiltration inoculation with *Psm* ES4326 resulted in no difference in host susceptibility comparing 22°C to 28°C (**Figure 3-3**, left). However, plants fertilized with NPK 5-7 days prior to inoculation with *Psm* ES436 show a significant difference in host susceptibility (**Figure 3-3**, right). These results indicate that host factors affected by plant nutritional status are required for increased *Psm* ES4326 proliferation at 28°C. Previous studies have confirmed that *B. napus* plants require NPK fertilization for optimal crop productivity (Yousaf et al., 2017).

CK Signaling Promotes HIS of *B. napus* to *Psm* ES4326

The results in **Figure 3-1** and **Figure 3-2** confirm that fertilized *B. napus* plants exposed to increased temperatures are more susceptible to *Psm* ES4326. However, whether this process is CK-mediated, as in HIS of Arabidopsis to *Pst* DC3000, is unclear. In order to assess whether CK is important for HIS, we first asked if CK content and/or signaling increases in response to high

temperature exposure. Previous work has supported that CK content and signaling in Arabidopsis increases in response to elevated ambient temperatures (Shigenaga et al., Chapter 2), but the effect in *B. napus* is unclear. To address this we compared the gene expression of *B. napus* plants at 22°C and 28°C, 48 hours post infiltration with a mock solution, 10mM $MgCl_2$, or *Psm* ES4326. We then tested the expression of genes encoding a CK biosynthesis enzyme (*BnIPT2*) and a gene encoding a CK degradation enzyme (*BnCKX1a*) (Song et al., 2015). Results indicate that exposure to high temperature leads to an increase in the expression of the CK biosynthesis gene, *BnIPT2* (**Figure 3-4**). Interestingly, the effect of high temperature on the CK degradation gene, *BnCKX2*, had the opposite effect, as this gene was down-regulated in response to high temperature and *Psm* inoculation (**Figure 3-4**). Together these results support that *B. napu*s shows an increase in CK-mediated processes in response to high temperature exposure, similar to the trend seen in Arabidopsis (Shigenaga et al., Chapter 2).

Next, we addressed if *B. napus* responses to exogenous application of CK were similar to those of Arabidopsis. Arabidopsis plants grown on MS media with CK have reduced primary root length (To et al., 2004). We thus used this CK root inhibition assay to test if *B. napus* has similar CK sensitivity. *B. napus* seedings were grown on vertical MS plates supplemented with DMSO or 100nM BA, a synthetic CK, at either 22ºC or 28ºC. Four days post germination plates were scanned, and the primary root length was measured. Seedlings grown at 22ºC exhibit CK-mediated root inhibition, as seedlings grown on BA had approximately a 79% decrease in primary root lengths (**Figure 3-5**). Similarly, seedlings grown at 28ºC also exhibited CK-mediated root inhibition with those grown on BA having an 87% decrease in root length (**Figure 3-5**). Additionally, *B. napus* seedlings grown at 28ºC had a 34% increase in root elongation compared to seedlings grown at 22ºC (**Figure 3-4**). These results support previous studies in

Arabidopsis showing that primary root elongation increases up to 40% in response to increased ambient temperature (21ºC to 26ºC) (Martins et al., 2017). Together these results suggest that *B. napus* has similar CK sensitivity as Arabidopsis, and that in our experimental conditions *B. napus* displays heat-induced growth phenotypes similar to those previously reported (Larkindale et al., 2005; Quint et al., 2016; Martins et al., 2017).

We then utilized a chemical approach to inhibit CK signaling and investigate whether CK plays a role in HIS of *B. napus* to *Psm* ES4326. Five-week-old *B. napus* plants were sprayed with PI-55, a CK signaling antagonist, or a mock 0.1% DMSO vehicle control solution, just before plants were subjected to heat treatment (28ºC) or maintained at control temperatures (22ºC). Plants were inoculated with *Psm* 24 hours later, and bacterial multiplication was assessed at 4 dpi. As seen in **Figure 3-6**, application of the CK signaling inhibitor PI-55 did not have an impact on *B. napus* susceptibility at normal growing conditions but led to decreased *Psm* ES4326 populations under high temperature conditions. This suggests that CK signaling promotes HIS of *B. napus* to *Psm* ES4326, similar to what has been observed in the Arabidopsis-*Pst* DC3000 pathosystem. However, differently from Arabidopsis, disease symptoms do not change with PI-55 application, even though *Psm* ES4326 populations were significantly diminished (**Figure 3-6**). This increase in chlorotic symptoms could be explained by the role CK plays in inhibition of senescence (Gan and Amasino, 1995; Zwack and Rashotte, 2013); if CK signaling is inhibited, senescence may no longer be repressed, thus leading to increased chlorosis.

In addition to demonstrating that CK has a role in HIS of *B. napus* to *Psm*, the results above also indicate that PI-55 could be used as a chemical strategy to combat HIS in *B. napus*, and perhaps other plant species. CK is a growth hormone that has long been implicated in important processes involved in development, such as cell division, energy allocation, and organ

development (Werner et al., 2001; Kieber and Schaller, 2018; Wu et al., 2021). Thus, application of a CK signaling inhibitor to a crop plant could have negative impacts on *B. napus* growth, potentially leading to yield penalties that outweigh losses to disease. To address if application of PI-55 could have a negative effect on yield, we measured the following vegetative growth parameters: plant height (in), fresh weight (g), dry weight (g), and hypocotyl length (in). These measurements were collected the same day that would correlate with 4 dpi of *Psm* ES4326 experiments, thus 5-days post a single application of PI-55. As seen in **Figure 3-7**, *B. napus* plants sprayed with PI-55 did not show a significant change in vegetative growth based on height, weight, or hypocotyl length. However, *B. napus* plants exposed to heat did exhibit increased shoot length or height (**Figure 3-7B**, top). Interestingly, although hypocotyl elongation is an early heat stress response in *Arabidopsis*, this phenotype is not a seen in our *B. napus* data (**Figure 3-7B**, bottom). Together these results suggest that although *B. napus* plants may respond to heat stress with some developmental phenotypes (i.e., increased height), if PI-55 is applied to heat stressed *B. napus* plants the developmental cost is no greater than *B. napus* plants grown under optimal temperature conditions.

3.5 Discussion

Due to an increase in disease susceptibility of *B. napus* to *Psm* ES4326 at 28°C (**Figure 3-1**), it was of interest to assess if HIS of *B. napus* was a CK-mediated process. The Brassicaceae family includes both Arabidopsis and *B. napus*, and although these two species belong to different lineages of the Brassicaceae family (Franzke et al., 2011) there have been studies linking conserved CK responses between the two species (Zuñiga-Mayo et al., 2018). Previous work showed that Arabidopsis exhibits HIS to the bacterial pathogen *Pst* DC3000 in a CK-

dependent manner (Shigenaga et al., Chapter 2), thus we assessed if HIS of *B. napus* was also driven by CK. First, we investigated if CK-mediated processes change in response to high temperature conditions in *B. napus*. Although the consensus of the role CK plays in heat stress is currently unclear (Todorova et al., 2005; Dobrá et al., 2015; Skalák et al., 2019; Prerostova et al., 2020), our results indicate that elevated temperature has a positive impact on CK-mediated processes leading to an increase in expression of the CK biosynthetic gene *BnIPT2* (**Figure 3-4**). To address if CK is promoting HIS of *B. napus* to *Psm* ES4326, we utilized a CK signaling chemical inhibitor due to the lack of CK signaling mutants in *B. napus*. Application of the CK signaling antagonist led to decreased susceptibility at 28°C, indicating that HIS of *B. napus* is partially dependent on CK signaling (**Figure 3-6**). Here we provide evidence of a novel chemical approach to alleviate the pressure of CK-mediated HIS in Brassicaceae crops.

HIS: a Threat to Agricultural Systems

As global temperatures continue to rise, important agricultural crops will become more susceptible to various environmental pressures, including pathogen stress (Chakraborty and Newton, 2011; Velásquez et al., 2018). Identifying factors contributing to HIS of important crop species, such as *B. napus* will act as a foundation for implementing more sustainable agricultural practices to alleviate the negative impact of high temperature on plant-pathogen interactions. Although some studies have shown that high temperature can lead to increased resistance of crop plants to pathogens (Carter et al., 2009; Fu et al., 2009; Webb et al., 2010; Zhu et al., 2010; Zhao et al., 2016; Cohen et al., 2017), this was not the scope of this study. In contrast, our work aimed to understand what processes are promoting increased susceptibility of *B. napus* to the bacterial pathogen *Psm* ES4326. HIS of other plant species has shown that outside of optimal growing

temperatures plants become more susceptible hosts (Moury et al., 1998; Webb et al., 2010; Prasch and Sonnewald, 2013; Zhao et al., 2016; Huot et al., 2017; Onaga et al., 2017). In these studies, the increase in susceptibility at elevated temperatures has been linked to either suppression of defense responses, such as decreased of Resistance (R) gene function (Wang et al., 2009; Prasch and Sonnewald, 2013), down-regulation of defense genes (Huot et al., 2017), or decreased abundance of intracellular immune receptors (Janda et al., 2019); as well as enhanced pathogen virulence by increased effector translocation (Huot et al., 2017) or increased expression of pathogen effector genes (Onaga et al., 2017). *B. napus* is a major oilseed crop that is highly sensitive to heat stress and is anticipated to show a decrease in crop productivity in response to climate change. Although, the impact of heat stress on reproductive structures of *B. napus* has been extensively studied (Huang et al., 2019; Chen et al., 2021; Mácová et al., 2021), the impact of increased temperature on response to biotic stress has not been characterized. A clear understanding of how *B. napus* becomes more susceptible to disease pressure under elevated temperatures can lead to new effective strategies that can be implemented in this agricultural system. Thus, we evaluated *B. napus* response to heat stress and infection with the bacterial pathogen *Psm* ES4326 to provide insight into this physiological process.

B. napus exhibits HIS to the bacterial pathogen *Psm* ES4326

We found that *B. nap*us plants exhibit increased susceptibility to the bacterial pathogen *Psm* ES4326 when exposed to heat stress, as determined by comparing host susceptibility of *B. napus* plants at 22ºC and 28ºC (**Figure 3-1, Figure 3-2**). To support increase in *Psm* ES4326 proliferation there is also an increase in disease symptoms of *B. napus* plants at 28ºC (**Figure 3-1**). These results support previous findings of Arabidopsis becoming more susceptible to *Pst*

DC3000 after exposure to elevated temperatures (Wang et al., 2009; Cheng et al., 2013; Huot et al., 2017). However, unlike Arabidopsis plants HIS of *B. napus* to *Psm* ES4326 is dependent on prior NPK fertilization (**Figure 3-3**). Previous studies have shown that *B. napus* plants require nitrogen fertilization due to its low nitrogen use efficiency (Bouchet et al., 2016) and requires NPK fertilization for optimal crop productivity (Yousaf et al., 2017). Additionally, nitrogen fertilization has been linked to *B. napus* susceptibility to fungal pathogens, although there are contrasting results with some studies showing an increase in susceptibility after fertilization and others showing a decrease (Söchting and Verreet, 2004; Veromann et al., 2013). Additionally, other studies have shown that nitrogen levels can impact the effectiveness of resistance genes to other important Brassicaceace pathogens, such as the clubroot disease causal agent *Plasmodiophora brassicae* (Laperche et al., 2017). Genotypes susceptible to clubroot show decreased susceptibility to this disease in the absence of nitrogen fertilization (Laperche et al., 2017). Thus, our results support evidence of the involvement of nitrogen fertilization on the outcomes of *B. napus*-pathogen interactions. Interestingly, nitrogen efficiency of *B. napus* has been linked to homeostasis of biologically active CKs in leaf tissue (Koeslin-Findeklee et al., 2015) and a link between nitrogen content and CK biosynthesis has been well documented in Arabidopsis (Sakakibara et al., 2006). It is then possible to imagine that nitrogen content may be needed for CK biosynthesis and/or homeostasis in *B. napus*, which would then lead to the initiation of CK-dependent HIS to *Psm* ES4326. Therefore, our results support that CK may indeed be playing an important role in HIS, and also that nitrogen may be an important player in this process (Shigenaga et al., Chapter 2).

CK contributes to high temperature responses of *B. napus*

To evaluate if CKs could be playing an important role in HIS of *B. napus* to *Psm* ES4326, we first addressed how expression patterns of CK-mediated genes change in response to high temperature conditions. The phylogenetic relationships between Arabidopsis and *B. napus* CK-dependent genes and expression profiles was previously characterized (Song et al., 2015). Our results show that the CK biosynthetic gene, *BnIPT2*, increases in expression in response to high temperature, with or without *Psm* ES4326 infection (**Figure 3-4**). The IPT enzyme of CK biosynthesis is responsible for synthesizing aromatic CKs in plants (Kamada-Nobusada and Sakakibara, 2009), thus these results suggest a role for increased CK content in heat-induced susceptibility. Various studies in crop species, including *B. napus*, have also shown that increasing *IPT* expression leads to improved abiotic stress tolerance (Peleg et al., 2011; Qin et al., 2011; Reguera et al., 2013; Kant et al., 2015). More specifically, an accumulation of CK content under high temperature conditions helps to maintain normal plant growth (Skalák et al., 2016). Additionally, our results show that expression of the CK degradation enzyme, *BnCKX1a*, is downregulated after exposure to high temperature conditions (**Figure 3-4**). Given that CKX genes irreversibly degrade bioactive forms of CK and their ribosides (Werner et al., 2006), this expression pattern supports that high temperature conditions promote CK biosynthesis. Our results support previous studies in Arabidopsis that show a down-regulation of *CKX* genes positively influenced acclimation to heat stress (Prerostova et al., 2020). Together these results support that under elevated temperatures, *B. napus* plants increase CK content, and presumably CK signaling, further supporting a role for CK in this heat-induced process.

Additionally, *B. napus* plants exhibited similar response to exogenous application of CK. Studies in Arabidopsis confirmed an inhibitory role of CK on root elongation (To et al., 2004;

Riefler et al., 2006), thus we wanted to test how this process was impacted under high temperature conditions. Our results show that *B. napus* plants exhibit CK-mediated root inhibition at normal and high temperature conditions (**Figure 3-5**), suggesting that *B. na*pus exhibits sensitivity to CK regardless of temperature treatment. Additionally, these root assays confirmed that *B. napus* plants exhibit heat-induced root elongation (**Figure 3-5**), a phenotype previously described in other plant species as a thermotolerance response (Gladish and Rost, 1993; Larkindale et al., 2005; Martins et al., 2017). Previous studies in bent grass show that exogenous application of CK during heat stress will result in similar responses as an increase in CK content, resulting in improved heat tolerance by decreasing the negative impacts of high temperature injury (Veerasamy et al., 2007; Xu and Huang, 2009). Together our results support that *B. nap*us plants exhibit similar high temperature phenotypes and CK sensitivity as Arabidopsis, and other plant species.

CK-based chemical approaches can be used to decrease HIS, with minimal effect to plant growth and yield

To address the role of CK in HIS, we utilized a chemical approach by exogenously applying the CK signaling antagonist PI-55 to decrease HIS of *B. napus* to *Psm* ES4326 (Spíchal et al., 2009). We hypothesized that applying a CK signaling inhibitor would phenocopy our previous results showing that Arabidopsis plants lacking CK signaling are less susceptible under high temperature conditions (Shigenaga et al., Chapter 2). A single application of PI-55 to *B. napus* plants 48 hours before inoculation resulted in significantly decreased susceptibility to *Psm* ES4326 at 28ºC (**Figure 3-6**).

Previous studies have shown that CK signaling mutants in Arabidopsis exhibit a developmental cost, because of CK's importance in growth and development (Riefler et al., 2006). Thus, we wanted to see if applying PI-55 would assert any developmental costs on *B. napus* plants at either 22ºC or 28ºC. Results show that exogenously applying the CK signaling inhibitor, PI-55, did not have any developmental costs to *B. napus* (**Figure 3-7A, B**). The only change in vegetative growth recorded was a decrease in plant height of PI-55 sprayed *B. napus* plants at 28ºC, compared to DMSO sprayed plants. However, PI-55 sprayed *B. napus* plants at 28ºC maintained similar heights to 22ºC (**Figure 3-7B**, top), thus it not being noted as a major developmental cost.

Together our results indicate that HIS of *B. napus* to *Psm* ES4326 is partially dependent on CK signaling. When the CK signaling inhibitor is applied to plants prior inoculation and heat stress exposure, this resulted in decreased *Psm* ES4325 populations. However, although the plants are less susceptible, the disease symptoms at 28ºC remain the same (**Figure 3-6**). Various studies have confirmed the role of CK in chlorophyll retention (i.e., delaying of senescence) (Gan and Amasino, 1995; Zwack and Rashotte, 2013), thus after applying a CK signaling inhibitor, *B. napus* plants would be expected to exhibit high amounts of chlorosis in response to *Psm* infection. Furthermore, studies have shown that increase in CK content through over-expression of *IPT* will lead to delayed senescence (Kant et al., 2015). Thus, although applying PI-55 will not alleviate disease symptoms, it would decrease host susceptibility without any negative fitness costs. Together these results confirm a role of CK in HIS of *B. napus* to *Psm* ES4326.

Understanding the factors that contribute to increased disease susceptibility under elevated temperatures will allow for new strategies to create more stress resilient crop species

and implement more sustainable disease management practices. Current disease control of Brassicaceae crops is heavily dependent on chemical applications throughout the season, due to lack of genetic tools available (Veromann et al., 2013). Here we provide a novel chemical approach to lower *B. napus* susceptibility to the bacterial pathogen *Psm* ES4326, without any major developmental costs, through the use of a plant hormone antagonist. However, the growth analysis done here was short term and the PI-55 chemical was only applied once. Longer term studies with various amounts of the CK signaling inhibitor applied will provide more insight to if this will be a viable option for disease management for Brassicaceae crops of bacterial pathogens under increased temperatures. Additionally, due to the scope of this study, no reproductive parameters were measured before or after application of the inhibitor. Considering *B. napus* is an important oilseed crop and CK has been linked to reproductive organ development (Zuñiga-Mayo et al., 2018), this would be a logical next step for analyzing whether application of PI-55 is a sustainable disease management solution. However, for long term disease resistance focus on identifying and breeding for Resistance (*R*) genes impacted by high temperature is of upmost importance. Although *B. napus* is an amphidiploid, as it was hybridized from two diploid species *B. rapa* and *B. oleracea* (Truco et al., 1996), R-genes for other important *Brassica* diseases have been identified (Laperche et al., 2017; Neik et al., 2017). Thus, future studies should focus on identifying R-genes that are impacting HIS of *B. napus* to *Psm* ES4326, as well as further characterizing what CK-mediated processes are contributing to HIS. Overall, the results from this study highlight the importance of CK-based approaches to alleviate negative pressures of climate change on host susceptibility and improve overall crop protection against biotic pressures under increased temperatures.

3.6 Figures

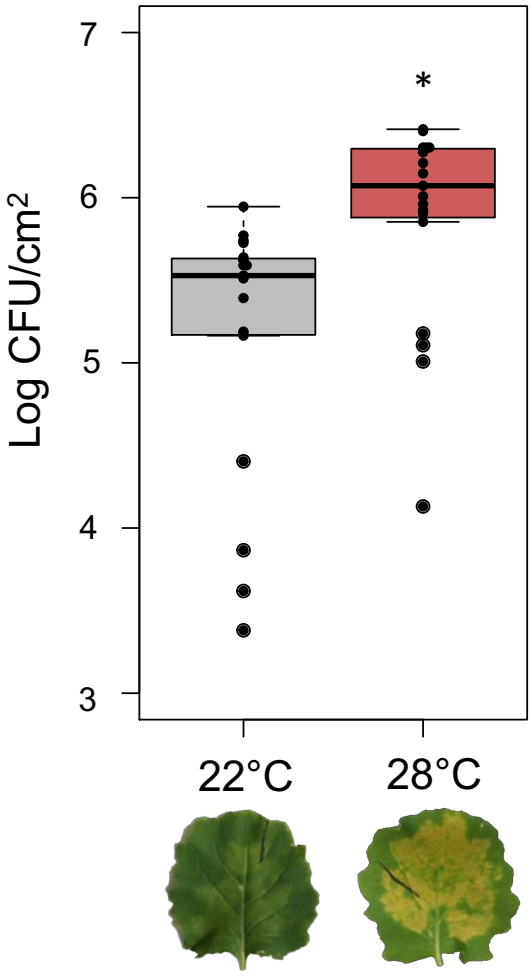

Figure 3-1: *B. napus* displays heat-induced susceptibility (HIS) to *Psm* ES4326 at 28°C. Host susceptibility was determined by quantifying *Psm* ES4326 colony forming units (CFU) per cm^2 of inoculated leaf tissue. A representative leaf exhibiting bacterial leaf speck disease symptoms exhibited below CFU/cm^2 counts. Asterisks indicate significant differences in host susceptibility as determined by a Student's t-test (p-value ≤ 0.05). Data represents pooled experiments (n=4), with 4 technical replicates per genotype and treatment.

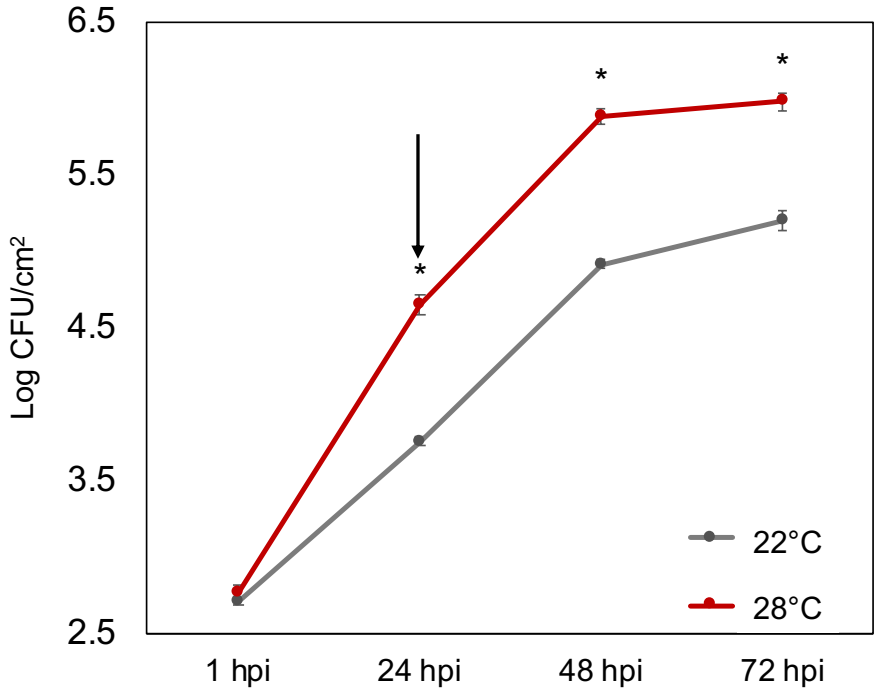

Figure 3-2: Temperature effects on *Psm* ES4326 proliferation overtime in *B. napus* plants. Growth curve of *Psm* ES4326 proliferation *in planta* over a three-day period post infiltration inoculation (1 x10^5 CFU/mL). Asterisks indicate a significant difference in host susceptibility between 22ºC and 28ºC at each timepoint based on Student's t-test (p-value ≤ 0.05). Arrow indicates earliest timepoint exhibiting statistically different host susceptibility phenotype. The error bars represent standard error (n=4) and this experiment was repeated at least three times with similar results.

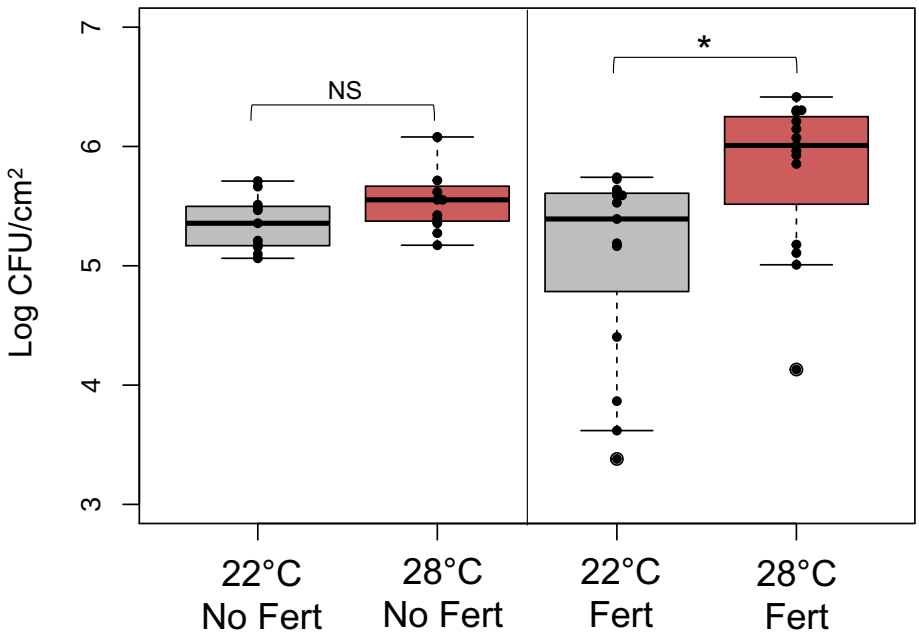

Figure 3-3: Heat-induced susceptibility of *B. napus* to *Psm* ES4326 at 28°C requires prior fertilization. Host susceptibility was determined by quantifying *Psm* ES4326 CFU/cm^2 of inoculated leaf tissue. Asterisks indicate significant differences in host susceptibility as determined by a Student's t-test (p-value ≤ 0.05), NS indicates no significant difference. Data represents pooled experiments (n=3), with 4 technical replicates per genotype and treatment.

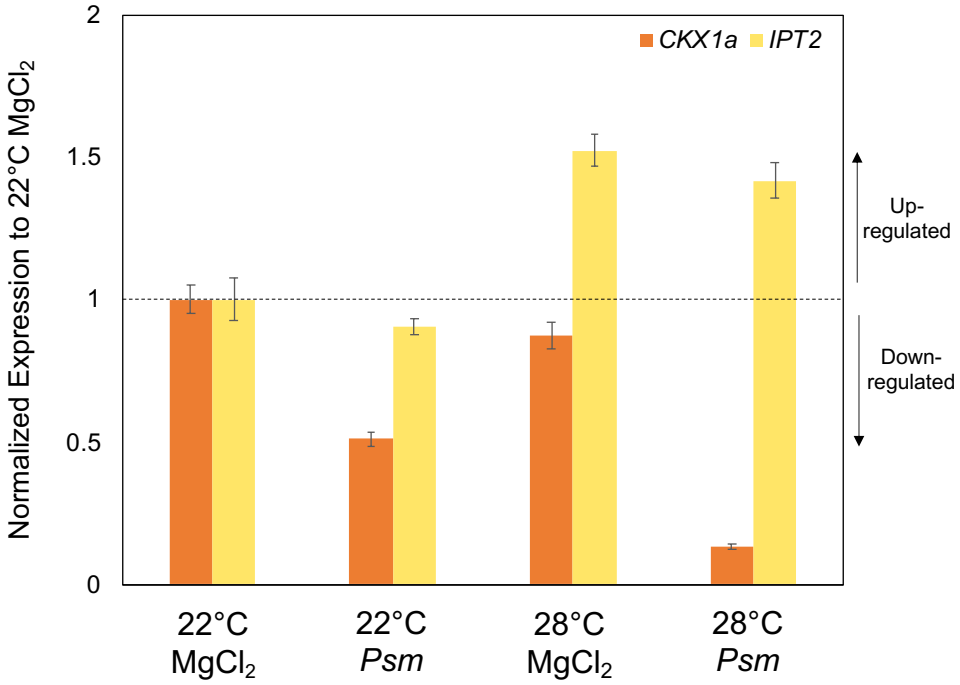

Figure 3-4: High temperature increases expression of a CK biosynthesis gene and down-regulation of a CK degradation gene. CK biosynthesis gene, *BnIPT2*, is upregulated in response to high temperature conditions, with or without *Psm* inoculation. CK degradation gene, *BnCKX1a*, is downregulated in response to high temperature conditions, with or without *Psm* inoculation. Tissue for qRT-PCR gene expression analyses were collected at 48 hours post stress,. Pooled data represents normalized expression to 22°C $MgCl_2$ for each gene tested. Gene expression experiments were conducted at least two times with similar results, error bars represent standard error (n=3).

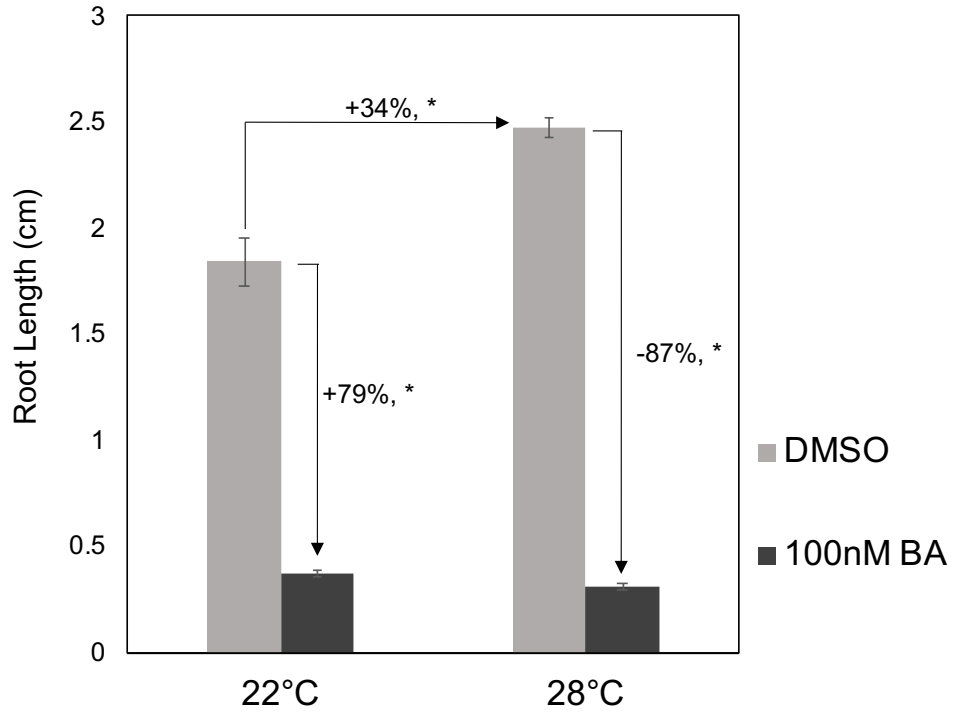

Figure 3-5: Temperature effect on CK-mediated root inhibition of *B. napus*. Seedings were grown on vertical 1X MS media plates at 22ºC and 28ºC. Germination was marked and root growth was measured after 4 dpg, at least 23 plants per treatment were measured. Arrows represent percent difference among biologically relevant samples. Asterisks indicate a significant difference based on Student's t-test when compared to appropriate control (p-value ≤ 0.05). Error bars represent standard error. Root growth experiments were repeated at least two times with similar results.

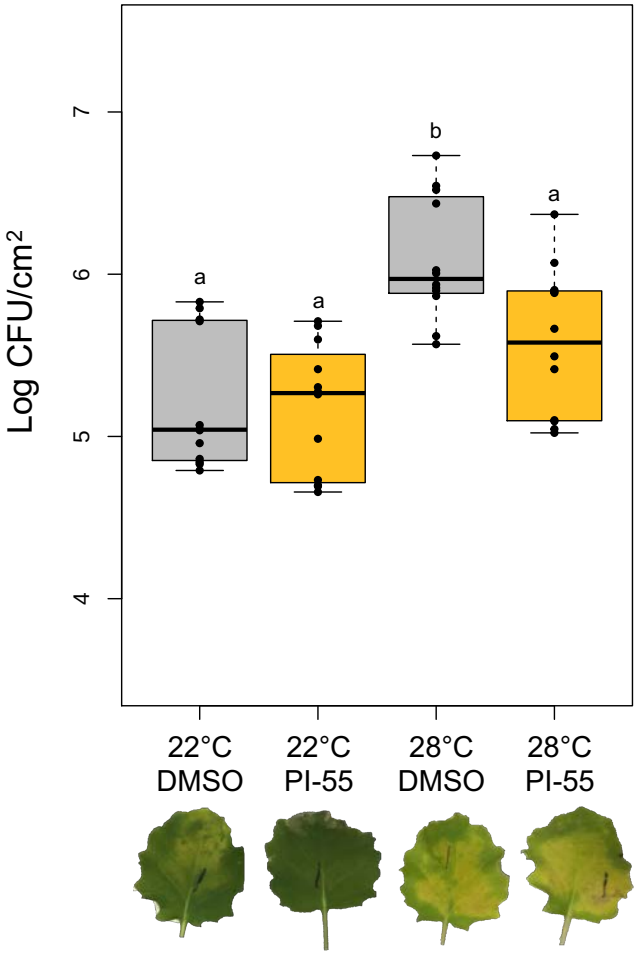

Figure 3-6: Inhibition of CK signaling through the use of a chemical inhibitor, PI-55, decreases *B. napus* susceptibility to *Psm* ES4326. Host susceptibility was measured by counting CFU/cm^2 of inoculated leaf tissue (n=4) at 4 dpi. Samples with different letters indicate significant differences in host susceptibility as determined by a Two-Way ANOVA, TUKEY HSD Post-hoc (p-value ≤ 0.05). Data represents pooled experiments, n=3 with 4 technical replicates per genotype and treatment. Representative leaves exhibiting bacterial leaf speck disease symptoms are shown below.

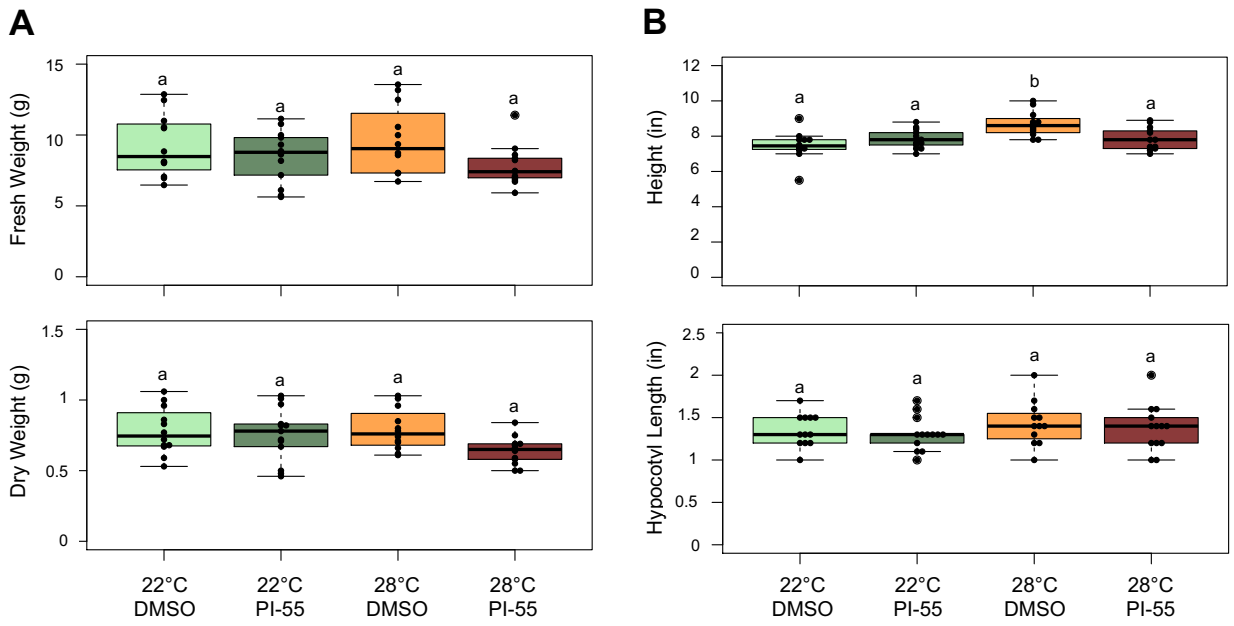

Figure 3-7: A single application of the PI-55 CK signaling antagonist does not adversely affect *B. napus* vegetative growth at 22ºC or 28ºC. A. Fresh weight was measured 5 days post application and dry weight was measured after 5 days of drying. **B.** Height and hypocotyl length were measured 5 days post application. Samples with different letters indicate significant differences in the measured vegetative growth parameter, as determined by a Two-Way ANOVA, TUKEY HSD Post-hoc (p-value ≤ 0.05). Data represents at least two pooled experiments, with at least 12 technical replicates per genotype and treatment. Four- to five-week-old *B. napus* plants were used for vegetative growth assays.

3-7 Tables

Table 3-1: Gene Specific Primers used for qRT-PCR Gene Expression Analysis. Gene specific *B. napus* primers from (Song et al., 2015).

Gene Name & ID	Forward (5' → 3')	Reverse (5' → 3')
BnIPT2, BnaC04g39280D	ACGTATCTCCCAGACACA AATAGCTC	TGTTGCATCAACGTGAT GGATATTC
BnCKX1a, BnaC04g01930D	CCACAGACAAAACAACAA GACTTTCCTC	GCCAAAGGTGGGAACT GGTATCT

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Chapter 4

Plant Disease & Climate Change: A Classroom Exercise Emphasizing Scientific Collaboration[3]

4.1 Summary

The proposed lesson, a model active-learning activity designed to give college students experience in synthesizing information and developing a solution, can be used to address socioscientific issues across fields. As a consequence of climate change, global temperatures are anticipated to rise. This rise in temperature is expected to have a negative impact on agricultural systems due in part to increased disease incidence and decrease in crop yields. This activity is written in the context of plant pathology and agricultural systems to emphasize the importance of collaboration and communication among scientists or experts in different fields to address global agricultural issues. Students will gain an understanding of the importance of agriculture on a global scale and work together to develop a solution through the development of an agricultural policy.

4.2 Introduction

Challenges of food security and climate change are current agricultural socioscientific issues that are important for creating a sustainable future. The instructional strategy described here aims to use the socioscientific issue of rice susceptibility to a bacterial pathogen to improve student understanding of (1) the interactions between abiotic and biotic factors currently decreasing rice yield, or total crop production; and (2) the importance of communication between different fields

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to produce solutions to major issues affecting global food security. Focusing this exercise on an international research station will allow students to understand the importance of agriculture on a global scale, while also emphasizing the importance of scientific research in decision making. Climate change affects many aspects of modern life, especially in industries that rely on environmental products, such as agriculture, fisheries, and conservation. Therefore, this lesson plan is meant to be used as a model that can be adapted to other disciplines to increase peer learning by having students analyze and interpret data to cooperatively develop solutions to issues surrounding climate change.

The lesson plan implements various active-learning approaches, as research has shown that active learning is more effective than traditional teaching approaches for science, technology, engineering, and mathematics (STEM) students (Freeman et al., 2014). Students will be asked to define problems, interpret and analyze data, design solutions, and engage in discussion, all while developing a model. These learning objectives coincide with Science and Engineering Practices of the *National Generation Sciences Standards* while emphasizing active learning and peer learning in a STEM college classroom (Springer et al., 1999; Smith et al., 2009; NGSS Lead States, 2013).

This lesson plan has been adapted from Constible et al., (2007), which focused on teaching concepts of penguin ecology in light of climate change. However, this adapted version not only aims to teach students about issues in agriculture surrounding climate change, but also requires students to develop a feasible policy to address the issues currently affecting global food security. For this activity, the students will be invited to a "Food Security Summit" at the International Rice Research Institution (IRRI), located in the Philippines. The Summit will act as a conference to bring together student experts from different fields to synthesize data and develop a policy to com-

bat the threat that climate change poses to rice production. The exercise presented here leads to an open-ended concept map, in which students will identify the most important data from their assigned field and how these data relate to other fields. The concept map will serve to illustrate the complexity of developing feasible solutions to produce a more sustainable future in terms of food security.

This in-class activity, though developed in an upper-level college plant physiology course, has been modified for an entry-level college classroom. Before the lesson, students should understand that (1) plants must respond to changes in their environment, (2) crops have been bred to specific environments, and (3) alterations in climate can affect crop yield. Modifications to the lesson plan and online resources for foundational plant biology and climate change knowledge have been provided in the "Instructor Notes" (**Appendix C, S4.1**).

4.3 Relevant Background Information

By 2050, the world's population is expected to have increased by 2.5 billion, reaching a total population of 9.8 billion people (United Nations, Department of Economic and Social Affairs, Population Division (U.N.D.o.E.a.S.A.), 2017). Therefore, the pressure to increase food productivity has intensified (Ray et al., 2013). Adverse environmental stress, including heat stress, has a devastating impact on agricultural systems, accounting for >50% of crop yield loss (Boyer, 1982; Wang et al., 2003; Zhao et al., 2017). In addition, each year, plant pathogens account for an estimated global yield loss of 10–16%, resulting in an economic loss equivalent to $220 billion (Strange and Scott, 2005; Oerke, 2006). Therefore, developing more stress-resistant crop varieties and implementing new agricultural policies is vital.

Rice is considered one of the most important staple crops in the world. IRRI is devoted to rice research and breeding for increased yields in major rice-producing countries. According to IRRI, plant diseases account for ~37% of all rice production losses in Asia (Rice Knowledge Bank). One of the major yield losses for rice grain is bacterial blight, a disease caused by a bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Niño-Liu et al., 2006; Mansfield et al., 2012), leading to yield losses of ≤ 70% (K. Reddy, 1979; Mew, 1993). *Xoo* thrives in warm temperatures with high humidity and is able to spread from plant to plant through water dispersal, contact, or wind (Mansfield et al., 2012). *Xoo* will infect a rice plant through natural openings in a leaf or through wounds and will quickly spread throughout the plant through the veins (Jiang et al., 2020). In order for disease to occur, there must be an optimal environment, susceptible rice plants, and an aggressive pathogen. As of 2019, *Xoo* has been reported to be widely found in a majority of rice-growing regions across the globe (Naqvi, 2019). Thus, as global temperatures are expected to rise, more rice-producing areas may experience the optimal environment for *Xoo* growth, increasing the chance of disease.

The activity described below is designed to take place over a three-day period: 15 minutes on day 1; 40 minutes on day 2; and 60 minutes on day 3. We recommend this activity for smaller classrooms, averaging ~30 students (but we include some modifications for large classrooms below).

4.4 Learning Objectives

Students will collaborate to determine how a changing climate will impact agriculture in the context of plant pathology. Students should be able to

- engage in discussion and cooperation,

- interpret scientific data points and facts,
- collaborate in small groups to make an interconnected concept map, and
- synthesize information and discussion points to develop a solution.

Students should demonstrate knowledge of

- how climate change impacts plant systems,
- how environmental factors impact plant–pathogen interactions,
- how international affairs and decision making impact agriculture, and
- how information from different fields can address agricultural challenges through policy changes.

4.5 Materials

All of the following are available in the Materials PDF (**Appendix C S4.2**) and Case Study Narrative PDF (**Appendix CS4.3**).

Instructor's Food Security Specialist Card

The instructor will play the role of a global food security specialist that has asked the other specialists to meet to solve a global emergency. The instructor will present students with information to use in their problem solving and concept maps.

- *Food Security Specialist* – Expert in organizing and implementing a food security program through policies and procedures with local and international government agencies. Card will depict a graph showing the negative relationship between increased temperatures and rice yield (**Figure 4-1**). The instructor will present the

graph to students when the Food Security Summit is introduced on day 1 of the activity.

Specialty Group Identity Cards

Each specialty group card contains one of four possible specialty options along with the role each specialist will play (**Figure 4-2**), which should be distributed evenly throughout the class:

- *Plant Pathologist* – expert on bacterial plant pathogens and plant defense responses
- *Climatologist* – expert on occurrences of tropical storms and factors contributing to changes in weather patterns
- *Agronomist* – expert on how to grow rice sustainably while also increasing grain production
- *Agricultural Economist* – expert on the monetary value of rice production and demand

Case Study Narrative

A case study narrative for each specialty group is provided (**Figure 4-3**). This case study will serve as background on how a similar agricultural issue has been solved; the example used will be the Hawaiian papaya ringspot virus epidemic. Each specialty group will have the same initial case study, presented from the perspective of their specialty and discussing how each specialty group contributed in responding to that epidemic.

Specialty Group Data Cards

Data cards are to be distributed on day 2 of the lesson. These cards outline data points and facts relevant to the given scenario (effect of climate change on agriculture in context of plant pathology; **Figure 4-4**). Each specialty group has its own data cards.

- *Plant Pathologist* – has facts and data points to show increase in disease symptoms and counts from field data
- *Climatologist* – has facts and data points to show increase in tropical storm probability and temperature changes over the years
- *Agronomist* – has facts and data points to show decrease in rice growth/yield in response to increases in temperature
- *Agricultural Economist* – has facts and data points to show that increase in temperature leads to drops in yield and increase in production costs/loss of profitability due to drops in yield

Concept Map Materials

Provide Post-It notes (7.6 × 7.6 cm) and Post-It note arrows along with large white self-stick chart paper (63.5 × 76.2 cm) for students to create easily edited concept maps for days 2 and 3 (**Figure 4-5**).

4.6 Game Play Specifics

Day 1: Introduction

The first day will require about 15 minutes of class time. The instructor should distribute specialty group identity cards equally throughout the class (i.e., in a class of 20, the instructor should provide five cards of each specialty). Each student must randomly choose a card from the

stack of cards to determine specialty positions. Alternatively, students can choose a number from 1 to 4 and the instructor can randomly assign each number with one specialty group. After specialty groups are determined, each student will be assigned the case study narrative (about the papaya ringspot virus epidemic) corresponding to their specialty.

Assessment: Students will read the case study narrative as homework and summarize how their assigned specialty group helped solve the agricultural problem presented in the papaya ringspot virus case study. Instructors can provide the case study narrative either through an online management system or as a hard copy for students.

Day 2: Specialist Group Summit

Goal: Student groups understand how their specialty group impacted agriculture for the current rice yield issue from their specialty lens.

1. Allow students to brainstorm (about 3–5 minutes). Instruct students to write down their thoughts incorporating what they have learned from the papaya case study and previous classroom knowledge regarding the relationships between climate change, agriculture, and plant disease.

• Ask students: How would you define climate change? What are some factors that can affect climate change? Do plants get sick? What factors do you think affect a plant's health?

2 Introduce the activity. Introduce the example of rice production in the Philippines, bacterial disease of rice, and the IRRI. To help with introduction, a video can be used to introduce the importance of rice and/or plant bacterial disease. For example, the IRRI video "Rice Is Life in Asia" could be used (about five minutes long).

3. Introduce food security specialist role and the Food Security Summit to which the students have been invited.

• Teacher introduction: “Welcome and thank you for meeting with us today, especially on such short notice! You have all been asked here today because of your help and expertise during the Hawaiian papaya ringspot virus incident. My name is _____ and I am a food security specialist working with the International Rice Research Institute. I have invited you all to meet here today because you are the top in your field and I would like to ask for your expertise, again, in helping us today. As a food security specialist, I study and anticipate when catastrophic food security events may occur and aim to stop this from happening. Recently, it has come to my attention that we are seeing more disease outbreaks in rice paddies in the Philippines and a decrease in rice yields. I fear that if rice yields continue to decrease this rapidly and we do not find a sustainable solution soon, countries that rely heavily on rice as a staple crop will be in danger of not being able to feed their people. As such, I hope that through collaboration of all your expertise we can identify the reasons for why we are seeing increased disease in rice production in the Philippines and come with solutions to solve this problem!”

4. Pass out specialty data cards to students based on the previously assigned specialties (~20 minutes). Ask students to take a moment to review their specialty cards. Designate different portions of the room for different specialty groups, then ask students to separate into specialty groups. Allow students to work together in their specialty groups to outline cause-and-effect relationships from the central idea of decreased rice yield. Information provided by students can be based on specialty group data card, previous knowledge, and/or assigned reading.

• Ask students: How does your specialty research impact rice yield in the Philippines? What factors have your specialty group identified that are contributing to the decrease in rice yield in the Philippines?

• Ask students to think in terms of cause and effect when interpreting their specialty research data points and facts.

• Ask student groups to develop a concept map for how their specialty is impacting rice production in the Philippines (i.e., the climatologist student group could correlate that increased temperature leads to lower yields). Provide students with an example for how to start a concept map (**Figure 4-6**).

Assessment: Ask students to list the factors that their specialty group decided on when creating a concept map. Then ask them to number these factors in order of importance to rice production in the Philippines (the greater the importance, the higher on the list). Inform students that they should bring this write-up for day 3.

Day 3: Food Security Summit

Goal: Student groups connect how changes in climate are impacting agriculture in the context of plant pathology, with specialty groups working together and sharing their knowledge to come up with a solution (i.e., determine possible policy solutions).

1. Interconnected concept maps (~30 minutes): Randomly split up students into new student groups composed of a minimum of one student specialist per group (four students total). For large classrooms, the maximum number of students per type of specialist should be two (eight students total per group).

• Allow each student to introduce their field of expertise or specialty. Then ask each student to describe the cause-and-effect relationships their specialty group outlined to the new group. Specialists in each group should have their assessment from day 2, which outlines, based on their specialty, the factors important to rice production in the Philippines.

• Ask student groups to create a concept map incorporating information from each "specialist" in relation to how this could be leading to a decrease in rice yield (**Figure 4-7**).

• Once student groups have created an interconnected concept map linking how each specialty impacts rice production, ask these groups to come up with a possible solution to stop this food security crisis. Ask students to write this separately from the concept map.

• Compare concept maps: Once all groups have completed the concept map, display the maps in the classroom and allow student groups to walk around to compare the different versions created.

3. Have a class discussion (~30 minutes) comparing and contrasting the interconnected concept maps. Prompt students to explore the importance of addressing the crisis and finding solutions.

• Ask students: How are environmental factors impacting the *Xoo* infection in rice? How is global climate change impacting rice production in the Philippines? Are there any ways we can reduce these expected negative impacts on agricultural systems? What are other aspects that we should be focusing on or considering? Why should we care about an epidemic that is happening in another country?

• Have each group propose a solution to the Philippines rice bacterial blight issue. As a class, determine pros/cons to each solution proposed. As a class, vote on the top two best (most feasible and thought-out) policy solutions.

Assessment: Assign students to write a brief paper, maximum one page, summarizing what they learned from the lesson and explaining the policy solution(s) their student group came up with during the class exercise and discussion.

4.7 Discussion

Here we have described an active-learning and peer-learning lesson plan that strives to teach students how to interpret and synthesize data from multiple disciplines to develop solutions to large problems. Throughout this exercise, students will learn that collaboration and data interpretation are key for developing a feasible solution to the proposed problem. By using an agricultural pathosystem, we aim to inform students how environmental conditions can impact economically important plant systems, the role scientists and experts play in policy making, and how international affairs are important for maintaining global food security.

This lesson plan was implemented in an upper-level plant physiology college laboratory course, and 35% of students (7 out of 20) reported coming into the exercise with deep prior knowledge of interactions between environmental or biotic stressors on plants. However, among students that came in with very little to some understanding, there was an 85% increase (11 of 13 students) in understanding these interactions after completing the exercise. As a result, the student groups developed multiple feasible policy changes to address the *Xoo*–rice scenario, including increasing biodiversity in rice fields, improving water management, and producing drought-tolerant rice to limit *Xoo* spread. Students also proposed improving international agricultural trade so that people can rely on rice production from various areas.

This activity can be applied to have students address issues across various fields. In an ecology course, the question of conservation of species can be addressed by using this model and synthesizing information from ecologists, conservationists, climatologists, and wildlife

organizations. Similarly, in a sociology course, students could ask how communities most impacted by climate change can adapt to sustain their livelihoods. Data can be interpreted by sociologists, economists, climatologists, and social workers. In any iteration of this model, the learning objectives of collaborative learning to interpret data and produce a solution are essential to addressing major questions affecting different fields in the context of climate change. Overall, this activity is an active-learning exercise designed to allow students not only to interpret data, but to develop a solution to the proposed problem through collaboration.

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4.8 Figures

Figure 4-1: Instructor's food security specialist card. The instructor will play the role of a specialist and act as a mediator in the Food Security Summit scenario.

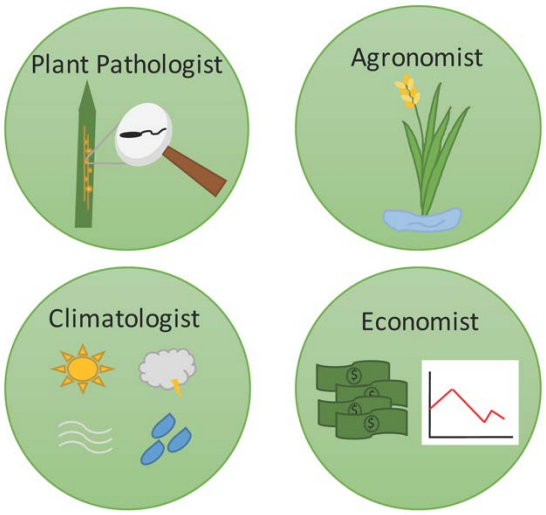

Figure 4-2: The four specialty group identity cards that will be assigned to students. Randomly assign students to a particular specialty. This will be the role they play in the Food Security Summit scenario.

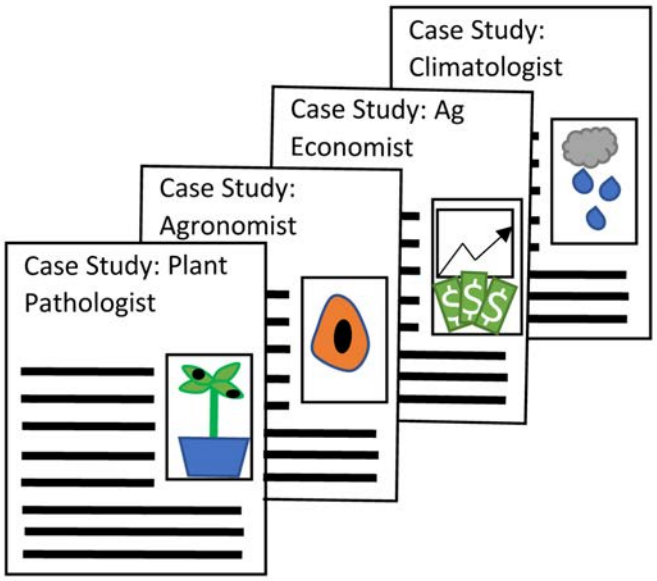

Figure 4-3: Examples of case study narrative for each specialty group. Each specialty will outline the Hawaiian papaya ringspot virus epidemic from the different specialty perspectives.

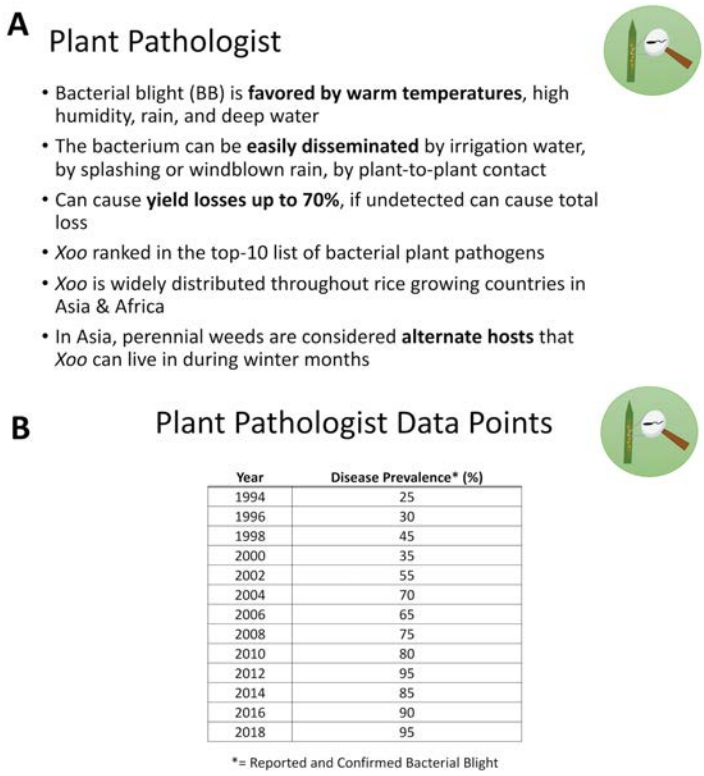

Figure 4-4: Example of a specialty group data card. Each specialty group will be given a data card with facts (**A**) and data points (**B**) specific to that specialty. Here is an example for the plant pathologist specialist group.

Figure 4-5: Example of a completed interconnected concept map by students. Providing Post-It notes for students allows the concept map to be more easily modified.

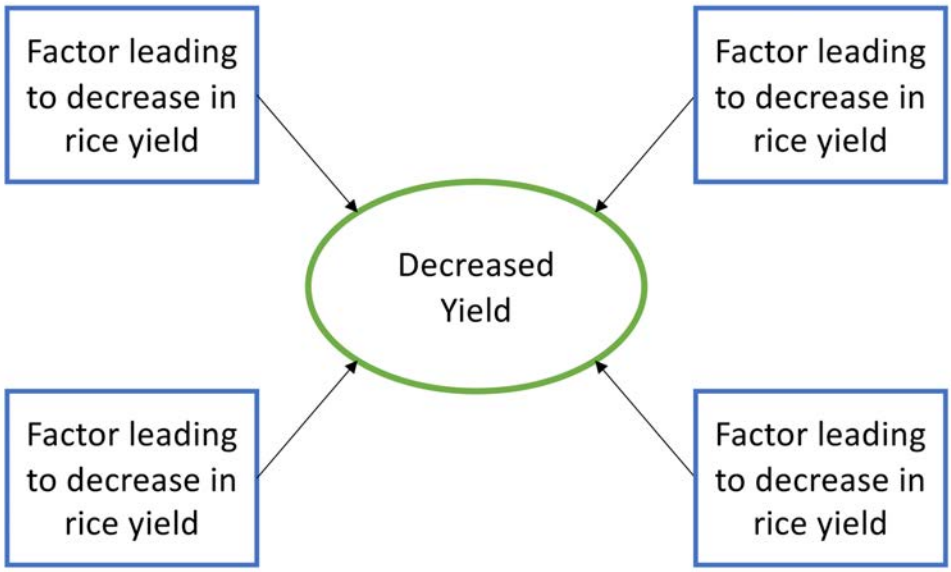

Figure 4-6: Example of how to start a concept map. After students have been separated into specialty groups, students will have to decide what factors most impact decreased yield from their specialty cards. factors most impact decreased yield from their specialty cards.

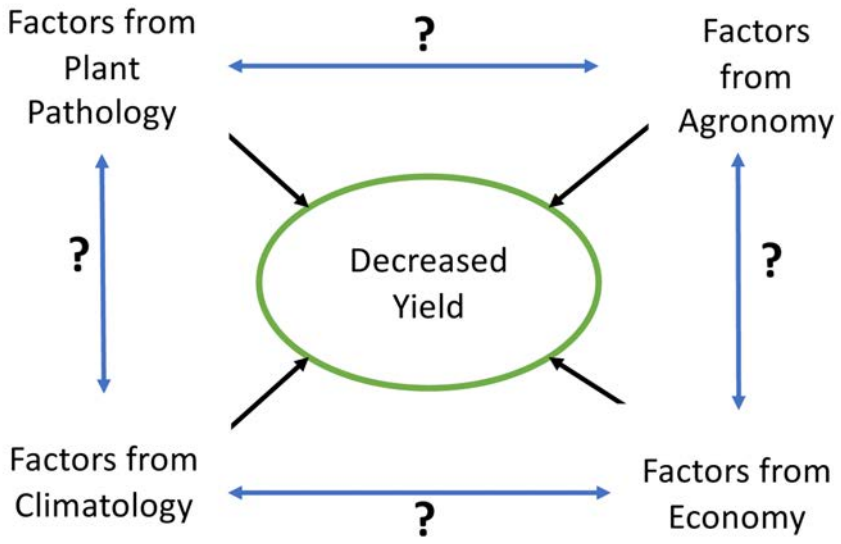

Figure 4-7: Example of how to form an interconnected concept map. Have students first determine which factors from each specialty group contribute to loss of rice yield, and second how these different factors interact between specialty groups. The model will help students conceptualize and visualize the purpose of the exercise.

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Chapter 5

Conclusions and Future Directions

As global temperatures continue to rise, agricultural plant systems are at risk of becoming more vulnerable to disease pressure. The observed increase in disease prevalence in response to increased temperature conditions is described here as heat-induced disease susceptibility. Without a clear understanding of how plants become more susceptible to disease pressure under high temperature conditions new management or engineering techniques cannot be implemented into our agricultural systems. This dissertation investigated the role the plant hormone cytokinin (CK) plays in the process of heat-induced disease susceptibility, to identify areas that future studies could target for loss of susceptibility approaches under high temperature conditions, as well as test a novel chemical approach. Additionally, this dissertation addresses using the topic of heat-induced disease susceptibly to teach future generations about this agronomic issue for implementation of improved agricultural practices and science policy.

In Chapter 2, a CK-signaling mutant was identified as less susceptible to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) infection under high temperature conditions. Simply put the CK-signaling mutant, *ahk2,3*, lost heat-induced disease susceptibility. Based on this, focus was placed on understanding the role CK was playing to promote heat-induced susceptibility of wild-type plants to the bacterial pathogen *Pst* DC3000. Pathogen *in vitro* and *in planta* assays showed that the increased *Pst* DC3000 proliferation at elevated temperatures is a plant-mediated process. When grown in liquid media *Pst* DC3000 proliferated more under normal temperature conditions. Whereas, *Pst* DC3000 proliferation *in planta* at 28ºC was consistent in wild-type plants, but plateaued at approximately 40 hours post inoculation in *ahk2,3* plants suggesting that this process is CK-dependent. Investigating the impact of high temperature

conditions on CK biosynthesis and signaling showed that the content of CK precursors and levels of signaling increased. CK signaling was also shown to increase based on gene expression analyses through qRT-PCR and RNA-Seq analysis, as well as utilizing a CK synthetic reporter line to visualize increases in CK-responding cells. CK signaling did not increase in response to high temperature and *Pst* DC3000 infection suggesting that the increase in CK signaling was an early heat stress response and may be priming the plant for pathogen infection. These findings were further supported by disease assays conducted in dexamethasone (DEX)-inducible CK degradation, *DEX::CKX2,* and CK biosynthesis, *DEX::IPT*, transgenic lines. Using these I was able to determine that an increase in CK-content is both necessary and sufficient for heat-induced susceptibility to occur, as constitutive breakdown of CK results in loss of heat-induced susceptibility but overproduction of CK is enough for this to occur. When investigating the underlying process that CK could be promoting to potentiate heat-induced disease susceptibility, I first assessed if defense responses such as stomatal immunity and defense gene expression were increased in the *ahk2,3* plants. My results indicate that Col-0 and *ahk2,3* plants exhibit a down-regulation of defense responses in response to high temperature conditions, suggesting that CK is promoting another physiological process. Further investigation through non-targeted metabolomics and autoradiography revealed that loss of susceptibility at 28ºC in *ahk2,3* is attributed to a decrease in available nutrients for *Pst* DC3000. Together our results support a model where CK promotes primary metabolic processes resulting in increased disease susceptibility under high temperature conditions and supports the inhibition of CK-mediated processes as a way to alleviate heat-induced disease susceptibility.

In Chapter 3, *Brassica napus* plants exhibited heat-induced disease susceptibility to the bacterial pathogen *P. syringae* pv. *maculicola* (*Psm* ES4326). Previous results from Chapter 2,

indicated that Arabidopsis, a member of the Brassicaceae family, exhibited heat-induced susceptibility to a bacterial pathogen, thus we addressed if this CK-mediated process was conserved in other Brassicaceae species. Thus, focus was placed on understanding if *B. napus* heat-induced susceptibility to *Psm* ES4326 was also dependent on the growth hormone CK. The results from these experiments showed that heat-induced susceptibility of *B. napus* is not only dependent on CK, but is also dependent on prior nitrogen fertilization. Plants not fertilized 5-7 days prior to inoculation with *Psm* ES4326, exhibited no difference in susceptibility at normal and high temperature conditions. When assessing if CK is important for this process *B. napus* plants exhibited similar gene expression patterns to Arabidopsis plants exposed to high temperatures. In response to 28ºC conditions, *B. napus* plants showed an increase in the CK-biosynthesis gene *BnIPT2* and a down-regulation of the CK-degradation gene *BnCKX1a*. Additionally, CK inhibition of root growth was seen in *B. napus* plants at normal and high temperature conditions, further supporting that *B. napus* plants respond similarly to CK application. To address the role of CK in *B. napus* I used a chemical approach, utilizing the CK-signaling inhibitor PI-55. Application of PI-55 lead to loss of susceptibility of *B. napus* at high temperature conditions and did not negatively affect vegetative growth. These results support the utilization of a CK-mediated chemical approach to alleviate the negative impacts of high temperature on *Brassica* crops.

In Chapter 4, using the agronomic issue of heat-induced disease susceptibility I created a lesson plan to teach younger generations about the negative impacts climate change can have on agricultural systems. The active learning lesson plan focused on using the example of increased rice susceptibility under high temperature conditions to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). For this activity, students are invited to a “Food Security Summit” at

The International Rice Research Institute, located in the Philippines. The Summit is meant to act as a conference, where students will come together as experts in their field (Plant Pathology, Climatology, Agronomy, Economics) and work together to synthesize data. The small group activity leads to a concept map that allows students to identify how their field of expertise relates with data of the other fields. Together students develop an agricultural policy to address the issues currently affecting global food security, based on the current heat-induced susceptibility scenario. Focusing this lesson plan on an international research station allow students to understand the importance of agriculture on a global scale, while also emphasizing the importance of collaboration and communication of scientists and experts in decision making.

Understanding how plants respond to high temperature conditions and the impact this has on plant-pathogen interactions is imperative. Future studies focusing on heat-induced susceptibility of plants to pathogens, should focus on a few target areas. These target areas include: (1) identifying resistance (*R*) genes with improved effectiveness under elevated temperature, (2) identifying susceptibility (S) genes activated by high temperature, and (3) optimizing use of hormone chemical inhibitor(s) for no fitness costs. Although not a scope of the work in this dissertation, previous studies have shown that high temperature conditions can lead to increased effectiveness of *R*-genes against various pathogens (Carter et al., 2009; Fu et al., 2009; Webb et al., 2010; Zhu et al., 2010; Zhao et al., 2016; Cohen et al., 2017). For example, the *R*-gene XA7 in rice that confers resistance to the bacterial pathogen *Xoo* is positively affected under high temperature conditions (i.e., is more robust and effective against *Xoo*) (Webb et al., 2010; Cohen et al., 2017). Thus, identifying effective *R*-genes under high temperature conditions, could be a viable breeding option for alleviating the negative impacts of climate change on agricultural systems.

Given the results in Chapter 2, we identified potential S genes, associated with primary metabolic pathways such as starch and trehalose, that could be important for heat-induced susceptibility of Arabidopsis to *Pst* DC3000. However, testing if disruption of these gene(s) results in loss of HIS is of upmost importance, as well as assessing if these genes have any developmental costs. Successful examples of identifying and targeting S genes, genes that facilitate pathogen infection (van Schie and Takken, 2014b), to achieve loss-of-susceptibility have been shown in various plants to different pathogens. For example, when the Arabidopsis S-gene *DMR6* (*DOWNEY MILDEW RESISTANCE 6*), previously identified to be required for susceptibility to the downy mildew pathogen *Hyaloperonospora parasitica* (van Damme et al., 2008), is disrupted this leads to broad spectrum resistance to various bacterial, oomycete, and fungal pathogens (Thomazella et al., 2021). Other examples have shown that disruption of the *MLO* (*MILDEW RESISTANCE LOCUS O*) gene provides barley plants with broad, durable resistance to the powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (Jorgensen, 1992; Büschges et al., 1997; Acevedo-Garcia et al., 2014) and provides pepper resistance to *Xanthomonas campestris* (Kim and Hwang, 2012). Additionally, other studies have shown that resistance to potato late blight disease increased after targeting and silencing multiple *S* genes important for *Phytophthora infestans* infection (Sun et al., 2016). Although targeting and disabling *S* genes is a promising alternative for breeding durable resistance, the fact that many *S* genes are also associated with other functions brings the possibility of pleiotropic effects, and therefore considerations of potential yield tradeoffs are also necessary (Pavan et al., 2010; van Schie and Takken, 2014b). Utilizing tissue specific promoters (Li et al., 2012) or inducible promoters (Kong et al., 2018; Leng et al., 2021) could be a viable way to activate loss-of-

susceptibility, by disabling *S* genes under high temperature conditions without major fitness costs to the plant (Lapin and Van den Ackerveken, 2013; van Schie and Takken, 2014a).

Given the possibility of pleiotropic effects of loss-of-susceptibility genetic approaches, other disease management techniques should be evaluated for ability to alleviate heat-induced susceptibility of crop plants. Given the results of Chapter 3, a single application of the PI-55 CK-signaling antagonist (Spíchal et al., 2009) prior to infection is enough to combat heat-induced disease susceptibility. However, although other studies in *B. napus* (Guo et al., 2017) and tomato (Costa et al., 2021) have confirmed the CK signaling inhibitory effect of PI-55, the long-term effects on plant growth and development has not yet been evaluated. Testing various PI-55 concentrations and growth parameters, including effect on reproductive tissues, will help elucidate if this is a viable approach for combating heat-induced disease susceptibility. Additionally, testing of other CK signaling inhibitors could also be promising. Use of the CK inhibitor LGR-991, 6-(2,5-Dihydroxybenzylamino)purine, developed after PI-55 and designed for broader specificity with less agonistic effects, might lead to a more optimized inhibitor to use for combating heat-induced disease susceptibility (Nisler et al., 2010).

Overall, breeding for loss-of-heat induced susceptibility will be of upmost importance as climate change is anticipated to have an overall negative effect on agricultural systems, making plants more vulnerable to disease. The identification of CK as a plant hormone involved in heat-induced disease susceptibility deepens our knowledge about this phenomenon in plants, and highlights CK-mediated metabolic processes that may be the target of future chemical and/or genetic approaches to combat plant diseases under conditions of climate change.

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Appendix A:

No Hormone to Rule Them All: Interactions of Plant Hormones During the Responses of Plants to Pathogens[4]

A.1 Summary

Plant hormones are essential regulators of plant growth and immunity. In the last few decades, a vast amount of information has been obtained detailing the role of different plant hormones in immunity, and how they work together to ultimately shape the outcomes of plant pathogen interactions. Here we provide an overview on the roles of the main classes of plant hormones in the regulation of plant immunity, highlighting their metabolic and signaling pathways and how plants and pathogens utilize these pathways to activate or suppress defence.

A.2 Introduction

Plant hormones, also known as phytohormones, are naturally occurring small, organic molecules that are not only important for plant developmental processes, but also play an integral role as signaling molecules in defence and immune responses. Salicylic acid, jasmonic acid and ethylene are the traditional hormones associated with defence responses against pathogens, but in the past decade several pieces of evidence demonstrate that abscisic acid, gibberellic acid, cytokinin, auxin and brassinosteroids, typically associated with abiotic stress or developmental processes, are also key components of the immune response of plants. It is now clear that no single hormone controls plant immunity; rather, plant hormones tend to act interdependently,

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through complex antagonistic or synergistic interactions. The results of these interactions are changes in plant physiology that culminate in an appropriate defence response against pathogen attack, or in the case of successful pathogens, to changes that benefit the invading pathogenic organism. Biotrophic pathogens, or those that acquire nutrients from living cells, have different host physiological requirements than necrotrophic pathogens, which use toxins and cell wall degrading enzymes to cause cell death and obtain their nutrients from dead tissue. Not surprisingly, the host hormonal balance required for resistance to pathogens of different lifestyles is distinct, and pathogens have evolved several different strategies to shift this balance to their benefit.

In this review we discuss the role of the major classes of plant hormones in plant immunity, and whether they act as positive or negative regulators of defence responses. Given the vast literature on this topic, we focus mainly on examples of action of hormones in plant immunity on the model plant species Arabidopsis, while also citing hormone action in other plant species as possible and appropriate. To further contribute to the understanding of the roles of plant hormones in immunity, we also discuss hormone biosynthesis and signal transduction pathways, as well as their manipulation by pathogen effectors.

A.3 The Master Rings: Key Hormones in Plant Immunity

Salicylic Acid

Salicylic acid (SA) is a phenolic compound with plant hormone activity, that is most recognized as an important endogenous signaling molecule in plant immunity. However, SA has also been documented to be indirectly involved in germination, flowering, mitochondrial electron transport and abiotic stress resistance, including thermotolerance (Metwally et al., 2003;

Clarke et al., 2004; Martinez et al., 2004; Norman et al., 2004; Rajjou et al., 2006). The first indication that SA was associated with tolerance to biotic stress came from studies where application of SA to tobacco plants led to increased resistance against *TOBACCO MOSAIC VIRUS* (TMV) and increased accumulation of pathogenesis-related (PR) proteins (Malamy et al., 1990). This protective effect of SA was observed not only on tobacco, but on several other monocotyledonous and dicotyledonous plant species against a variety of biotrophic plant pathogens (Klessig and Malamy, 1994). In addition, *in vitro* experiments demonstrated that this activity was due to plant-specific processes, rather than a direct killing activity of SA on pathogens (Mills and Wood, 1984). SA levels were also found to accumulate at sites of pathogen infection, and a correlation was observed between SA accumulation and resistance to pathogenic attack. The similarity between the effects of SA application and pathogen attack on plant physiology led to the suggestion that SA was a signal for activation of defence against plant viruses (Malamy et al., 1990). These findings were later extended to other pathosystems and SA was determined a signal for defence to biotrophic pathogens in general (Vlot et al., 2009).

SA is derived from the primary metabolite chorismate, by way of two major enzymatic pathways, one involving the phenylalanine ammonia lyase pathway, and another which involves a two-step process metabolized by the enzymes ISOCHORISMATE SYNTHASE (ICS), which converts chorismate to isochorismate, and ISOCHORISMATE PYRUVATE LYASE (IPL), which catalyzes the conversion of isochorismate into SA (Strawn et al., 2007). During the response to pathogens, plants preferentially employ the isochorismate pathway (Wildermuth et al., 2001). Once formed, SA accumulates both at the site of infection and systemically (Metraux et al., 1990; Ward et al., 1991; Uknes, 1993). SA and/or a derivative of SA is typically required for innate immune responses (Pathogen-Associated Molecular Patterns (PAMP)-triggered

immunity or PTI; Effector-Triggered Immunity or ETI) (Dodds and Rathjen, 2010), localized resistance responses such as expression of *PATHOGENESIS-RELATED* (*PR*) genes and activation of programmed cell death, as well as for systemic acquired resistance (SAR), a form of broad-spectrum resistance to biotrophic pathogens that can act in both local and distal plant tissues (Gaffney et al., 1993; Ryals et al., 1996). In Arabidopsis, mutant plants lacking a functional ISOCHORISMATE SYNTHASE (ICS1) enzyme, *sid2*/*eds16*, fail to accumulate SA during pathogenic interactions, indicating that this enzyme is necessary for the majority of pathogen-induced SA biosynthesis (Dewdney et al., 2000; Wildermuth et al., 2001). While SA is biosynthesized in the chloroplasts, after biosynthesis most SA can be readily converted into a biologically inactive form, SA β-glucoside (SAG) (Hennig et al., 1993), by a pathogen-inducible SA β-GLUCOSYLTRANSFERASE (SAGT) in the cytosol (Dean et al., 2003). SAG biosynthesis is followed by transport to the vacuole (Dean et al., 2003; Dean and Mills, 2004; Dean et al., 2005), where it is stored until conversion back to biologically active SA(Hennig et al., 1993). SA can also be methylated into an inactive volatile form, methyl SA (MeSA), through the enzymes SA METHYL TRANSFERASE (SAMT) and SA/BENZOIC ACID METHYL TRANSFERASE (BSMT) (Chen et al., 2003; Liu et al., 2010).

The first studies to demonstrate the importance of SA in plant immunity used transgenic tobacco and Arabidopsis plants expressing the *nahG* transgene, encoding the bacterial SA-degrading enzyme salicylate hydroxylase (Gaffney et al., 1993; Delaney et al., 1994). *nahG* plants failed to accumulate SA and displayed increased susceptibility to biotrophic pathogens. Further, these plants failed to active SAR, implicating SA accumulation in systemic resistance to pathogens (Delaney et al., 1994). Exogenous application of SA or SA analogues to *nahG* plants restored resistance both locally and systemically, as well as the expression of *PR-1*, a known

marker of disease resistance to biotrophic pathogens (Delaney et al., 1994). In the early 1990's, several genetic screens for Arabidopsis mutants impaired in SAR, showing increased susceptibility to pathogens or displaying altered responses to SA led to the identification of different alleles of the *NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1)*, now known to be a master regulator of SA-mediated defence responses (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). *nahG* and *npr1* plants both showed increased susceptibility to biotrophic pathogens, including TMV, the oomycetes *Hyaloperonospora arabidopsidis* (*Hpa*, formerly *Peronospora parasitica*) and *Phytophthora parasitica*, as well as several bacterial pathogens such as *Pseudomonas syringae* pv. *tabaci* and *Pseudomonas syringae* pv. *maculicola* ES4326 (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996), but decreased susceptibility to necrotrophic pathogens such as the fungi *Botrytis cinerea* and *Alternaria brassicicola* (Thomma et al., 1998). Collectively, these results established a model where SA is an important positive regulator of immunity to biotrophic pathogens, but a negative regulator of immunity to necrotrophic pathogens.

The identification of NPR1 was a first step in the elucidation of the SA signaling pathway. Cloning of the *NPR1* gene revealed that it encoded a protein with ankyrin repeats, as well as BTB/POZ repeats (Cao et al., 1997; Ryals et al., 1997), domains known to mediate protein-protein interactions. Yeast two-hybrid screens identified proteins from the TGA family of bZIP transcription factors and the family of nuclear localized NIMIN1 proteins as NPR1-interacting proteins (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Weigel et al., 2001; Kim and Delaney, 2002), implicating a function for NPR1 in the control of gene expression. Further studies solidified the function of NPR1 as a transcription co-activator of defence gene expression. Upon pathogen perception, SA biosynthesis leads to activation of

thioredoxins, which act in the reduction of conserved cysteine residues of NPR1, changing its conformation from an oligomeric to a monomeric state, and leading to its re-localization from the cytosol to the nucleus (Kinkema et al., 2000; Mou et al., 2003; Tada et al., 2008). In the nucleus NPR1 can interact with TGA factors, which play a mostly redundant function at the genetic level, but have individual differing roles in defence activation (Kesarwani et al., 2007). For example, TGA3 and TGA6 have been shown to increase SA-dependent *PR-1* gene expression, while TGA2 will bind to the *PR-1* promoter region through the help of NPR1 and SA to repress gene expression (Despres et al., 2000; Johnson et al., 2003). NINIM proteins act mostly as negative regulators of NPR1 function, since their overexpression results in decreased SA-mediated immune responses, possibly through their EAR (ERF-Associated Amphiphilic Repression) repression domain, and the opposite effect is seen in *nimin* mutants (Weigel et al., 2005). Finally, turnover of nuclear NPR1 by the 26S proteasome pathway was shown to be essential for its co-activator function. This fine-tunes NPR1 availability to transcription factors, therefore controlling SA-dependent defence gene expression (Spoel et al., 2003).

While increasingly more was learned about the function of NPR1 in SA signaling, the identity of any SA receptors remained elusive. Several SA-binding proteins were identified through biochemical approaches, however their function as SA receptors could not be supported. A breakthrough came when NPR1 paralogues NPR3 and NPR4, and NPR1 itself, were found to bind SA and function as SA receptors (Fu et al., 2012; Wu et al., 2012). In a search for possible regulators of NPR1 proteosomal degradation, the authors considered the NPR1 paralogues NPR3 and NPR4 as possible candidates, because both contained BTB and ankyrin domains, which are typical of CUL3 ubiquitin E3-ligase substrate adaptors in SKP1-Cullin-F-box (SCF) complexes that participate in proteasomal degradation (Fu et al., 2012; Wu et al., 2012). Analysis of the

npr3 npr4 plants revealed that the NPR1 protein accumulated in much higher levels in these double mutants than in wild type plants and yeast two-hybrid analyses proved that these three proteins physically interacted. Given the conserved theme in plant biology of hormone receptor-driven proteasomal degradation of negative regulators, the authors then hypothesized that NPR3 and NPR4 acted as SA-receptors, which upon binding to SA would mediate NPR1 turnover through the proteasome. This hypothesis was later confirmed through biochemical assays, where NPR3 and NPR4 were shown bind to SA with low and high affinities, respectively (Fu et al., 2012). Further, biochemical assays using equilibrium dialysis showed that NPR1 could also bind to [^{14}C]-labeled SA, with affinities similar to other plant hormone ligand-receptor interactions (Wu et al., 2012). Mutations affecting the BTB/POZ domain or in two conserved cysteine residues ($Cys^{521/529}$), which were previously known to affect NPR1 activity *in vivo* (Rochon et al., 2006), also abolish the ability of NPR1 to bind SA *in vitro* (Wu et al., 2012). The binding of SA to NPR1 protein was determined to occur through the $Cys^{521/529}$ residues, and to require a transition metal cofactor (Wu et al., 2012).

Given its importance to plant immunity, the SA pathway is commonly targeted by pathogens that manipulate the signaling or synthesis of SA in order to increase virulence and lower host resistance (Tanaka et al., 2015). Many Gram-negative bacteria use type III secretion systems (T3SS) to directly inject type III secreted effectors (T3SEs) into the cytoplasm of the host plants, targeting various components of plant defence pathways (Lewis et al., 2009). The highly conserved T3SEs AvrE and HopM1 from *Pseudomonas* spp. are important suppressors of SA responses to biotrophic pathogens. Loss-of-function mutations in these effectors lead to decreased bacterial virulence (DebRoy et al., 2004). The conservation of this effector family in a variety of pathogenic bacteria suggests that suppression of the SA pathway is a common

virulence strategy. Similarly, XopJ, an effector from the biotrophic bacterial pathogen *Xanthomonas euvesicatoria*, can interfere with NPR1 activity by interacting with the host protease RPT6, an important component of the proteasome pathway that regulates NPR1 turnover, and consequently hinder SA signaling (Ustun et al., 2013). In addition, a toxin secreted by some strains of *Pseudomonas syringae* pv. *syringae*, syringolin A, suppresses the SA pathway by acting as a proteasome inhibitor that inhibits NPR1 turnover (Schellenberg et al., 2010).

Effectors from pathogenic oomycetes and fungi also target the SA pathway. A study by Caillaud et al. (2012) identified 15-nuclear *Hpa* RxLR effectors, HaRxLs, which both directly and indirectly associate with nuclear components to suppress PTI and increase pathogenic growth of *Hpa* (Caillaud et al., 2012). HaRxLs also target components of the Mediator complex (MED), a protein complex that functions as a bridge between specific transcription factors and the core transcriptional machinery in eukaryotes (Kidd et al., 2011). Given their key role in transcription regulation, MED complex subunits play an integral role in SA-mediated defence gene expression (Canet et al., 2012; Zhang et al., 2012; Zhang et al., 2013), and therefore are targets of effector proteins (Canet et al., 2012; Zhang et al., 2012; Zhang et al., 2013). The *Hpa* effector HaRxL44 decreases *PR-1* expression in cells infected with *Hpa* by interacting with the Mediator complex subunit MED19a and targeting it for degradation through a proteasome-dependent pathway, decreasing SA-mediated responses and enhancing *Hpa* pathogenicity (Caillaud et al., 2013).

Other effectors target SA biosynthetic pathways instead of SA signaling. For example, the biotrophic smut fungus, *Ustilago maydis*, interferes with the SA-pathway through the Cmu1 effector. Cmu1 functions as a chorismate mutase enzyme that coverts chorismate to prephenate, depleting chorismate levels for conversion to SA by ICS, and ultimately preventing the host from

accumulating SA in response to infection (Djamei et al., 2011). Interestingly, Liu et al. (2014) showed that other fungal (*Verticillium dahliae*), and oomycete species (*Phytophthora sojae*) contain isochorismatase effectors, Vdlsc1 and Pslsc1, respectively (Liu et al., 2014). In order to achieve full virulence the fungal and oomycete species break down isochorismate to interrupt the SA metabolic pathway and repress SA-mediated host immunity (Liu et al., 2014).

Jasmonic Acid:

Jasmonic acid (JA) is a lipid-derived signaling molecule involved in various developmental and defence plant processes (Pieterse et al., 2012; Santino et al., 2013). Early studies revealed that exogenous application of JA or JA-derivatives, but not SA or SA-derivatives, resulted in an over-production of the defence-related proteins defensins and thionins, which are normally induced by necrotrophic pathogens (Epple et al., 1995; Penninckx et al., 1996; Penninckx et al., 1998). Studies with Arabidopsis mutants with impaired JA signaling also validated the importance of the JA signaling pathway in conferring resistance to necrotrophic pathogens, reaffirming that JA is a positive regulator of immunity in regards to necrotrophic pathogens and a negative regulator in response to biotrophic pathogens (Thomma et al., 1998).

The first step in JA biosynthesis is the release of α-linolenic acid from chloroplast membranes, followed by oxygenation by LIPOXYGENASE (LOX) enzymes (Wasternack and Hause, 2013). Wounding or pathogen attack is followed by significant up-regulation of JA-responsive genes, including *LOX* genes (Wasternack and Hause, 2013; Ranjan et al., 2015). Once oxygenated, α-linolenic acid is converted into JA, which is then rapidly modified into several JA-derivatives (Wasternack and Hause, 2013). One of these derivatives, (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), synthesized through isoleucine conjugation to JA by the enzyme

JASMONOYL ISOLEUCINE CONJUGATE SYNTHASE (JAR1)/ JA AMINO SYNTHASE (Staswick and Tiryaki, 2004), was shown to act as the endogenous biologically active jasmonate in plant cells (Fonseca et al., 2009). JA can also be converted into the plant volatile methyl-jasmonate (MeJA), through methylation by the enzyme JA CARBOXYL METHYLTRANSFERASE (JMT) (Seo et al., 2001). Plants overexpressing the gene encoding JMT display increased resistance to the necrotrophic fungus *B. cinerea*, and up-regulation of the JA-responsive genes such as *PLANT DEFENSIN1.2* gene (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*), providing evidence that MeJA acts as a signaling molecule in plant immunity to necrotrophic pathogens (Seo et al., 2001).

JA perception is mediated by the E3-ligase SCF F-box protein CORONATINE INSENSITIVE 1 (COI1), which functions as the JA receptor, along with the JASMONATE ZIM-domain (JAZ) transcriptional repressor proteins (Yan et al., 2009; Sheard et al., 2010). In the absence of JA, JAZ proteins function as transcriptional repressors by association with the adaptor proteins NINJA (NOVEL INTERACTOR OF JAZ) and the general transcriptional repressor TOPLESS (TPL) (Pauwels et al., 2010). After JAZ associates with TPL through the EAR domain, chromatin modification is initiated through recruitment of histone modifying enzymes HISTONE DEACETYLASE 6 (HDA6), HISTONE DEACETYLASE 9 (HDA9) and HISTONE METHYLTRANSFERASE (HMT), to repress JA-dependent gene expression. The JAZ-NINJA-TPL complex also associates with JA-regulated basic helix-loop-helix transcription factors (MYC2, MYC3 and MYC4), preventing them from activating JA-responsive genes (Fernandez-Calvo et al., 2011). Upon pathogen induction, binding of bioactive JA to COI1 recruits JAZ proteins to the SCF^{COI1} complex, resulting in JAZ degradation through the 26S proteasome pathway. This results in the release of MYC transcription factors, which initiate

transcription activity of JA-regulated genes such as *VSP2* (Thines et al., 2007). Another branch of the JA pathway regulates the expression of the JA-responsive genes involved in defence against necrotrophic pathogens, such as *PDF1.2*. This part of the JA pathway is regulated by transcription factors from the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors, such as the OCTADENOID-RESPONSIVE ARABIDOPSIS59 (ORA59) and ERF1, in conjunction with components of ethylene signaling (discussed in detail in the Ethylene section) (Zhu et al., 2011). Lastly, JAZ repressor proteins can directly bind through their Jas motif to a subunit of the Mediator complex, MED25, which participates in the transcription process of JA-responsive genes during pathogen attack (Kidd et al., 2009; Cevik et al., 2012; Chen et al., 2012; Zhang et al., 2015). The JAZ –MED25 interaction inhibits MYC3 from directly binding to MED25 and initiating the transcription of JA response genes (Zhang et al., 2015).

During immunity to biotrophic pathogens, JA is mostly known for its antagonistic action with the SA pathway. The interaction of these two hormones is considered the hormone backbone of plant immune responses to pathogens, shifting defence responses to either the SA or JA pathway, depending on the lifestyle of the particular invading pathogen (Pieterse et al., 2012). Consequently, biotrophic pathogens have evolved to target host proteins to positively regulate the JA pathway, repressing the SA pathway to decrease host resistance and increase virulence. A well-researched example is the phytotoxin coronatine (COR), produced by several *P. syringae* pathovars, which is an essential component of *P. syringae* pathogenicity and virulence (Brooks et al., 2005; Uppalapati et al., 2007; Zheng et al., 2012). COR is also a JA-Ile structural mimic, and therefore can act as competitor with JA for binding to the COI receptor. Binding of COR to COI1 initiates JAZ protein degradation, up-regulating JA signaling and repressing the SA

pathway (Katsir et al., 2008). Several *Pseudomonas syringae* pathovars also use this JA mimicry strategy to regulate stomatal immunity. Closing of stomata is an SA-mediated PTI response and secretion of COR stimulates the reopening of the stomata allowing bacteria to enter the host (Melotto et al., 2006). Recently, the COR action on SA was discovered to be mediated by three NAC transcription factors, ANAC019, ANAC055 and ANAC072, which are targets of the JA-regulated transcription factor MYC2. Activation of MYC2 leads to the initiation of a transcriptional cascade involving ANAC019, ANAC055 and ANAC072, which act to down-regulate the expression of *ICS1*, and up-regulate the expression of the methyltransferase *BSMT1*, ultimately resulting in decreased levels of biologically active SA (Zheng et al., 2012).

Pathovars of *P. syringae* that do not produce COR can similarly subvert the JA pathway for their own benefit. An interesting example is the conserved effector protein HopX1, a cysteine protease present in several COR-lacking *P. syringae* pathovars, including *P. syringae* pv. *tabaci*. Upon secretion into plant cells, HopX1 targets JAZ proteins for degradation through its protease activity, resulting in the activation of JA signaling in plant cells (Gimenez-Ibanez et al., 2014). Similarly, some effectors can target JA signaling independently of COR. HopZ1, a *P. syringae* effector with putative acetyltransferase activity, can interact with the ZIM domain of JAZ proteins, leading to their acetylation and proteasome degradation, activating JA signaling to increase pathogen virulence (Jiang et al., 2013). Finally, pathogens other than *P. syringae* have successfully developed tactics to use the JA pathway and antagonize SA responses to their advantage. For example, Brodhun et al. (2013) showed that the hemi-biotrophic fungal pathogen *Fusarium oxysporum* can produce bioactive forms of JA through an iron 13S-LIPOXYGENASE similar to the LOX enzymes utilized by plants for JA biosynthesis, allowing the fungus to inhibit the SA pathway and SA-regulated defences (Brodhun et al., 2013).

Ethylene:

The gaseous plant hormone ethylene (ET) is considered an important component of the immune response of plants to pathogens. ET is synthesized from the amino acid methionine, which is converted to S-adenosylmethionine (SAM) by the enzyme SAM SYNTHASE. SAM is then converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC SYNTHASE (ACS), in what is considered the most rate-limiting step in ET biosynthesis. ACC is then converted to ET in a reaction catalyzed by ACC OXIDASE (ACO) (Argueso et al., 2007). Pathogen infection can alter ET biosynthesis at several levels. For instance, *B. cinerea* infection of Arabidopsis can promote ET production through the activity of isoforms of ACC SYNTHASE (ACS2, ACS6), through a mechanism controlled by mitogen activated protein kinases MAPK3/6 (Han et al., 2010). In addition, genes encoding ACO can also be transcriptionally up-regulated, resulting in increased ET biosynthesis. This is the typical case during fruit development in tomato, and also true in response to pathogen attack (Argueso et al., 2007). In Arabidopsis, genes encoding ACO are up-regulated by infection with *B. cinerea*, but down-regulated following inoculation with *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Cohn and Martin, 2005; Broekaert et al., 2006).

ET signaling in plants is initiated by binding of ET to one or more of its receptors, ETHYLENE RESPONSE 1 (ETR1), ETHYLENE RESPONSE 2 (ETR2), ETHYLENE RESPONSE SENSOR 1 (ERS1), ETHYLENE RESPONSE SENSOR 2 (ERS2) and ETHYLENE INSENSITIVE 4 (EIN4), which are hybrid histidine kinases localized on the endoplasmic reticulum membrane. Binding of ET to receptors inhibits a downstream protein Ser/Thr kinase, CTR1, which acts as a negative regulator of ET signaling. Inhibition of CTR1 leads to a suppression of its ability to phosphorylate ETHYLENE INSENSITIVE 2 (EIN2), a

key positive regulator of the ethylene pathway. Finally, lack of EIN2 phosphorylation leads to the translocation of its C-terminal part to the nucleus, resulting in the stabilization of ETHYLENE INSENSITIVE 3 (EIN3)/ EIN3-LIKE (EIL) transcription factors that are responsible for the transcription of ethylene-regulated genes (Merchante et al., 2013). Studies with the ethylene signaling mutants *etr1* and *ein2* showed that similarly to JA, ET has a negative, albeit modest, effect on the resistance of Arabidopsis to the biotrophic pathogen *Hpa* (Lawton et al., 1994; Lawton et al., 1995). These results suggest that the main role of ET in response to biotrophs is primarily associated to its synergistic interaction with JA, and consequent antagonistic action on the SA pathway. The first evidence of a synergistic action by JA and ET in immunity came from analyses of the expression of *PDF1.2*. Expression of *PDF1.2* is induced by necrotrophic pathogens, such as *A. brassicicola*, as well as by exogenous application of either ET or JA, however this induction was either reduced or abolished in ethylene receptor mutants (Penninckx et al., 1996; Penninckx et al., 1998). Further, concomitant application of JA and ET induces expression of JA- and ET-regulated genes to a much higher level than application of either hormone alone (Penninckx et al., 1998). The mechanisms mediating the synergy between ET and JA are beginning to be uncovered. JAZ proteins are negative regulators of EIN3/EIL transcription factors, in a mechanism involving the histone deacetylase HDA6. JAZ degradation upon JA perception releases HDA6 repression on EIN3/EIL1, leading to the activation of JA-regulated transcription factors, such as ERF1 and ORA59, and synergistic activation of ET- and JA-regulated genes, including *PDF1.2* (Zhu et al., 2011). ET and JA pathways can also work independently of each other. The increase in expression of the JA-regulated gene *VSP2* by the necrotrophic pathogen *Pectobacterium carotovorum* (formerly *Erwinia carotovora*) is independent of ethylene signaling (Norman-Setterblad et al., 2000), relying on the MYC2

pathway. Similarly, genome-wide expression studies have shown a significant, but not complete, overlap between genes regulated by these hormones and by necrotrophic pathogens (Schenk et al., 2000).

Similar to other phytohormones ET has become a target of pathogens in an attempt to reduce host defences. In *Ralstonia solanacearum*, the HrpG protein acts as a key regulator of transcription of T3SS components (Valls et al., 2006). In addition, HrpG also regulates the expression of T3SS-independent targets, including genes involved in bacterial ethylene production. Production of ethylene by *R. solanacearum* was shown to down-regulate plant defence genes, ultimately increasing pathogen virulence (Valls et al., 2006). Further, the T3SE XopD from *Xanthomonas euvesicatoria* targets the ethylene-inducible tomato transcription factor SlEFR4 to suppress ethylene production and increase pathogen virulence (Kim et al., 2013). Studies have also shown that the *Pst* effectors AvrPto and AvrPtoB stimulate virulence by increasing ethylene production after infection, suppressing innate defence pathways through activation of the ACO-encoding genes *LeACO1/2* (Cohn and Martin, 2005). In support for a role for ethylene in pathogen virulence, several *R. solanacearum* and many *P. syringae* pathovars produce ET conjugates both *in vitro* and *in planta*, and mutants in these ET-forming genes have reduced ability to cause disease (Weingart and Volksch, 1997; Weingart et al., 1999; Weingart et al., 2001).

A.4 Between an Axe and a Sword: Stress and Growth Hormones in Plant Immunity

As plants respond to pathogen attack by activating the SA and JA/ET pathways and turning on defence responses, other hormone signaling pathways also get activated. These pathways, regulated by hormones most often associated with growth and abiotic stress responses,

are believed to fine-tune the responses to pathogens, turning on the appropriate physiological responses required to fight the specific invading organism. As pathogens manipulate host physiology to create conditions that enhance their chances of success and reproduction, they are also likely to target plant developmental pathways controlled by growth hormones, generating a parallel response in these developmental pathways by the plant. In the sections below we highlight the role of the plant hormones most known for the function in plant growth and abiotic stress response, in the context of plant-pathogen interactions.

Abscisic Acid

The plant hormone abscisic acid (ABA) has long been associated with responses to abiotic stresses, especially drought and salinity. However, only in the last decade has its function in biotic stress been elucidated. ABA biosynthesis occurs mostly in plastids, and is initiated by the conversion of the carotenoid zeaxanthin to *trans*-violaxanthin, by the enzyme ZEAXANTHIN EPOXIDASE (ZEP) (Marin et al., 1996; Schwartz et al., 1997). An yet unidentified enzymatic activity catalyzes the conversion of all-*trans*-violaxanthin to 9-*cis*-violaxanthin (North et al., 2007). This step is then followed by cleavage into xanthoxin by 9-*cis*-EPOXYCAROTENOID DIOXYGENASES (NCED), in what is considered the rate-limiting step of ABA biosynthesis (Iuchi et al., 2001). In the cytosol, xanthoxin is converted to abscisic aldehyde by a SHORT CHAIN DEHYDROGENASE (SDR), and then oxidized to ABA by the enzyme ALDEHYDE OXIDASE (AAO) (Seo et al., 2000). The last step in the ABA biosynthetic pathway consists on the addition of a molybdenum cofactor to ABA, by the enzyme MOLYBDENUM COFACTOR SULFURASE (MOCO/ABA3) (Bittner et al., 2001).

ABA signaling in plants involves perception by a receptor complex formed by

PYRABACTIN RESISTANCE 1 (PYR) and PYR1-LIKE (PYL) proteins, also known as REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR). In the absence of ABA, PYR/PYL/RCAR proteins form dimers, which are dissociated upon binding to ABA. Dimer dissociation leads to conformational changes on PYR/PYL/RCAR proteins and the formation of a binding site for PHOSPHATASE TYPE 2 C (PP2Cs) proteins. Binding of PP2C to PYR/PYL/RCAR is followed by the release of PP2C targets SNF1-RELATED KINASES (SnRK2s). Released SnRK2s can then move to the nucleus and phosphorylate the ABA RESPONSIVE ELEMENT BINDING FACTOR/PROTEINS (ABF/AREB) class of bZIP transcription factors that activate the expression of ABA-regulated genes, positively regulating ABA physiological outputs (Hauser et al., 2011).

The first indication of a role for ABA in defence responses to pathogens came from observations of altered host susceptibility to pathogens on plants treated with ABA. Application of ABA to potato plants led to reduced phytoalexin production and consequently to decreased resistance to *Phytophthora infestans* (Henfling et al., 1980). Similarly, application of ABA led to increased susceptibility of tobacco plants to *Peronospora tabacina* (Salt et al., 1986). Further experiments showed that ABA application could regulate defence gene expression, as in the treatment of tobacco cell cultures with ABA, which leads to down-regulation of the *PR-2* gene (Rezzonico et al., 1998). These initial studies led to the conclusion that ABA acted by increasing plant susceptibility to pathogens, especially to fungi.

Additional experiments with Arabidopsis revealed a more complex role of ABA in plant immunity. Application of ABA to Arabidopsis plants leads to increased growth of *Pst* DC3000, and a similar increase in susceptibility was observed in mutants with increased ABA signaling and content(de Torres-Zabala et al., 2007; Fan et al., 2009). Other studies, however, reported that

application of ABA led to decreased susceptibility to *Pst* DC3000 (Mohr and Cahill, 2003) or that it did not have any effect on susceptibility to this pathogen (Yasuda et al., 2008). Similarly, ABA can have either a positive and negative role in the regulation of JA signaling and resistance to necrotrophs. Application of ABA to plants leads to reduced expression of *PDF1.2* and the JA- and ET-induced *LEGUME LECTIN-LIKE PROTEIN (LEC AT3G15356)* (Anderson et al., 2004), but to increased callose deposition and resistance to *A. brassicicola* (Flors et al., 2008). Arabidopsis *abi4* mutants impaired in ABA signaling due to a mutation of the AP2/DREB transcription factor involved in ABA signaling, as well mutants in the genes encoding the ABA biosynthetic enzymes AAO and SDR, show increased susceptibility to both *Pythium irregulare* and *A. brassicicola,* but not to another necrotrophic pathogen, *B. cinerea* (Adie et al., 2007).

These confounding results on the role of ABA on resistance to biotrophic and necrotrophic pathogens may be explained as consequences of different environmental conditions during experiments, as it was shown that abiotic stress mediated by ABA can suppress SA-mediated defence responses(Yasuda et al., 2008; Kusajima et al., 2010). Increased SA content or signaling was also found to have a negative effect on ABA sensitivity and responses (Mosher et al., 2010; Kim et al., 2011). Chemical genetics screens also revealed that SA interferes with ABA signaling downstream of ABA perception by PYR/PYL/RCAR proteins, by disrupting cytosolic Ca^{2+} signaling activated by ABA perception (Kim et al., 2011). Together these results suggest an antagonistic interaction between ABA and SA, and a parallel interplay between SA-mediated biotic and ABA-mediated abiotic stresses.

How exactly ABA controls responses to both biotic and abiotic stresses is still unknown. It is interesting to note that both drought stress and pathogen attack lead to the up-regulation of genes encoding NCED enzymes (Iuchi et al., 2001; de Torres-Zabala et al., 2007; Fan et al.,

2009), pointing to a crosstalk between biotic and abiotic stresses at the level of transcriptional regulation of ABA biosynthesis. The nature of regulators involved in the reciprocal repression of SA- and JA-mediated responses by ABA is unknown, although some candidates have been uncovered, such as the rice MAPK OsMAPK5. Silencing of *OsMAPK5* leads to increased defence response and disease resistance, but also to decreased abiotic tolerance (Xiong and Yang, 2003), implicating it as a key switch in the inverse regulation of biotic or abiotic stress by ABA (Xiong and Yang, 2003). Most recently, the Mediator complex subunit MED25, involved in the positive regulation of JA signaling through association with MYC2 (Cevik et al., 2012), was shown to also physically interact with the bZIP transcription factor ABA-INSENSITIVE 5 (ABI5) and repress the transcription of ABA-regulated genes (Chen et al., 2012). Therefore, MED25 can be considered an interaction node between the JA and ABA signaling pathways. How this interaction could affect SA-dependent responses is unclear, however it is possible to hypothesize that recruiting of MED25 away from the JA pathway, and towards the ABA signaling pathway, could result in less signaling through the JA pathway, and consequently in the up-regulation of SA-dependent responses.

Finally, another important role for ABA in the regulation of defence responses to pathogens comes from its pivotal function in the control of stomatal aperture during plant transpiration. ABA-mediated stomatal closure is a common plant defence response to PAMPs and pathogens, aimed at preventing pathogen entry into plant tissues (Melotto et al., 2006; Zhang et al., 2008; Zeng and He, 2010). As in the case of other plant hormones, pathogens have learned to modulate ABA responses by the way of toxins and effectors. COR production by *Pst* DC3000 leads to increased ABA synthesis, followed by decreased SA synthesis through inhibition of *ICS1* expression (de Torres-Zabala et al., 2009), and consequently to increased stomatal aperture

allowing pathogen entry, followed by decreased defence responses. *In planta* conditional expression of *avrPtoB* also leads to increased levels of ABA biosynthesis in plants through activation of the NCED3 enzyme involved in ABA biosynthesis, accompanied by decreased defence gene expression upon bacterial inoculation (de Torres-Zabala et al., 2007). Similarly, biosynthesis and secretion of ABA is an important virulence mechanism of the rice fungal pathogen *Magnoporthe oryzae* (Spence et al., 2015) and a comparable role in pathogen virulence has also been proposed for the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Xu et al., 2013).

Cytokinins:

Although mostly known for their role in regulating cell division, cytokinins (CK) constitute a group of hormones with varied functions in plants, including the control of meristem function, chloroplast development, senescence and sink-source relationships (Argueso et al., 2009). A role for CKs in biotic stress was initially suggested based on experiments where exogenous application of CK led to altered susceptibility to pathogens, however the effect of CK on pathogen growth was variable (Levin, 1984; Clarke et al., 1998; Babosha, 2009).

Experiments establishing the role of CK in plant immunity were achieved in Arabidopsis, where it was shown that application of high concentrations of CK (in the micromolar range) led to decreased growth of the biotrophic pathogens *Hpa* and *Pst*, in a manner dependent on the cytokinin receptors, and therefore dependent of CK-regulated physiological processes (Choi et al., 2010; Argueso et al., 2012). Since then, CKs have been shown to be both positive and negative regulators of immunity to biotrophs- depending on the concentration of CK at the infection site. High levels of CK lead to decreased pathogen growth and activation of defence

responses, while lower CK concentrations (in the nanomolar range) result in increased pathogen growth (Argueso et al., 2012; Hann et al., 2014). The importance of CK to biotrophs goes beyond bacterial and fungal/oomycete pathogens. A recent report has demonstrated that an intact CK signaling pathway is necessary for successful nematode infection of Arabidopsis (Shanks et al., 2016). Application of CKs or use of genotypes with increased CK content/signaling also leads to activation of defence responses and reduced nematode infection (Shanks et al., 2016).

In plants, the majority of bioactive CKs are isoprenoid-derived (Kudo et al., 2010). Isoprenoid CKs are synthesized by the addition of an isoprene moiety to the N^6 position of adenine derivatives ATP, ADP or AMP, a step catalyzed by the rate-limiting enzyme ISOPENTENYL TRANSFERASE (IPT) (Kakimoto, 2001). Further, *trans*-hydroxylation by the cytochrome P450 mono-oxygenases CYP735A1 and CYP735A2 yield zeatin ribotides (Takei et al., 2004). Finally, hydroxylation by the LONELY GUY (LOG) phosphoribohydrolase leads to bioactive isoprenoid CKs (Kurakawa et al., 2007; Kudo et al., 2010).

The CK signaling pathway in plants involves a two-component system, similar to signaling systems ubiquitously found in bacteria and fungi (Argueso et al., 2009). In Arabidopsis, this encompasses a phosphorelay pathway where HISTIDINE KINASE (AHK) receptors bind to CK and undergo autophosphorylation, leading to the phosphorylation of HISTIDINE PHOSPHOTRANSFER proteins (AHP), and resulting in phosphorylation and activation of RESPONSE REGULATOR proteins (ARR) in the nucleus. ARR proteins can have transcription factor activity and act as positive regulators of CK outputs (type-B ARRs), or lack DNA binding domains and function as negative regulators of the pathway (type-A ARRs) (Argueso et al., 2009). The effect of high levels of CK on defence to biotrophic pathogens is, at least in part, mediated by SA, and components of the CK pathway play a prominent role in this

regulation (Choi et al., 2010; Argueso et al., 2012). Activation of SA-dependent gene expression by CK involves the interaction of a type-B ARR, ARR2, directly binding to a complex composed of the SA-transcription factor TGA3 and NPR1, to activate *PR-1* gene expression (Choi et al., 2010). Type-A ARRs, on the other hand, negatively regulate SA-dependent gene expression (Argueso et al., 2012). A synergistic interaction between cytokinin and SA, and a negative interaction between auxin and SA during defence activation has also been demonstrated (Naseem et al., 2012; Naseem and Dandekar, 2012).

The dual function of CK in plant immunity, at times facilitating pathogen infection and at times promoting resistance, has undoubtedly been exploited by pathogens. For example, *Rhodococcus fascians,* a CK-secreting biotrophic pathogen, uses CK derivatives to manipulate host defences to promote bacterial growth *in planta*, resulting in increased disease symptoms (Pertry et al., 2009). This action by *R. fascians* requires the CK receptors AHK3 and AHK4, indicating that CK-mediated alteration of host physiology are likely to result in better cellular conditions for pathogen growth. Likewise, the biotrophic fungus *M. oryzae* is able to synthesize and secrete CK during infection of rice, and this ability is necessary for full virulence (Chanclud et al., 2016). Interestingly, the increased virulence of *M. oryzae* is not due to decreased defence activation, but possibly to increased accumulation of nutrients at sites of infection (Chanclud et al., 2016). These examples are reminiscent of the role of CK on green island formation after biotrophic pathogen attack (Walters and McRoberts, 2006), but whether they are dependent on an interplay with the SA pathway is unknown. In any case, pathogen regulation of CK content/signaling for impairment of SA-dependent pathways has also been demonstrated, as in the case of the *Pst* T3SE HopQ1. HopQ1 acts as a negative regulator of PTI by increasing CK levels in the host. This results in lower levels of the PTI receptor FLAGELLIN SENSING 2

(FLS2), and culminates in reduced host immune responses and increased bacterial growth (Hann et al., 2014).

Auxin:

Auxin (AUX) is a phytohormone most often associated with cell expansion and growth-promoting processes, which include activation and control of plant meristems. The indole-3-acetic acid (IAA) constitutes the most common form of auxin found in plants, however several other natural active auxin species have been identified (Tivendale et al., 2014). IAA synthesis is typically originated from the essential amino acid tryptophan (Trp), through one of four Trp-dependent AUX biosynthetic pathways, which differ in the auxin intermediate originated immediately downstream of Trp. Analysis of Arabidopsis and maize Trp auxotrophic mutants that were still able to synthesize IAA helped lead to the discovery of a Trp-independent IAA biosynthetic pathway in plants (Wright et al., 1991; Normanly et al., 1993). Further, interconversion of IAA into its storage form, indole-3-butyric acid (IBA), is a limiting step for cellular IAA availability (Korasick et al., 2013). In addition to IBA, other natural forms of IAA, including methyl-IAA, IAA-amino acid and IAA-sugar conjugates, can act as different forms of auxin storage (Korasick et al., 2013). The contribution of each of these pathways to the IAA pools in plant cells is tightly regulated, and their predominance seems to vary among plant species (Tivendale et al., 2014). In addition to IAA biosynthesis, cell-specific distribution of IAA has a major function in the control of auxin-regulated processes. IAA is produced in plant apexes and then transported to other parts of the plant through the phloem, or through directed cell-to-cell polar transport. Polar transport of auxin is mediated by the asymmetric sub-cellular distribution of members of auxin efflux carrier protein families (PIN-FORMED, or PIN, and

ATP-BINDING CASSETE TRANSPORTER, or ABC), creating the IAA gradients ultimately responsible for the developmental patterns of plant growth. In addition to plants, some microbes can also synthesize IAA, through either Trp-dependent or -independent pathways (Normanly, 2010).

Once synthesized and distributed throughout the plant, auxin signaling at the cellular level involves proteasomal degradation of transcriptional repressors, a common theme and signaling strategy in plant hormone signaling pathways. The auxin receptors are part of a family of F-box proteins, consisting of TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and the AUX F-box (AFB) proteins, all of which are part of an E3-ligase SCF complex. Binding of auxin to the TIR/AFB proteins increases the affinity of this $SCF^{TIR/AFB}$ complex for the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors, leading to their degradation through the 26S proteasome. Degradation of Aux/IAA repressors results in the release of the transcriptional activators AUXIN RESPONSE FACTORS (ARF), normally blocked by AUX/IAA proteins under conditions of low auxin. Finally, release of ARF transcription factors culminates in the activation of auxin-dependent genes (Wang and Estelle, 2014).

In the context of immunity to biotrophic pathogens, auxin works mainly to promote susceptibility to pathogens. After successful biotrophic pathogen infection the concentration of IAA increases significantly, resulting in the up-regulation of auxin-responsive genes such as Aux/IAA and GRETCHEN HAGEN 3 (GH3) (Zhang et al., 2007; Gonzalez-Lamothe et al., 2012). In Arabidopsis, exogenous application of auxin to plants leads to increased virulence of biotrophic pathogens (*Pst* and *Hpa*) and promotion of disease (Chen et al., 2007; Wang et al., 2007). Correspondingly, the down-regulation of auxin signaling seems to be part of the plant

immune response to biotrophic pathogen attack. Perception of the PAMP flg22 by the pattern recognition receptor (PRR) FLS2 leads to the up-regulation of the microRNA *miR393*, whose targets are auxin receptor *TIR1* transcripts. *TIR1* transcript degradation by *miR393*-driven post-transcriptional gene silencing leads to reduced auxin perception and signaling, preventing biotrophic pathogen growth (Navarro et al., 2006). In addition, and perhaps alongside flg22 perception, increases in SA levels upon pathogen attack lead to another strategy of auxin signaling attenuation, through increased stabilization of Aux/IAA repressor proteins, as well as down-regulation of *TIR1* transcripts through a *miR393*-independent mechanism (Wang et al., 2007).

With regards to necrotrophic pathogens, auxin interacts synergistically with JA to promote resistance. Qi et al (2012) showed that levels of both JA and IAA increase upon infection with *A. brassicicola*, and mutants in auxin biosynthesis or transport display increased susceptibility to necrotrophic pathogens (Llorente et al., 2008; Qi et al., 2012). The same Aux/IAA proteins that are stabilized by SA in the interaction of Arabidopsis and biotrophic pathogens (Wang et al., 2007) are targeted for enhanced degradation in response to necrotrophic pathogen attack (Qi et al., 2012). Interestingly, concomitant application of a JA derivative (MeJA) and a bioactive form of auxin (IAA) to plants results in increased potentiation of JA-regulated defence marker *PDF1.2* (Qi et al., 2012), which is reminiscent of the synergistic response triggered by high levels of CK and SA in defence response to biotrophic pathogens (Choi et al., 2010; Argueso et al., 2012; Naseem et al., 2012).

Many studies show that pathogens have learned to target the auxin pathway to manipulate its synthesis and signaling in order to increase virulence. For example, the *Pst* T3SE AvrRpt2 promotes auxin signaling *in planta* (Chen et al., 2007), through targeted proteasomal degradation

of Aux/IAA proteins, which increases pathogen virulence (Cui et al., 2013). During penetration of host cells, the oomycete *Phytophthora parasitica* secretes penetration specific effectors (PSE), one of which, PSE1, targets auxin transport through enhanced activation of *PIN* transcription and sub-cellular localization. Thus, PSE1 promotes increased auxin content at sites of infection, culminating in suppression of SA-mediated defence responses, such as cell death and increased pathogen growth (Evangelisti et al., 2013).

Gibberellins:

Gibberellins, or gibberellic acid (GA), are a large family of tetracyclic diterpenoid hormones that aid in the regulation of both plant growth and immune responses (De Bruyne et al., 2014). GA was first identified when rice infected with a necrotrophic fungal pathogen, *Gibberella fujikuroi*, showed unnatural elongation. This elongation was later discovered to be caused by a GA mimic secreted by the pathogen (Yabuta and Sumiki, 1938). In plants, the most common biologically active forms of GA are the derivatives GA_1, GA_3 and GA_4 (Olszewski et al., 2002). While fungal pathogens have a distinct biosynthetic pathway for GA (Bomke and Tudzynski, 2009), in plants GA synthesis is initiated in plastids from the GA precursor geranylgeranyl diphosphate. In the first step of GA biosynthesis, geranylgeranyl diphosphate (GGDP) is converted to *ent*-kaurene (Olszewski et al., 2002). In the second step, *ent*-kaurene is converted into G_{12} by cytochrome P450 monooxygenases, *ent*-KAURENE OXIDASE (KO) and *ent*-KAURENOIC ACID OXIDASE (KAO). Then G_{12} can be converted into G_{53} by the 13-hydroxylation pathway. The final step of GA synthesis involves converting G_{12} and G_{53} into bioactive forms of GA, through oxidation steps by GA 20-OXIDASES (GA20ox) and -3 OXIDASES (GA3oX), to trigger GA-dependent responses.

GA signaling in plants occurs by binding of GA to its nuclear receptor protein GID1 (GIBBERELLIN INSENSITIVE DWARF 1). GA binding leads to conformational changes to GID1, favoring the recruitment of DELLA proteins, which are proteins with conserved N-terminal DELLA (Asp-Glu-Leu-Leu-Ala) motifs that act as negative regulators of GA responses [165]. Formation of the GID1-GA-DELLA complex promotes ubiquitylation and degradation of DELLA proteins through the 26S proteasome, which is achieved by E3-ligase SCF complex with F-box proteins SLY1 (SLEEPY1) in Arabidopsis and GID2 (GIBBERELLIN INSENSITIVE DWARF2) in rice (Daviere and Achard, 2013). The targeted degradation of DELLA proteins during GA signaling ultimately relieves the negative regulation on the GA pathway, activating transcription factors that promote GA responses (Daviere and Achard, 2013).

In Arabidopsis, plants harboring mutations in four of the five genes encoding DELLA proteins show increased resistance to *Pst*, accompanied by increased levels of SA (Navarro et al., 2008). Given that GA responses are increased in the *della* mutant background, these results suggest a positive and synergistic interaction between SA and GA in the regulation of resistance to biotrophic pathogens (Navarro et al., 2008). The *della* mutants also showed increased susceptibility to *A. brassicicola* (Navarro et al., 2008), possibly through a mechanism involving deregulation of production of reactive oxygen species (ROS) (Achard et al., 2008). These results point to a model where, at least in Arabidopsis, GA has a synergistic relationship with SA, contributing to defence against biotrophs, and a consequently antagonistic effect on the JA and ET pathways, responsible for susceptibility to necrotrophs (Navarro et al., 2008; De Bruyne et al., 2014). Support for this model comes from data showing that in the absence of GA, DELLA proteins will actively compete with the JA-regulated transcription factor MYC2 for binding to JAZ proteins, which results in increased MYC2 availability for activation of resistance to

necrotrophic pathogens (Hou et al., 2010). Therefore, if GA is present, then DELLA proteins will be degraded allowing for JAZ and MYC2 to interact, resulting in blocking of the JA-signaling. Similar examples of JA and GA crosstalk have been uncovered. Analyses of *coi1* RNAi lines and mutants in rice and Arabidopsis, respectively, showed that absence of JA signaling increases signaling in the GA pathway, indicating that in wild type plants JA antagonizes GA signaling (Yang et al., 2012). This antagonistic effect of JA on the GA pathway is mediated by JAZ proteins, which function to delay DELLA protein degradation, thus inhibiting GA responses that activate plant growth, such as the activation of plant growth promoting transcription factors from the PHYTOCHORME INTERACTING FACTOR (PIF) family (Yang et al., 2012). A similar interaction also occurs in response to insects, in the up-regulation of genes involved in the biosynthesis of sesquiterpenes, which act as important olfactory cues in plant-insect interactions. The expression of both *SESQUITERPENE SYNTHASE 21* and *11* (*TPS21* and *TPS11)* is regulated by GA and JA, through direct transcriptional activity of MYC2 (Hong et al., 2012). Before insect attack, MYC2 is repressed by physical interaction with the DELLA protein REPRESSOR OF GA1 (RGA). An insect-induced increase in the levels of JA and GA leads to RGA degradation, allowing MYC2 to initiate *TPS* transcription for sesquiterpene biosynthesis (Hong et al., 2012).

Rice plants, however, show a different regulation of immunity by GA. Exogenous application of GA leads to increased susceptibility to biotrophic pathogens, as do mutations that lead to increased accumulation of bioactive Gas (Yang et al., 2008). For example, the *ELONGATED UPPERMOST INTERNODE* (*EUI*) gene encodes a P450 monooxygenase that deactivates biologically active GAs. A mutation in the *EUI* gene causes rice plants to be more susceptible to two biotrophic pathogens, *X. oryzae* and *M. oryzae* (Yang et al., 2008) . Similarly,

increased transcription of the biosynthetic gene *OsGA20Ox3* also leads to enhanced susceptibility to these pathogens (Qin et al., 2013). Thus, rice and Arabidopsis plants seem to have different hormonal requirements for defence responses, likely caused by differences in plasticity of hormonal pathways in these plant species (De Vleesschauwer et al., 2014).

Similar to other phytohormones involved in immune response, GA has become a target of invading pathogens to increase host susceptibility. *G. fujikuroi* (teleomorph *Fusarium moniliforme*) increases pathogenicity through secretion of a GA mimic, to repress JA-defence signaling and improve conditions for necrotrophic growth. After infection with *G. fujikuroi*, rice plants display phenotypes similar to plants with an accumulation in endogenous GA levels (Bomke and Tudzynski, 2009). This has been demonstrated to be an evolutionary advantage for *G. fujikuori* because many other *Fusarium* species are unable to synthesize GA, and therefore are easier to control in regards to infection (Wiemann et al., 2013). The *Xanthomonas campestris* effector protein $XopD_{Xcc8004}$ inhibits GA-degradation of a DELLA protein, resulting in DELLA-mediated repression of GA-defence response to promote tolerance to the biotic stress (Tan et al., 2014). Zhu et al. (2005) demonstrated that the *RICE DWARF VIRUS* (RDV) P2 protein interacts with the GA biosynthetic enzyme *ent*-kaurene oxidase, causing lower levels of bioactive GA_1 synthesis and increasing susceptibility to RDV (Zhu et al., 2005). When exogenous bioactive GA_3 was applied to diseased plants with RDV the host resistance was restored, confirming that RDV P2 was blocking GA-synthesis to promote disease (Zhu et al., 2005).

Brassinosteroids:

Brassinosteroids (BR) compose a family of plant-derived polyhydroxylated steroidal compounds structurally related to animal steroid hormones, but with important functions in plant

development and responses to environmental changes. BRs have been shown to act as both positive and negative regulators of immunity to a variety of pathogens on a wide range of hosts (Nakashita et al., 2003). Several studies have shown that exogenous application of BR increases plant immune responses to biotrophic pathogens, including studies in tobacco and Arabidopsis. Recently, Canales et al. (2016) demonstrated that exogenous application of a BR-derivative, epibrassinolide, to infected citrus plants significantly reduced the bacterial growth of *Candidatus* Liberibacter asiaticus, the Haunglongbing bacterial pathogen responsible for the citrus greening disease, suggesting that exogenous application of BR can act as a potential management tool for this devastating citrus disease (Canales et al., 2016). In the case of both tobacco and Arabidopsis, the effect of BR on boosting disease resistance was found to be independent of SA, as *nahG* and *npr1* plants continued to show increased resistance or increased defence gene expression after BR treatment (Canales et al., 2016). Even though BR-induced resistance to biotrophs has been demonstrated, De Vleesschauwer showed that treatment of rice plants with BR can increase susceptibility to the necrotrophic pathogen *Pythium graminicola* (De Vleesschauwer et al., 2012), through a mechanism that involves the plant hormone GA. Application of BR leads to stabilization of the single DELLA protein in rice, SLENDER RICE 1 (SLR1), ultimately blocking GA signaling and GA-mediated defence responses and increasing susceptibility to *P. graminicola*.

The biosynthetic pathways for BR synthesis are complex. While the most active form of BR, brassinolide (BL), is synthesized from the common sterol precursor campesterol, many other BR derivatives are derived from a general sterol biosynthesis pathway from isoprenoids (Yokota, 1997; Clouse, 2011). During BL synthesis, campesterol is converted into BL by enzymes DET2 and DWF4, followed by either an early or late C6 oxidation pathway, ultimately leading to

castasterone. The last step of the pathway is the synthesis of BL from castasterone by the cytochrome P450 monoxygenase CYP852A (Noguchi et al., 2000; Clouse, 2011).

The BR signaling pathway is a tightly regulated process and, as in the case of other plant hormones, is intertwined with plant immunity. BR signaling is initiated through perception of BR by the LRR receptor kinase (LRR-RK) BRASSINOSTEROID INSENSITIVE 1, BRI1, which acts as the main BR receptor (Belkhadir and Jaillais, 2015). Binding of bioactive forms of BR to BRI1 initiates a phosphorylation cascade that activates the GS3-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2). BIN2 regulates the function of downstream transcription factors BRASSINAZOLE-RESISTANT 1 (BRZ1) and BR-INSENSITIVE EMS SUPPRESSOR 1 (BES1), which together coordinate the transcription of BR-regulated genes (Belkhadir and Jaillais, 2015). Initial recognition of BR also involves the co-receptor BAK1 (BRI1-ASSOCIATED KINASE), another LRR-RK, also known as SOMATIC EMBROGENESIS RECEPTOR KINASE 3 (SERK3). Binding of BR to BRI1 increases the affinity of this receptor-ligand complex for BAK1, breaking the association of BAK1 to a negative regulator of the pathway, the membrane bound receptor BIK1 (BRI1 KINASE INHIBITOR). Association of BAK1 to the BR-BRI1 complex potentiates the BR response, positively regulating the pathway (Belkhadir and Jaillais, 2015).

Apart from its role in BR signaling, the BR co-receptor BAK1 is also a major component of PTI. Recognition of flg22 and elf18 by their specific PRRs leads to association of BAK1 to the corresponding ligand-receptor complexes, resulting in the activation of PTI responses (Chinchilla et al., 2007; Schulze et al., 2010). The requirement for BAK1 in both PTI and BR signaling led to the hypothesis that BR content or signaling levels could fine-tune PTI. Given the role of BR in plant development, BAK1 was also suggested as a possible regulator in the growth

trade-offs observed in plant immunity. Using mutants and transgenic lines altered in BR signaling and content, Belkhadir et al. (2012) demonstrated that contrasting levels of BR signaling can have a similar negative effect on PTI activation, and this modulation of PTI levels by BR is dependent on the recruitment of BAK1 by the BR receptor BRI1 (Belkhadir et al., 2012). In contrast, another study demonstrated that extended exposure of plants to a BR derivative, epibrassinolide, leads to inhibition of PTI. However this response is independent of BAK1 and can be triggered by PAMPs that elicit PTI in a BAK1-independent manner, such as chitin, suggesting that the negative regulation of PTI by BR happens downstream of BIK1(Albrecht et al., 2012). The contrasting responses from these two studies may reflect the different levels of BR signaling activation obtained with the use of mutants and transgenic lines altered in BR signaling/biosynthesis and those obtained through exogenous treatment of plants with BR, and a distinct requirement for BAK1 at these different levels of BR signaling.

Several pathogens target the BR pathway in order to surpass host defence responses and increase pathogenicity. As mentioned above, De Vleesschauwer et al. (2012) demonstrated that *P. graminicola* exploits BR as a virulence factor to hijack BR signaling pathways to initiate indirect stabilization and accumulation of DELLA proteins to repress GA signaling, decreasing host resistance (De Vleesschauwer et al., 2012). *Pseudomonas syringae* strains have been shown to use various T3SEs to manipulate BR signaling to increase disease symptoms. Shan et al. (2008) demonstrated that *Pst* effectors AvrPto and AvrPtoB directly target BAK1 in order to repress BR and PTI signaling responses. When AvrPto or AvrPtoB bind to BAK1 it does not change the receptor conformation or phosphorylation, but instead block FLS2 or EFR from binding BAK1 and induces PTI (Shan et al., 2008). In addition, BAK1 is also the target of the *Pst* DC3000 T3SE HopF2, in order to suppress PTI responses. In mutants lacking the BAK1

receptor, *bak1*, disease symptoms of *Pst* strains containing HopF2, AvrPto, or AvrPtoB were significantly decreased, corroborating that BAK1 is indeed the target of these bacterial effectors to increase host susceptibility (Zhou et al., 2014). Yamaguchi et al. (2013) showed that the *X. oryzae* T3SE Xoo2875 targets the analogous BAK1 receptor in rice, OsBAK1, to inhibit PTI responses (Yamaguchi et al., 2013). The association between OsBAK1 and Xoo2875 was confirmed through protein-protein interaction experiments, verifying that this T3SE also acts as a blocking mechanism in order to reduce basal plant defences (Yamaguchi et al., 2013). Whether the action of these T3SEs is restricted to changing BR signaling components in the context of PTI, or whether they also have a direct effect on BR signaling during plant development, is not known.

A.5 Many Battles to Win a War: Hormonal Crosstalk in Plant Immunity

Plant hormones play an important role in mediating immune responses to varying biotic stresses. The last two decades have provided a wealth of information on the regulation of plant immunity by plant hormones, thanks mostly to work on Arabidopsis, and the availability of mutants and other genetic tools for this model plant species. It is now clear that no single plant hormone is responsible for mediating all plant immune responses to pathogens. Rather, it is a complex and interconnected combination of hormonal interactions that modulates plant immunity, referred to as hormonal crosstalk (**Figure A-1**).

One of the main lessons from studies focusing on the role of plant hormones in immunity is the importance of pathogen lifestyle to the hormonal requirements for defence. The plant hormones SA and JA have long been associated as antagonistic counterparts in regards to plant immune response. While SA is generally required for defence to biotrophic pathogens, the

hormones JA and ET are necessary for resistance to necrotrophs. These two pathways act mostly antagonistic to each other, and together comprise the nexus, or backbone, of the hormonal regulation of plant immunity. This antagonistic interaction suggests the triggering of different defence responses after attack by pathogens of different lifestyles, capable of stopping pathogenic organisms with widely distinct pathogenicity and virulence strategies. However, while pathway-specific gene expression markers do exist (e.g. *PR-1*, *PR-2* and *PR-5* for the SA pathway, *PDF1.2* and *Thi2.1* for the JA/ET pathway), transcriptomics analyses have shown that a mostly overlapping set of genes is activated by exogenous treatment of plants with either JA or SA (Schenk et al., 2000). Rather than indicate that similar responses can be effective against dissimilar pathogens, these results most likely underscore the importance of modest changes in host physiology and defence response activation to the outcome of plant-microbe interactions.

Although the SA/JA-ET pathways compose the major nexus in the hormonal regulation of plant immune responses, other hormones such as CK, AUX, GA, BR, and ABA have also been shown to be important players (**Figure A-1**). The function of these hormones, which may be considered auxiliary hormones in plant immunity, appears to be the shifting of the hormonal balance towards SA or JA-ET, through either direct or indirect action on the components of the SA and JA/ET signaling pathways, fine-tuning the required hormonal signals to mediate an appropriate immune response to the specific invading pathogen (**Figure A-1**). This fine-tuning of immunity is likely to be particularly relevant to hemi-biotrophic pathogens, such as *Pst*, where the importance of the SA and JA/ET pathways will vary according to the phase of infection, being it biotrophic or necrotrophic. Several of these hormones also have a fundamental role in the regulation of plant growth and development, and in this context are likely to have a major

function in the control of the growth trade-offs involved in immunity activation produced by the stimulation of the SA-JA/ET backbone (Yang et al., 2012).

Hormonal signaling pathways in plants are usually complex, and include a high degree of genetic redundancy, thus providing the combinatorial plasticity that plants require to adapt to ever changing environmental conditions. In addition to plants, pathogens have also learned to take advantage of these pathways to manipulate the immune status of plants during infection (**Figure A-2**). It is interesting to note that, despite the existence of multiple regulatory steps in plant hormone signaling pathways, the hormone signaling proteins that are targeted by effectors seem to be conserved among effectors of different pathogens. A recurrent theme on hormonal signaling regulation in plants, the proteasomal degradation of negative regulators of hormonal pathways (Santner and Estelle, 2010), is also a conserved strategy of effectors from a variety of pathogens (Duplan and Rivas, 2014). This is best exemplified by the DELLA proteins, which act as negative regulators of GA signaling. Rather than promoting DELLA degradation and activation of SA signaling, pathogen effectors can stabilize DELLA proteins, maintaining repression of the GA pathway and indirectly suppressing activation of the SA pathway (Navarro et al., 2008). In rice, stabilization of DELLA proteins to prevent immune activation can also be indirectly achieved by BR, leading to suppression of resistance to necrotrophic pathogens in this case (De Vleesschauwer et al., 2012). Similar to their action in hormone signaling, effectors tend to act on enzymes catalyzing committed rate-limiting steps of hormone biosynthesis, or to activate catabolism reaction (**Figure A-3**). Therefore, in their subverting of the plant immune system, pathogens target conserved hubs of hormone signaling and metabolism, to achieve the perfect host physiological conditions for success.

As discussed in this review, multiple hormones interact in a complex manner to modulate the immune response of plants to various pathogens. Thus, given the vast amount of hormonal interactions uncovered in the relationships between different plants and pathogens, a far-reaching understanding of the roles of plant hormones in plant immunity will require approaches that can handle the analysis of large amounts of data, through methods involving computational biology. Such systems biology approaches have been elegantly utilized in recent studies, as in the modeling of quantitative disease data in different plant hormonal genetic backgrounds (Kojima et al., 2009; Tsuda et al., 2009; Sato et al., 2010; Kim et al., 2014), as well as by *in silico* approaches to hormonal interactions (Naseem et al., 2012). Additionally, most of the information obtained about hormonal crosstalk in plant immunity has relied on the quantification of plant hormones obtained in a few time-points during the progression of infection/pathogen attack. In this regard, the advent of new methods of high-throughput plant hormone quantification through high definition mass spectrometry (Kojima et al., 2009) will be an essential tool for the elucidation of the dynamic modulations of plant immunity by plant hormones, and their ultimate role in shaping the outcome of specific plant-pathogen interactions.

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A.6 Figures

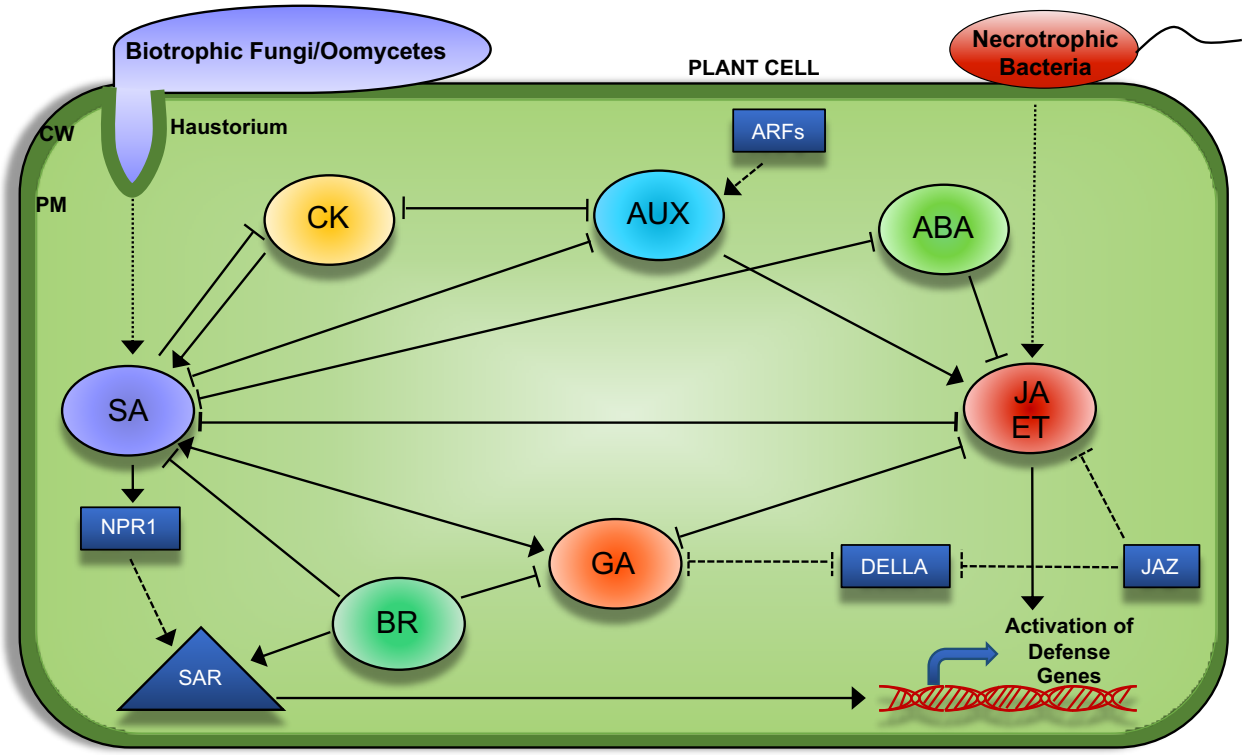

Figure A-1: A conceptualized overview of the phytohormone crosstalk in plant immunity. In general, in response to biotrophic pathogens SA is up-regulated, whereas in response to necrotrophic pathogens JA and ET are up-regulated. To fine-tune the host immune response other abiotic stress and growth hormones contribute to immunity such as: ABA, CK, AUX, GA, and BR. These hormones typically contribute to host immunity through up-regulation or down-regulation of either the SA or JA/ET branches. The different phytohormones are indicated by various colors. Dark blue shapes with white font represent transcription factors or processes that are activated in the nucleus. CW= cell wall and PM= plasma membrane. Solid lines represent hormone mediated up-regulation (arrow) or inhibition (blunt-end line). Dotted lines represent pathogen identification by the host. Dashed lines represent some transcription factors or processes involved in hormone crosstalk. (SA- salicylic acid; JA- jasmonic acid; ET- ethylene; CK- cytokinin; AUX- Auxin; GA- gibberellins; BR-brassinosteroids)

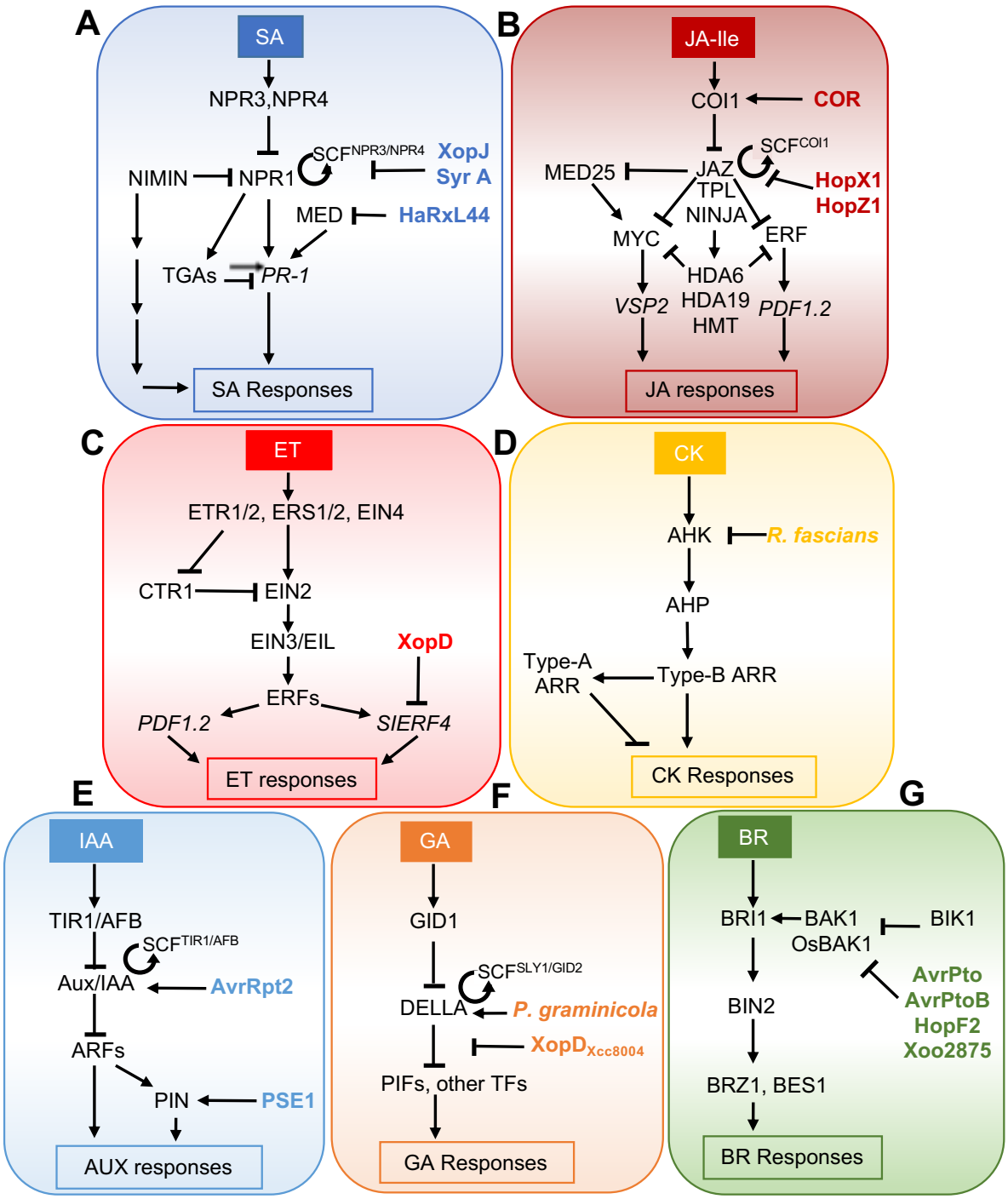

Figure A-2: Effector manipulation of various phytohormone signaling pathways. For explanation of pathways, please see main text. Arrows indicate positive interaction; blunt-end indicates negative interaction (inhibition), three quarter circle arrow indicates degradation by 26S proteasome through SCF complex. **A. SA signaling pathway**. SA- salicylic acid; NPR1 – NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1; NPR3 – NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 3; NPR1 – NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 4; SCF $^{NPR3/NPR4}$- SCF E3 ubiquitin ligase complex with FNPR3 and NPR4; NIMIN- NIM1-INTERACTING proteins; TGA- TGA transcription factors; MED 19- Mediator

complex subunit 19; *PR-1- PATHOGENESIS-RELATED 1* gene; XopJ- *Xanthomonas euvesicatoria* effector; SyrA: Syringolin A- *Pseudomonas syringae pv. syringae* toxin; HaRXL44- *Hyaloperonospora arabidopsidis* effector. **B. JA signaling pathway**. JA-Ile- (+)-7-*iso*-jasmonoyl-L-isoleucine; COI1- CORONATINE INSENSITIVE 1; JAZ- JASMONATE ZIM-domain proteins; SCF^{COI1} SCF E3 ubiquitin ligase complex with F-box proteins COI1; TPL- TOPLESS transcriptional repressor; NINJA- NOVEL INTERACTOR OF JAZ protein; HDA6- HISTONE DEACETYLASE 5; HDA19- HISTONE DEACETYLASE 19; HMT- HISTONE METHYLTRANSFERASE; MED25- Mediator complex subunit 25; MYC- basic helix-loop-helix transcription factors; ERF- ETHYLENE RESPONSE FACTOR transcription factors; *VSP2- VEGETATIVE STORATE PROTEIN 2* gene; *PDF1.2- PLANT DEFENSIN 1.2* gene; COR- Coronatine, *Pseudomonas syringae* phytoxin; HopX1- *Pseudomonas syringae* effector; HopZ1- *Pseudomonas syringae* effector. **C**. **ET signaling pathway**. ET- ethylene; ETR1/2- ETHYLENE RESPONSE 1/2; ERS1/2- ETHYLENE RESPONSE SENSOR 1/2; EIN4- ETHYLENE INSENSITIVE 4; EIN2- ETHYLENE INSENSITIVE 2; CTR1- Ser/Thr Kinase; EIN3/EIL-ETHYLENE INSENSITIVE 3/ EIN2-LIKE transcription factors; ERFs- ETHYLENE RESPONSE FACTOR transcription factors; PDF 1.2- PLANT DEFENSIN 1.2; SIERF4- Ethylene inducible tomato transcription factor; XopD- *Xanthomonas euvesicatoria* effector. **D**. **CK signaling pathway**. CK- cytokinin; AHK- HISTIDINE KINASE receptors; AHK- HISTIDINE PHOSPHOTRANSFER proteins; Type-A ARR- Negative RESPONSE REGULATOR proteins; Type-B ARR- Positive RESPONSE REGULATOR proteins; *R. fasciens*- CK-secreting *Rhodococcus fascians*. **E. AUX signaling pathway**. IAA- Indole-3-acetic-acid; TIR- TRANSPORT INHIBITOR RESPONSE 1; AFB- AUX F-box proteins; $SCF^{TIR/AFB}$- E3-ligase SCF complex with TRANSPORT INHIBITOR RESPONSE 1/AUX F-box proteins; Aux/IAA- AUXIN/INDOLE-3-ACETIC ACID transcriptional repressors; ARFs- AUXIN RESPONSE FACTORS transcriptional activators; PIN- PIN-FORMED proteins; AvrRpt2- *Pseudomonas syringae* pv. *tomato* effector; PSE1- *Phytophthora parasitica* effector. **F. GA signaling pathway**. GA- gibberellins; GID1- GIBBERELLIN INSENSITIVE DWARF 1; $SCF^{SLY1/GID2}$- SCF E3 ubiquitin ligase complex with F-box proteins SLEEPY1 and GID2; DELLA- proteins with conserved N-terminal Asp-Glu-Leu-Leu-Ala motifs; $XopD_{Xcc8004}$- *Xanthomonas campestris* effector. **G.** BR-brassinosteroids; BRI1- BRASSINOSTEROID INSENSITIVE 1; BAK1- BRI1-associated kinase; OsBAK1- BAK1 receptor in rice; BIN2- BRASSINOSTEROID INSENSITIVE 2; BRZ1- BRASSINAZOLE-RESISTANT 1; BES1- BR-INSENSITIVE EMS SUPPRESSOR 1; AvrPto- *Pseudomonas syringae* pv. *tomato effector*; AvrPtoB- *Pseudomonas syringae* pv. *tomato* effector; HopF2- *Pseudomonas syringae* pv. *tomato* effector; Xoo2875- *Xanthomonas oryzae* effector.

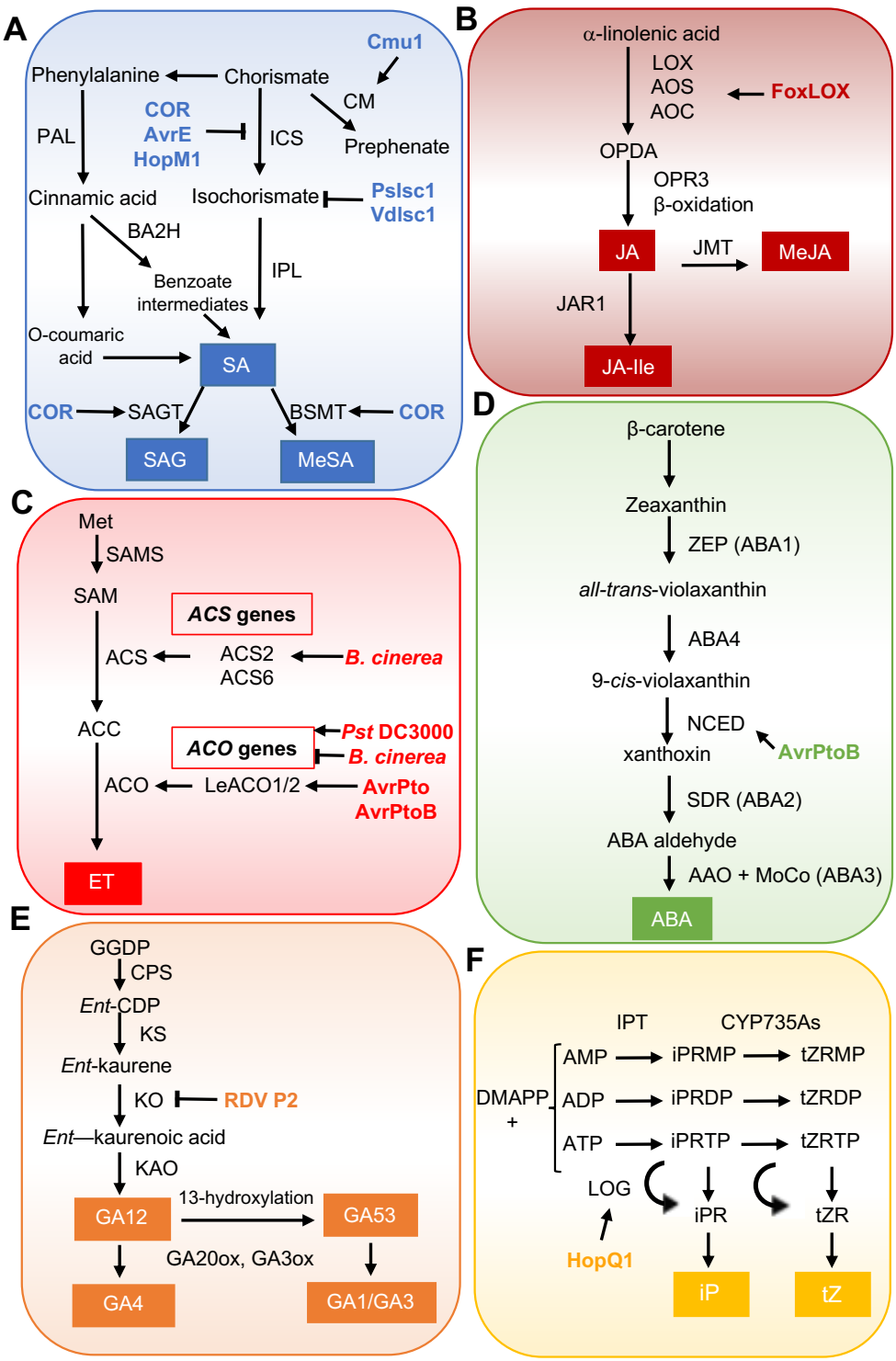

Figure A-3: Effector manipulation of various phytohormone biosynthetic pathways. For explanation of pathways, please see main text. Arrows indicate positive interaction; blunt-end indicates negative interaction (inhibition). **A. SA biosynthetic pathway** SA- salicylic acid; SAG- SA O-β-glucoside; MeSA- methyl salicylate; ICS- ISOCHORISMATE SYNTHASE; PAL- PHENYLALANINE AMMONIA LYASE; IPL- ISOCHORISMATE PYRUVATE LYASE; BA2H- BENZOIC ACID-2-HYDROXYLASE; SAGT- SA

GLUCOSYLTRANSFERASE; BSMT- BENZOIC ACID SA METHYLTRANSFERASE; Cmu1- *Ustilago maydis* effector; CM- CHORISMATE MUTASE; COR- *Pseudomonas syringae* phytotoxin; Pdlsc1- *Phytophthora sojae effector;* Vdlsc1- *Verticillium dahliae* effector. **B. JA biosynthetic pathway**. JA- jasmonic acid; JA-Ile- (+)-7-*iso*-jasmonoyl-L-isoleucine; MeJA- methyl jasmonate; LOX- LIPOXYGENASE; AOS- ALLENE OXIDE SYNTHASE; AOC- ALLENE OXIDE CYCLASE; OPDA- 12-oxo-phytodienoic acid; OPR3- OPDA REDUCTASE; JMT- JA CARBOXYL METHYLTRANSFERASE; JAR1- JASMONOYL ISOLEUCINE CONJUGATE SYNTHASE; FoxLOX- *Fusarium oxysporum* induced pathway.
C. ET biosynthetic pathway. ET- ethylene; Met- methionine; SAM- S-adenosyl-methionine; ACC-1-aminocyclopropane-1-carboxylic acid; SAMS- SAM SYNTHETASE; ACS- ACC SYNTHASE; ACO- ACC OXIDASE; AvrPto & AvrPtoB- *Pseudomonas syringae* pv. *tomato* DC3000 effectors. **D. ABA biosynthetic pathway**. ABA- abscisic acid; BCH1/BCH2-β-CAROTENE HYDOXYLASEs; ZEP (ABA1)- ZEAXANTHIN EPOXIDASE; VDE- VIOLAXANTHIN DE-EPOXIDASE; ABA4- ABA protein that is directly involved in trans-neoxanthin production; NCED3- 9-*CIS*-EPOXYCAROTENOID DIOXYGENASE; SDR (ABA2)- DEHYDROGENASE/REDUCTASE-like enzyme; AAO + MoCo(ABA3)- ABA ALDEHYDE OXIDASE+MOLYBDENUM COFACTOR SYNTHASE; AvrPtoB- *Pseudomonas syringae* pv. *tomato* DC3000 effector. **E. GA biosynthetic pathway**
GA12, GA53- gibberellin derivatives; GA1, GA3, GA4, GA7- bioactive forms of gibberellin; GGDP- geranylgeranyl diphosphate; *Ent*-CDP- *ent*-copalyl diphosphate; CPS- COPALYL-DIPHOSPHATE SYNTHASE; KS- *ent*-KAURENE SYNTHASE synthase; KO- *ent*-KAURENE OXIDASE; KAO- *ent*-KAURENOIC ACID OXIDASE; GA20ox- GA20 OXIDASES; GA3ox- GA3 OXIDASES; RDV P2- *Rice Dwarf Virus* P2 protein. **F. CK biosynthetic pathway.** DMAPP- dimethylallyl diphosphate; AMP, ADP and ATP- Adenosine mono, di- or tri-phosphate; IPT- ISOPENTENYL TRANSFERASE; iPRMP, iPRDP, iPRTP- isopentenyl riboside mono-, di-, tri-phosphate; CYP735A- cytochrome P450 monoxygenase; tZRMP, tZRDP, tZRTP- *trans*-zeatin riboside mono-, di-, tri-phosphate; iPR- isopentenyl riboside; *t*ZR, *trans*-zeatin riboside; iP- isopentenyl adenine; *t*Z- *trans*-zeatin; HopQ1- *Pseudomonas syringae* pv. *tomato* DC3000 effector

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Appendix B

Towards Engineering of Hormonal Crosstalk in Plant Immunity[5]

B.1 Summary

Plant hormones regulate physiological responses in plants, including responses to pathogens and beneficial microbes. The last decades have provided a vast amount of evidence about the contribution of different plant hormones to plant immunity, and also of how they cooperate to orchestrate immunity activation, in a process known as hormone crosstalk. In this review we highlight the complexity of hormonal crosstalk in immunity and approaches currently being used to further understand this process, as well as perspectives to engineer hormone crosstalk for enhanced pathogen resistance and overall plant fitness.

B.2 Introduction

Plant hormones are small signaling molecules with important regulatory roles in various plant processes. Molecules with plant hormone activity have historically been classified based on their chemical structures, and also on their primary function in physiological processes, such as growth, biotic or abiotic stress hormones. Such functional classifications are now considered loose categorizations, as many hormones first thought to be only associated with growth processes have now been linked to immune responses against pathogens (Pieterse et al., 2012; Shigenaga and Argueso, 2016) . The complex network of communication among plant hormone

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signaling pathways is often referred to as hormone crosstalk, and is employed in many plant processes, not just in the case of immune responses. Such interplay among different hormonal pathways is presumed to confer advantages to plants, allowing for the concomitant regulation of different hormone-regulated physiological processes, thus leading to increased ability to respond to different types of pathogens as well as beneficial organisms, and changing developmental and environmental conditions. This review will cover the importance of hormonal crosstalk in immune responses, approaches to understand complex hormonal networks such as quantification of hormones and hormonal signaling, mathematical modeling, and synthetic biology, and how hormonal crosstalk could be engineered to increase plant overall fitness.

B.3 Hormonal Crosstalk and Plant Immunity

Plant hormones are essential regulators of the responses of plants to microbes. The hormones salicylic acid (SA) and jasmonate (JA) are recognized as the most important hormones for plant immune responses. These two classes of hormones are believed to form the hormonal backbone of plant immune responses to pathogens (Pieterse et al., 2012). Defense against biotrophic pathogens, which keep the host alive to obtain nutrients, requires SA biosynthesis and signaling, whereas JA biosynthesis and signaling are required for resistance against necrotrophic pathogens, which acquire nutrients from decaying host tissue (Glazebrook, 2005). In many plant-pathogen interactions involving JA, ethylene (ET) has been shown to assist in defense responses, resulting in increased resistance to necrotrophic pathogens(Penninckx et al., 1998). In the last two decades, various studies have shown that other plant hormones important for growth, such as auxins (AUX), cytokinins (CK), brassinosteroids (BR), gibberellins (GA) and strigolactones (SL), as well as the abiotic stress hormone abscisic acid (ABA), participate in plant defense

(Shigenaga and Argueso, 2016). These hormones typically function through action on the SA and JA pathways in plant immunity. Such crosstalk is frequently exploited by pathogens through manipulations of their metabolic and signaling pathways by pathogen effectors (reviewed in (Kazan and Lyons, 2014; Ma and Ma, 2016; Shigenaga and Argueso, 2016)). In addition, several pathogens and beneficial microbes are able to synthesize and secrete plant hormones, or mimics thereof, affecting the SA-JA hormonal backbone and influencing host physiological status. A well-studied example is the phytotoxin coronatine (COR), which is secreted by several *Pseudomonas syringae* pathovars and functions as a JA-mimic, resulting in inhibition of SA-regulated defense responses and decreased host resistance to biotrophic pathogens(Brooks et al., 2005; Uppalapati et al., 2007; Zheng et al., 2012). Nonetheless, pathogenic organisms can also secrete other classes of plant hormones, such as ET (Weingart and Volksch, 1997; Weingart et al., 2001), AUX (Glickmann et al., 1998), GA (Bomke and Tudzynski, 2009), ABA (Gong et al., 2014) and CK (Pertry et al., 2009; Chanclud et al., 2016), although it is unclear whether this virulence mechanism is used to increase host susceptibility or counteract defense responses. Similarly, beneficial microbes are also able to produce plant hormones, using them to promote plant immunity and shape community composition (Lebeis et al., 2015; Großkinsky et al., 2016), thus adding another level of complexity to hormone crosstalk in plant-microbe interactions.

With a variety of both plant and pathogen-derived molecules with hormone activity affecting plant immunity, crosstalk among the different hormonal networks is often observed. The antagonistic nature of the crosstalk between the SA and JA pathways in plant immune responses was first suggested in experiments involving exogenous hormone treatment of plants, followed by measurements of gene expression or gene product activity of SA- or JA/ET-specific markers (Vanderhee et al., 1990; Uknes et al., 1993; Penninckx et al., 1996; Penninckx et al.,

1998). Activation of the SA or JA sectors was triggered depending on whether Arabidopsis plants were inoculated with a (hemi-)biotrophic or necrotrophic pathogen. A trade-off between these two pathways was demonstrated by experiments in which inoculation with a hemi-biotrophic pathogen (*P. syringae* pv. *tomato* DC3000) rendered plants more susceptible to the necrotrophic pathogen *Alternaria brassicicola,* and led to decreased expression of the JA/ET markers *PDF1.2*, *HEL* and *CHI-B* (Spoel et al., 2007). Similarly, exogenous application of an SA analogue reduced JA-regulated defenses against *A. brassicicola* (Spoel et al., 2007). This reciprocal antagonistic crosstalk between the SA and JA pathways, initially demonstrated in Arabidopsis, is also observed in other plant species, and phylogenetic studies indicate that it may have evolved with the development of angiosperms (Berens et al., 2017). Nonetheless, evidence exists for deviations of this antagonism, particularly in the case of monocotyledonous plants (Thaler et al., 2012; Ding et al., 2016a). Antagonistic or synergistic interactions of other plant hormones with the SA-JA backbone, as well as among themselves, also contribute to plant immunity (**Table B-1**). For example, high levels of CK potentiate the SA pathway (Choi et al., 2010; Argueso et al., 2012). Interestingly, in some cases, different levels of the same hormone will lead to opposing physiological/resistance responses (Babosha, 2009; Argueso et al., 2012; Ding et al., 2016a).

B.4 Understanding Hormone Crosstalk in Plant Immunity

The contributions of the different plant hormones to hormone crosstalk during pathogen attack have been determined predominantly by the use of mutants impaired in the signaling/metabolism of single hormones. However, such analyses may be misleading because they reveal the effects of not only the loss of the disrupted hormone signaling sector, but also the

loss of hormone crosstalk (Tsuda et al., 2009; Hillmer et al., 2017). This caveat can be overcome through the use of higher order mutants, as well as exhaustive combinatorial mutants followed by modeling-based analysis of quantitative crosstalk outputs, such as pathogen growth or changes in pathogen-regulated gene expression. This approach has been successfully applied to reveal the individual and combined contributions of the SA, JA and ET signaling sectors to hormone crosstalk in relation to resistance against *P. syringae* and *A. brassicicola* (Tsuda et al., 2009) and to genome-wide transcriptional changes triggered by pathogen elicitors(Hillmer et al., 2017). An expanded analysis using multiple immune outputs at two time points followed by a multiple regression or Bayesian network model showed a temporally dynamic plant hormone signaling network model during pattern-triggered immunity (PTI) with high predictability (Kim et al., 2014). These modeling-based studies revealed important network properties of plant hormone signaling networks in plant immunity, such as network robustness and tunability, and can generate sometimes-unexpected hypotheses that can be further tested. For instance, with a generated hypothesis as the starting point, a follow-up study uncovered the molecular detail for positive and negative effects of JA on SA accumulation during PTI (Mine et al., 2017).

Systems biology and mathematical modeling are powerful approaches to disentangle complex biological systems. The high degree of complexity observed in hormonal crosstalk during plant immunity renders it particularly suitable for such analyses. For instance, analyses of genome-wide transcriptional responses of plants to exogenously applied plant hormones, immune elicitors or pathogen infection, have contributed significantly to our holistic understanding of plant hormone signaling networks (Windram and Denby, 2015). Co-expression analyses using publicly available transcriptome data have revealed *cis*-regulatory elements that are implicated in hormone crosstalk during plant immunity (Tully et al., 2014; Deb et al., 2016).

Through integrated analysis of protein-protein interaction and transcriptomic responses during pathogen infection, Jiang et al. identified components of auxin response as hubs in a defense network (Jiang et al., 2016). Similarly, *in silico* dynamic simulations using Boolean network approaches of various information reported in the literature have been used to predict synergism between CK and SA and antagonism between CK and AUX in plant immunity (Naseem et al., 2012), confirming interactions previously identified in biological studies (Choi et al., 2010; Argueso et al., 2012). However the predominance of single mutant studies in the literature, rather than the more informative approaches based on double and higher mutant analyses, could also produce a bias in literature-based *in silico* examinations. Nevertheless, the above-mentioned approaches have been successfully used to understand hormone crosstalk, as well as generate new hypotheses.

Dynamics in plant hormone accumulation influence signaling outputs mediated by a given plant hormone, as well as any resulting hormone crosstalk. For example, antagonism between ABA and SA during plant immunity is dependent on both the hormone concentration and the timing of hormone application (Yasuda et al., 2008; Ding et al., 2016b). Thus, measuring phytohormone concentrations, as well as the resulting signaling, is vital to understanding hormone crosstalk. One approach for the measurement of hormone signaling relies on the use of hormone-specific transcriptional fluorescent reporters (Waadt, 2015). These reporters, such as the auxin reporter DR5 (Ulmasov et al., 1997) and the CK reporter TCSn (Zurcher et al., 2013), are driven by synthetic promoters engineered to contain *cis*-elements targeted by central transcription factors in hormone signaling, thus bypassing hormone-independent regulatory elements. This synthetic approach may represent an advantage over the use of native promoters of hormone marker genes for measuring activity of hormone signaling. For instance, the

expression of the classical SA marker gene *PATHOGENESIS-RELATED 1* (*PR-1*) can be regulated independently of SA (Tsuda et al., 2013), pointing to a need for caution in using marker gene expression to measure hormonal activity.

Similarly, understanding of hormonal crosstalk can benefit from direct measurements of plant hormones levels. Several mass spectrometry-based high-throughput protocols to simultaneously quantify multiple classes of plant hormones have been established. Such methods have already been used, for example, in systemic analysis of hormonal crosstalk during abiotic stress responses (Maruyama et al., 2014). Other methods based on immunological detection have also been used, although with limited sensitivity (Mertens et al., 1983). Genetically encoded plant hormone sensors, which exploit native hormone recognition mechanisms and allow for quantitative measurement of hormones *in vivo*, have recently been developed and provide extraordinary sensitivity. While such hormone sensors for ABA (Jones et al., 2014; Waadt et al., 2014), AUX (Brunoud et al., 2012; Wend et al., 2013), JA (Larrieu et al., 2015) and SL (Samodelov et al., 2016) have been established, sensors for other plant hormones such as SA, ET, CK, GA and BR await method development. Although the use of fluorescent and genetically encoded hormone sensors in the context of plant immunity has so far been limited (Patkar et al., 2015), these approaches should provide excellent opportunities to understand temporal and spatial/cellular dynamics of plant hormone activities and hormone crosstalk. This is an important consideration given that hormone crosstalk in plant immunity is not only pathogen-specific, but also spatially-regulated (Spoel et al., 2007).

B.5 Engineering of Hormone Crosstalk in Plant Immunity

Hormone crosstalk is believed to balance between defense response activation and plant development and abiotic stress tolerance. In nature, plants are frequently exposed to multiple abiotic and biotic stresses at the same time. In such conditions, the involvement of hormone crosstalk is evident, as reported by transcriptome analysis and genome-wide association studies (Atkinson and Urwin, 2012; Vos et al., 2015; Coolen et al., 2016; Davila Olivas et al., 2017). Increased understanding of the role of hormones and hormonal interplay during plant immunity paves the way for attempts at engineering of hormonal crosstalk to generate plants with increased disease resistance and abiotic stress tolerance, maintaining overall plant fitness.

The mechanisms that mediate hormonal crosstalk have not been completely defined. While examples of co-regulation of transcriptional targets can be found, in general hormone-regulated transcriptional networks are mostly non-overlapping (Nemhauser et al., 2006). One common theme in hormone crosstalk is the sharing of signaling proteins. For example, NONEXPRESSOR OF PR GENES1 (NPR1) is a known regulator of SA signaling. Given the antagonistic relationship between SA and JA, a negative regulation of NPR1 by post-translational mechanisms also functions as mechanism of JA signaling activation (Spoel et al., 2003; Tada et al., 2008; Spoel et al., 2009; Saleh et al., 2015). In another example, DELLA proteins, known negative regulators of GA signaling, can positively regulate the JA pathway. In the absence of GA, DELLAs can actively compete with the JA-regulated transcription factor MYC2 for binding to the negative regulators of JA signaling Jasmonate-ZIM-domain proteins (JAZ), thus enabling crosstalk between the GA and JA pathways (Hou et al., 2010). NPR1 and DELLA proteins can thus be considered hormone crosstalk hubs, or proteins that mediate the interplay between different hormonal signaling pathways (**Figure B-1A, B**). Such hubs are

integral for hormone crosstalk and may be engineered to shift hormonal networks to the desired physiological responses, and theoretically lead to outcomes of enhanced pathogen resistance without plant fitness reduction. The recent demonstration that growth tradeoffs and defense activation can be unlinked (Campos et al., 2016) indicates that biotic trade-offs can be overcome, paving the way for the manipulation of hormonal pathways not only for increased defense to pathogens, but also for enhanced abiotic stress tolerance and plant growth.

So far, many hormone crosstalk hubs in plant immunity have been identified through genetic screens, however the computational and systems biology approaches mentioned above are likely to reveal not only patterns of crosstalk, but also hormone crosstalk hubs. Another possible strategy for the identification of hormonal crosstalk hubs lies on pathogen effector targets. Several pathogen effectors are known to target plant hormone signaling or metabolic pathways. Effective manipulation of hormonal networks to favor conditions for pathogenicity and virulence requires overcoming of the multiple regulatory steps commonly present in plant hormone signaling/biosynthetic pathways, as well as the genetic redundancy often observed in these pathways. Thus, effector targets with hormone regulatory activity are likely to substantially change host physiological status and function as hormone crosstalk hubs. For example, effectors are known to target NPR1 function (Schellenberg et al., 2010; Ding et al., 2016b), a suitable hormone crosstalk hub for target as it plays a key role in the SA-JA antagonism. *P. syringae* pathovars also secrete effectors to target JAZ proteins for degradation and activate JA signaling (Jiang et al., 2013b; Gimenez-Ibanez et al., 2014). Recent efforts of effector target identification through high-throughput yeast two-hybrid approaches have been very successful and reveal that the hormone signaling proteins targeted by pathogen effectors are conserved even among effectors from different pathogens (Mukhtar et al., 2011; Wessling et al., 2014). Finally, natural

plant genetic diversity can also be used to determine hormone crosstalk hubs with different levels of crosstalk activity. Natural genetic variation in relation to hormone crosstalk pathways has been demonstrated not only among different plant species, but also among accessions/varieties of the same species (Thaler et al., 2012; De Vleesschauwer et al., 2014; Berens et al., 2017). In Arabidopsis, ecotypes treated with SA- or JA-derivatives responded differently, suggesting the existence of natural genetic variation and phenotypic plasticity in relation to hormone crosstalk (Kliebenstein et al., 2002; Ekengren et al., 2003; Zhao et al., 2003; van Leeuwen et al., 2007), thus opening possibilities for genetic mapping of genes with functions in hormone crosstalk regulation.

One expected limitation of a hormone crosstalk engineering approach is the high level of interconnectivity of hormonal networks (**Figure B-1A**). Because hormonal networks control several physiological responses, engineering efforts may lead to undesired pleiotropic effects. Thus, the same plasticity that allows plants to respond to varying stimuli can also be a constraint in engineering hormone crosstalk for increased fitness. However, recent advances in synthetic biology may allow for the development of synthetic hormonal crosstalk networks with tunable properties that may help circumvent pleiotropic effects. Such approaches may make use of synthetic promoters, harboring *cis*-regulatory elements to drive the expression of particular hormone crosstalk hubs under specific conditions (Dey et al., 2015), as well as more complex genetic circuits driven by tunable switches. Recent efforts to quantitatively characterize genetic parts for plant synthetic biology have been promising (Braguy and Zurbriggen, 2016), including synthetic small RNAs, repressible promoters/repressors pairs (Schaumberg et al., 2016) and chemically or optogenetically inducible switches (Stahl et al., 2013; Mueller et al., 2014). Moreover, effective recapitulation of a hormonal signaling pathway has been demonstrated in

yeast (Pierre-Jerome et al., 2014), indicating the feasibility of engineering synthetic hormonal pathways, albeit in a far less complex system. The use of tissue-specific regulatory elements may also allow for activation of localized defense responses, likely diminishing fitness costs frequently associated with constitutive/systemic defense activation. This tissue-specific approach was successfully used to express NPR1, creating plants with increased resistance to biotrophs without negative effects on plant fitness (Molla et al., 2016).

B.6 Conclusions

Hormonal crosstalk is ubiquitous to plant life, and as such is an essential feature of the plant immune response. Genetic analysis of plant hormone signaling components clearly shows the importance of hormone crosstalk in plant immunity, which is also supported by frequent exploitations of hormone crosstalk by pathogens to increase virulence. Although it is assumed that hormone crosstalk is beneficial for plants, we are far from fully understanding this process, especially in regards to how and when crosstalk contributes to increased plant fitness. Recent improvements in computational biology approaches, coupled to improved methods of quantification of hormone levels and signaling, will aid in our ability to understand hormonal crosstalk in plant defense, and the ramifications of this crosstalk to plant fitness. Further, as the use of beneficial microbes in agriculture becomes more prevalent, the need to understand the hormonal crosstalk between plants and beneficial microbes becomes more pressing.

Domestication and breeding of crop plants may have altered patterns of hormonal crosstalk, favoring those that enhance domestication traits, such as yield production, however selecting against crosstalk interactions that may help stress tolerance. Advancements in synthetic biology may allow the development of synthetic hormonal networks with different

functionalities, thus allowing fine-tuning of plant responses according to the types of invading pathogens, interacting beneficial microbes or prevailing environmental conditions (**Figure B-1 B**). Such approaches may lead to the development of advanced crops with increased yield, enhanced stress tolerance abilities, as well as improved capacity to associate with beneficial microbes for increased plant health.

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B.7 Figures

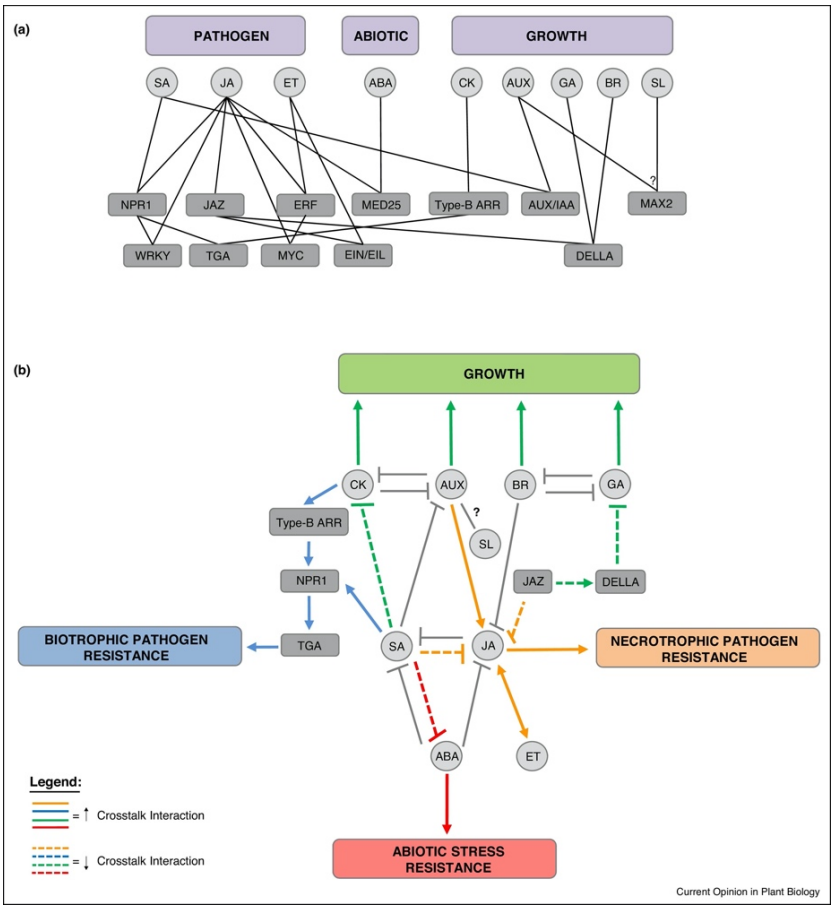

Figure B-1: **Complex hormone crosstalk interactions in response to biotic stress, abiotic stress and during plant growth. (a).** In response to pathogen stress, each plant hormone is involved in the deployment of an appropriate immune response depending on the pathogen perceived. Each line indicates an established interaction between a plant hormone with various signaling proteins as potential hormone crosstalk hubs (dark-gray boxes). Purple boxes indicate general hormone classifications. Gray circles represent the major classes of plant hormones SA- salicylic acid; JA- jasmonate; ET- ethylene; ABA- abscisic acid; AUX- auxin; CK- cytokinin; GA- gibberellin; BR- brassinosteroids; SL- strigolactones. A "?" indicates an unclear interaction in hormone crosstalk. For explanation of crosstalk shown, see Table 1 and reference (Shigenaga and Argueso, 2016). **(b). Potential crosstalk engineering of hormonal pathways to improve biotic and abiotic stress tolerance and plant growth.** Although hormone crosstalk is highly complex, there is potential to engineer hormone crosstalk for particular outcomes. Each line represents an established crosstalk interaction among various hormones in relation to biotic and abiotic stress responses, as well as growth. Colored lines represent pathways that could be engineered by certain crosstalk signaling hubs (dark-gray boxes) to increase certain responses/processes (colored boxes), such as pathogen resistance or plant growth. An arrow indicates a positive/synergistic interaction, while a blunt end indicates a negative/antagonistic interaction. Solid lines represent engineering for increased crosstalk interaction. Dotted lines represent engineering for a decreased crosstalk interaction. Blue lines and boxes represent engineering for increased resistance to biotrophic pathogens. Orange lines and boxes represent

engineering for increased resistance to necrotrophic pathogens. Red lines and boxes represent engineering for increased abiotic stress tolerance. Green lines and boxes represent engineering for increased plant growth. A “?” indicates an unclear interaction in hormonal crosstalk.

B.8 Tables

Table B-1: Reports of hormone crosstalk interactions in relation to biotic stresses in various plant species. SA- salicylic acid; JA- jasmonate; ET- ethylene; ABA- abscisic acid; AUX- auxin; CK- cytokinin; GA- gibberellin; BR- brassinosteroids; SL- strigolactones. "Synergistic" indicates a positive interaction between hormones involved in crosstalk, while "Antagonistic" indicates a negative interaction between the hormones. "One-way" indicates that the first hormone listed is either positively or negatively interacting with the second hormone listed in the "Hormone Crosstalk" column, resulting in a unique outcome or pathogen response. "Unclear" indicates a possible synergistic or antagonistic relationship that has not yet been confirmed. (Penninckx et al., 1996; Penninckx et al., 1998; Lorenzo et al., 2003; Anderson, 2004; Lorenzo et al., 2004; Wang et al., 2007; Navarro et al., 2008; Yasuda et al., 2008; Choi et al., 2010; De Vleesschauwer et al., 2010; Hou et al., 2010; Argueso et al., 2012; De Vleesschauwer et al., 2012; Naseem et al., 2012; Qi et al., 2012; Thaler et al., 2012; Jiang et al., 2013a; Nahar et al., 2013; Xu et al., 2013; Großkinsky et al., 2014; Piisilä et al., 2015)

Hormone Crosstalk	Interaction	Species	Pathogen Response	Reference
SA – JA/ET	Antagonistic	Conserved among various monocot and eudicot species	SA-responses increase resistance to (hemi-)biotrophic pathogens; JA-responses increase resistance to necrotrophic pathogens; in some species SA & JA are effective against both biotrophic and necrotrophic pathogens	Reviewed in [24]
JA – ET	Synergistic	*Arabidopsis thaliana*	Increased resistance to necrotrophic pathogens	[4,21,85,86]
SA – CK	One-way Antagonism	*Arabidopsis thaliana*	Increased susceptibility to biotrophic pathogens	[27]
CK – SA	One-way Synergism	*Arabidopsis thaliana* *Oryza sativa*	Increased resistance to (hemi-)biotrophic pathogens	[26,27,84]
CK– JA	Unclear	*Arabidopsis thaliana*	Unknown, may increase resistance to necrotrophic pathogens	[26]
CK – ABA	Antagonistic	*Nicotiana tabacum*	Increased resistance to (hemi)biotrophic pathogens	[83]
ABA – JA/ET	Antagonistic	*Arabidopsis thaliana*	Increased resistance to herbivory, but compromises resistance to necrotrophic pathogens	[81]
ABA – ET	Antagonistic	*Oryza sativa*	Increased resistance to necrotrophic pathogens	[82]
ABA – SA	Antagonistic	*Arabidopsis thaliana* *Oryza sativa*	Increased susceptibility to biotrophic pathogens	[39,92]
AUX – JA	One-way Synergism	*Arabidopsis thaliana*	Increased resistance to necrotrophic pathogens	[90]
AUX – SA	Antagonistic	*Arabidopsis thaliana*	SA-responses increase resistance to (hemi-)biotrophic pathogens; AUX-responses increase susceptibility	[91]
AUX – CK	Antagonistic	*Arabidopsis thaliana*	CK-responses increase resistance to biotrophic pathogens, AUX-responses increases susceptibility	[37]
GA – JA/ET	Antagonistic	*Arabidopsis thaliana*	Increased susceptibility to necrotrophic pathogens	[62,88]
GA – SA	Synergistic	*Arabidopsis thaliana*	Increased resistance to biotrophic pathogens	[88]
BR – GA	Antagonistic	*Oryza sativa*	Decreased resistance against necrotrophic pathogen	[93]
BR – SA	Antagonistic	*Oryza sativa*	Decreased resistance against necrotrophic pathogen	[93]
BR – JA	Antagonistic	*Oryza sativa*	Decreased resistance to nematode	[87]
SL – AUX	Unclear	*Arabidopsis thaliana*	Increased resistance to bacterial pathogens	[89]

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Appendix C

Supplemental Materials

C.1 Chapter 4 Supplemental Materials

S4.1 "Instructor Notes" PDF

1. For online resources regarding foundational information on plant biology and climate change please refer to the sources below. These resources contain basic prior knowledge students should have learned prior to their undergraduate career, for both specified topics.

- 12 Principles of Plant Biology (American Society of Plant Biology (ASPB)): aspb.org/education-outreach/k12-roots-and-shoots/the-12-principles-of-plant7 biology-2/#toggle-id-1
- Climate Change Education: Essential Information for Educators (National Education Association (NEA)): www.nea.org/climatechange
- Resources for Educators (U.S. Global Change Research Program): www.globalchange.gov/browse/educators

2. During the activity you will be acting as a food security specialist working for IRRI in the Philippines. During Day 2, after giving students the opportunity to brainstorm & passing out the specialty cards, you will introduce the students as to why they have been called to this meeting.

3. Before asking students to create a concept map, give students an outline of how to begin a concept map (**Figure 4-6**).

4. Throughout the activity walk around to check on groups, keep reminding them of the question they are trying to solve: what are the effects of warming on Philippines rice system status?

Continuation S4.1 "Instructor Notes" PDF

5. When assigning students to make an interconnected concept map that is incorporating facts from each specialty give them an example of how to start (**Figure 4-7**).

6. If a group is focusing on one specialty remind them that 23 this is a collaborative effort and different specialties have different knowledge and skills. Make sure each group is incorporating information from all fields.

7. Classroom discussion comparing interconnected concept maps between groups: act as the leader of the discussion.

- First: Ask students to point out some comparisons they noticed between the maps: what is the same and what is different? Write these down for the students to follow.

- Second: Ask if they agree or disagree with these contrasts? How did it change their view on the topic?

- Third: Ask student groups to share a solution they came up with, then ask if other groups had similar solutions or different.

- *Questions to pose for students*: Are there any ways we can reduce these negative impacts expected on agricultural systems? What are other aspects that we should be focusing on?

8. While this lesson plan is intended for an entry-level science college course, it would also work well in an introductory plant biology college course or upper-level biology college course. For use in an upper-level biology course, it is recommended that the instructor assigns peer reviewed review articles to each specialty group in place of or alongside the papaya ring-spot virus case study. References for examples of peer review articles that are useful for this exercise are listed below:

Continuation S4.1 "Instructor Notes" PDF

- *Plant Pathologist*: Noctor, G., Mhamdi, A. (2017) Climate change, CO2, and defense: The metabolic, redox, and signaling perspectives. *Trends in Plant Science*, 22, 857-870. doi: 10.1016/j.tplants.2017.07.007

- *Climatologist*: Naumann, G., Alfieri, L 46 ., Wyser, K., Mentaschi, L., Betts, R.A., Carrao, H., Spinoni, J., Vogt, J., Feyen, L. (2018) Global change in drought conditions under different levels of warming. *Geophysical Research Letters*, 45. doi: 10.1002/2017GL076521

- *Agricultural Economist*: Stevanovic, M., Popp, A., Lotze-Campen, H., Dietrich, J.P., Muller, C., Bonsch, M., Schmitz, C., Bodirsky, B.L., Humpenoder, F., Weindl, I. (2016) The impact of high-end climate change on agricultural welfare. *Science Advances*, 2, e1501452. doi: 10.1126/sciadv.1501452.

- *Agronomist*: Altieri, M.A., Nicholls, C.I. (2013) The adaptation and mitigation potential of traditional agriculture in a changing climate. *Climate Change*, 140, 33-45. doi: 10.1007/s10584-013-0909-y

9. *If desired*, Days 1 and 2 can be combined into one day if instructor has time for each student to individually read the case study in class.

10. *If desired*, the case study & lesson plan can be easily modified for teaching other scientific topics. This lesson plan is aimed to teach students about plant pathology, agriculture, and climate change but many aspects can be modified for other important scientific topics in different fields.

11. *Want to make the lesson more fun?* Provide students with costumes or props to be used depending on their specialty group. Can also provide certain specialty groups with "breaking

Continuation S4.1 “Instructor Notes” PDF

news”, this will give the students new information to use when creating a concept map and provide a more “urgent” or “emergency” feel.

S4.2 Materials PDF: includes Specialty Group Identity Cards, Specialty Group Data Cards, and Instructor Cards.

Specialty: Plant Pathologist

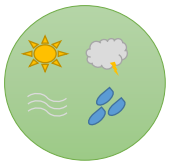
- Expert in bacterial diseases of rice plants
- Focuses on the bacterial pathogen: *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)
- *Xoo* causes bacterial blight of rice
- Understands what factors are important for the disease to start

Specialty: Agronomist

- Expert in soil management and crop production
- Focuses specifically on the production of rice
- Understands what components rice plants need for proper growth and production

Specialty: Climatologist

- Expert on effects of changes in temperature and why it could be rising
- Focuses on tropical storm occurrences
- Understands what factors are important for severe changes in the weather patterns

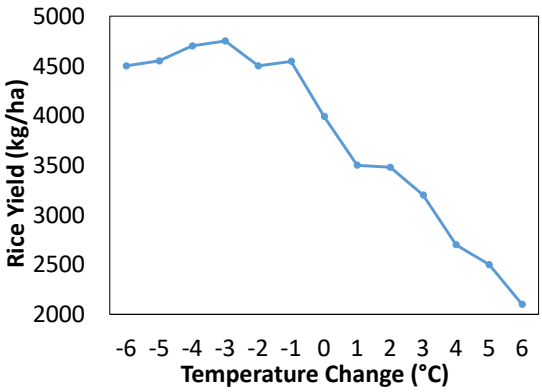


Specialty: Economist

- Expert on the monetary value of rice production
- Middleman in financial situations for rice acquisition from farmers to interested buyers
- Understands what factors are important for changes in crop demand

Food Security Specialist

Expert in organizing and implementing a food security program through policies and procedures with local and international government agencies



Plant Pathologist

- Bacterial blight (BB) is **favored by warm temperatures**, high humidity, rain, and deep water
- The bacterium can be **easily disseminated** by irrigation water, by splashing or windblown rain, by plant-to-plant contact
- Can cause **yield losses up to 70%**, if undetected can cause total loss
- *Xoo* ranked in the top-10 list of bacterial plant pathogens
- *Xoo* is widely distributed throughout rice growing countries in Asia & Africa
- In Asia, perennial weeds are considered **alternate hosts** that *Xoo* can live in during winter months

Plant Pathologist Data Points

Year	Disease Prevalence* (%)
1994	25
1996	30
1998	45
2000	35
2002	55
2004	70
2006	65
2008	75
2010	80
2012	95
2014	85
2016	90
2018	95

*= Reported and Confirmed Bacterial Blight

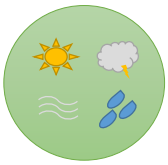
Agronomist

- Optimal conditions for rice growth
  - Temperature (day & night): **29/21°C**
  - Moisture: **High**
  - Soil requirements: **Grows on a variety of soils**
    - If nutrient content is too high can lead to plants being more susceptible to pests
- If grown outside of optimal conditions rice plants **do not** grow at the normal rate of a healthy rice plant (**will not reach high yields such as 5000 kg/ha**)
- More specifically, high nighttime temperature has been shown to **negatively impact growth**

Agronomist Data Points:

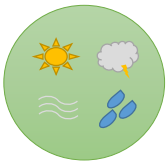
Year	Yield (kg/ha)
1994	4800
1996	4850
1998	4700
2000	4750
2002	4600
2004	4545
2006	3990
2008	3500
2010	3480
2012	3200
2014	2700
2016	2500
2018	2000

Climatologist



- Air temperatures have been slowly increasing over the years
- Tropical **storm incidences** have been increasing steadily, storm duration is hard to identify
- As tropical storms approach, the severity of **wind and precipitation** becomes more unpredictable
- 2017 marks the **highest annual temperature** and the 41st consecutive year that global temperatures have risen
- By 2050, global temperatures are expected to **increase approx. 3°C**
- **Night temperatures are increasing**, leading to plants being exposed longer to high temperature conditions
- As temperatures increase, humidity becomes more **unpredictable**

Climatologist Data Points



Year	Tropical Storm Probability (1- low, 6-high)
1994	1
1996	2
1998	1
2000	3
2002	4
2004	4
2006	5
2008	4
2010	5
2012	5
2014	6
2016	4
2018	5

Agricultural Economist

- Over the years rice yield has declined substantially, up to **40% crop loss seen on average** with some years worse than others
- As yield decreases, demand increases leading to prices increasing
- Due to increase in production price and demand the **average cost for consumer has increased**
- The average price for rice is **~20 pesos/kg**, however these prices have been steadily increasing

Agricultural Economist Data Points

Year	Rice Prices (pesos/kg)
1994	5
1996	5
1998	6
2000	8
2002	8
2004	10
2006	15
2008	20
2010	35
2012	38
2014	35
2016	37
2018	40

S4.3 Case Study Narratives PDF

Case Study: Eradication of Papaya Ringspot Virus on Hawaii: Agricultural Economist

Papaya ringspot virus (PRV) is a major concern worldwide in papaya production because it is a disease that causes production of smaller, diseased fruit and smaller overall tree size. PRV is a disease that affects the whole papaya tree. PRV has been especially problematic in Hawaiian papaya production. While PRV was known to be present in Hawaii since the 1940's as a relatively mild viral infection, the virus mutated and became more aggressive in the 1950's leading to the Hawaii papaya industry almost being destroyed. While PRV was thought to be gone in 1975 from Hawaii, it resurfaced in the late 1990's.

Papaya trees that have the PRV disease grow less and have less fruit production which leads to less profits. This would suggest that as papaya trees become more sick with PRV, the cost of production will increase, but the amount of fruit produced will likely decrease (Figure 1).

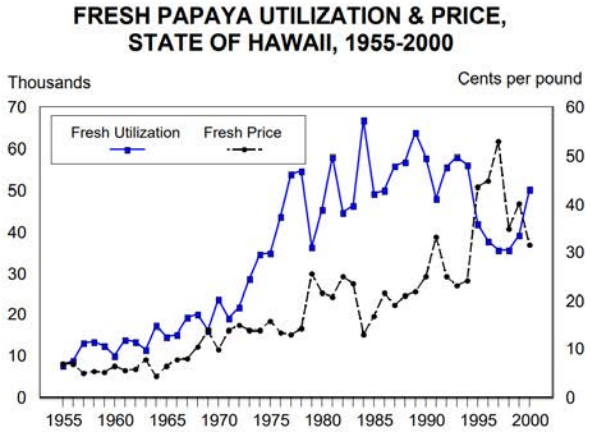


Figure 1: Price of Hawaii papaya (black) and the amount of papaya produced (blue). During the height of the PRV infection in the early 1990's papaya production decreased while price increased.

When PRV was widespread in Hawaii, the cost of papaya increased for consumers before 1998 when a solution was found. With the US being a large exporter of papaya, and most US produced papaya being grown in Hawaii, the impact of the disease was going to lead to a huge

financial loss for the industry. During the 1990s, there was a 50% decline in US papaya production due to this devastating virus. When the disease was at its worse, an average of 5,000 pounds of fruit per acre was produced, compared to the normal production size of 125,000 pounds of fruit per acre.

Scientists, agronomists, and farmers attempted various ways to decrease disease in Hawaiian papaya farms, such as removal of infected material, avoiding diseased fields, and aphid control but none of these solutions worked for long. To save the papaya industry in the US, the government allowed for genetically modified papaya to be produced. The genetically modified papaya, named Rainbow Papaya, although slightly different in color, is similar in flavor and size, giving customers the same experience. The price of papaya dropped by 22 cents/lb after the introduction of the genetically modified papaya, as Rainbow Papaya was able to produce more fruit per acre than the non-genetically modified papaya in light of PVR. Additionally, the genetically modified papaya is now being sold for 1 dollar at most Hawaiian farmer's markets. The average price for Hawaiian papayas in 2011 was approximately $2.00/lb.

Continuation S4.3 Case Study Narratives PDF

Case Study: Eradication of Papaya Ringspot Virus on Hawaii: Plant Pathologist

Papaya ringspot virus (PRV) is a major concern worldwide in papaya production because it is a disease that causes production of smaller, diseased fruit and smaller overall tree size. PRV is a disease that affects the whole papaya tree. PRV has been especially problematic in Hawaiian papaya production. While PRV was known to be present in Hawaii since the 1940's as a relatively mild viral infection, the virus mutated and became more aggressive in the 1950's leading to the Hawaii papaya industry almost being destroyed. While PRV was thought to be gone in 1975 from Hawaii, it resurfaced in the late 1990's.

PRV is a disease caused by a plant virus and is most common in tropical and subtropical environments. Specifically, PRV symptoms include wilted and spotted leaves and spotted fruits. Symptoms vary based on stage of disease, but most commonly farmers will notice yellowing leaves, less growth, and spotted fruits (Figure 1). Leaf symptoms are more severe in cold

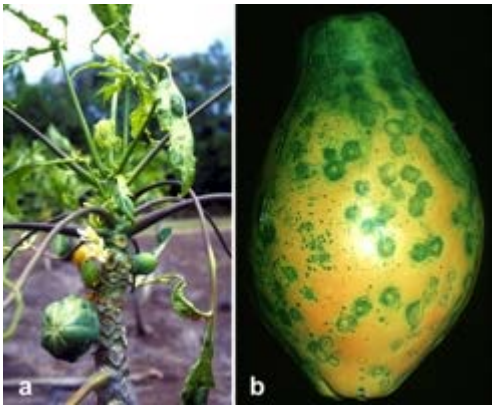


Figure 1: Papaya ringspot virus produces smaller and mis-shaped papayas that have rings on the outside.

temperatures. The virus is able to spread through the entire plant, thus if a plant is exhibiting PRV symptoms it is very hard to cure. The virus is spread by aphids, planting of infected

seedlings, and by use of contaminated tools. The virus has not been shown to spread through the seeds.

PRV was first found in Hawaii in 1992 and quickly spread to take over the entire area in only five years. Scientists, agronomists, and farmers attempted various ways to decrease disease in Hawaiian papaya farms, such as removal of infected material, avoiding diseased fields, and aphid control but none of these solutions worked for long. The only way to successfully get rid of the disease was by planting genetically engineered papaya that was resistant to PRV, meaning the virus was unable to infect these trees. A Hawaiian native research scientist, Dr. Gonsalves, got approval from the US Government to make and use the genetically engineered papaya called Rainbow Papaya. Farmers of Hawaiian papaya plantations began planting Rainbow Papaya in diseased fields and the resistance to the virus held strong. As a result, the Rainbow Papaya was widely adopted by the Hawaiian papaya farmers. Without the use of the Rainbow Papaya the Hawaiian papaya industry would have been lost.

Continuation S4.3 Case Study Narratives PDF

Case Study: Eradication of Papaya Ringspot Virus on Hawaii: Agronomist

Papaya ringspot virus (PRV) is a major concern worldwide in papaya production because it is a disease that causes production of smaller, diseased fruit and smaller overall tree size. PRV is a disease that affects the whole papaya tree. PRV has been especially problematic in Hawaiian papaya production. While PRV was known to be present in Hawaii since the 1940's as a relatively mild viral infection, the virus mutated and became more aggressive in the 1950's leading to the Hawaii papaya industry almost being destroyed. While PRV was thought to be gone in 1975 from Hawaii, it resurfaced in the late 1990's.

PRV results in much smaller plant and leaf size compared to a healthy plant. The decreased leaf size leads to lower fruit yield. In Hawaii, papaya is grown year-round. PRV is more infectious during the colder months, leading to less fruit production during cold month planted papaya. The disease is known to be spread by aphids, but using pesticides was useless in the height of the outbreak. Aphids are spread to papaya plants by strong wind, which is also required for papaya plant pollination. Scientists, agronomists, and farmers attempted various ways to decrease disease in Hawaiian papaya farms, such as avoiding specific growing areas and keeping plants to one specific location, but none of these solutions worked for long. There was not a good option to bring in a different variety of papaya because the papaya being grown had been bred to thrive in the high rainfall and volcanic, low nutrient soils of Hawaii. For most papaya varieties, too much water can cause root rot and ultimately death of the tree. However, the high rainfall in Hawaii, mixed with the high drainage lava rock soils provide a perfect water regimen to the papaya bred for production on Hawaii.

Continuation S4.3 Case Study Narratives PDF

To get rid of PRV on Hawaii, the government allowed for regulated production and use of genetically modified papaya that was resistant to PRV, known as Rainbow Papaya. Today, about 70% of papaya grown on Hawaii are Rainbow Papaya which has saved farmers livelihoods. The Rainbow papaya is much larger than an infected non-genetically modified papaya and therefore, can produce more papaya fruit (Figure 1). The use of the genetically

Figure 1: Genetically modified Rainbow papaya (left) compared to a non-genetically modified papaya (right) after infection by PRV.

modified plants alongside non-genetically modified papaya also help to increase the diversity of papaya in Hawaii which could help to protect more varieties if PRV is able to infect Hawaii papaya again. Concerns first associated with the use of genetically modified papaya have now lessened as the Hawaiian population has accepted the Rainbow Papaya due to the advantages it brought to the papaya industry.

Continuation S4.3 Case Study Narratives PDF

Case Study: Eradication of Papaya Ringspot Virus on Hawaii: Climatologist

Papaya ringspot virus (PRV) is a major concern worldwide in papaya production because it is a disease that causes production of smaller, diseased fruit and smaller overall tree size. PRV is a disease that affects the whole papaya tree. PRV has been especially problematic in Hawaiian papaya production. While PRV was known to be present in Hawaii since the 1940's as a relatively mild viral infection, the virus mutated and became more aggressive in the 1950's leading to the Hawaii papaya industry almost being destroyed. While PRV was thought to be gone in 1975 from Hawaii, it resurfaced in the late 1990's.

PRV results in less papaya growth and fruit production. Symptoms of the disease include spots on the leaves, stem and fruit. The fruit may also be distorted in shape. Once infected with the virus, the plant cannot recover, and papaya plantations become decimated (Figure 1). In

Figure 1: Hawaiian papaya field devastated by the Papaya ringspot virus.

Hawaii, papaya is grown year-round. PRV is more infectious during the colder months, leading to less fruit production during cold month planted papaya. While the disease is less infectious during warm months, the papaya can stop producing fruit during warm weather and decrease yield. Thus, changing the growing season was not a good option. The disease is spread by aphids which do not normally choose to feed on papaya, but if other plants that aphids do feed on, such as melon, are in the same field, an aphid infestation in papaya is possible. Aphids are not flying

organisms so they would slowly infect other plants. However, in the presence of high wind, aphids can easily be carried to new plants to infect.

Scientists, agronomists, and farmers attempted various ways to decrease disease in Hawaiian papaya farms, such as avoiding specific growing areas and keeping plants to one specific location, but none of these solutions worked for long. There was not a good option to bring in a different variety of papaya because the papaya being grown had been bred to thrive in the volcanic, low nutrient soils of Hawaii. Because the wind and growing season temperatures helped to increase the prevalence of the disease, PRV moved too quickly to determine which plant would be susceptible to the virus infection. To get rid of PRV, the government allowed for regulated production and use of genetically modified papaya that contained a gene for resistance to the PRV, known as Rainbow Papaya. Rainbow Papaya is now thriving in the Hawaiian papaya production, making up 70% of the market.

Continuation S4.3 Case Study Narratives PDF

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***Climatologist, agronomist, and pathologist Figure 1 from this reference**

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***Economist figure 1 from this reference**

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