TEMPERATURE MODULATION OF SALICYLIC ACID-MEDIATED SIGNALING AND *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000 PATHOGENESIS IN *ARABIDOPSIS THALIANA*

By

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ABSTRACT

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A predominant issue of global concern is increasing agricultural output to meet the steady rise in global demand. One of the most significant challenges to meeting this objective is overcoming crop loss due to disease and adverse weather. While individual biotic and abiotic stresses are damaging to plants, they can have catastrophic affects when combined, as most often occurs in the field. It has long been observed that environmental conditions, such as temperature and humidity, play a determining role in the outcome of plant-pathogen interactions. Both low and high temperatures have been shown to promote disease depending on the pathosystem involved. Salicylic acid (SA) is a plant hormone important for protection against a broad spectrum of crop-relevant pathogens. However, the direct effect of elevated temperature on SA-mediated defense is unknown. The aims of the research described here were to determine 1) what impact elevated temperature has on SA biosynthesis and signaling, 2) whether observed effects are a direct result of temperature on the host or are also pathogen-dependent and 3) how observed temperature effects on the plant and pathogen interact to determine the final disease outcome. Using the model *Arabidopsis thaliana* and *Pseudomonas syringae* pv. *tomato* DC3000 plant-pathosystem, I present evidence demonstrating that loss of SA biosynthesis and enhanced delivery of bacterial type III effector (T3E) proteins into the plant cells at elevated temperature (30°C) both contribute to enhanced disease.

In the host, both SA biosynthesis and signaling are affected in a pathogen-independent manner resulting in enhanced susceptibility. Global transcriptome profiling revealed a temperature-sensitive bifurcation in the SA signaling pathway, with 66% of benzothiadiazole (BTH)-regulated genes, including *ISOCHORISMATE SYNTHASE 1* (*ICS1*) and the widely-used SA marker genes *PATHOGENESIS RELATED 1* (*PR1*), *PR2* and *PR5*, showing compromised expression at 30°C. Surprisingly, BTH-mediated protection against disease is maintained at elevated temperature in spite of the loss of the temperature-sensitive *PR1*/*ICS1* branch of SA-signaling. Exploration of a potential mechanism for SA-mediated protection revealed a novel role of SA in restricting translocation of bacterial T3E into host cells, as translocation was increased in SA-deficient mutants and reduced in BTH-treated plants at 23°C. However, there also seems to be a direct effect of temperature on the pathogen, as T3E translocation was increased more in response to elevated temperature than SA-deficiency.

Taken together, these findings support a model whereby elevated temperature acts on both the host, resulting in loss of SA biosynthesis, and on the pathogen, resulting in increased secretion of T3E proteins into plant cells, to promote enhanced bacterial multiplication and disease. Provision of an SA signal, such as BTH, is sufficient to reduce translocation of effector proteins to confer protection against disease. As BTH is used commercially as a crop protectant, the discovery of preserved BTH-mediated protection at elevated temperatures is agriculturally relevant. Furthermore, exploration of the temperature-sensitive and -insensitive branches of SA signaling may also be used to inform genetic approaches to achieve plant resilience to disease under adverse environmental conditions.

This dissertation is dedicated to my family, Scott and Carla Caughel. I would not have achieved this without you.

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KEY TO ABBREVIATIONS

ABA	abscisic acid
BTH	benzothiadiazole
COR	coronatine
DEG	differentially expressed gene
ET	ethylene
ETI	effector triggered immunity
JA	jasmonate
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	pattern triggered immunity
SA	salicylic acid
SAG	glucosylated SA
SAR	systemic acquired resistance
T3E	type III effector
T3SS	type III secretion system
TF	transcription factor
WT	wild type

Chapter 1

Turning up the heat: the role of elevated temperature in plant-pathogen interactions

A special thank you to Scott A. Caughel for help with the figures in this chapter.

Abstract

One of the greatest challenges scientists face in the 21st century is how to increase crop yields to meet rising global food demands. This challenge is made more difficult by global changes in weather that may increase the severity of crop loss due to combined abiotic and biotic stresses. To meet these challenges, a more thorough understanding of how environmental factors affect the plant host, the pathogen and their interaction is needed. In this chapter, I outline some of the known effects of an important climate condition, elevated temperature, on plant-pathogen interactions, as well as relevant crosstalk between salicylic acid and other hormones, such as jasmonic acid, abscisic acid and auxin, that may be involved in determining the disease outcome under this condition.

Introduction

It was the perfect storm – a genetically limited, susceptible host (potato), an exotic pathogen (*Phytophthora infestans*) and the cool, moist conditions ideal for sporulation. The outcome? A famine that ravaged Ireland from 1845 to 1852, killing a million people[1]. While not typical, severe crop losses due to biotic and abiotic stresses can be as high as 40% and 50%, respectively, even with modern disease prevention strategies[2, 3]. When these stresses are combined, the losses can be catastrophic, especially in poor and developing countries that often rely on a single staple crop as their primary source of nutrition[1]. Unfortunately, breeding efforts to optimize yield typically reduce genetic diversity, resulting in a general loss of crop plant resistance that will likely be exacerbated by adverse environmental conditions[4]. With global demand for food, fuel and other plant products on the rise, one of the primary challenges for plant scientists in the 21st century will be increasing crop yield in the face of increasingly severe and dynamic weather conditions[1, 2].

Most plant pathogens cause disease by secretion of virulence factors, such as effector proteins, into host cells to shut down basal plant defense responses[5] (Fig. 1b). One of the most widely used methods of engineering crop resistance has been breeding for plant resistance (R) proteins, which function by recognizing, either directly or indirectly, the presence of pathogen effector proteins resulting in effector-triggered immunity (ETI)[6]. ETI is associated with a specific type of programmed cell death called the hypersensitive response (HR), and can result in a broad spectrum, long lasting systemic defense response, systemic acquired resistance (SAR)[6]. Because ETI involves recognition of a single effector protein by a single R protein, it is also referred to as “gene-for-gene resistance,” and the gene encoding the recognized effector was

previously known as an "avirulence" gene[7]. The downside of this type of defense response is that the pathogen can, over time, modify or eliminate the avirulence gene to restore its virulence[6].

Within the last several years a novel approach for engineering a longer-lasting, broad-spectrum resistance has been shown to be successful. This approach involves the introduction of pattern recognition receptors (PRRs) into crop plants to broaden their pathogen detection capabilities[8]. PRRs are membrane-localized receptors that activate defense responses, referred to as pattern-triggered immunity (PTI), upon detection of conserved microbial patterns, such as fungal chitin or bacterial flagellin[9, 10] (Fig. 1b). PTI is transient and elicits a less intense defense response compared to ETI; however, because PTI involves recognition of conserved components, the pathogen cannot easily modify them to avoid detection[6].

Activation of ETI or PTI results in the accumulation of defense-associated hormones, including salicylic acid (SA)[11-13] (Fig. 1b). SA is important for both local and systemic acquired resistance against hemi/biotrophic microbes that feed from living plant tissues[14]. SA-mediated protection is broad-spectrum—it is effective against various types of pathogens, including bacterial, viral, fungal and oomycete[14]. Examples of economically important pathogens against which SA provides protection are *Fusarium graminearum*, which is a fungal pathogen that causes blight in wheat, barley and maize[15, 16]; *Ralstonia solanacearum*, which is a bacterial pathogen that causes wilt in a large host range including potato and soybean[17, 18]; *Xanthomonas oryzae* pv. *oryzae*, which is a bacterial pathogen that causes blight in rice[18, 19]; and *Phytophthora infestans*, which is the oomycete responsible for late blight resulting in the Irish potato famine[1, 20, 21].

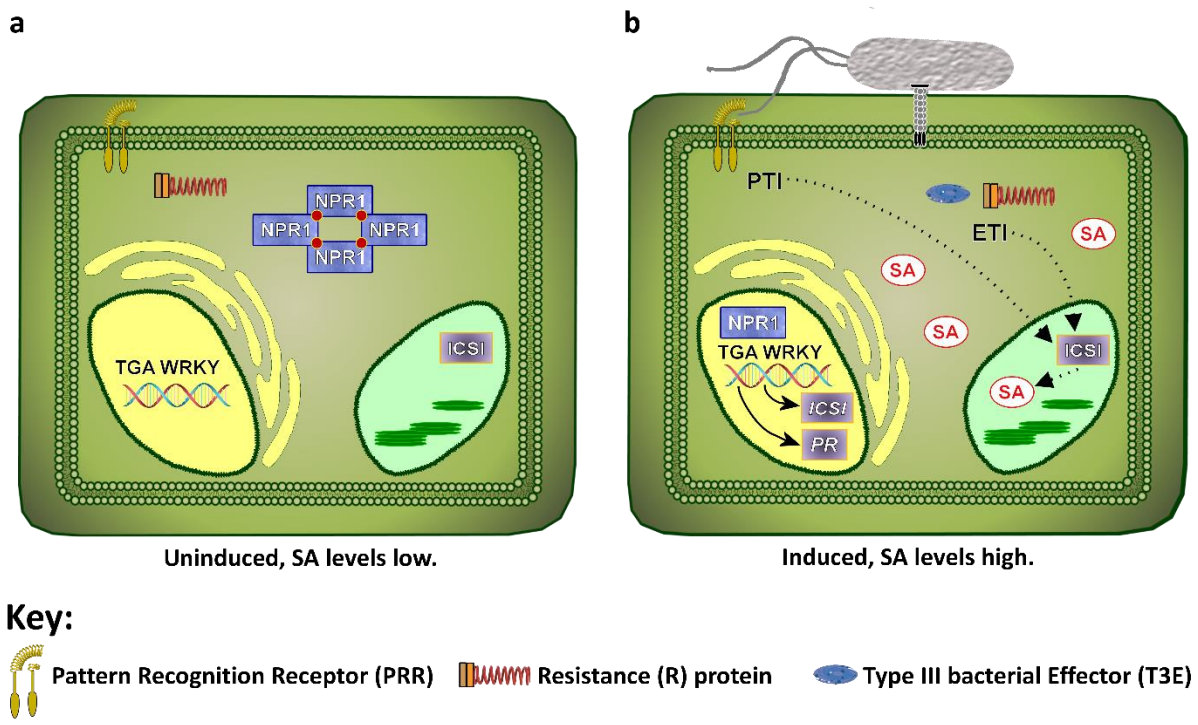

Figure 1. Overview of Arabidopsis perception of and responses to *Pst* DC3000. (**a**) In an uninduced state, SA levels are low and NPR1 is sequestered in the cytosol via disulfide bonds between conserved cysteine residues. (**b**) Virulent strains of *Pst* DC3000 can be detected by PRR recognition of conserved microbial patterns, such as flagellin, to induce PTI. *Pst* DC3000 secretes type III effector proteins into the host cell to shut down plant defense responses. Avirulent strains of *Pst* DC3000 can be detected by host R protein recognition of specific bacterial effector proteins to induce ETI. Upon induction of either PTI or ETI, SA is produced in the chloroplast via the isochorismate pathway, and is then transported into the cytosol. Following SA accumulation, reducing conditions within the cytosol enable reduction of NPR1 disulfide bonds by thioredoxin activity. Upon monomerization, NPR1 protein accumulates in the nucleus where it interacts with various TGA and WRKY TFs to facilitate SA-dependent transcriptional reprogramming. PR proteins are produced and secreted into the apoplast (not shown) where they presumably inhibit bacterial multiplication.

Due to its broad-spectrum protection, activation of SA-mediated defense, either through genetic modifications of plants or application of the SA synthetic analog, benzothiadiazole (BTH), has been shown to be an effective crop protection strategy[22-25]. However, the resilience of this important pathway to the combination of an important climate condition, elevated temperature, and pathogen stress is unknown. The purpose of this dissertation research is to improve our understanding of how elevated temperature affects SA biosynthesis and signaling, with the hope that this information may prove useful in developing strategies for robust crop resistance under increasingly dynamic environmental conditions.

To explain the basis for specific approaches and lines of inquiry pursued during this research project, this literature review will provide 1) examples of temperature affects on plant diseases for which SA-mediated defense is known to be important, 2) what is known regarding the effect of elevated temperature on pathogen growth and virulence, 3) a brief background regarding plant responses to elevated temperature and 4) possible hormonal crosstalk that may be important in the context of disease progression at elevated temperature.

Temperature is a key conditioning factor for plant disease

Temperature is a critical conditioning factor for disease; however, there is no "one size fits all approach" to mitigating temperature affects on plant disease as conditions that cause disease in one pathosystem may not in another[26]. In 1928, Dickson and Holbert described the effect of temperature on disease progression in two major crop plants – wheat and maize[27]. Using the same fungal pathogen, *F. graminearum*, to infect both plant species, they discovered that temperature played a determining role in the disease outcome; however, the temperature range favoring disease varied. Wheat, which has an optimal growing temperature of 4°C to

12°C, was resistant to the fungus around 8°C and susceptible at temperatures above 12°C. On the other hand, maize, which has an optimal growing temperature of 20°C to 24°C, was resistant at temperatures above 24°C and susceptible at temperatures below its optimal growing temperature range. Further investigation into this phenomenon revealed that the cellular composition of the wheat and maize cells was similarly altered when grown outside of their optimal temperature ranges. In both cases, cells had a reduction in cell wall reinforcement, enabling higher penetration efficiency of the fungus, and higher levels of carbohydrates shown to enable optimal growth of the fungus *in vitro*[27].

In this 1928 study, the authors cite similar observations for enhanced disease potential of fungal wilt pathogens infecting plants outside of the host's optimal growing temperature[27]. In these cases, all the plant species assessed were more susceptible at elevated temperatures, but the temperature threshold was shifted up depending on the optimal growing temperature of the plant. For example, cotton and tomato, which are both high temperature grown plants, have a 5°C higher threshold of resistance to fungal wilt than the low temperature plants, flax and cabbage[27]. A more recent study investigating the effect of environmental conditions on sporulation and lesion expansion caused by the fungal pathogen *Cercospora zeae-maydis* in maize found that both were greater at 25°C and 30°C relative to either 20°C and 35°C[28].

The phenomenon of enhanced disease at elevated temperature is not restricted to fungal infections. In fact, some of the earliest temperature-related studies were done to improve methods for infecting tobacco with viral pathogens for the purpose of studying this specific plant-pathogen interaction[29, 30]. One study assessed the effect of pre- and post-incubation of *Nicotiana glutinosa* plants at elevated temperature (36°C) infected with five different viruses.

Although the disease outcome varied for each virus when plants were infected first and then incubated at elevated temperature, increased host susceptibility to all five viruses was observed following pre-incubation at elevated temperature[30]. Both resistant and susceptible sweet potato varieties as well as cotton have been observed to be more susceptible to nematode infection at elevated temperatures (28°C, sweet potato; 35°C, cotton)[31, 32], and *Arabidopsis thaliana* (hereafter Arabidopsis) and *Nicotiana benthamiana* have been shown to be more susceptible to both bacterial and oomycete pathogens in this temperature range as well[33-35].

Within the last ten years, a series of studies from the Hua lab have begun to uncover the molecular mechanisms underlying host susceptibility at elevated temperature specific to R protein-mediated defense. The first study identified that the dwarf plant and disease resistance phenotypes of the *bonzai1* (*bon1*) mutant were due to constitutive activation of the R gene, *SUPPRESSOR OF NPR1-1, CONSTITUTIVE1* (*SNC1*)[34]. Both the growth and defense phenotypes were suppressed by crossing the *bon1* mutant with mutants compromised in SA-mediated defense or by growing plants at temperatures above 28°C[34]. They next explored the effect of elevated temperature on both basal defense and ETI, and found both to be compromised; however, the temperature-sensitive component resulting in enhanced susceptibility remained elusive[35].

To gain further insight regarding the temperature-sensitive component in ETI, the *snc1* and *bon1* constitutive defense mutants were each mutagenized and the resulting populations screened for retention of disease resistance at elevated temperature. Based on characterization of one of these mutants, Zhu and colleagues (2010) identified that loss of SNC1 nuclear

localization at elevated temperature was likely responsible for loss of disease resistance associated with this protein[36]. A specific mutation within the *SNC1* gene restored both SNC1 nuclear localization and disease resistance at 28°C[36]. Interestingly, a similar mutation within the *NECROSIS* (*N*) gene also enabled HR in *Nicotiana tobaccum* when co-expressed with a viral elicitor protein at 30°C, indicating that loss of R protein nuclear localization may be an important underlying cause and that naturally occurring genetic variants or genetic modification may be used to overcome it[36]. Another mutant identified as retaining disease resistance at elevated temperature was found to be affected in the ABA biosynthetic gene, *ABA2*[37]. ABA deficiency caused by this mutation enabled increased nuclear accumulation of both the SNC1 and RPS4 R proteins at 28°C, indicating a role for ABA in loss of R protein nuclear localization at elevated temperature[37]. However, ABA signaling mutants did not have the same effect, making the role of ABA in suppression of R proteins unclear[37]. In addition to protein localization, transcription of several *R* genes is suppressed at elevated temperatures, suggesting there are multiple levels of inhibition of ETI in response to this environmental condition[38, 39].

It is interesting to note that R proteins in every category have been found to be temperature-sensitive, indicating this may be a conserved trait. The majority of R proteins contain nucleotide-binding and leucine-rich repeat (NB-LRR) domains, and are further categorized based on the presence of a Toll interleukin 1 receptor homology (TIR) or coiled-coil (CC) domain[40]. Wang and colleagues (2009) showed loss of HR-induction and disease resistance mediated by three TIR-NB-LRRs – N, SNC1 and RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4) – and three CC-NB-LRRs – RESISTANCE TO P. SYRINGAE PV MACULICOLA1 (RPM1), RPS2 and Rx[35]. Temperature-sensitivity has also been observed for the CLADOSPORIUM FULVUM4

(Cf-4) and Cf-9 R proteins[41], which are LRRs with transmembrane domains (LRR-TM), and RESISTANCE TO POWDERY MILDEW8 (RPW8)[33], which is a TM-CC R protein. However, based on the studies discussed here, the effect of temperature on disease outcome is affected by the pathosystem involved, the temperature used and the length and timing of temperature exposure. For example, Wang and colleagues (2009) observed loss of both HR and disease resistance in Arabidopsis infected with *Pst* DC3000 carrying AvrRpt2[35]. In this study, they used 3-week-old plants acclimated for one week to elevated temperature (28°C) for HR assays and 10 to 14-day-old seedlings grown at constant elevated temperature for disease assays[35]. Conversely, a more recent study using a 24 h acclimation period to elevated temperature (30°C) showed loss of HR but retention of disease resistance in Arabidopsis plants infected with *Pst* DC3000 carrying either HopZ1a or AvrRpt2[42].

The temperature-sensitivity of *N*-mediated resistance in tobacco was used to correlate plant resistance to an avirulent pathogen with induction of SA, which was prevented when infected plants were kept at 32°C and was dramatically elevated upon shifting plants back to 22°C[43]. However, as the N protein has since been shown to be temperature-sensitive in terms of nuclear localization[36], it is likely that loss of SA in these cases is an indirect effect of temperature on the R protein behavior. The few studies specifically investigating the effect of elevated temperature on SA signaling and resistance show conflicting results depending on whether plants were kept at elevated temperature for the duration of the experiment or not. For example, exogenous application of aspirin (acetylsalicylic acid) or SA to tobacco plants at 32°C conferred protection against tobacco mosaic virus in both resistant and susceptible cultivars, as measured by lesion size after shifting plants back to 20°C[44, 45]. However, in another

study, exogenous application of aspirin to tobacco did not provide protection when plants were kept at 32°C[46]. The effect of elevated temperature on PTI (a major component of basal defense) was also investigated recently[47]. Several PTI-associated defense responses, including marker gene induction and MAP kinase phosphorylation, appear to be enhanced rather than suppressed at elevated temperatures[47]. This has been suggested to result from plant adaptation to temperature-regulation of plant pathogens, whose growth (and therefore production of PAMPs) is generally enhanced at elevated temperatures[47].

Elevated temperature affects *Pst* DC3000 virulence

Due to the differential effects of temperature on various plant pathogens, I focus here on what is known regarding the effect of elevated temperature on aspects relevant to *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 that I studied. The basic life cycle of *Pst* DC3000 begins with a brief epiphytic stage on the leaf surface followed by entry into the leaf interior via natural openings, such as stomata, or wounds[48]. *Pst* DC3000 multiplies in the intercellular spaces within the leaf, and successful colonization requires the use of various virulence factors to shut down the plant's basal defenses[48]. Two important virulence mechanisms used by *Pst* DC3000 involve the phytotoxin coronatine (COR), which mimics the hormone JA-isoleucine (JA-Ile), and the type III secretion system (T3SS), which is used to secrete bacterial type III effectors (T3E) into plant cells[48]. COR is a polyketide comprised of coronafacic acid (CFA) and coronamic acid (CMA), and genes involved in COR biosynthesis are encoded in the *cfa* and *cma* operons, respectively[48]. Infiltration of tomato plants with a COR-deficient strain showed necrosis but no chlorosis, indicating that COR is important for this disease symptom development[49]. The T3SS is a syringe-like structure comprised of multiple components encoded by the *hrp* (hypersensitive

response and pathogenicity) and *hrc* (hrp conserved) genes[50]. Loss of the *hrcC* gene prevents assembly of the T3SS apparatus[50]. Although COR production is still present in a *Pst* DC3000 *ΔhrcC* mutant strain, it is considered a non-pathogenic strain due to its inability to multiply and cause normal disease symptoms[49]

A majority of studies show a negative impact of elevated temperature on bacterial virulence[51]. For example, production of COR was found to be negatively affected in *P. syringae* pv. *glycinea* grown at elevated temperature (28°C) due to a down-regulation of COR biosynthetic gene expression as well as a decrease in stability of COR biosynthetic proteins[52]. Similarly, down-regulation of *hrp* genes in *Erwinia amylovora* and decreased secretion of T3E proteins by *P. syringae* strains were both observed at 28°C and 30°C, respectively[53, 54]. However, as these studies were done *in vitro*, it is possible they do not reflect what occurs within the plant. For example, a study using a promoter-reporter system to assess expression of COR biosynthetic genes *in planta* showed that induction of these genes was impaired at elevated temperature in *P. syringae* pv. *glycinea* but not in *Pst* DC3000[55]. Additionally, bacterial genes involved in auxin biosynthesis were observed to be upregulated at elevated temperature in *P. syringae* pv. *syringae*[56]. Auxin and other growth hormones are known to be used by plant pathogens to suppress host immune responses[57]; therefore, more information is needed to determine the effect of elevated temperature on bacterial virulence within the host.

Plant perception and responses to elevated temperature

Plants have the ability to sense temperature changes as small as 1°C, and there is evidence to support the role of active signaling vs. temperature-induced changes in enzymatic activity in regulating plant responses to temperature[58]. Based on responses in Arabidopsis, temperature

ranges are typically categorized as warm ambient (22°C to 27°C), moderately elevated or high (27°C to 30°C) and extremely high or heat shock (37°C to 42°C)[59]. Some of the known temperature-induced changes include alterations in membrane fatty acid composition to regulate membrane fluidity, as well as temperature-sensitive exclusion of the histone variant, H2A.Z, to facilitate transcriptional responses affecting cell elongation and flowering time[60, 61]. Although many of these temperature-induced responses have been well characterized, the identity of the plant temperature sensor(s) has been an outstanding question until now. Two recent studies showed that, in addition to its role as a photoreceptor, phytochrome B (phyB) also functions as a temperature receptor[62, 63].

Phytochromes are red/far-red light photoreceptors in plants that exist in two interconvertible forms, an inactive, red-light (R ~660 nm) absorbing form (P_r) and an active, far-red light (FR ~ 730 nm) absorbing form (P_{fr})[60]. It was recently shown that the spontaneous reversion of the light-activated P_{fr} form to the inactive P_r form is accelerated in response to increased ambient temperature, resulting in a reduction in both the pool of active PhyB and its localization to nuclear bodies[63]. Systematic analyses of wild type, *phyB* deficient and phyB thermo-stable plants under different light and temperature conditions were used to demonstrate the dual function of phyB as both a light and temperature receptor in plants[63].

Dual functionality of phytochromes was previously hypothesized based on similar morphological and developmental changes in Arabidopsis in response to vegetative shade and elevated temperature, including elongation of the hypocotyl and petioles, raising of the petioles (hyponasty) and accelerated flowering[58, 60, 64, 65]. These responses are regulated by several PHYTOCHROME INTERACTING FACTORs (PIFs), with PIF4 playing a major role in response to

elevated temperature[66, 67]. PhyB negatively regulates PIFs to restrict hypocotyl growth and promote cotyledon expansion upon transitioning from dark to light conditions[68]. It was recently shown that PhyB represses the expression of PIF1-, 3-, 4- and 5-regulated genes by binding to the same G-box *cis* element within gene promoters[62]. However, PhyB promoter-binding was diminished at elevated temperature (27°C) due to the accelerated depletion of active PhyB in warm temperature conditions, thereby resulting in a de-repression of PIF-activated genes[62].

At ambient temperatures (17°C), PIFs are suppressed in a fluence-dependent manner by the LONG-HYPOCOTYL 5 (HY5) TF, resulting in a reduction in hypocotyl length with increasing fluence rates[67]. However, at elevated temperature (27°C), PIF-mediated hypocotyl elongation is promoted with increasing fluence due to accumulation of auxin[67]. PIF4 binding to promoters of auxin biosynthetic genes, including *YUCCA 8*, is increased at elevated temperatures, resulting in an increase of free indole-3-acetic acid (IAA)[69-71]. Additionally, a recent study showed that the co-chaperones, HEAT SHOCK FACTOR 90 (HSP90) and SUPPRESSOR OF G2 ALLELE SKP 1 (SGT1) physically interact with the auxin co-receptor, TRANSPORT INHIBITOR REPONSE 1 (TIR1), resulting in stabilization and accumulation of the TIR1 protein at elevated temperature[72]. The combination of increased PIF4-mediated auxin biosynthesis and HSP90-SGT1-mediated TIR1 stability at elevated temperature enable up-regulation of auxin signaling and promotion of cell elongation and extension under this condition. Two other growth-promoting hormones, gibberellins (GA) and brassinosteroids (BR) have also been implicated in morphological responses to elevated temperature, although to a somewhat lesser extent than auxin[73]. The positive role of GA most likely occurs via removal of the growth-repressing DELLA proteins, which are known inhibitors of PIFs[74], whereas the BR TF, BZR1, has been shown to physically

interact with PIF4 to co-regulate nearly 2,000 genes[73]. Among the genes co-regulated by BZR1 and PIF4 are the family of PACLOBUTRAZOL RESISTANCE (PRE) helix-loop-helix factors[75], which are positive regulators of cell elongation in Arabidopsis[76].

Plants exhibit both basal and acquired tolerance to heat shock, and various hormones have been shown to contribute to both[77]. SA was found to be important for basal thermotolerance based on increased electrolyte leakage, reduced seedling survival and increased oxidative damage in mutants compromised in either SA accumulation or signaling relative to wild type plants[78, 79] . Plants treated with SA or mutants with elevated endogenous SA exhibited increased basal thermotolerance, further supporting a role for SA in this response. ET and abscisic acid (ABA) were also shown to contribute to basal thermotolerance, although ABA seems to play a larger role in acquired thermotolerance[79]. The role of SA in acquired thermotolerance is a bit unclear. In two separate studies, mutants compromised in either SA accumulation or signaling were evaluated to determine a role of SA on acquired thermotolerance. In the first study, SA accumulation and signaling were determined to not be required for acquired thermotolerance based on hypocotyl elongation and electrolyte leakage similar to wild type plants following temperature acclimation and heat stress treatments[78]. However, the authors of the second study concluded SA may play a minor role in acquired thermotolerance based on impaired seedling survival and increased oxidative damage in the SA mutants relative to wild type plants following temperature acclimation and heat shock treatment[79].

Salicylic acid signaling in Arabidopsis

SA is a phenolic hormone shown to affect many plant processes including growth, development, senescence and stress responses[12, 80]. While SA is generally credited for its role in biotic stress responses, it has also been shown to alleviate oxidative stress caused by adverse abiotic conditions, such as drought and salinity[80, 81]. Both freezing and heat shock conditions induce SA biosynthesis[78, 82, 83]. Although SA accumulation was shown to not be required for freezing tolerance[84], it has been shown to potentiate thermotolerance as described above[78, 79, 85]. However, the effect of SA on plant tolerance to most abiotic stresses is concentration dependent, with low concentrations of SA alleviating oxidative stress and high concentrations of SA, such as occur in response to biotic stress responses to facilitate cell death, exacerbating abiotic stress tolerance[80, 81].

Plant biosynthesis of SA occurs in the chloroplast via modification of a chorismate precursor through either the PHENYLALANINE AMMONIA LYASE (PAL) pathway or the ISOCHORISMATE SYNTHASE (ICS) pathway[86]. SA levels rise significantly in both local and systemic tissues following induction of either PTI or ETI[12, 87, 88], and roughly 90% of this increase is due to ICS1 activity[89]. Free SA is quickly metabolized in the cytoplasm, primarily by conjugation with glucose (SAG), and sequestered in the vacuole[90]. Experiments using plants genetically altered in SA biosynthesis or accumulation as well as wild type plants treated with pathogen or exogenous application of SA have shown that SA is required for full induction of local resistance and is also both necessary and sufficient for establishment of SAR[88, 89, 91-93].

Multiple components, including the lipase like proteins PHYTOALEXIN DEFICIENT 4 (PAD4)[94, 95] and ENHANCED DISEASE SUSCEPTBILITY 1 (EDS1)[96, 97], the CAM-BINDING PROTEIN 60-LIKE G

(CBP60g) and SAR DEFICIENT 1 (SARD1) proteins[98], and the WRKY28 TF[99] have been shown to positively regulate *ICS1* gene expression to promote accumulation of SA. CALMODULIN-BINDING TRANSCRIPTION ACTIVATORS (CAMTAs) are negative regulators that modulate SA biosynthesis in a temperature-dependent manner[84, 100]. Whereas CAMTA-mediated suppression of SA accumulation is alleviated by long-term (1-week) exposure to low temperatures (4°C)[84], the dwarf phenotype of the *camta3* mutant, which was shown to be associated with elevated basal SA, was restored to wild type by growing plants at an elevated temperature (25°C to 27°C)[100]. CAMTA3 was shown to directly suppress two positive regulators of SA biosynthesis, *EDS1* and *NON RACE-SPECIFIC DISEASE RESISTANCE 1* (*NDR1*), by binding to their gene promoters[100, 101]. Although up-regulation of *CBP60g*, *SARD1* and *ICS1* gene expression was correlated with both cold-induced de-activation and genetic removal of CAMTA TFs, it is unclear whether CAMTAs act directly or indirectly to regulate expression of these genes. However, a possible mechanism for indirect regulation may be via CAMTA3-mediated promotion of *ETHYLENE INSENSITIVE 3* (*EIN3*) gene expression[101]. EIN3 and EIN3-LIKE 1 (EIL) are both negative regulators of *ICS1* gene expression, with EIN3 functioning by directly binding to the *ICS1* gene promoter[102].

Multiple genetic screens led to the identification of NONEXPRESSOR OF PATHOGENESIS RELATED PROTEINS 1 (NPR1, aka NIM1, SAI1), which is a key regulator of SA signaling[103-106]. In the non-induced state, NPR1 proteins oligomerize in the cytoplasm due to S-nitrosylation of highly conserved cysteine residues resulting in the formation of intermolecular disulfide bonds[107]. Cellular redox changes in response to SA accumulation activate cytoplasmic thioredoxins, which reduce the disulfide bonds to release active NPR1 monomers that then

translocate to the nucleus and activate defense gene expression[107-109]. Nuclear localized NPR1 is phosphorylated and poly-ubiquitinated by the CULLIN 3 (CUL3) E3 ubiquitin ligase complex resulting in its degradation by the 26S proteasome[110]. This process of NPR1 protein turnover is promoted during SAR and is required for full induction of NPR1-mediated transcriptional reprogramming[110]. After years of searching, two recent studies have proposed NPR1 and its paralogs, NPR3 and NPR4, to act as SA receptors[111-113]. NPR3 and NPR4 function as adaptors for the CUL3 ubiquitin E3 ligase, with NPR4 serving to remove NPR1 to prevent defense gene activation when SA levels are low and NPR3 facilitating NPR1 turnover when SA levels are high[111]. Specific post-translational modifications of the NPR1 protein regulate its interactions with NPR3 and NPR4 and help switch its role from a transcriptional repressor to a transcriptional activator depending upon accumulation of SA[114].

NPR1 regulates gene expression through physical interaction with TGA transcription factors (TFs), which bind to promoters of *PATHOGENESIS RELATED* (*PR*) genes to activate expression in the presence of SA and repress expression in the absence of SA[13, 115]. *PR* genes encode small proteins, some of which have been shown to possess antimicrobial or antifungal properties *in vitro*[116]. Of the many *PR* genes identified, *PR1*, *PR2* and *PR5* have been shown to be induced by SA and have long been used as markers of SA signaling in Arabidopsis[13]. Other genes identified as direct targets of NPR1 include WRKY TFs and components required for the synthesis and secretion of PR proteins[117]. WRKYs are involved in both NPR1-dependent and NPR1-independent SA signaling and, as in the case of PTI, include both positive and negative regulators of SA-mediated defense[117-119].

Crosstalk with potential to suppress SA-mediated defense at elevated temperature

As sessile organisms, plants must perceive, integrate and respond appropriately to multiple environmental and developmental cues for successful growth and reproduction[57, 120]. Both spatial and temporal modulation of various hormones, calcium and other signals enables fine-tuning of these responses[121, 122]. Due to the need for this delicate and dynamic balance, there are examples of both synergistic and antagonistic interactions between SA and other hormone-signaling pathways[123]. Here I will discuss negative crosstalk between SA and auxin, JA and ABA, with an emphasis on specific examples of *Pst* DC3000 hijacking of plant hormone signaling to shut down SA biosynthesis and signaling.

Negative crosstalk between SA and growth-promoting hormones, such as auxin, are best illustrated by the dwarf plant phenotype typical of mutants with constitutively elevated SA[124, 125]. Application of SA analogues, such as BTH, or extended exposure to cold temperatures are also known to inhibit growth due to elevated SA biosynthesis and/or signaling[83, 126]. However, the dwarf phenotype associated with constitutive SA mutants is typically lost at elevated temperature due to a loss of SA accumulation under these conditions[127]. As described above, auxin plays a primary role in response to elevated temperature; therefore, it is possible that up-regulation of auxin biosynthesis and signaling may contribute to suppression of SA-regulated responses under this condition (Fig. 2). SA-mediated defense has been shown to be affected by auxin, as transgenic overexpression of the *AFB1* gene, which enhances auxin signaling, led to a reduction in pathogen-induced SA biosynthesis relative to wild type plants[128]. However, transgenic overexpression of the *YUCCA 1* gene showed that elevation of auxin levels alone can promote plant disease without affecting SA levels or signaling[129].

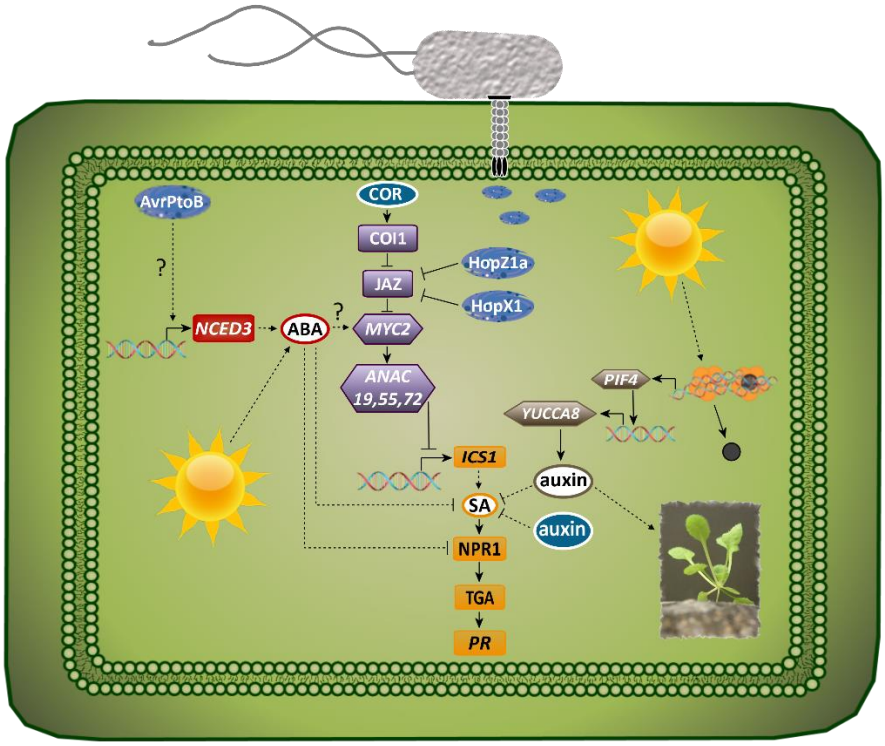

Figure 2. *Pst* DC3000- and temperature-induced hormone crosstalk with potential to suppress SA. *ICS1* gene induction results in increased SA biosynthesis, which activates NPR1. NPR1 interaction with TGAs and other TFs facilitates SA-dependent transcriptional reprogramming. Both ABA and auxin are up-regulated in response to elevated temperature (depicted with sun). In the case of auxin (white with brown outline), the H2A.Z histone variant (black ball) is excluded from nucleosomes as temperatures rise, enabling increased *PIF4* gene expression. The PIF4 TF binds the *YUCCA 8* gene promoter to enhance its expression, resulting in auxin biosynthesis and signaling. Increased auxin levels result in hypocotyl and petiole elongation, as well as leaf hyponasty as shown by the Arabidopsis plant in the insert. This plant was grown at 23°C, shifted to 30°C at 2-weeks-old and photographed 4 d later. Pathogen produced auxin (blue with white outline) may also contribute to suppression of SA via unknown mechanisms. ABA inhibits SA both upstream and downstream of its biosynthesis. It is possible that ABA inhibition of SA upstream of NPR1 occurs via MYC2, however, this has not been shown. ABA also inhibits *NPR1* gene expression and NPR1 protein stability to suppress SA-mediated signaling. T3E-induction of ABA biosynthesis occurs by induction of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) gene expression, possibly due to AvrPtoB activity. COR-induction of *MYC2* occurs in a CORONATINE INSENSITIVE 1 (COI1)-dependent manner, and results in upregulation of three ANAC TFs, which bind the *ICS1* gene promoter to suppress its expression. In addition, two T3E proteins, HopZ1a and HopX1, target the JASMONATE ZIM-DOMAIN (JAZ) repressor proteins to relieve suppression of JA-mediated signaling to enable suppression of SA-mediated defense. Solid lines depict confirmed, direct connections, whereas dashed lines depict indirect/multiple or missing/unknown steps between components. Bent arrows next to DNA molecules depict gene promoters. Arrowheads show positive and blunted lines show negative interactions. Circles depict metabolites, hexagons depict TFs.

Auxin positively regulates expansins, which are involved in cell wall loosening, to promote growth[130, 131], and the ability of *Xanthomonas oryzae* pv. *oryzae* to induce expansins in rice was shown to be important in determining the outcome of the plant-pathogen interaction[131]. In fact, many pathogens, including *P. syringae* and *Agrobacterium tumefasciens*, can directly synthesize auxin or manipulate auxin synthesis and signaling in plants to promote disease[132] (Fig. 2). Microorganisms primarily synthesize IAA from tryptophan, and, in some cases, the genes encoding the enzymes required for this process are located on a pathogen virulence plasmid [133]. Analysis of plant transcriptional reprogramming following some pathogen infections has shown a general de-repression of the auxin pathway including promotion of auxin biosynthetic genes and repression of *AUX/IAA* genes resulting in enhanced plant susceptibility[134, 135]. Furthermore, virulence of *Pst* DC3000 can be enhanced by treatment with synthetic auxins prior to pathogen inoculation[136, 137]. Together these studies indicate a dual function for auxin in direct interference with SA-mediated defense and in positive regulation of physiological changes that aid pathogen proliferation in the plant.

Pst DC3000 employs various other virulence mechanisms to specifically target and suppress SA. Because of their specialized functions, signaling regulated by SA and JA/ET is generally antagonistic[121, 138]. JA-Ile is perceived by a co-receptor complex formed with the F-box protein CORONATINE INESENSTIVE 1 (COI1) and the JASMONATE ZIM-DOMAIN (JAZ) family of transcription repressors[139]. The JAZ-family proteins repress JA signaling by directly binding to the MYC family of TFs required for the expression of JA-responsive genes[140-142]. An increasing concentration of JA-Ile promotes physical interaction between COI1 and JAZ proteins, which leads to ubiquitination and subsequent degradation of JAZs through the 26S proteasome,

thereby relieving the repression on MYC TFs and initiating the expression of JA-responsive genes[140, 141, 143]. One of the best examples of SA-JA antagonism is the use of COR by *Pst* DC3000 to activate JA signaling. COR is more efficient than JA-Ile in promoting interaction between COI1 and JAZ proteins, resulting in MYC2 activation[143, 144]. MYC2 then induces the expression of several genes encoding ANAC TFs, which bind to the *ICS1* promoter to suppress its expression, resulting in loss of SA accumulation[144]. In addition, two T3E proteins, HopZ1a and HopX1, directly target and promote degradation of JAZ repressor proteins to activate JA signaling resulting in suppression of SA-mediated defense[145, 146].

MYC2 is also involved in ABA-mediated responses, which are also targeted by *Pst* DC3000 to suppress SA[147, 148]. ABA is considered the primary hormone associated with abiotic stress tolerance[149]. Although examples of synergism between SA and ABA exist, such as in pathogen-induced stomatal closure[150], interactions between these two pathways are generally antagonistic. For example, SA-mediated defense was increased in tomato mutants with reduced ABA[151], whereas exogenous application of ABA resulted in enhanced susceptibility to pathogens in both tomato and Arabidopsis[151-153]. The use of chemicals that induce SA signaling either upstream or downstream of SA biosynthesis were used to show that ABA-mediated antagonism occurs both upstream of *ICS1* induction and downstream of NPR1 activation[154]. While the effect of exogenous ABA on SA is independent of JA/ET-mediated signaling[154], an activation-tagged line with elevated endogenous ABA also showed increases in JA levels and resistance to necrotrophic pathogens concomitant with suppression of SA and enhanced susceptibility to biotrophic pathogens[153]. This may indicate a synergistic or additive effect of ABA and JA antagonism resulting in suppression of SA in these plants. In rice, ABA was shown to

suppress pathogen-induction of *WRKY45* and *NPR1*, which are important for SA-mediated signaling and defense in rice[155]. Recently it was shown that one of the mechanisms for ABA suppression of SA signaling involves promotion of NPR1 degradation[156]. In addition, *Pst* DC3000 uses T3Es to induce the expression of the ABA biosynthetic gene, *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*), to promote ABA accumulation and signaling, which results in suppression of SA biosynthesis and enhanced susceptibility[157, 158]. It is possible, therefore, that the combination of *Pst* DC3000 infection and elevated temperature would promote ABA accumulation, which would in turn suppress SA-mediated defense.

Summary and conclusions

With global food demand on the rise, mitigating crop loss due to abiotic and biotic stresses is imperative[1, 159]. There are many examples of elevated temperature promoting plant disease[27, 28, 30, 31]; however, genetic variation for temperature-sensitivity of various defense responses have been observed[27, 160, 161]. While progress has been made in elucidating the molecular mechanisms underlying loss of ETI-mediated defense, the factors contributing to loss of basal defense remain unknown. As SA-mediated defense is important for protecting crop plants against multiple economically important diseases[15, 19, 20, 162-164], a more thorough understanding of how elevated temperature specifically effects SA accumulation, signaling and defense will provide an avenue for engineering robust, broad-spectrum resistance in plants that will be resilient to both current and anticipated changes in global climate.

Chapter 2

Dual impact of elevated temperature on plant defense and bacterial virulence in Arabidopsis

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Abstract

Salicylic acid (SA) is a plant hormone critical for local and systemic resistance against pathogens. I investigated the impact of an important climate condition on SA-mediated defense in Arabidopsis, and found that loss of ICS1-mediated SA biosynthesis at 30°C largely accounts for enhanced susceptibility to *Pseudomonas syringae* pv. *tomato*. Application of an SA analogue, benzothiadiazole (BTH), confers protection against disease at both 23°C and 30°C; however, global transcriptome analysis revealed a clear bifurcation in the SA signaling network, with a majority (66%) of BTH-regulated genes constituting the temperature-sensitive, *PR1*/*ICS1* branch. Contrary to previous *in vitro* analyses showing down-regulation of virulence-associated genes at elevated temperature, I observed increased translocation of bacterial type III effectors (T3E) into the plant cell at 30°C. Enhanced translocation of T3E into SA-deficient plants at 30°C vs 23°C reveals previously unrecognized molecular interplays between temperature, SA signaling and the function of a central bacterial virulence system.

Introduction

Plant diseases represent one of the most important causes of crop loss worldwide[1]; therefore, understanding the mechanisms underlying disease development is critical for generating effective disease control measures as part of global efforts to enable crop yields commensurate with increasing demand[1, 2]. Climate plays a large role in determining the outcome of plant-pathogen interactions, and it has been noted that disease epidemics are more likely to occur when environmental conditions are suboptimal for the plant[26, 27]. Responding to combined stresses (e.g., abiotic plus biotic) is challenging for plants because the response needed to mitigate one stress often can exacerbate another[165, 166]. Breeding efforts to enhance yield typically reduce genetic diversity, which increases vulnerability to disease and is also likely to negatively impact the resilience of plant immunity under adverse environmental conditions[1, 167]. Increasing our understanding of how specific environmental factors affect the host and the pathogen as well as their interactions can inform strategies for developing robust crop resistance under increasingly unpredictable climate conditions.

The hormones salicylic acid (SA), jasmonate (JA) and ethylene (ET) are important regulators of plant defense responses, with SA generally facilitating defense against biotrophs, which feed off of living plant tissue, and JA/ET primarily defending against necrotrophs, which feed off of the nutrients released upon killing plant cells[168]. In Arabidopsis, pathogen induction of SA biosynthesis occurs predominantly through the isochorismate pathway involving ICS1[89]. Following SA induction, the master regulator, NPR1, accumulates in the nucleus where it interacts with various TGA and WRKY transcription factors (TFs) to promote transcriptional

reprogramming to activate SA-mediated defense[169]. Among the many genes induced by SA, *PR1* is one of the most widely used markers for SA signaling in Arabidopsis[116, 170, 171].

SA is important for both local and systemic resistance against pathogens[13]. SA-mediated defense has been well established as a crop protectant; for example, an SA synthetic analogue, benzothiadiazole (BTH), is used commercially to provide resistance resulting in increased yield in multiple crops, including wheat[22] and maize[23]. Additionally, over-expression of *NPR1* has been shown to improve fitness in field-grown Arabidopsis[25] and to improve disease resistance in rice[172]. As both basal defense against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and induction of SA during effector-triggered immunity (ETI) have been shown to be compromised at elevated temperature[35, 43], SA-mediated defense may be compromised as well. However, it is unclear whether either of these outcomes results from a direct impact of temperature on SA, as SA-deficient mutants were reported to retain temperature sensitivity during basal defense[35], and loss of ETI-induced SA may be an indirect effect resulting from temperature-mediated loss of upstream R protein function. In addition, SA plays a role in pattern-triggered immunity (PTI)[87], which is not suppressed at elevated temperature[47]. Overall, whether there is a direct impact of elevated temperature on the SA-mediated defense network remains unclear. The aim of this study is to address this important biological question.

Our findings reveal a direct effect of elevated temperature on both SA-mediated defence in the host and type III secretion in the pathogen resulting in enhanced disease. Additionally, in spite of loss of the *PR1*/*ICS1* temperature-sensitive branch of BTH-regulated genes, BTH-mediated protection against *Pst* DC3000 persisted at 30°C. Together, these results shed light on

the enigmatic interplays in host-pathogen interactions, and demonstrate the potential use of BTH as a crop protectant even at elevated temperatures.

Materials and Methods

Plant materials and growth conditions

Arabidopsis Columbia-0 (Col-0) WT and mutant plants (in Col-0 background) were soil-grown (2:1 "Arabidopsis mix":perlite covered with standard Phiferglass mesh) for 3 to 4 weeks at 12 h light (85 ± 10 µmol m^{-2} s^{-1}), 12 h dark, 23°C and 60% relative humidity. Two-week-old plants were used for confocal microscopy to minimize the loss of age-related decrease in yellow fluorescent protein (YFP)-associated fluorescence. The *tga2 tga5 tga6*, *sid2-2* (*ics1*), *ein2-1*, *aba2-1* and *camta2 camta3* mutants were previously characterized[84, 89, 115, 173-176]. The *myc2 myc3 myc4* mutant was generated by combining the previously described single mutants: *myc2-1* (SALK_040500)[177], *myc3-1* (GK-445B11)[178], and *myc4-1* (GK-491E10)[178]. The *npr1-6* (SAIL-708F09) T-DNA insertion mutant was obtained from the Arabidopsis Biological Resource Centre (ABRC) at The Ohio State University. T-DNA insertion mutants were genotyped using the REDExtract-N-Amp Plant PCR kit (Sigma) following the manufacturer's protocol. Primers used for genotyping are listed in Table 1.

Temperature and chemical treatments

Test chambers were set to either 23°C (control) or 30°C (test), with all other conditions the same as above. Plants were moved to test chambers 2 h after lights on and acclimated for 48 h before pathogen infiltration. For experiments with chemical pre-treatment, plants were temperature-acclimated for 24 h before spraying with either mock (0.1% DMSO, 0.01% Silwet L-77) or benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester (BTH, Chem Service Inc.; 100 µM, 0.1% DMSO, 0.01% Silwet). For callose assays, flg22 (200 nM in 0.1% DMSO) served as a

positive control, and all solutions were infiltrated into leaves using a needleless syringe. Subsequent assays were performed 24 h after chemical treatment.

Table 1. Primer sequences used in this study.

AGI Number (Gene name)	Primer name	Primer sequence (5'-3')	Purpose
NA	SAIL_LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC	Genotyping
AT1G64280 (*NPR1*)	SAIL708F09_LP	ATTTGTTTGAAGCACACCTGC	Genotyping
	SAIL708F09_RP	CTCTCAAAGGCCGACTATGTG	Genotyping
NA	SALK_LBb1.3	ATTTTGCCGATTTCGGAAC	Genotyping
AT1G32640 (*MYC2*)	MYC2_GT_LP	GCTACAACCAACGATGAATC	Genotyping
	MYC2_GT_RP	TCATCAACAGCGTCATCCGA	Genotyping
NA	GABI Kat_LB1	ATAACGCTGCGGACATCTACATT	Genotyping
AT5G46760 (*MYC3*)	MYC3_GT_LP	GTTAGATCAGCTGCGAATGATTCGG	Genotyping
	MYC3_GT_RP	CTCCGACTTTCGTCATCAAAGCAAC	Genotyping
AT4G17880 (*MYC4*)	MYC4_GT_LP	GGATCCATGTCTCCGACGAATGTTCAAGTA	Genotyping
	MYC4_GT_RP	TCTCTCACAACTTGATCCAGCTAA	Genotyping
AT1G64280 (*NPR1*)	NPR1_F	agaattcATGGACACCACCATTGATGGA	cloning
	NPR1_R	agtcgacCCGACGACGATGAGAGARTTTAC	cloning
NPR1 promoter	pNPR1_F	cgcggccgcTCGTTTGTTTTCCGTTTTGTTCTGA	cloning
	pNPR1_R	agaattcCAACAGGTTCCGATGAATTGAAAT	cloning
AT1G64280 (*NPR1*)	NPR1-NT F	AGGATCCATGGACACCACCATTGATGG	RT-PCR
	NPR1-CT R	AGCGGCCGCTCACCGACGACGATGAGAGA	RT-PCR
At2g14610 (*PR1*)	PR1-RT-F	GTTCACAACCAGGCACGA	RT-PCR
	PR1-RT-R	CACCTCACTTTGGCACATCC	RT-PCR
AT4G05320 (*UBQ10*)	UBQ10-RT-F	ACCCTCCACTTGGTCCTCA	RT-PCR
	UBQ10-RT-R	AGTTTTCCCAGTCAACGTCTT	RT-PCR
At1G13320 (*PP2AA3*)	PP2AA3_qRT_F1	GGTTACAAGACAAGGTTCACTC	qPCR
	PP2AA3_qRT_R1	CATTCAGGACCAAACTCTTCAG	qPCR
At2g14610 (*PR1*)	PR1_qRT_F1	GGCTAACTACAACTACGCTG	qPCR
	PR1_qRT_R1	TCTCGTTCACATAATTCCCAC	qPCR
At1g74710 (*ICS1*)	SID2_qRT_F2	ACTTACTAACCAGTCCGAAAGACGA	qPCR
	SID2_qRT_R2	ACAACAACTCTGTCACATATACCGT	qPCR
AT1G64280 (*NPR1*)	NPR1_qRT_F1	CCGCCGCTAAGAAGGAGAAA	qPCR
	NPR1_qRT_R1	GCCAAAACAGTCACAACCGA	qPCR
AT4G18170 (*WRKY28*)	WRKY28_qRT_F	CTCCTTCTAATTCTTCCTCTAGTG	qPCR
	WRKY28_qRT_R	TCTCTTTGTTTCTTCACCTCAG	qPCR
AT4G17880 (*MYC2*)	MYC2_qRT_F1	GAAACTCCAAATCAAGAACCAG	qPCR
	MYC2_qRT_R1	ATCTTCACTTCAATCTCCATCC	qPCR
At5g13220 (*JAZ10*)	JAZ10_qRT_F1	GTAGTTTCCGAGATATTCAAGGTG	qPCR
	JAZ10_qRT_R1	GAACCGAACGAGATTTAGCC	qPCR

Phytohormone extraction and quantification by liquid chromatography-tandem mass spectrometry (LC/MS)

Phytohormones were extracted and quantified as previously described[179] with some modifications. Leaf tissue between 10 - 50 mg (fresh weight, FW) was flash-frozen in liquid nitrogen, ground and extracted at 4°C overnight (~16 h) using 0.3 – 0.5 mL of ice-cold extraction buffer (methanol:water (80:20 v/v), 0.1% formic acid, 0.1 g L^{-1} butylated hydroxytoluene, 100 nM ABA-d_6). After filtering and transferring to autosampler vials, plant hormones were quantified using an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters Corporation, Milford, MA) as previously described[179], except the capillary voltage, cone voltage, and extractor voltage were set to 3.5 kV, 25 V, and 5 V, respectively, and the desolvation gas and cone gas were set to flow rates of 600 L h^{-1} and 50 L h^{-1}, respectively. Selected ion monitoring (SIM) was conducted in the negative ES channel for salicylic acid (SA; m/z 137>93), SA glucoside (SAG; m/z 299.1>137) and the internal ABA-d_6 standard (m/z 269.1>159.1). Parent>daughter SIM pairs, as well as the optimal source cone and collision energy voltages for each compound monitored were determined using Quan-Optimize software. Analyte responses based on peak area integrations relative to the internal standard was determined using QuanLynx v4.1 software (Waters, Milford, MA). Both the SA and SAG analytes were quantified based on the SA standard curve to calculate the sample concentrations (nM), which were converted to ng using the molecular weight of the compound and the extraction volume, and were then normalized by sample FW in g.

RNA extraction and qPCR

RNA was extracted from flash-frozen, ground leaf tissue with the ToTally RNA kit following the manufacturer's protocol (Ambion). Samples were digested with DNaseI (Roche) to remove

any genomic DNA contamination, and then purified using the RNeasy Mini kit (Qiagen). M-MLV reverse transcriptase (RT, Life Technologies) was used to synthesize cDNA. For all genes of interest, approximately 1.5 ng of cDNA template was used for quantitative PCR (qPCR), with expression normalized to the *PROTEIN PHOSPHATASE 2A SUBUNIT A3* (*PP2AA3*) internal control gene using the equation $2^{-\Delta CT}$, where ΔC_T is $C_{T\ target\ gene} - C_{T\ PP2AA3}$ (see Table 1 for primer sequences). All qPCR reactions were performed using the SYBR® Green master mix (Life Technologies) and 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, California), with three technical replicates and a minimum of three biological replicates per experimental treatment.

RNA sequencing and data analysis

Extracted RNA was checked for purity using a BioAnalyzer Agilent 2100 and three biological replicates of each treatment type were selected based on having a RNA Integrity Number (RIN) score around 7. The final twelve samples were submitted to the Research Technology Support Facility at Michigan State University for preparation of next-generation sequencing libraries. Pooled samples were loaded on two lanes of an Illumina HiSeq 2500 Rapid Run flow cell (v1) and sequenced in a 1 x 50 bp single end format using Rapid SBS reagents. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.61 and output of RTA was de-multiplexed and converted to FastQ format using Illumina Bcl2fastq v1.8.4. Due to inadvertent exclusion of sample 6, a second pool was generated and run on one lane of a Rapid Run flow cell, and sample 6 was also run individually on a MiSeq flow cell. RNA-seq reads were cleaned and trimmed using Trimmomatic[180] and were aligned to the Arabidopsis genome assembly (TAIR10) using the STAR alignment program, allowing only unique alignments[181]. Read counts were

obtained for each gene using the featureCounts function from the Rsubread package in R[182], and subsequent count data were normalized using TMM using the limma package in R[183]. Genes with average counts less than 10 across all samples were discarded. The count data were further normalized using voom within the limma package[183]. The expression data were fit to a linear model (treatment:temp + replicate), and differentially expressed genes were identified with a FDR < 0.01 for four specific contrasts (23°C mock vs. 30°C mock; 23°C BTH vs 30°C BTH; 23°C BTH vs 23°C mock; 30°C BTH vs 30°C mock) using the eBayes function within the limma package. Results were then filtered for those genes that exhibited a $\log_2$-fold change greater than 2 for at least one of the tested contrasts. Gene ontology analysis was done for genes in each cluster using The Database for Annotation, Visualization and Integrated Discovery (DAVID)[184].

Callose accumulation

Following temperature acclimation and chemical treatment, leaves were harvested and cleared in 100% ethanol overnight. Cleared leaves were fixed with a 75% ethanol, 25% acetic acid solution for 2 h, after which leaves were washed consecutively with 75% ethanol, 50% ethanol, and 150 mM K_2HPO_4 pH 9.5 for 15 min. Finally, leaves were stained in an aniline blue solution (0.1%, 150 mM K_2HPO_4 pH 9.5) overnight at 4°C. Callose deposits were visualized using an Olympus IX71 inverted microscope with a 120-watt metal halide lamp (X-Cite series 120) using a DAPI filter (Semrock, excitation 377/50 and emission 447/60). Images shown are at 10X-magnification. Callose counts were processed using ImageJ (Rasband W.S., National Institutes of Health, U.S.A.). Images were first converted to 32-bit grayscale, after which the threshold of the image was adjusted so that only callose deposits were visible over the background. Callose

deposits were then counted using the analyse particles tool. Four callose measurements were collected per leaf; each individual leaf was collected from a different plant. Six to eight plants were evaluated per treatment.

Confirmation of a T-DNA knock-out allele for *NPR1*

An *npr1* mutant allele, SAIL_708F09, was identified as an NPR1 knock-out allele, which was named *npr1-6*. This allele contains a T-DNA insertion in the third exon of the *NPR1* gene (Fig. 8a). RT-PCR was used to confirm this allele has a complete loss of *NPR1* transcript and loss of *PR1* gene induction by BTH (Fig. 8d). Bacterial growth in mock- and BTH-treated plants was assessed to confirm enhanced susceptibility relative to WT and loss of BTH-mediated protection (Fig. 8c).

Preparation, selection and complementation analysis of transgenic lines

The full length coding sequence of *NPR1* without the stop codon was PCR-amplified using NPR1_F and NPR1_R primers (Table 1) and cloned into *Eco*RI/*Xho*I sites of pENJAZ9C[185] to create pEN*NPR1C*, a Gateway compatible entry vector. Next, a 2.3kb DNA fragment containing the *NPR1* promoter was PCR-amplified using pNPR1_F and pNPR1_R primers (Table 1) and cloned into *Not*I/*Eco*RI sites of pEN*NPR1C* to create pEN*pNPR1C::NPR1*. Then, the *pNPR1::NPR1* construct was transferred by LR recombination into the binary expression vector pGWB540 to create the *pNPR1::NPR1-YFP* construct (Fig. 3b). The correct construct was confirmed by sequencing and introduced into *Agrobacterium tumefaciens* (GV3101) by electroporation. GV3101 clones containing the gene fusion construct were selected on LB medium containing rifampicin (Rif, 100 mg L^{-1}), spectinomycin (50 mg L^{-1}), and gentamycin (25 mg L^{-1}) antibiotics and used to transform *npr1-6* by floral dipping. T1 seeds were plated on ½X Murashige and

Skoog, 5 mM MES, 0.7% Bacto agar plates (1/2 MMS) containing hygromycin and resistant seedlings were transplanted to soil. Ten T1 plants were selected for protein extraction, and transgene expression was assessed using Western blot analysis using an α-GFP primary antibody (1:5,000, Abcam, data not shown). T2 seeds were collected from the ten T1 lines and ~100 seeds each were sown on ½ MMS plus 1% sucrose (1/2 MMSS) plates containing hygromycin (25 mg L^{-1}) to ascertain segregation ratios. Resistant T2 seedlings from lines exhibiting a 3:1 segregation ratio were then screened for induction by BTH using confocal microscopy (data not shown). Lines showing strong induction were transplanted to soil. Homozygous T3 lines were selected by screening for 100% resistance to hygromycin. BTH protection assays were conducted in three independent lines to confirm complementation of the *npr1-6* knockout mutation (Fig. 3c). RT-PCR was also used to confirm recovery of *NPR1* expression and BTH-induction of *PR1* in the NPR1-Y1 line used for experiments (Fig. 3d).

For generation of *p35S::YFP* transgenic plants, the GATEWAY cassette in pEARLEYGATE104[186] was removed by *XmaI* digestion, and the resulting linearized vector was re-ligated to create *pJYP35S:YFP*. This construct was used for Agrobacterium-mediated transformation of Arabidopsis Col-0 wild-type plants by floral dipping[187]. A homozygous T3 line with the single T-DNA insertion was selected for the further experiments.

Disease and BTH protection assays

Pst DC3000 was streaked from a frozen glycerol stock onto a LM (10.0 g Bacto Tryptone, 6.0 g Bacto yeast extract, 1.5 g K_2HPO_4, 0.6 g NaCl, 0.4 g $MgSO_4$ * 7 H_2O L^{-1}) + Rif (100 mg L^{-1}) plate and grown in the dark for two days at room temperature until single colonies were formed. Colonies from this plate were streaked onto a fresh LM+Rif plate and grown in the dark for one

day at room temperature, after which 100 µl sterile LM media was added to the plate and the cells were spread evenly and kept in the dark at room temperature overnight to form a lawn. Cells were scraped from this lawn plate and re-suspended in 0.25 mM $MgCl_2$ by incubating at room temperature for 5 min and then vortexing vigorously. A DU800 Spectrophotometer (Beckman Coulter, Inc, Fullerton, California) was used to measure the optical density (OD) of the culture at an absorbance wavelength of 600 nm (OD_{600}), and an inoculation culture was prepared by first adjusting the starting culture to 1 x 10^8 colony forming unites (cfu) ml^{-1} (OD_{600} of approximately 0.1) and then preparing 1:10 dilutions to reach the desired inoculum concentration of ~1 – 3 x 10^6 cfu ml^{-1} (OD_{600} of approximately 0.001). For vacuum infiltration, Silwet (0.005%) was added to the culture to enhance wetting of the leaves. Serial dilutions of inoculum were plated to determine the actual cfu ml^{-1} of culture used in each experiment.

Following temperature acclimation and chemical treatments (see above), syringe- or vacuum-infiltration was used to inoculate plants as previously described[188]. Following infiltration, plants were immediately returned to the test chambers where the leaves were allowed to dry completely before covering with transparent domes to maintain high humidity. Bacterial quantification was done by harvesting and grinding leaf discs in 0.25 mM $MgCl_2$ and preparing serial dilutions, which were then plated on LM+Rif plates and kept for 24 h at 30°C. Cfus were counted and the cfu cm^{-2} calculated as (cfus * total dilution)/(vol plated)/leaf area harvested.

Effector translocation assay

Following temperature and/or chemical treatments, plants were infiltrated using a needless syringe with a high inoculum (2 – 4 x 10^7 cfu mL^{-1}, 0.25 mM $MgCl_2$) of *Pst* DC3000 or Δ*hrcC*

mutant carrying the P_{nptII}*::avrPto-cyaA* construct[189]. To offset the effect of BTH on bacterial populations, *Pst* DC3000 P_{nptII}*::avrPto-cyaA* inoculum was adjusted to 2 x 10^7 cfu mL^{-1} for mock-pre-treated plants and 4 x 10^7 cfu mL^{-1} for BTH-pre-treated plants. Leaf discs were harvested using a biopsy punch 6-7 hpi for both bacterial population quantification and cAMP quantification, which was normalized by total plant protein. cAMP was extracted and quantified using the Direct cAMP ELISA kit (ENZO) according to the manufacturer's protocol. Total protein was quantified using a Quickstart Bradford assay (BioRad) according to the manufacturer's protocol.

Nuclear fractionation and western blotting

Following temperature and chemical treatments, a minimum of 0.5 g (FW) leaf tissue was harvested and the mass recorded prior to flash freezing in liquid nitrogen. After grinding, cell lysate was isolated using the CelLytic PN Isolation/Extraction Kit (Sigma), using the semi-pure fractionation method according to the manufacturer's protocol. Following isolation and fractionation, the whole cell lysate and cytosolic fractions were diluted with an equal volume of 4X SDS Laemmli sample buffer (125 mM Tris-HCl, pH 6.8; 4% (w/v) SDS; 20% glycerol; 0.02% bromophenol blue; 5% (v/v) β-mercaptoethanol) while the nuclei pellet was resuspended in 100 µl of 2X SDS Laemmli sample buffer, resulting in a nuclear fraction sample with an 8-fold higher concentration than the whole cell or cytosolic fraction samples. All samples were boiled for 10 min at 95°C, equal volumes of each were loaded in 4-12% SDS-PAGE gradient gels (NuPAGE, Novex) and run for 40 – 50 min at 200 V. Proteins were transferred to PVDF membranes at room temperature for 1 h at 25 V. Primary antibodies used were α-GFP (1:7,500, Abcam), for detection of NPR1-YFP; α-PR1 (1:5,000, gift from Xinnian Dong, Duke University); α-

UGPase (1:3,000, Agrisera) and α-H3 (1:10,000, Agrisera). The secondary antibody used for all blots was a goat α-rabbit (1:20,000, Thermo).

Confocal microscopy

Images were taken on a Zeiss 510 Meta Confocal Laser Scanning system configured on a Zeiss AxioObserver.Z1 inverted microscope (Carl Zeiss Microscopy, Thornwood, NY) using a Zeiss 63x C-Apochromat water immersion objective (NA 1.2) objective. Sequential imaging with a Kalman averaging of 4 and pinhole set to 120 µm was used to capture images from a single confocal plane. Bright-field (BF) images were recorded using an Argon 514 nm laser. YFP was excited with an Argon 514 nm laser set at 20% and fluorescence emission was recorded using a 535-565 band pass filter. Aim Image Browser Software (Zeiss LSM) was used to add scale bars and for adjustments to brightness and contrast, which were as follows: *NPR1-Y1* images, contrast 60%; *npr1* images, contrast 65%; *35S::YFP* YFP images, brightness 52%, contrast 55%; *35S::YFP* BF images, brightness 48%, contrast 55%. Images were also adjusted for sharpness and contrast using Microsoft PowerPoint as follows: all images, sharpness +50%; *npr1* BF images, contrast (+20%); *35S::YFP* YFP images, -40% contrast; *35S::YFP* BF images, +20% contrast.

Statistical analysis

A minimum of three independent experiments were done for all assays unless otherwise indicated. Statistical significance was determined using a Student's *t*-test (Excel) for pairwise comparisons or by conducting a 2x2 factorial analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test (RStudio (https://www.rstudio.com/) for multi-variate analyses. In the case of unequal variances as determined by the Brown-Forsythe test ($\alpha \leq 0.05$, Prism 6, GraphPad Software, Inc.), data were $\log_{10}$-transformed prior to conducting the ANOVA.

Results

Elevated temperature causes loss of SA biosynthesis and enhanced disease susceptibility

Arabidopsis thaliana (hereafter Arabidopsis) is more susceptible to *Pst* DC3000 when grown continuously at elevated temperatures (28°C)[35]. However, these plants also exhibit dramatically different morphology relative to plants grown at 23°C, including exaggerated hypocotyl and petiole elongation, due to PHYTOCHROME INTERACTING FACTOR 4 (PIF4)-induction of auxin[190]. To minimize the impact of physiological differences confounding my study, I first assessed the effect of a short-term temperature acclimation period on infection of Arabidopsis plants by the virulent pathogen *Pst* DC3000. Four-week-old plants were acclimated to test chambers at 23°C (control) or 30°C (test) for 48 h prior to syringe infiltration with *Pst* DC3000. Although leaf hyponasty (raising of leaves) was observed within 24 h after shifting plants to 30°C, morphological differences were greatly reduced between test and control plants acclimated for 48 h relative to those acclimated for 7 d (Fig. 3a – c). A 30-fold increase in bacterial growth as well as a dramatic increase in disease-associated chlorosis was observed in plants at 30°C relative to those kept at 23°C (Fig. 4a, b). Based on these results, I concluded that a 48 h acclimation to 30°C prior to inoculation results in enhanced plant susceptibility with minimal differences in plant morphology.

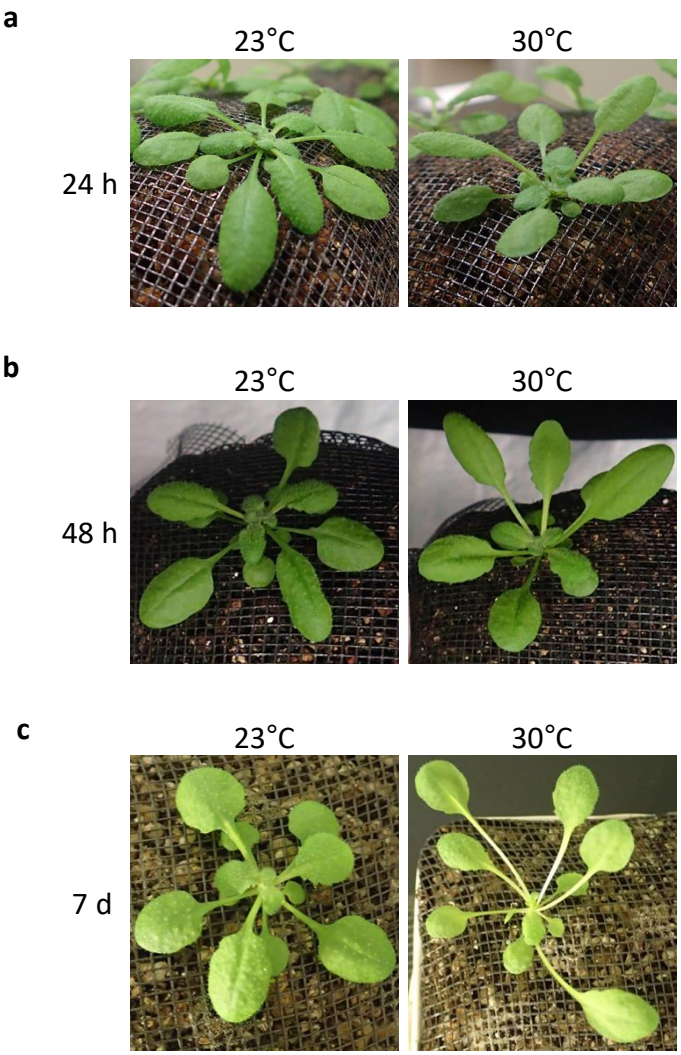

Figure 3. Morphological response of Arabidopsis plants to elevated temperature. (**a**) Four-week-old plants were shifted to test chambers at 23°C or 30°C. Pictures were taken 24 h or (**b**) 48 h after temperature shift. (**c**) Two-week-old plants were shifted to test chambers at 23°C or 30°C. Pictures were taken 7 d after temperature shift.

As SA-mediated defense plays a major role in resistance of Arabidopsis against virulent *Pst* DC3000, I investigated the possibility that increased susceptibility of plants at 30°C is caused by this pathway being compromised. I measured *ICS1* and *PR1* marker gene expression as well as free and glucosylated SA (SAG) 24 h post infiltration (hpi) with mock or *Pst* DC3000. I observed significant induction of both *ICS1* (7-fold) and *PR1* (60-fold) by *Pst* DC3000 in plants kept at 23°C, whereas neither gene was induced by the pathogen at 30°C (Fig. 4c). Similarly, both SA metabolites were induced by *Pst* DC3000 to levels roughly 8-fold higher than in mock-infiltrated plants at 23°C with no significant difference at 30°C (Fig. 4d). Finally, I tested bacterial growth in both wild type (WT, Col-0) and *sid2-2* (hereafter, *ics1*) mutant plants. As previously reported[35], *ics1* plants were more susceptible to *Pst* DC3000 than WT plants at 23°C, showing 40-fold more bacterial growth 3 dpi (Fig. 4e); however, under my experimental conditions, there was no difference in bacterial growth between the WT and *ics1* mutant plants at 30°C and a minimal difference (2.5-fold) between the *ics1* mutant plants at 23°C vs. 30°C (Fig. 4e, 3 dpi). To confirm temperature sensitivity of the *ics1* mutant was not missed due to bacterial saturation at 3 dpi, I also measured bacterial growth at 2 dpi with similar results (Fig. 4e). Based on these results, I concluded that pathogen-induction of SA is compromised at 30°C in association with enhanced disease susceptibility. Furthermore, loss of SA production via the ICS1 pathway appears to be the primary cause for this enhanced susceptibility, as there is no further enhanced bacterial growth in the *ics1* mutant relative to WT plants at 30°C.

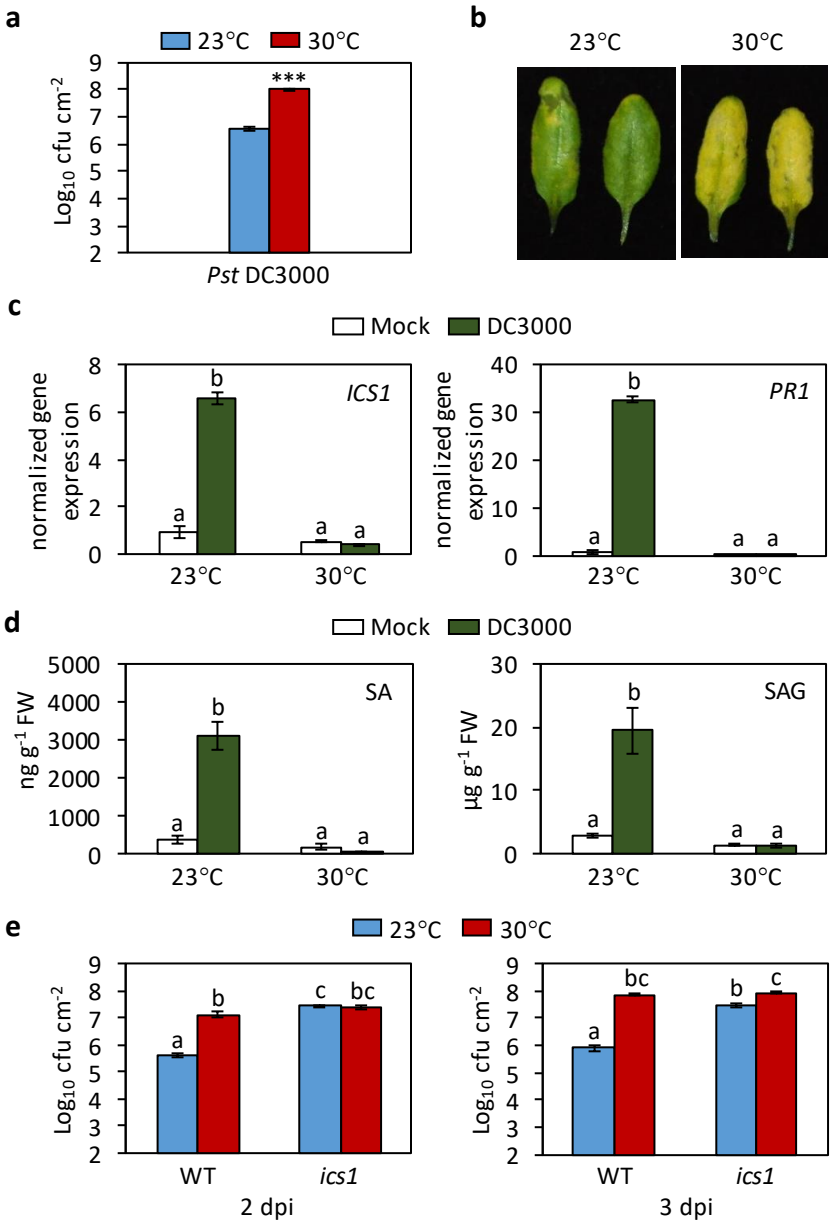

Figure 4. Increased susceptibility of Arabidopsis to *Pst* DC3000 at 30°C is correlated with a loss of SA marker gene expression and SA metabolite accumulation. (**a**) Bacterial growth in temperature acclimated plants (n = 4) three days after syringe infiltration with *Pst* DC3000. (**b**) Disease symptoms three dpi for plants in (**a**). (**c**) SA marker gene expression (n = 3) and (**d**) SA metabolite quantification (n = 4) in temperature acclimated plants 24 h after vacuum infiltration with mock or *Pst* DC3000. qPCR was used for gene expression analysis, with expression of *ICS1* and *PR1* normalized to the expression of *PP2AA3*. SA and SAG metabolites were quantified using LCMS. An SA standard curve was used to determine the SA and SAG metabolite concentrations (nM) within each sample, which were then converted to ng and normalized by sample fresh weight (FW) mass (g). (**e**) Bacterial growth in WT and *ics1* mutant plants (n = 4). Experimental conditions are the same as in (**a**). All data are representative of

Figure 4. (cont'd)
three independent experiments, except the 2 dpi data in (**e**), which was collected in two of the three independent experiments. All graphical data are presented as the mean ± standard error of the mean (SEM), with n = biological replicates. Asterisks indicate statistical significance based on a Student's *t*-test (*** $P<0.001$). Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis ($P<0.05$); samples sharing letters are not significantly different.

Enhanced disease at 30°C is correlated with increased pathogen virulence

To test whether or not enhanced multiplication of *Pst* DC3000 *in planta* at 30°C requires specific virulence factors, growth of two bacterial mutant strains, *Pst* DC3000 Δ*hrcC* (Δ*hrcC*) and *Pst* DC3118, was compared with growth of *Pst* DC3000 in Arabidopsis plants kept at 23°C or 30°C. The Δ*hrcC* mutant lacks a functional type III secretion system (T3SS) for translocation of disease-promoting type III effector (T3E) proteins into host cells[49, 191], and the *Pst* DC3118 mutant is deficient in the production of the coronatine (COR) toxin[192]. While disease-associated leaf chlorosis was greatly reduced in plants infiltrated with the *Pst* DC3118 mutant, growth of this strain was 400-fold higher at 30°C than that at 23°C (Fig. 5a, b). The Δ*hrcC* mutant strain had no detectable increase in growth at 30°C (Fig. 5a), indicating that enhanced growth of *Pst* DC3000 *in planta* at 30°C requires a functional T3SS but not COR.

The observed dependence on a T3SS for the positive effect of elevated temperature on *Pst* DC3000 growth seems to contradict previous *in vitro* analyses, which showed a negative impact of elevated temperature on T3SS gene expression[51, 53, 54]. I used a *Pst* DC3000 strain carrying a plasmid containing a P_{nptII}*::avrPto-cyaA* reporter construct to quantify T3E translocation into the plant cells to assess if this process is affected at 30°C[189]. A Δ*hrcC* mutant strain carrying the same plasmid was used as a negative control. I observed a 3-fold increase in T3E translocation 6 hpi of plants at 30°C relative to plants infiltrated at 23°C (Fig. 5c) with no detectable difference in bacterial populations at this time point (Fig. 6a). A significant increase in bacterial populations within plants at 30°C vs. 23°C was confirmed at 24 hpi (Fig. 6b). Therefore, increased *Pst* DC3000 virulence at 30°C is linked to increased translocation of T3E proteins prior to and likely resulting in a difference in bacterial populations.

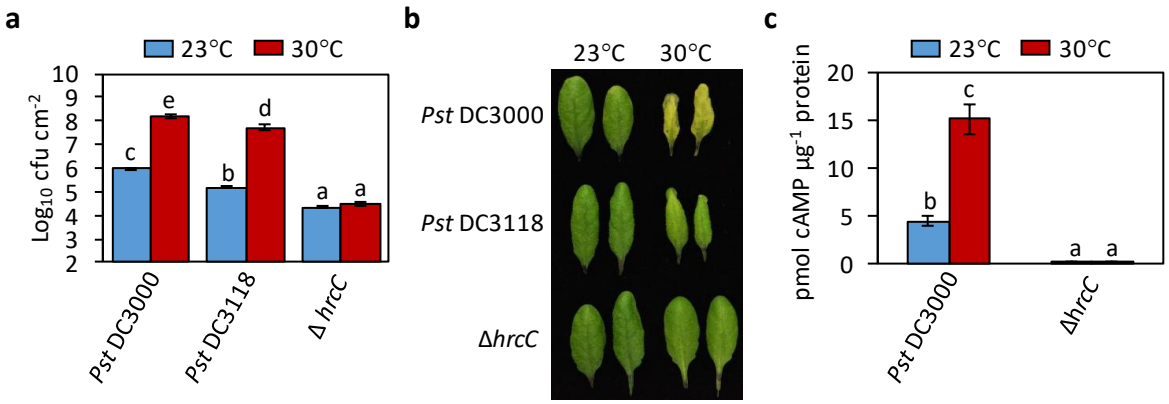

Figure 5. Enhanced growth of *Pst* DC3000 *in planta* at 30°C requires a functional type III secretion system and results in elevated levels of effector translocation. (**a**) Bacterial growth in temperature acclimated plants (n = 4) three days after syringe infiltration with *Pst* DC3000, *Pst* DC3118 (coronatine deficient mutant) or Δ*hrcC* (T3SS-deficient mutant) strains at 1 x 10^6 cfu mL^{-1}. (**b**) Disease symptoms three dpi for plants in (**a**). (**c**) Amount of cyclic AMP (cAMP) generated in temperature acclimated plants (n = 4) syringe infiltrated with *Pst* DC3000 P_{nptII}*::avrPto-cyaA* or *Pst* DC3000 Δ*hrcC* P_{nptII}*::avrPto-CyaA* strains at 2 x 10^7 cfu mL^{-1}. Tissue was collected at 6 hpi for quantification of cAMP, which was normalized by total protein. Higher levels of cAMP indicate more translocation of bacterial effectors. All data are representative of three independent experiments. All graphical data are presented as the mean ± SEM, with n = biological replicates. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis (P<0.05); samples sharing letters are not significantly different.

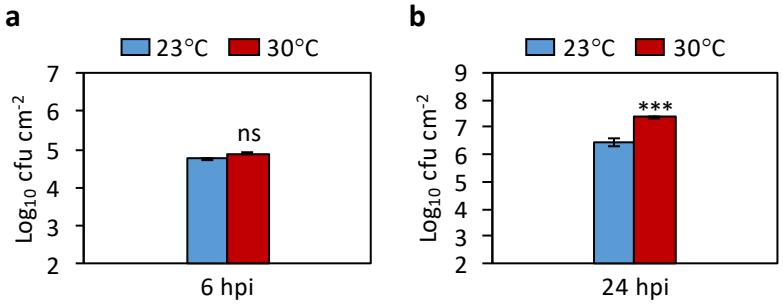

Figure 6. Elevated temperature does not affect bacterial growth at 6 hpi. Bacterial growth in temperature acclimated plants 6 h (**a**) and 24 h (**b**) after syringe infiltration with *Pst* DC3000 P_{nptII}*::avrPto-cyaA* (2 x 10^7 cfu ml^{-1}). Data are presented as the mean (n = 4) ± SEM, and are representative of three independent experiments. Asterisks indicate statistical significance based on a Student's *t*-test (*** P< 0.001); "ns" indicates no statistical significance.

BTH-induction of SA accumulation, marker gene expression and callose deposition is impaired at 30°C

BTH was used to directly test the effect of elevated temperature on SA biosynthesis and signaling in a pathogen-free system. BTH is widely used as a surrogate for the SA signal and is a potent inducer of SA response genes in Arabidopsis[193, 194]. BTH significantly induced both *ICS1* (10-fold) and *PR1* (>2,500-fold) gene expression as well as SA (5-fold) and SAG (2-fold) metabolite levels in plants kept at 23°C with no significant induction in plants treated at 30°C (Fig. 7a, b). Similarly, an 8-fold increase in BTH-induced callose was observed at 23°C, whereas no significant accumulation of callose was observed following BTH treatment at 30°C (Fig. 7c, d). In contrast, although reduced at elevated temperature, flg22 was able to elicit a strong callose response at both 23°C (90-fold) and 30°C (50-fold; Fig. 7e, f), indicating that the observed effect of temperature on BTH-induced callose deposition is likely due to compromised SA signaling rather than an effect on the callose synthase enzyme.

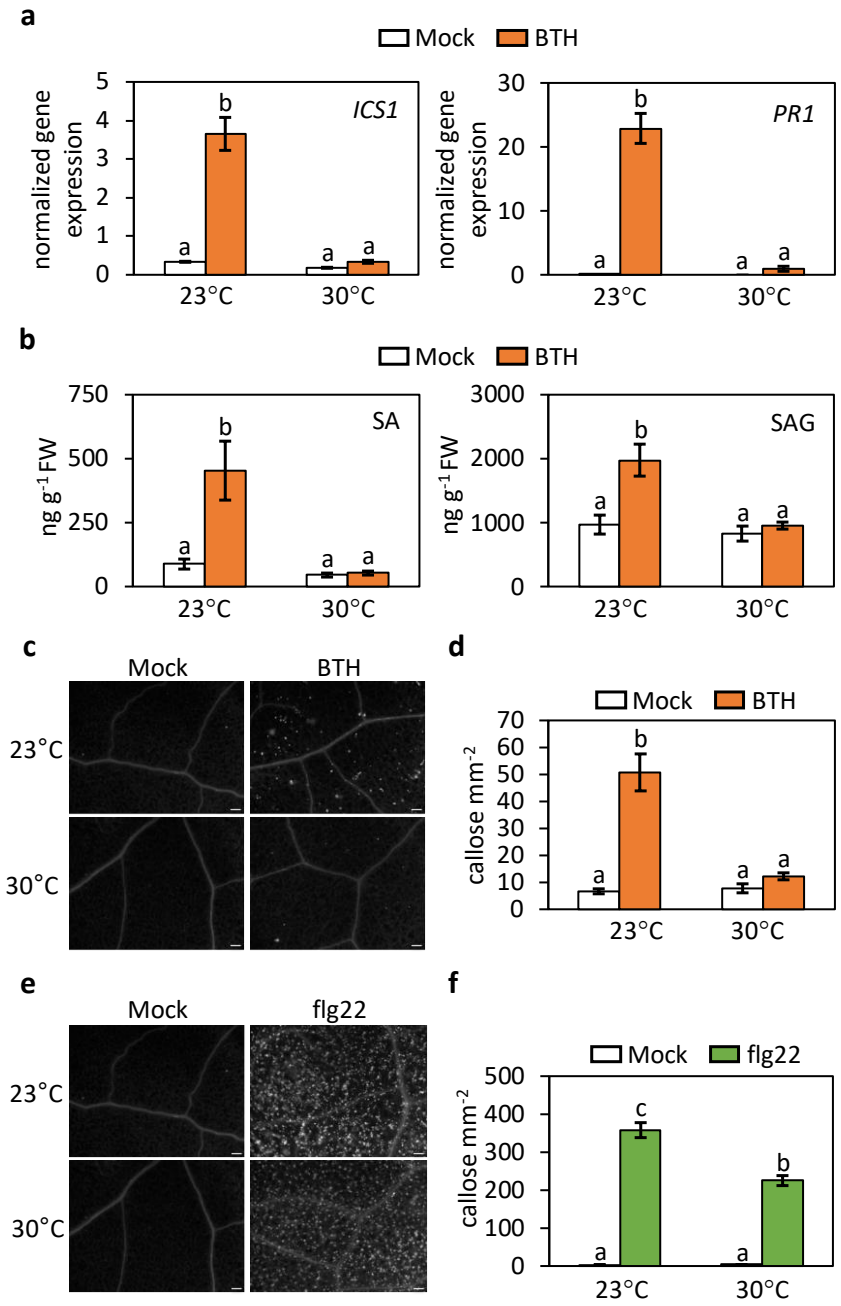

Figure 7. BTH-induction of SA marker genes, SA metabolite accumulation and callose deposition is compromised at 30°C. (**a**) SA marker gene expression (n = 6) and (**b**) SA metabolite quantification (n = 8) in temperature acclimated plants 24 h after spraying with mock or BTH. qPCR was used for gene expression analysis, with expression of *ICS1* and *PR1* normalized to the expression of *PP2AA3*. SA and SAG metabolites were quantified using LCMS. An SA standard curve was used to determine the SA and SAG metabolite concentrations (nM) within each sample, which were then converted to ng and normalized by sample fresh weight (FW) mass (g). (**c**) Representative images of callose accumulation 24 hpi with mock, BTH or (**e**) flg22 of temperature acclimated plants. Callose deposits were stained with aniline blue and visualized with an epifluorescent microscope using a DAPI filter (excitation 377/50 and emission

Figure 7. (cont'd)
447/60). Scale-bar length represents 100 µm. (**d**), (**f**) Quantification of callose accumulation from plants (n = 6) treated as described in (**c**) and (**e**), respectively. Callose assays conducted by André C. Velásquez. All data are representative of three independent experiments. All graphical data are presented as the mean ± SEM, with n = biological replicates. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis ($P<0.05$); samples sharing letters are not significantly different.

The master regulator of SA-signaling, NPR1, retains nuclear localization at 30°C

Zhu *et al.* (2010) showed that loss of R protein nuclear localization contributes to compromised R-mediated defense at elevated temperature (28°C)[36]. NPR1 is a key regulator of SA signaling and accumulates in the nucleus upon SA signal perception[169, 195]. As nuclear localization of NPR1 is required for *PR1* gene induction[109], it is possible that loss of NPR1 nuclear localization results in loss of *PR1* gene induction at 30°C. To test this, I generated transgenic lines expressing a functional NPR1 protein tagged at the C-terminal end with YFP under control of the native *NPR1* promoter (*pNPR1::NPR1-YFP* hereafter, *NPR1-Y1*; Fig. 8b – d) in a confirmed *npr1* knock-out mutant (Fig. 8a, c, d). Transgenic lines expressing the YFP protein under the control of the constitutive *35S* promoter (*p35S::YFP*) were also generated as controls. A nuclear YFP signal was observed in both mock- and BTH-treated *NPR1-Y1* plants at both 23°C and 30°C, although the signal was extremely weak in mock-treated plants at both temperatures (Fig. 9a). There was no observable effect of treatment (chemical or temperature) on YFP signal detected in the *p35S::YFP* control, and no YFP signal was detected in the parent *npr1* plants (Fig. 9b, c).

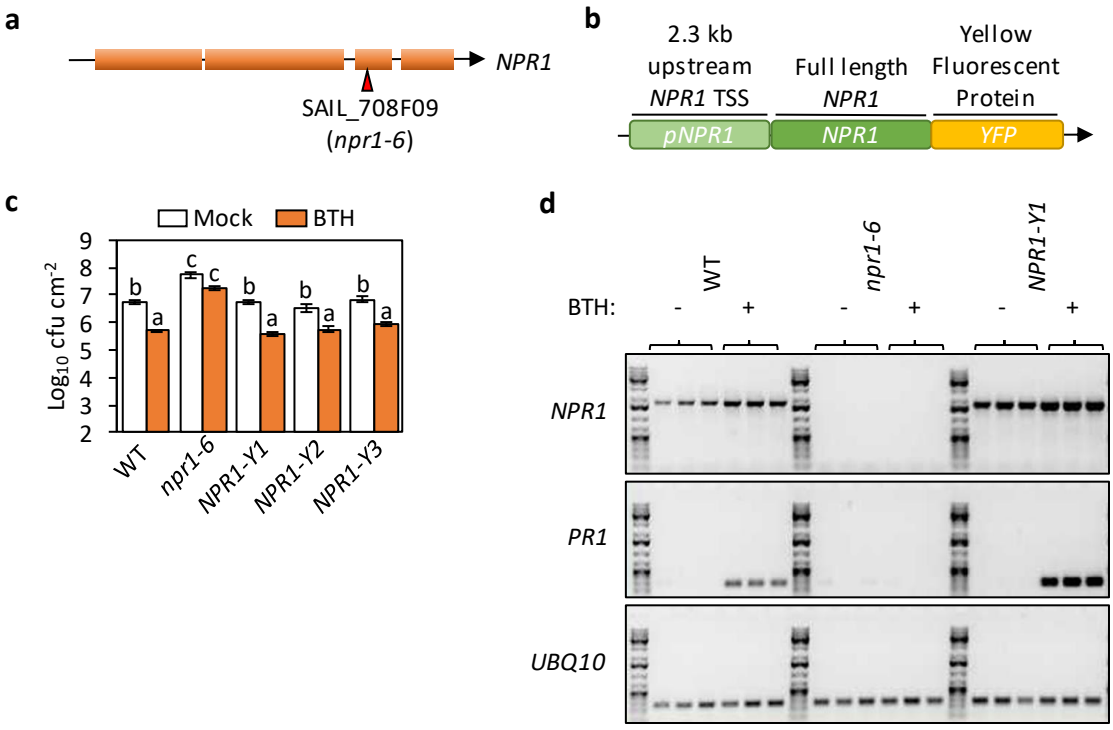

Figure 8. Characterization of *npr1-6* knock-out and *pNPR1::NPR1-YFP* transgenic lines. (**a**) Model of T-DNA insertion in *NPR1* for the SAIL_708F09 allele, named here *npr1-6* and referred to as *npr1*. (**b**) Model of *pNPR1::NPR1-YFP* construct. The NPR1 promoter used was 2.3 kb upstream of the NPR1 transcriptional start site (TSS). (**c**) Bacterial growth in five-week-old mock- or BTH-pre-treated plants three days after syringe infiltration with *Pst* DC3000. Data are presented as the mean (n = 3) ± SEM, and are representative of three independent experiments. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis ($P<0.05$); samples sharing letters are not significantly different. (**d**). Five-week-old plants (n = 3) were sprayed with mock or BTH 24 h prior to harvesting tissue for RNA extraction. Semi-quantitative gene expression analysis was used to determine the expression levels of *NPR1* (35 cycles) and *PR1* (25 cycles) with *UBQ10* (25 cycles) expression used as an internal control. Data are representative of two independent experiments. Primer sequences are provided in Table 1.

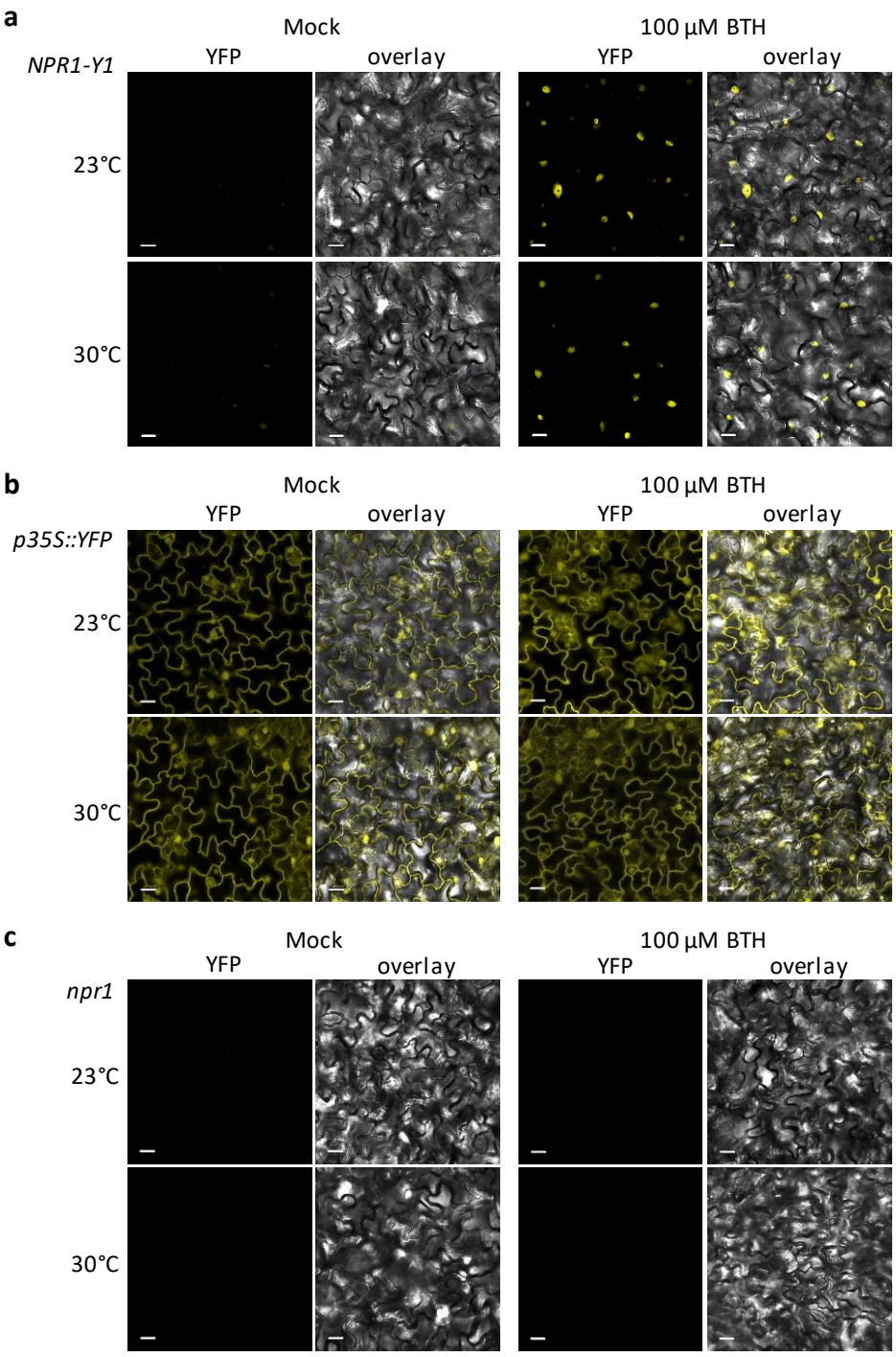

Figure 9. BTH-induction of NPR1 nuclear localization is retained at 30°C. Representative confocal microscopy images of temperature acclimated *NPR1-YFP* (**a**) *35S::YFP* (**b**) and *npr1* mutant (**c**) plants (n = 4) 24 h after spraying with mock or BTH. Images are of YFP (yellow) alone or YFP overlaid on Brightfield (gray-scale). Brightfield images were recorded using an Argon 515 nm laser. YFP was excited with an Argon 515 nm laser and fluorescence emission was recorded using a 535-565 band pass filter. Scale bar length represents 10 µm.

To independently confirm NPR1 nuclear localization, subcellular fractionation experiments were also conducted. Total protein was extracted from fully expanded leaves pooled from four *NPR1-Y1* plants following temperature acclimation and BTH treatment. Samples were then separated into nuclear and non-nuclear (referred to as cytosolic) fractions, and western blotting using a GFP antibody was used to determine the presence or absence of the NPR1-YFP protein in each fraction. Whole cell lysate extracted from *npr1* plants treated with BTH at 23°C was used as a negative control. NPR1-YFP was observed in whole cell lysate and both fractions of BTH-treated samples at both temperatures (Fig. 10a). Western blotting of UDP-GLUCOSE PYROPHOSPHORYLASE (UGPase) and HISTONE 3 (H3) proteins, which were used as cytosolic- and nuclear-fraction specific markers, respectively, showed significant enrichment within their respective fractions (Fig. 10a). The confocal and nuclear fractionation data both show that elevated temperature does not prevent BTH-induced nuclear accumulation of the NPR1 protein. To confirm the loss of BTH-induction of PR1 protein accumulation, western blot analysis using an anti-PR1 antibody was also conducted with fractionated protein samples confirmed to have NPR1 localized to the nucleus. Similar to *PR1* gene expression (Fig 7a), PR1 protein was only detectable in BTH-treated plants at 23°C (Fig. 10b).

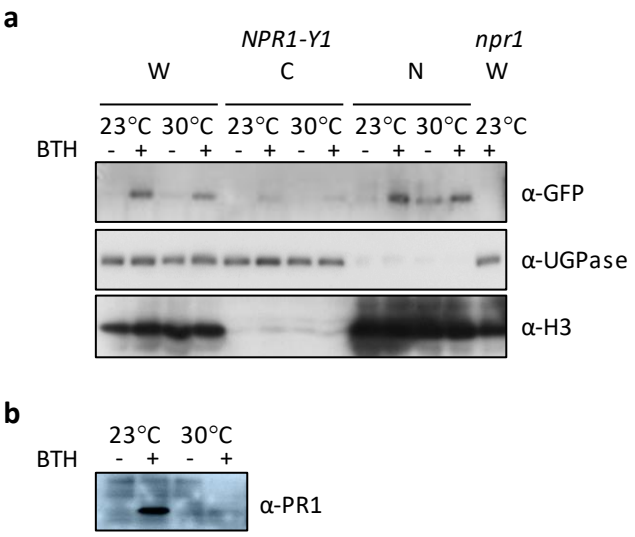

Figure 10. BTH-induction of NPR1 nuclear localization does not enable PR1 protein accumulation at 30°C. (**a**) Western blots of whole cell lysate (W), and non-nuclear (C, cytosolic) and nuclear (N) enriched fractions isolated from leaves pooled from four *NPR1-Y1* transgenic plants treated with mock (-) or BTH (+) solutions at 23°C or 30°C. Equal volumes (10 µl) of each protein sample were loaded and run in two separate 4-12% gradient SDS-PAGE gels. Following transfer to PVDF membranes, one blot was probed with α-GFP primary antibody to detect the NPR1-YFP protein (expected MW ~90 kD) while the other blot was cut in two and the upper portion probed with the α-UGPase cytosolic protein control and the lower portion probed with the α-H3 nuclear protein control. Whole cell lysate extracted from *npr1* plants treated with BTH at 23°C was used as the negative control for the NPR1-YFP band. (**b**) Western blot of non-nuclear fraction isolated from leaves pooled from four, temperature acclimated *NPR1-Y1* plants treated with mock (-) or BTH (+). Equal volumes (10 µl) of each protein sample were loaded and run a 4-12% gradient SDS-PAGE gel. Following transfer, the PVDF membrane was probed using a α-PR1 primary antibody (expected MW ~16 kD). NPR1 nuclear localization was confirmed in the same samples. Data are representative of three independent experiments.

Discovery of temperature-sensitive and -insensitive sectors of the SA-regulated transcriptome

As BTH-induction of the canonical SA marker genes, *PR1* and *ICS1,* is compromised at 30°C but NPR1 nuclear accumulation is not, I conducted RNA sequencing (RNA-seq) using the Illumina HiSeq 2500 platform to determine the extent of elevated temperature's impact on BTH-mediated global transcriptional reprogramming. Four separate contrasts (23°C Mock vs. 30°C Mock, 23°C BTH vs. 30°C BTH, 23°C Mock vs. 23°C BTH and 30°C Mock vs. 30°C BTH) were used to identify differentially expressed genes (DEGs, 4-fold cut-off, $P<0.01$), resulting in a total of 2,820 DEGs. K-means cluster analysis (k = 9) was conducted using fold change values (BTH/mock) for all DEGs at both temperatures. Clusters 1, 2 and 3 contain genes induced by BTH at 23°C but compromised in BTH-induction at 30°C, cluster 4 contains genes similarly induced at both temperatures, clusters 5, 6 and 7 contain genes induced more by BTH at 30°C than at 23°C and clusters 8 and 9 contain genes suppressed by BTH at 23°C but compromised in suppression at 30°C (Fig. 11).

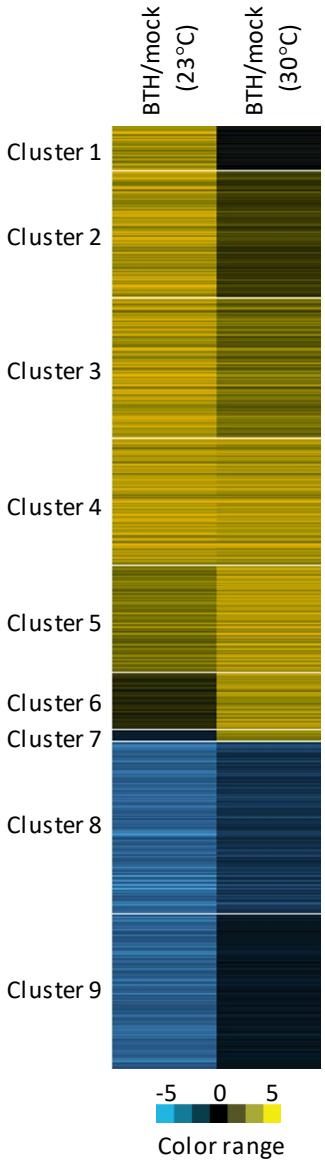

Figure 11. The effect of elevated temperature on the BTH-regulated transcriptome. Differentially expressed genes were identified from RNA-seq data and k-means clustering was conducted as described in the Methods and Results. The heat map shows a visual representation of gene expression patterns based on fold change (BTH/mock) within each cluster. Down-regulation of expression between the mock- and BTH-treated plants at each temperature is denoted by the level of blue color, up-regulation by the level of yellow color and no change by black as indicated by the color scale. DEG identification, k-means clustering and heat map generation all done by Dr. Kenichi Tsuda.

To gain insight into the types of genes found in each cluster, expression patterns were analysed using publicly available microarray datasets and functional annotations using gene ontology (GO) analysis. Together, these analyses show that genes in clusters 1 – 7 are involved in various biotic or abiotic stress responses and genes in clusters 8 and 9 are involved in photosynthesis and growth-related processes (Fig. 12 – 14, Table 2). As growth-defense trade-offs are known to occur[57], it is possible that the compromised suppression of cluster 8 and 9 genes at 30°C either contributes to or is the consequence of compromised induction of SA-associated defense signaling. The same may be true for genes in clusters 5 and 6 (uniquely induced by BTH at 30°C), which were functionally annotated as being involved in ET-, JA- and abscisic acid (ABA)-regulated responses (Table 2), all of which are known to be antagonistic with SA[102, 144, 154].

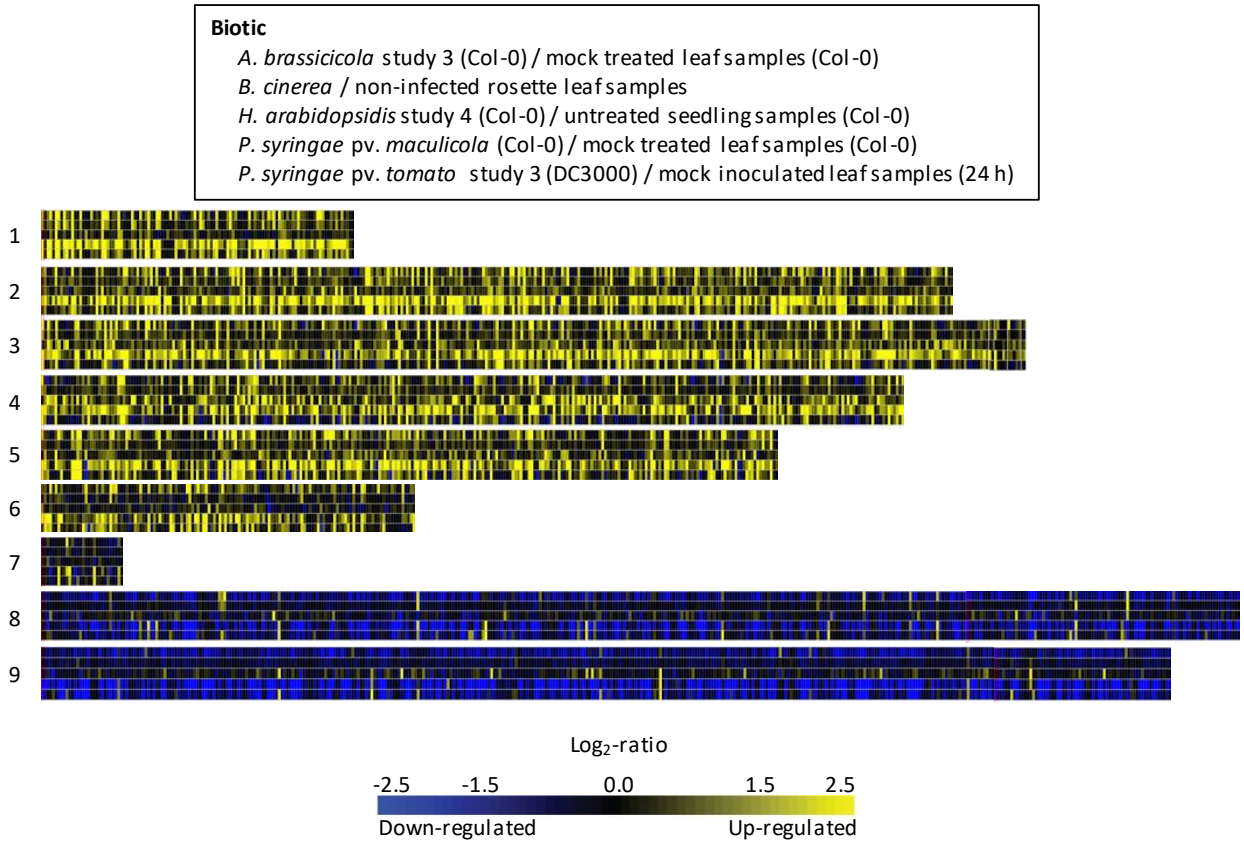

Figure 12. Genevestigator analysis using publicly available microarray expression data of genes in response to biotic stress. Of the pathogens listed under biotic stress, *Alternaria brassicicola* and *Botrytis cinerea* are necrotrophic pathogens, *Hyaloperonospora arabidopsidis* is an obligate biotrophic pathogen and both strains of *Pseudomonas syringae* are hemi-biotrophic pathogens. Numbers to the left of each panel indicate the cluster to which that set of genes is assigned. Expression values are $\log_2$ ratios of treated vs. untreated or mock controls, with down-regulation of expression denoted by the level of blue color, up-regulated denoted by the level of yellow color and no change in expression denoted by black color as indicated by the color scale. Genevestigator analysis conducted by Dr. Kenichi Tsuda.

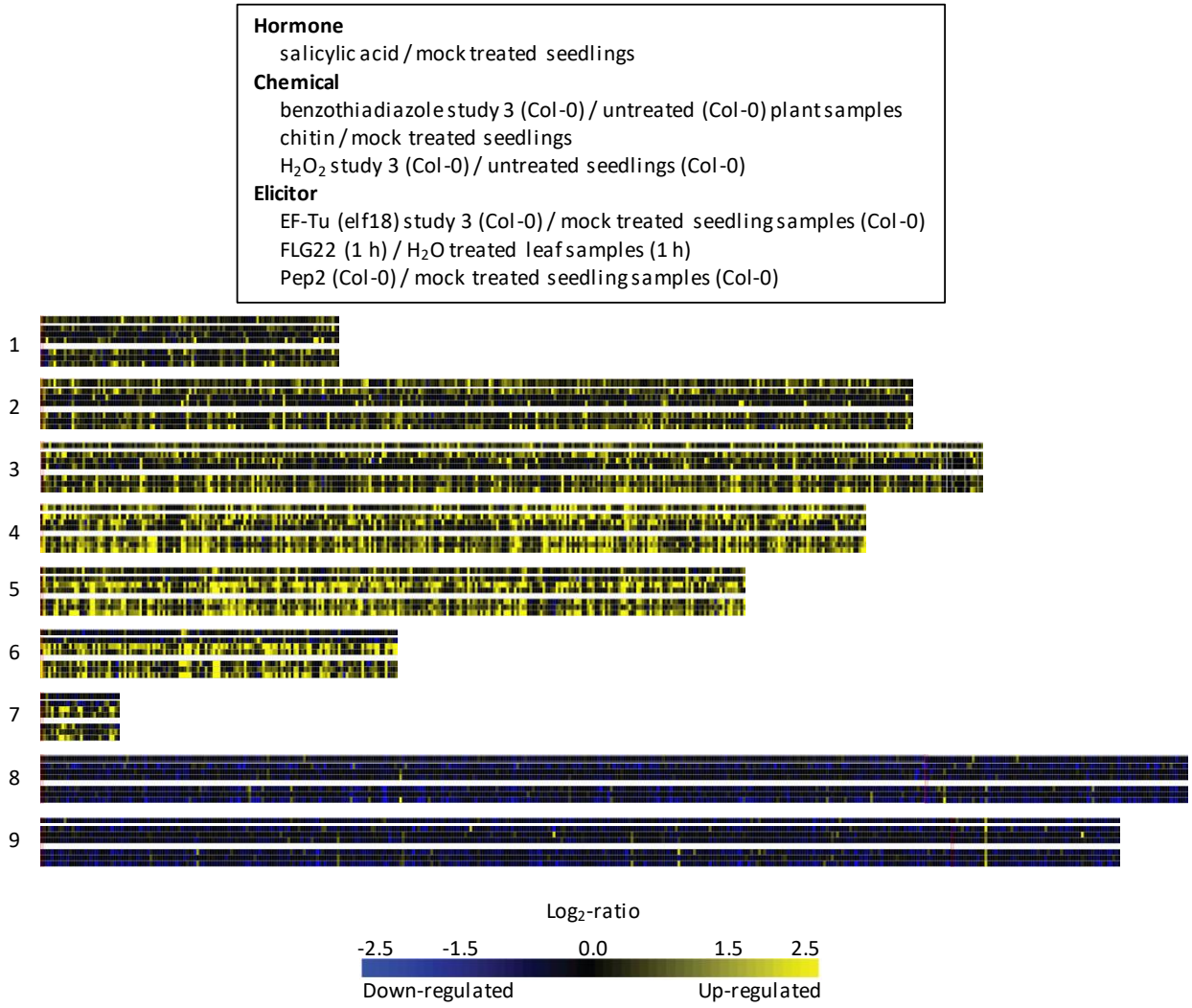

Figure 13. Genevestigator analysis using publicly available microarray expression data genes in response to SA/BTH or microbe-associated elicitors. Numbers to the left of each panel indicate the cluster to which that set of genes is assigned. Expression values are log_2 ratios of treated vs untreated or mock controls, with down-regulation of expression denoted by the level of blue color, up-regulated denoted by the level of yellow color and no change in expression denoted by black as indicated by the color scale. Genevestigator analysis conducted by Dr. Kenichi Tsuda.

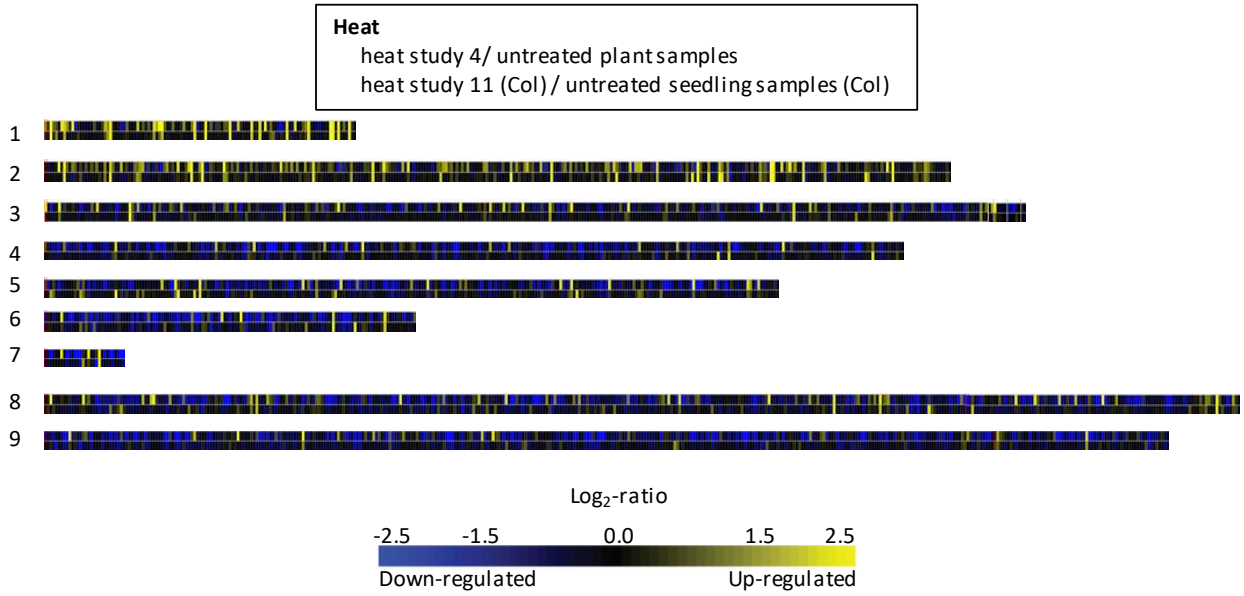

Figure 14. Genevestigator analysis using publicly available microarray expression data of genes in response to heat stress. Numbers to the left of each panel indicate the cluster to which that set of genes is assigned. Expression values are log_2 ratios of treated vs untreated or mock controls, with down-regulation of expression denoted by the level of blue color, up-regulated denoted by the level of yellow color and no change in expression denoted by black as indicated by the color scale. Genevestigator analysis conducted by Dr. Kenichi Tsuda.

Table 2. Subset of functional annotations for differentially expressed genes in RNA-seq dataset. The GO term is provided in the second column. Subsequent columns provide the number of unique genes within each cluster containing the corresponding GO term. GO analysis was conducted using DAVID (https://david.ncifcrf.gov/) by Dr. Jane A. Pulman.

Category	GO term	1	2	3	4	5	6	7	8	9
Biotic Stress	Defense			40	53	54	21			
	Immune			20	26	31	8			
	Cell death			20	20	21				
	Bacterium	7	13	15	17	14				
	Chitin				16	32	20	3		
	SA/SAR			17	19					
	ET					9	17			
	JA					8				
Abiotic stress	Abiotic stress					8	17			
	ABA				13		7			
	heat	7	8							
Growth	Chloroplast part								34	45
	photosynthesis									26
	auxin								10	12

To identify TFs that may be involved in regulation of genes within each cluster, Analysis of Motif Enrichment (AME)[196] was conducted using 1,000 bp upstream of each gene. Overall these results matched those based on the microarray and GO analyses. Genes in clusters 2, 4 and 5 had an over-representation of the *as-1* element bound by TGA TFs[197], which are known regulators of genes involved in biotic stress responses[198], and genes in clusters 2, 3, 4, 5 and 6 had an over-representation of the W-box motif bound by WRKY TFs[119], which are known regulators of genes involved in both biotic and abiotic stress responses[119, 198] (Table 3). Additionally, genes in cluster 6 have an over-representation of *cis* elements bound by TFs involved in ABA signaling as well as the CAMTA2 and CAMTA3 TFs, both of which are negative regulators of SA[100, 148]. Finally, clusters 8 and 9 genes show an overrepresentation of *cis* elements bound by PIFs, which are growth-promoting TFs involved in responses to high temperature, red- and far red-light and shade avoidance[199], and MYCs, which are involved in both ABA- and JA-mediated signaling[147].

Table 3. Transcription factors implicated in regulation of genes within each cluster. Analysis of Motif Enrichment (AME; http://meme-suite.org/tools/ame) was conducted by cluster as described in the Methods. For each cluster analysis, a subset of TFs, as implicated by enrichment of the motif to which they bind, was compiled based on known relevance to SA, ABA, JA and growth-related processes. No enriched motifs were identified for clusters 1 and 7. AME analysis conducted by Dr. Kenichi Tsuda.

Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 8	Cluster 9
TGA2	WRKY (x23)	TGA5	TGA5	CAMTA2	PIF1	PIF1
TGA5		TGA6	TGA6	CAMTA3	PIF3	PIF3
TGA6		WRKY (x25)	WRKY (x24)	ABF1	PIF4	PIF4
WRKY (x13)				ABF2	PIF5	PIF5
				ABF3	MYC2	MYC2
				ABI5	MYC3	MYC3
				PIF3	MYC4	
				WRKY (x19)	JAM2	

To take a closer look at genes well-characterized for their involvement as positive or negative regulators of either SA biosynthesis and/or signaling, genes were grouped based on the differences of expression levels in BTH-treated plants at 23°C vs. 30°C. BTH-induced genes (clusters 1 – 7; 1,833 genes) were categorized into three groups as follows: Group A, expression ≤ 2-fold lower at 30°C vs. 23°C (956 genes; 52% of BTH-induced genes); Group B, expression similar at 30°C and 23°C (725 genes; 40% of BTH-induced genes); Group C, expression ≥ 2-fold higher at 30°C vs. 23°C (152 genes; 8% of BTH-induced genes; Fig. 15a). Using the same 2-fold cut-off criteria, 810 out of 987 (82%) of BTH-suppressed genes at 23°C were compromised in suppression at 30°C; therefore, BTH-suppressed genes were kept in a single Group D (Fig. 15a). If genes regulated by BTH at 23°C (Groups A, B and D) are defined as the typical SA signaling network, a clear temperature-sensitive bifurcation is revealed, with 66% (1,766) of genes misregulated and 34% (902) retaining similar expression levels at 30°C. Based on this analysis, known positive regulators of SA biosynthesis, including *EDS1*, *PAD4*, *CBP60g* and *SARD1*[13], are predominantly in the *PR1*/*ICS1* branch (Group A), while negative regulators, such as *WRKY54*, *MYC2* and several MYC2-regulated *ANACs*[13], are predominantly in Groups B and C (Fig. 15b, c). *NPR1* and two of its known regulators, *NPR3*[13] and *SUMO3*[114], are in Group B (Fig. 15b), further supporting my finding that NPR1 induction and localization are not impaired at 30°C. Downstream of NPR1, many SAR-related genes, including *PR1*, *PR2*, *PR5*, *ALD1* and *FMO1*[13], appear in Group A; however, several positive regulators of SA signaling, including WRKY54[13] and GRX480[200] are in Group B (Fig. 15b, c).

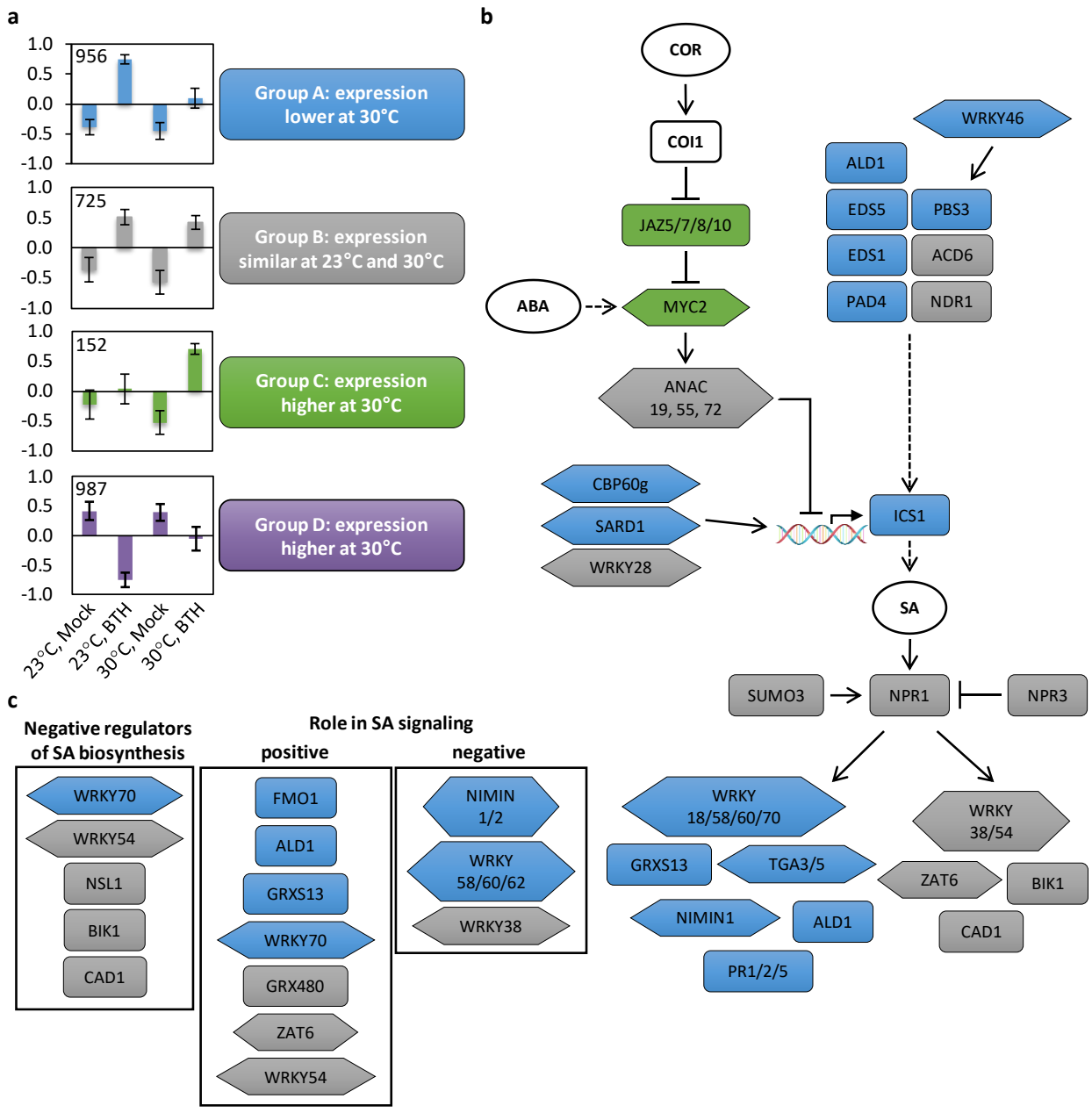

Figure 15. Global transcriptome analysis of BTH-regulated genes reveals a temperature-sensitive bifurcation in the SA-signaling network. (**a**) RNA-seq gene expression profiles. Genes were grouped based on a 2-fold change difference in expression between BTH-treated samples at 30°C vs. 23°C. Data are represented as the mean ± SEM of the centered, normalized expression values for each sample type (23°C, Mock; 23°C, BTH; 30°C, Mock; 30°C, BTH) within each group. The number of genes within each group is shown in the upper left corner of each graph. (**b**) and (**c**) Graphical depiction of genes involved in promotion or suppression of SA biosynthesis via regulation of the *ICS1* gene as well as genes involved in the SA signaling pathway. Genes are color-coded based on the gene group (**a**) to which they belong. Shapes without fill denote metabolites and *COI1*, which was not a DEG in this study. Metabolites are

Figure 15. (cont'd)
also denoted by oval shapes; TFs are denoted by hexagon shapes. The DNA helix with bent arrow depicts the *ICS1* gene promoter. Solid lines depict confirmed, direct connections, whereas dashed lines depict indirect/multiple or missing/unknown steps between components. Bent arrows next to DNA molecules depict gene promoters. Arrowheads show positive and blunted lines show negative interactions. References for each gene function depicted are provided in Table 4.

Table 4. RNA-seq differentially expressed genes involved in SA biosynthesis and/or signaling. Genes are identified based on their Arabidopsis Genome Initiative (AGI) number and commonly used gene name. Cluster numbers are based on k-means clustering. Group classifications are based on differences in fold change (2-fold cut-off) between the BTH-treated samples at 23°C and 30°C.

Process	AGI Number	Gene Name	Cluster	Group	References
Positive regulation of SA biosynthesis	AT3G52430	PAD4	3	A	94, 95, 201
	AT3G48090	EDS1	4	A	96, 97
	AT3G20600	NDR1	5	B	202
	AT5G13320	PBS3	2	A	203, 204
	AT5G26920	CBP60G	4	A	98
	AT1G73805	SARD1	4	A	98
	AT4G18170	WRKY28	5	B	99
	AT2G46400	WRKY46	4	A	99
	AT1G74710	ICS1, SID2	1	A	89, 205
	AT4G39030	EDS5, SID1	1	A	205
	AT4G14400	ACD6	4	B	206
	AT2G13810	ALD1	2	A	207, 208
	AT1G64280	NPR1, SAI1, NIM1	4	B	201
Negative regulation of SA biosynthesis	AT2G40750	WRKY54	4	B	117
	AT3G56400	WRKY70	4	A	117
	AT1G29690	CAD1	5	B	209
	AT2G39660	BIK1	4	B	210
	AT1G28380	NSL1	5	B	211
	AT1G64280	NPR1, SAI1, NIM1	4	B	105, 201
	AT1G52890	ANAC019	5	B	144
	AT3G15500	ANAC055	5	B	144
	AT4G27410	ANAC072	5	B	144
	AT1G32640	MYC2	7	C	144
Positive role in SA signaling/SAR	AT1G28480	GRX480	4	B	200, 212
	AT1G03850	GRXS13	4	A	200
	AT1G64280	NPR1, SAI1, NIM1	4	B	103, 105, 106
	AT5G55170	SUMO3	4	B	114
	AT1G22070	TGA3	3	A	213, 214
	AT5G06960	TGA5, OBF5	2	A	115
	AT4G31800	WRKY18	4	A	117
	AT4G23810	WRKY53	5	C	117
	AT2G40750	WRKY54	4	B	117
	AT3G56400	WRKY70	4	A	117
	AT2G13810	ALD1	2	A	207, 215

Table 4. (cont'd)

Process	AGI Number	Gene Name	Cluster	Group	References
Positive role in SA signaling/SAR, cont.	AT1G19250	FMO1	3	A	216
	AT2G14610	PR1	2	A	170
	AT3G57260	PR2, BGL2	4	A	170
	AT1G75040	PR5	4	A	170
Negative role in SA signaling/SAR	AT5G45110	NPR3	4	B	111, 217
	AT1G02450	NIMIN1	4	A	218, 219
	AT3G25882	NIMIN-2	4	A	218, 219
	AT4G31800	WRKY18	4	A	220
	AT5G22570	WRKY38	4	B	117, 221
	AT3G01080	WRKY58	4	A	117
	AT2G25000	WRKY60	3	A	220
	AT5G01900	WRKY62	3	A	221, 222
	AT5G04340	ZAT6	6	B	223

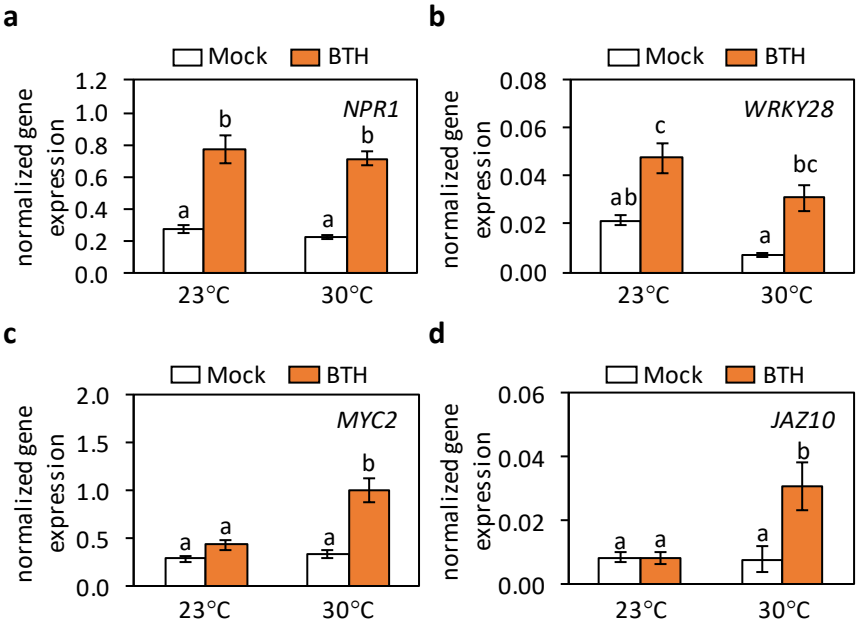

Figure 16. Validation of RNA-seq gene expression patterns. qPCR was conducted to validate the expression patterns of RNA-seq Groups (Fig. 15a) (**a**) NPR1 is a positive regulator of SA signaling that also acts in both positive and negative feedback loops regulating SA biosynthesis. (**b**) WRKY28 is a positive regulator of SA biosynthesis that binds the *ICS1* promoter to promote its expression. (**c**) MYC2 promotes expression of *ANAC* TFs that bind and suppress *ICS1* expression. (**d**) *JAZ10* is induced by activation of the JA signaling pathway, which is known to antagonize SA signaling. All genes were normalized to the expression of *PP2AA3*. Data are presented as the mean (n = 6) ± SEM, and are representative of three independent experiments. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis ($P<0.05$); samples sharing letters are not significantly different.

Gene expression profiles were confirmed using qPCR for several genes in each group of BTH-induced genes. While Group A (*ICS1*, *PR1*) and B (*NPR1*, *WRKY28*) genes showed reproducible expression profiles across several independent experiments, Group C genes (*MYC2*, *JAZ10*) did not (Fig. 15a, Fig. 16). However, the overall expression values for these two genes as measured by qPCR were low, which may contribute to their variability between experiments.

JA, ET and ABA do not contribute to enhanced susceptibility at 30°C

As mentioned above, JA, ET and ABA are known to antagonize SA biosynthesis and signaling[102, 144, 154], and all three of these pathways were implicated as being up-regulated in response to BTH at 30°C. To test the possibility that one or more of these hormone pathways might contribute to loss of SA accumulation at elevated temperature resulting in enhanced susceptibility, I conducted disease assays at 23°C and 30°C using mutants affected in either the biosynthesis and/or signaling of these hormones. The JA mutants tested were *dde2-2* (hereafter *aos*), which is JA-deficient due to a defect in the *ALLENE OXIDE SYNTHASE* gene[224], and *myc2 myc3 myc4* (hereafter *myc2/3/4*), which is compromised in three TFs involved in JA-mediated signalling[178]. The ET mutants tested were *ein2-1* (hereafter *ein2*), which is compromised in ET-mediated signaling[173], and the *ein3 eil1* double mutant, which is defective in two TFs known to promote ET signaling and negatively regulate *ICS1* gene expression[102]. I also tested *aba2-1* (hereafter *aba2*), which is an ABA biosynthetic mutant having roughly 20-25% of ABA present in WT plants[174, 176]. All five mutants showed a slight (5 to 10-fold) but significant reduction of *Pst* DC3000 growth at 23°C compared to WT plants (Fig. 17a – e). However, based on the retention of temperature sensitivity in the mutant plants and the similar level of susceptibility between

each mutant and the WT plants at 30°C (Fig. 17a – e) none of these signaling pathways is likely responsible for enhanced susceptibility to *Pst* DC3000 at elevated temperature.

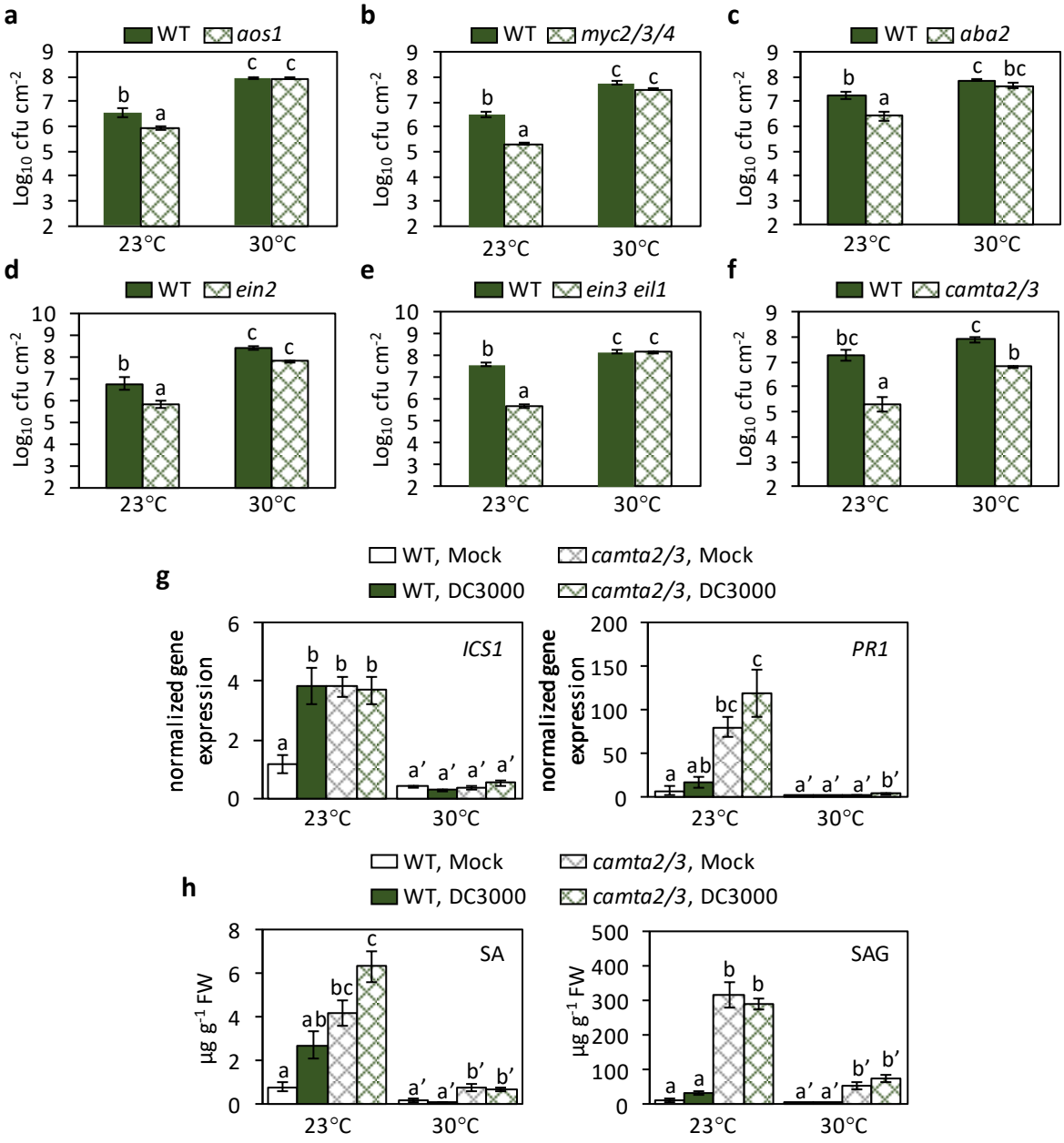

Figure 17. CAMTAs contribute to enhanced susceptibility but are not responsible for loss of SA induction at 30°C. Bacterial growth in temperature acclimated WT vs. (**a**) *aos1,* (**b**) *myc2/3/4*, (**c**) *aba2*, (**d**) *ein2*, (**e**) *ein3 eil1* and (**f**) *camta2/3* mutant plants three days after vacuum infiltration with *Pst* DC3000. (**g**) SA marker gene expression and (**h**) SA metabolite quantification 24 h after vacuum infiltration with mock or *Pst* DC3000. qPCR was used for gene expression analysis, with expression of *ICS1* and *PR1* normalized to the expression of *PP2AA3*. SA and SAG metabolites were quantified using LCMS. An SA standard curve was used to determine the SA and SAG metabolite concentrations (nM) within each sample, which were then converted to ng and normalized by sample fresh weight (FW) mass (g). Data are presented as the mean (n = 4) ± SEM, and are representative of three independent experiments. Letters

Figure 17. (cont'd)
indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis (P<0.05); samples sharing letters are not significantly different. Data in (**g**) and (**h**) were analyzed in two groups based on temperature as indicated by the prime symbol (').

Loss of CAMTA2 and CAMTA3 enables resistance but not recovery of SA biosynthesis at 30°C

CAMTA1, CAMTA2 and CAMTA3 are TFs that function redundantly in repressing SA biosynthesis at temperatures between 19°C and 22°C[84, 100]. This repression is relieved upon pathogen perception or in response to cold temperatures (~4°C), enabling SA accumulation in these conditions[84, 225]. As expected, the *camta2 camta3* double mutant (hereafter *camta2/3*) showed enhanced resistance against *Pst* DC3000 relative to WT plants at 23°C (140-fold less growth; Fig. 17f). Unlike the other mutants tested, the *camta2/3* mutant retained heightened resistance at 30°C (80-fold less growth; Fig. 17f), indicating that CAMTAs may contribute to enhanced susceptibility at elevated temperature by suppressing SA biosynthesis. Although *ICS1* and *PR1* gene expression was constitutively elevated in the *camta2/3* mutants at 23°C, expression of these genes remained compromised at 30°C (Fig. 17g), indicating that loss of SA biosynthesis at elevated temperature is not due to CAMTA2/3-mediated suppression. SA and SAG levels were observed to be constitutively elevated in the *camta2/3* mutants relative to WT at both temperatures; however, there was a significant decrease in the amount of both SA and SAG in mutant plants at 30°C vs. 23°C (Fig. 17h).

Provision of an SA signal restores resistance to *Pst* DC3000 at 30°C

Although greatly depleted, it is possible that the residual levels of endogenous SA/SAG and/or priming effects associated with plant development in the presence of constitutively elevated SA may be sufficient to confer the protection against disease observed in the *camta2/3* mutants at 30°C. This would support the hypothesis that loss of SA biosynthesis is the cause of enhanced susceptibility at elevated temperature. However, it is intriguing that this disease resistance exists in the absence of the *PR1/ICS1* branch of SA signaling, suggesting it

may be SA-independent. To investigate whether provision of an exogenous SA signal can confer disease protection at elevated temperature, I conducted BTH-protection assays in WT plants at 23°C and 30°C. Similar to the *camta2/3* mutants, BTH-treated WT plants had 150 to 200-fold less bacterial growth and showed no disease symptoms relative to the mock-treated controls at both temperatures (Fig. 18a, b). Furthermore, no induction of the *ICS1* and *PR1* marker genes or of the SA and SAG metabolites was observed in plants pre-treated with BTH and infiltrated with *Pst* DC3000 relative to the control (mock-pre-treatment + mock infiltration) plants at 30°C (Fig. 18c, d). Full transcriptome analysis of pathogen- and BTH-treated plants at both temperatures would be needed to determine whether the entire *PR1*/*ICS1* branch identified in the RNA-seq analysis also remains compromised. However, it is possible that both BTH-mediated protection and the enhanced resistance of the camta2/3 mutant plants at 30°C are SA-dependent, but occur in the absence of this important branch of SA-mediated signaling.

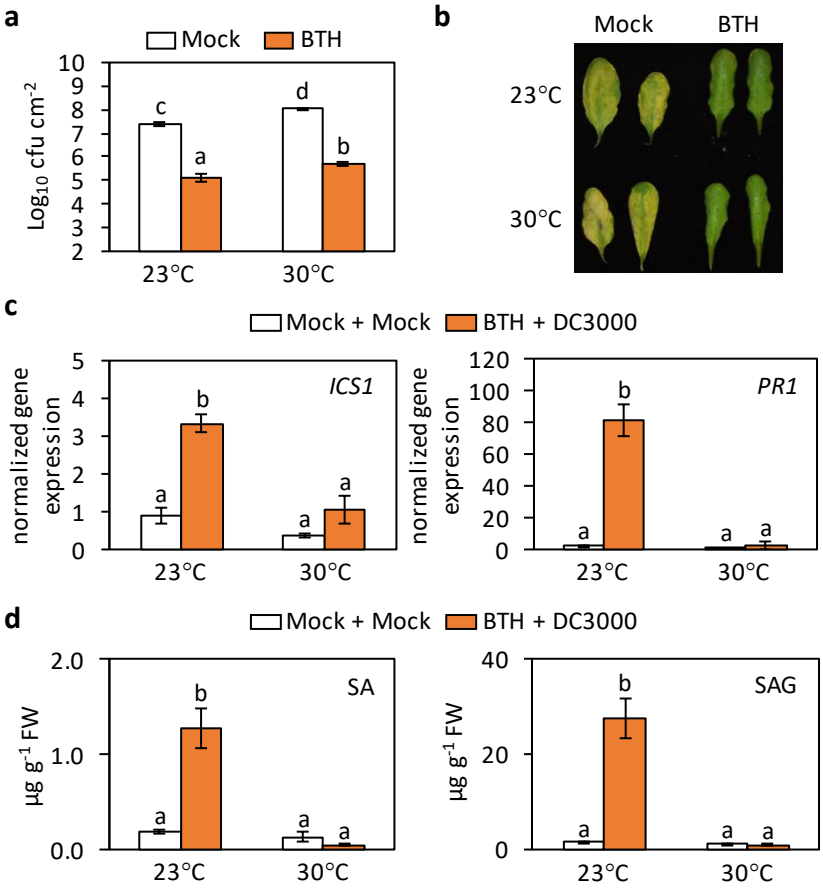

Figure 18. BTH-mediated protection against *Pst* DC3000 persists at 30°C in the absence of *PR1*, *ICS1* and SA metabolite accumulation. (**a**) Bacterial growth in temperature acclimated, mock- or BTH-pre-treated plants (n = 4) three days after vacuum infiltration with *Pst* DC3000. (**b**) Disease symptoms three dpi in plants used for BTH protection assay in (**a**). Tissue was collected 24 hpi from the same plants in (**a**) as well as mock-infiltrated plants for quantification of (**c**) SA marker gene expression (n = 3) and (**d**) SA metabolites (n = 4). qPCR was used for gene expression analysis, with expression of *ICS1* and *PR1* normalized to the expression of *PP2AA3*. SA and SAG metabolites were quantified using LCMS. An SA standard curve was used to determine the SA and SAG metabolite concentrations (nM) within each sample, which were then converted to ng and normalized by sample fresh weight (FW) mass (g). All data are representative of three independent experiments. All graphical data are presented as the mean ± SEM with n = biological replicates. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis (P<0.05); samples sharing letters are not significantly different.

To determine if NPR1 is required for BTH-mediated protection at 30°C, I conducted BTH-protection assays in WT and *npr1* mutant plants. The *npr1* mutant plants had 30-fold higher bacterial growth relative to WT plants at 23°C, but no difference in bacterial growth was observed between the two genotypes at 30°C (Fig. 19a). Whereas BTH-mediated protection was once again observed in the WT plants at both temperatures, no protection was observed in *npr1* plants at either temperature (Fig. 19a), indicating that NPR1 is required for BTH-induced protection at both 23°C and 30°C.

The combined loss of TGA2, TGA5 and TGA6 TFs is known to compromise SA-mediated defense at 23°C[115]. To further investigate the utilization of the core SA-signaling pathway in BTH-mediated protection at elevated temperature, I repeated the BTH-protection assay at 23°C and 30°C using WT and *tga2 tga5 tga6* (hereafter *tga2/5/6*) triple mutant plants. The *tga2/5/6* mutant plants had 10-fold more bacterial growth than WT at 23°C, but were slightly more resistant (2-fold less bacterial growth) than WT at 30°C (Fig. 19b). While some protection was observed in BTH-treated *tga2/5/6* mutants at 23°C, it was compromised relative to WT plants (5-fold vs. 55-fold) and completely absent at 30°C, indicating that TGA2, TGA5 and TGA6 are required for BTH-mediated protection at elevated temperature (Fig. 19b).

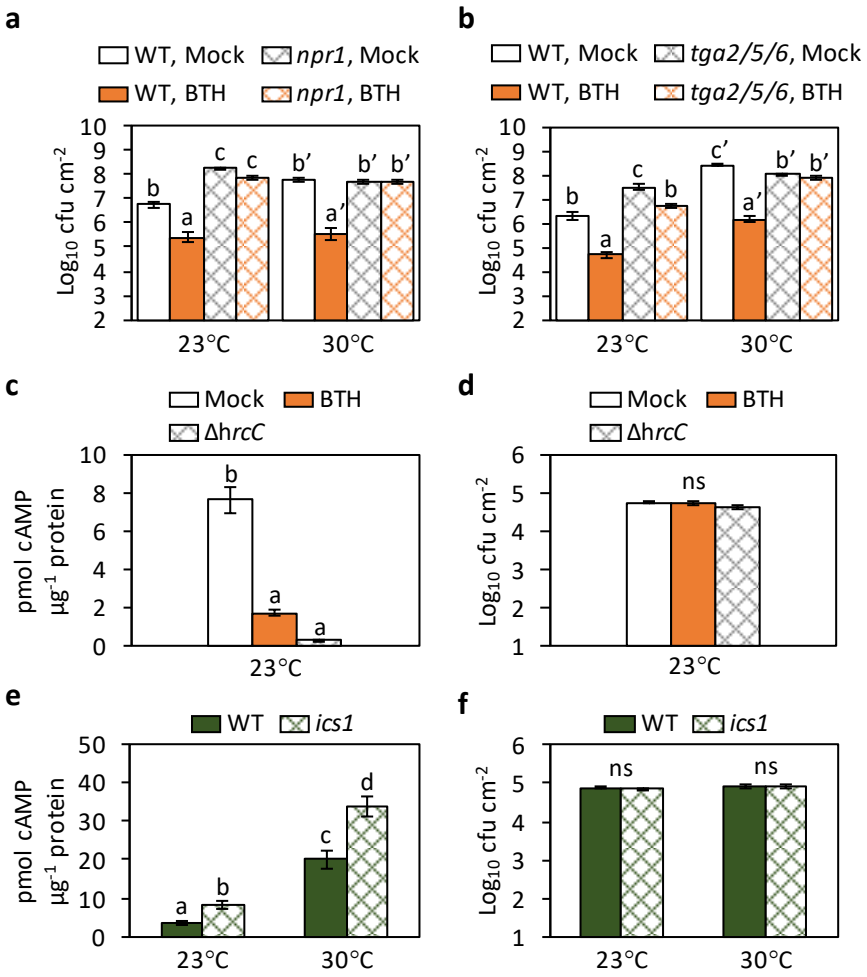

Figure 19. BTH-mediated protection at 30°C requires NPR1 and the TGA2, TGA5, TGA6 TFs and involves restriction of bacterial T3E. (**a**) Bacterial growth in temperature acclimated, mock- or BTH-pre-treated WT and *npr1* mutant plants and (**b**) *tga2/5/6* mutant plants three days after vacuum infiltration with *Pst* DC3000. The effect of BTH (**c, d**) and SA-deficiency (**e, f**) on translocation of bacterial effectors (**c, e**) and bacterial multiplication (**d, f**) 6 hpi of temperature acclimated plants with *Pst* DC3000 P_{nptII}*::avrPto-cyaA* or *Pst* DC3000 Δ*hrcC* P_{nptII}*::avrPto-CyaA* strains. cAMP levels were normalized by total protein, and higher levels of cAMP indicate more translocation of bacterial effectors. All data are representative of three independent experiments. All data are presented as the mean (n = 4) ± SEM. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis (P<0.05); samples sharing letters are not significantly different. Data in (**a**) and (**b**) were analyzed in two groups based on temperature as indicated by the prime symbol (’).

BTH-mediated protection against disease involves reduction in effector protein translocation

Because BTH-mediated protection remains intact in spite of increased T3E translocation by *Pst* DC3000 at elevated temperature, it is possible that a component of the SA/BTH-protection mechanism is to restrict T3E translocation. To test this, I quantified T3E translocation in mock- and BTH-treated WT plants at 23°C as well as in WT and *ics1* mutants at 23°C and 30°C. As before, bacterial populations were assessed at 6 hpi to confirm differences in T3E translocation are not due to differences in bacterial population at this time point (Fig. 19d, f). I observed a significant reduction (4-fold) in T3E translocation into BTH-treated plants relative to the mock-treated controls (Fig. 19c) as well as a significant increase (2-fold) in T3E translocation into *ics1* vs. WT plants at 23°C (Fig. 19e), indicating that SA does play a role in restricting T3E translocation into host cells. However, the effect of elevated temperature on T3E translocation is larger than that of SA, as I observed significantly more translocation into both WT and *ics1* mutant plants at 30°C relative to 23°C (~5-fold; Fig. 19e). This indicates that, at elevated temperature, both loss of SA biosynthesis in the host and increased translocation of T3E by the pathogen contribute to enhanced disease.

Discussion

According to the long-standing "disease triangle" dogma in plant pathology, plant-pathogen interactions can only be fully understood within the context of environment[226]. Elevated temperature has been shown to promote disease in many plant-pathosystems[227], from fungal infection of wheat[27] to viral infection of tobacco[29]. While some insight regarding the molecular mechanisms involved in loss of ETI-mediated defense has been gained[127], the underlying cause for a potentially general reduction of basal defense was unknown. Wang *et al.* (2009) found that 10- to 14-d-old SA-deficient mutant seedlings grown continuously at elevated temperature (28°C) retained temperature sensitivity in terms of enhanced bacterial growth at elevated temperature[35]. Because extended growth at elevated temperature results in dramatically altered plant morphology (and likely altered plant physiology) compared to 48 h treatment (Fig. 3b, c), future research is needed to determine the causes for the apparently SA-independent effect of long-term treatment of elevated temperature on disease susceptibility of seedlings, as observed by Wang and colleagues[20].

I provide evidence showing that enhanced basal susceptibility of Arabidopsis to *Pst* DC3000 is correlated with a loss of SA biosynthesis and downstream defense gene expression following a brief (48 h) acclimation to elevated temperature (Fig. 4a - d). Furthermore, I show that the SA-deficient mutant, *ics1*, has greatly diminished temperature sensitivity relative to WT plants and exhibits similar susceptibility as WT plants at 30°C (Fig. 4e). Additionally, provision of the SA analogue, BTH, confers similar levels of protection against disease at both temperatures (Fig. 18a). Based on these data, I conclude that loss of SA is the primary temperature-sensitive component in the host responsible for enhanced disease.

I considered three potential models to determine how elevated temperature affects the SA-mediated host-pathogen interaction to enable enhanced disease. Based on previous *in vitro* analyses showing a negative effect of elevated temperature on the expression of virulence-associated genes[51-54, 56] and my data showing loss of pathogen-induced SA biosynthesis (Fig. 4b), Model 1 predicts that elevated temperature negatively affects both pathogen virulence and SA-mediated defense. In this model, enhanced disease would result from increased host susceptibility in spite of compromised bacterial pathogenicity. However, the inability of the non-pathogenic Δ*hrcC* mutant strain to grow more in plants at elevated temperature indicates that a functional T3SS is required for enhanced bacterial growth at 30°C (Fig. 5a). Also, contrary to the *in vitro* studies, my results show that effector translocation *in planta* is more efficient at 30°C (Fig. 5c), effectively refuting Model 1.

Alternatively, Model 2 proposes that elevated temperature enhances bacterial virulence, which then causes loss of SA biosynthesis to promote disease. *Pst* DC3000 production of COR, which is known to specifically target and antagonize SA biosynthesis[144], was previously shown to be unaffected by elevated temperature *in planta*[55]. Although symptom development was greatly reduced in plants infected with the COR-deficient mutant strain, *Pst* DC3118, growth of this strain was enhanced similarly to that of *Pst* DC3000 at 30°C (Fig. 5a), thereby eliminating COR-mediated suppression of SA biosynthesis as the potential mechanism. As T3E translocation is increased at 30°C, it is possible that an effector-mediated process may contribute to loss of SA biosynthesis at elevated temperature. However, the loss of BTH-induced SA production at 30°C (Fig. 7b), which is a pathogen-free treatment, prompts rejection of Model 2.

Therefore, I propose a third model whereby elevated temperature directly antagonizes SA accumulation in the host as well as promotes virulence in the pathogen resulting in enhanced disease (Fig. 20). Increased T3E translocation into the *ics1* mutant relative to WT plants at 23°C (Fig 19e) combined with the discovery of a novel function of BTH in restricting T3E translocation (Fig. 19c) suggests that loss of SA may result in increased permeability of the cell to T3SS-mediated pathogenesis. This enhanced permeability coupled with temperature-mediated promotion of T3E translocation would result in the observed temperature-sensitivity of the *ics1* mutant to effector delivery (Fig. 19e).

One outstanding question to be addressed is what is the mechanism responsible for loss of SA at elevated temperature? Based on *in vitro* activity assays, which show the ICS1 enzyme maintains >90% maximal activity from 4 to 37°C[228], and my data showing complete loss of *ICS1* gene induction at 30°C, it seems likely that loss of SA biosynthesis and accumulation occurs upstream of *ICS1* gene expression. However, ICS1 protein abundance and/or modifications occurring *in planta* at elevated temperature may affect ICS1 activity in addition to loss of *ICS1* gene expression. In this study, I focused on testing various known negative regulators of ICS1-mediated SA biosynthesis, including components of JA-[144], ET-[102] and ABA-[154, 157]mediated signalling, to determine if one or more of these hormones could be responsible for the loss of SA production and subsequent enhanced susceptibility at 30°C. However, the *aos* JA-deficient mutant, the *myc2/3/4* JA-signaling mutant, the *ein2* and *ein3 eil1* ET-signaling mutants and the *aba2* ABA biosynthesis mutant showed similar levels of susceptibility as the WT plants at 30°C (Fig. 17a - e), indicating that none of these pathways is solely responsible for SA suppression at elevated temperature. While the *camta2/3* mutant exhibited elevated resistance compared to

WT plants at 30°C (Fig. 17f), *ICS1* gene induction remained compromised (Fig. 17g), indicating that CAMTA2 and CAMTA3 are not responsible for suppression of SA biosynthesis at elevated temperature. It is possible that loss of SA biosynthesis is due to loss of a positive regulator rather than enhanced suppression by a negative regulator. The high number of positive regulators that are compromised in induction at 30°C lends support to this theory. EDS5 is a multidrug and toxin extrusion transporter that functions in export of SA out of the chloroplast[229]. Although a mechanism has yet to be demonstrated, loss of stress-induced SA in the *eds5* mutant has been proposed to occur due to autoinhibitory feedback loop[229]. As BTH-induction of *EDS5* is compromised at elevated temperature, it is possible that loss of SA transport from the chloroplast to the cytosol is blocked at elevated temperature resulting in loss of SA biosynthesis.

Retention of resistance in the *camta2/3* mutants at 30°C in the absence of *PR1*/*ICS1* gene induction could implicate involvement of an SA-independent process. However, I observed the same uncoupling between resistance and BTH-induction of *PR1*/*ICS1* gene expression at 30°C (Fig. 18a, c). The observed retention of BTH-induced NPR1 nuclear accumulation (Fig. 9a, 10a), as well as the requirement for NPR1 and the TGA2, TGA5, TGA6 TFs (Fig. 19a, b) indicates that the core SA signaling pathway is utilized to facilitate this resistance at 30°C. Global transcriptome analysis of BTH-regulated genes revealed a temperature-sensitive bifurcation in the SA signaling pathway, with 66% of genes in the canonical *PR1*/*ICS1* temperature-sensitive branch, and 34% of genes in the temperature-insensitive branch (Fig. 15a, 20). Future research should investigate whether plants treated with both BTH and *Pst* DC3000 exhibit a similar temperature-sensitive bifurcation in the SA signaling network at elevated temperature.

However, based on the lack of induction of *PR1* and *ICS1* by either BTH or *Pst* DC3000 alone (Fig. 4c, Fig. 7a) or in combination (Fig. 18c), it is possible that a major part of the SA-regulated transcriptome is dispensable for BTH-mediated protection against *Pst* DC3000 (Fig 20). This surprising result indicates that either the temperature-insensitive branch of the SA transcriptome and/or a gene expression-independent process underlies BTH-mediated resistance against *Pst* DC3000 at 30°C. It is also possible that the set of genes uniquely induced by BTH at elevated temperature (Group C) may compensate for the loss of the *PR1*/*ICS1* temperature-sensitive branch to enable protection (Fig. 15a, 20).

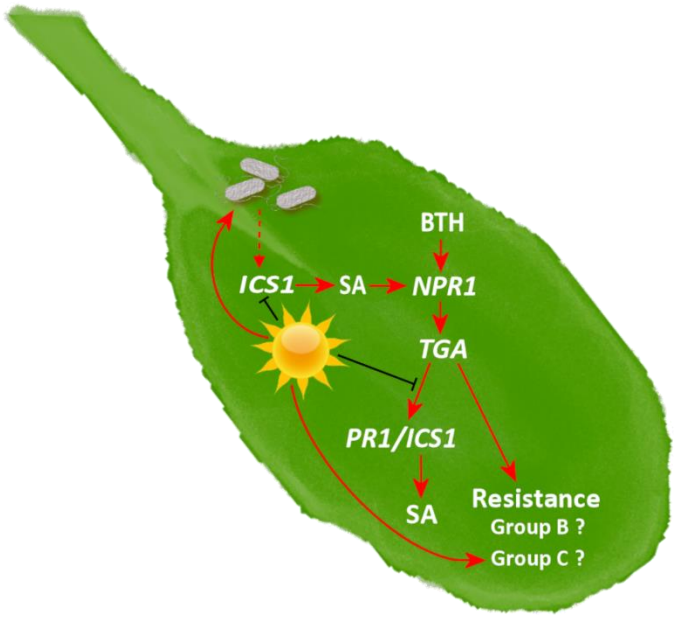

Figure 20. Model for Arabidopsis – *Pst* DC3000 interaction at elevated temperature. At elevated temperature (depicted by sun), pathogen-induction of SA biosynthesis via the ICS1 enzyme is blocked and translocation of bacterial T3E proteins is enhanced to promote disease. SA signaling induced by the synthetic analogue, BTH, is also affected, with the *PR1*/*ICS1* branch (group A in Fig. 15a) no longer induced. However, BTH-mediated resistance against *Pst* DC3000 is still conferred at elevated temperature in a NPR1- and TGA2/5/6-dependent manner. It is possible that genes in the temperature-insensitive branch (Group B) and/or genes in the elevated temperature + BTH-induced branch (Group C) are involved in BTH-mediated resistance under this condition. → represent direct positive interactions, ⇢ represents induction of *ICS1* in response to pathogen detection and ⊣ represent inhibitory interactions.

In summary, I have studied the impact of one of the most important climate conditions, elevated temperature, on a model compatible plant-pathogen interaction. My results highlight pathogen-induced SA production as a key temperature-vulnerable step in the SA defense network. In addition, my study revealed a surprisingly positive effect of elevated temperature on T3E translocation *in planta*, which challenges the long-standing notion based on previous *in vitro* studies that an efficient T3SS requires a low temperature (e.g., 18°C to 20°C)[51]. Although not required for enhanced bacterial growth, COR-associated virulence is likely also increased at elevated temperature based on the dramatic increase in disease-associated chlorosis. Surprisingly, in spite of the increase in pathogen virulence and loss of the *PR1*/*ICS1* branch of SA-dependent signaling at elevated temperature, I still observed BTH-mediated protection against *Pst* DC3000 infection. Although much of the SA-signaling network has been described[13], little is known regarding the actual mechanism of protection against disease. My research also uncovered a novel function of BTH in restriction of bacterial T3E protein translocation into host cells, presumably as an important part of its protection mechanism. Because the SA pathway is an integral component of the plant immune system, I hope that the fundamental insights gained from this study will stimulate future research to uncover additional temperature-sensitive and -insensitive nodes of the plant immune system. This information should prove useful for genetic manipulation of climate-relevant components of the plant immune system to enhance plant resilience to combined adverse abiotic and biotic conditions.

Chapter 3

Future perspectives

There are many interesting questions to pursue regarding how environmental conditions affect plant-pathogen interactions. Of special interest would be repeating experiments with fluctuating day/night temperature cycles that more closely resemble natural conditions to determine whether lower temperatures during the night would enable SA biosynthesis in response to a pathogen in spite of elevated day time temperatures. As SA biosynthesis is light-dependent[230], it is possible that day time temperatures are more relevant than night time temperatures, but it would be necessary to test this directly. Another line of inquiry would be to determine whether loss of the *PR1*/*ICS1* branch of SA signaling at elevated temperature affects the broad-spectrum nature of BTH-mediated protection. This could be tested using different pathogens in Arabidopsis and/or more crop-relevant species. Some plants, such as rice, have elevated basal levels of SA and so have differences in regulation and signaling[231]. As a model monocot that is also an important crop, it would be interesting to repeat some of these experiments in rice to see what similarities and/or differences exist relative to Arabidopsis relating to the effect of elevated temperature on basal SA levels and induction of SA-signaling.

While there are many more such questions one might wish to pursue, in this final section, I will focus on potential strategies for addressing two main questions that are a more direct follow-up to the research I conducted for my dissertation: 1) What is the factor upstream of SA biosynthesis responsible for loss of *ICS1* induction and SA accumulation at elevated temperature? and 2) How does BTH restrict T3E translocation to protect against *Pst* DC3000 infection?

Strategies for identifying the component upstream of *ICS1* responsible for loss of SA biosynthesis at elevated temperature

It is currently unknown whether loss of *ICS1* gene induction at elevated temperature is due to loss of a positive regulator or increased suppression by a negative regulator. During my dissertation research, I focused primarily on negative regulators of SA biosynthesis and/or signaling, and so assessed Jasmonic acid (JA)-biosythetic and signaling mutants, two ethylene (ET)-signaling mutants, an abscisic acid (ABA)-deficient mutant and the *camta2/3* (CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR) mutant. Although not included in the preceding chapter, a mutant deficient in *PIF1*, *PIF3*, *PIF4* and *PIF5* (*pifq*), was assessed to determine whether growth-defense tradeoffs are involved in loss of SA biosynthesis at elevated temperature. Based on similar levels of susceptibility of the *pifq* mutant and wild type plants to *Pst* DC3000 at 30°C, and lack of recovery of BTH-induction of *ICS1* gene expression (see Appendix A, Fig. 22), I concluded that PIF-mediated growth responses are not responsible for enhanced susceptibility or loss of SA biosynthesis at elevated temperature.

It remains possible that the actual cause for loss of SA biosynthesis at elevated temperature is loss of a positive regulator. To test this, over-expression lines of known positive regulators, such as EDS1, PAD4, CBP60g, SARD1, etc., may be acquired or constructed to determine if *ICS1* is expressed at 30°C. Another candidate that would be interesting to test is phytochrome B (phyB). Two recent Science papers have finally revealed phyB as a temperature sensor in addition to its function as a red-light photoceptor[62, 63]. SA-induction of *PR1* is negatively affected in the *phyB* mutant, and is positively correlated with increasing light fluence. Although SA accumulation was also observed to be positively regulated by light, it was not affected in the *phyB* mutant, so it remains unclear whether phyB itself or another light-regulated factor may be

involved in regulation of SA biosynthesis. It would be interesting, however, to test both the *phyB* mutant and phyB temperature stable transgenic plants to determine whether this newly identified temperature sensor contributes in any way to loss of SA biosynthesis at 30°C.

In addition to the candidates mentioned above, several studies have been published recently identifying new regulatory components upstream of SA, including TFs that bind to its promoter[232-234]. As testing potential candidates one by one can be costly and time-consuming, an alternative approach would be to use a method like chromatin immunoprecipitation (ChIP) followed either by sequencing or PCR of known *cis* regulatory elements bound by TFs such as CBP60g or WRKY28[235]. This may reveal how temperature affects which positive and negative regulators bind the *ICS1* gene promoter to regulate its expression. Of course, it is possible that the temperature-sensitive component is not a TF and so would not be identified with this method, or there could be multiple temperature-sensitive components involved. The latter case is probable based on the additional affects I observed on BTH-mediated signaling at elevated temperature. A more comprehensive approach would be to obtain or generate a reporter line, such as luciferase driven by the *ICS1* gene promoter. This line could then be mutagenized and the resulting population screened for recovery of *ICS1* induction at 30°C.

One thing to keep in mind is that induction with BTH is not the same as induction with a pathogen. Although SA biosynthesis and signaling is activated by the plant as a defense mechanism, the pathogen will also induce genes, such as those involved in JA-, auxin- and/or ABA- signaling, to antagonize SA and promote susceptibility. It is possible, therefore, that a specific regulator may be compromised in response to pathogen but not BTH treatment, and vice versa. To help with this issue, it would be interesting and informative to compare the

global transcriptional profile following pathogen treatment with that observed following BTH-treatment at 23°C and 30°C. As indicated by the heat map generated using RNA-sequencing data from mock- or BTH-treated plants at 23°C and 30°C (Fig. 11), there is a gradient effect of temperature on BTH-induced genes. For instance, of the upstream regulators of SA biosynthesis that were in Group A (compromised in expression levels following BTH treatment at 30°C relative to 23°C), many were still induced by BTH, but to a much less extent.

Determining the mechanism for BTH-mediated restriction of T3E translocation

The identification of T3E restriction by BTH is exciting, but more information is needed as to how this restriction occurs. Although not shown in the chapter above, I did confirm that restriction of T3E translocation occurs at 30°C and that it is NPR1-dependent (See Appendix A, Fig. 24). Unfortunately, it is difficult to conduct this experiment in such a way that the bacterial populations are equal within the BTH-treated plants at both temperatures, so these analyses require additional experimentation. Some of the outstanding questions are: Is the process transcription-dependent or -independent? Does it involve cell wall reinforcements to provide an extra physical barrier against penetration of the T3SS or does BTH act by suppressing the expression of virulence genes within the pathogen?

In regards to transcriptional dependence, I identified a large number of genes, which comprise the *PR1*/*ICS1* temperature-sensitive branch, that are not required for BTH-mediated protection against *Pst* DC3000. While I did observe that NPR1 is required for BTH-mediated reduction in T3E (Fig. 24), it is possible that the role of NPR1 in this process does not involve transcriptional changes. One way to determine this would be to use the NPR1-GR (glucocorticoid receptor) fusion transgenic line, which requires dexamethasone treatment for

nuclear accumulation of NPR1[117]. This line was used previously to show that NPR1 plays a role in antagonizing JA-signaling that does not require NPR1 nuclear localization[236], so it remains possible that there are other cytosolic functions of the NPR1 protein.

The primary type of pathogen-induced cell wall fortification is callose deposition[237]; however, BTH-induction of callose deposition is blocked at 30°C (Fig. 7c, d). BTH-mediated reduction in T3E translocation was slightly compromised at 30°C relative to 23°C, but I still observed a significant decrease relative to mock-treated plants (Fig 24). It is possible that this negative effect on the efficiency of BTH at elevated temperature is due to loss of callose deposition; however, it is unlikely that callose deposition alone is responsible for the observed effect of BTH on T3E translocation. Lignin is also used to reinforce plant cell walls during defense[238]. The *CAROTENOID AND CHLOROPLAST REGULATION 2* (*CCR2*) gene in Arabidopsis was shown to be specifically induced in response to pathogen detection. Interestingly, this gene is in Group C of my RNA-seq dataset, showing higher expression levels in response to BTH at 30°C relative to 23°C. Histochemical staining can be used to determine if BTH-induction of lignin occurs[239], and, if so, how it is affected at elevated temperature. Lignin-compromised mutants could then be used to determine the role it may play in BTH-mediated restriction of T3E effectors.

Finally, it is possible that BTH acts by suppressing the expression of T3E-associated genes within the pathogen. This is something I have wanted to test, but which has proven technically difficult. It is possible to isolate bacterial RNA from plant tissue, but the concentration is low, and, until very recently, there have been no reliable bacterial “house-keeping” genes available to use for normalization of genes of interest for the purposes of qPCR. Over the last year, a

former member of the He lab has worked to identify and validate several such house-keeping genes, so this may be a feasible approach in the near future. In addition to measuring bacterial gene expression within the host, it would also be useful to look at protein abundance of bacterial T3E proteins for which antibodies are available, like AvrPto. Using these approaches in combination with the cAMP method for quantifying effector translocation may enable us to distinguish whether BTH acts strictly in preventing T3E entry into host cells and/or affects the quantity of T3E that are produced, thereby reducing overall translocation.

In conclusion, having a basic understanding of the molecular mechanisms governing plant-pathogen interactions, especially in the context of adverse climate conditions, is crucial for crop yield optimization. It is my hope that the work I have done during my graduate studies may help provide a foundation on which others, including those within the new Plant Resilience Center here at Michigan State University, can build towards achieving this goal.

APPENDICES

APPENDIX A

Additional data

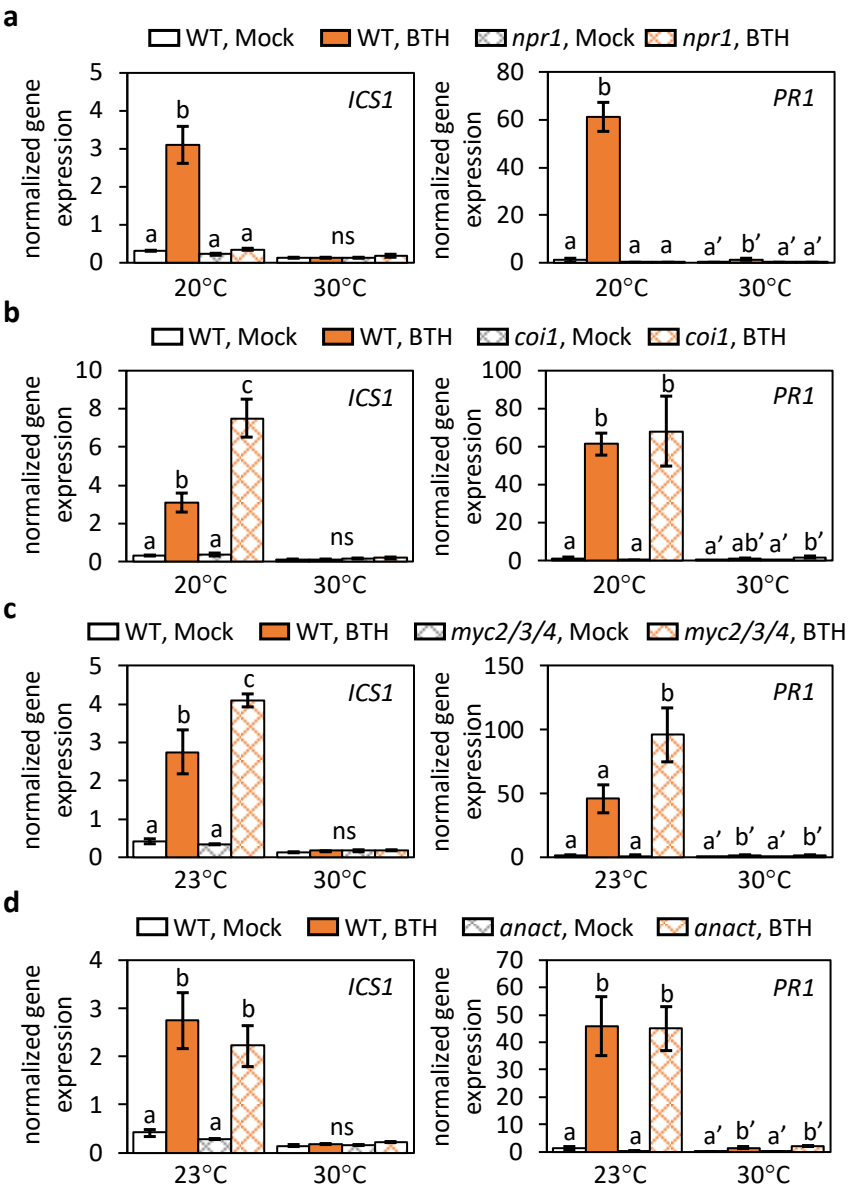

Figure 21. Effect of temperature on SA marker gene induction in WT, *npr1*, *coi1*, *myct2/3/4*, and *anac19/55/72* mutant plants. SA marker gene expression in WT vs. *npr1* (**a**) *coi1* (**b**) *myc2/3/4* (**c**) and *anac19 anac55 anac72* (*anact*) mutant plants. Plants were grown at 20°C (**a**, **b**) or 23°C (**c**, **d**) and temperature acclimated in test chambers at either the growth temperature (20°C/23°C) or 30°C 24 h before spraying with mock or BTH. Tissue for RNA extraction was collected 24 h after chemical treatment. qPCR was used for gene expression analysis, with expression of *ICS1* and *PR1* normalized to the expression of *PP2AA3*. Data are representative of one (**c**, **d**) or two (**a**, **b**) independent experiments, and are presented as the mean (n = 4) ± SEM. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis ($P<0.05$); samples sharing letters are not significantly different. Data were analyzed in two groups based on temperature as indicated by the prime symbol (').

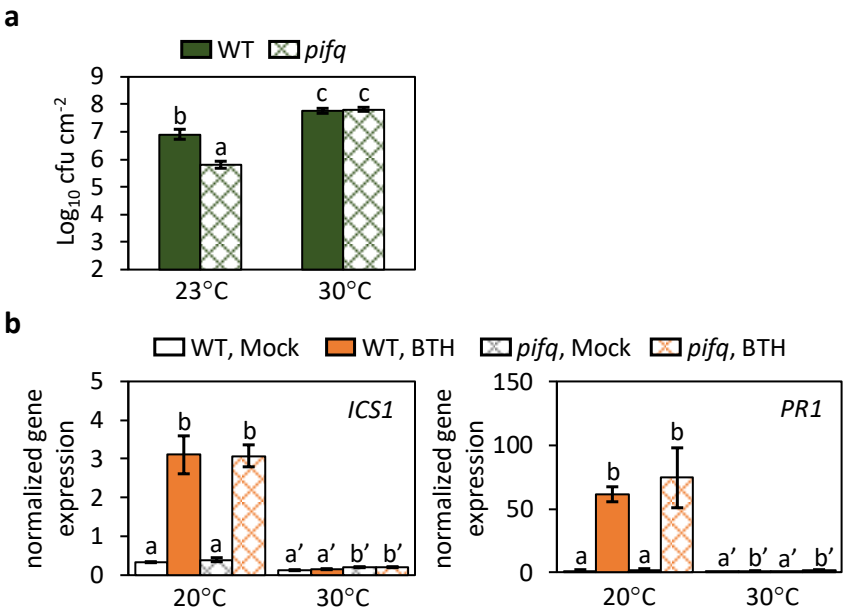

Figure 22. PHYTOCHROME INTERACTING FACTORs (PIFs) are not involved in enhanced susceptibility or loss of SA marker gene induction at elevated temperature. (**a**) Bacterial growth in temperature acclimated WT vs. *pifq* mutant plants three days after vacuum infiltration with *Pst* DC3000. (**b**) SA marker gene expression in WT vs. *pifq* mutant plants. Plants were grown at 20°C and temperature acclimated to test chambers at either 20°C or 30°C for 24 h, then sprayed with mock or BTH. Tissue for RNA extraction was collected 24 h after chemical treatment. qPCR was used for gene expression analysis, with expression of *ICS1* and *PR1* normalized to the expression of *PP2AA3*. Data are representative of two independent experiments, and are presented as the mean (n = 4) ± SEM. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis ($P<0.05$); samples sharing letters are not significantly different. Data in (**b**) were analyzed in two groups based on temperature as indicated by the prime symbol (').

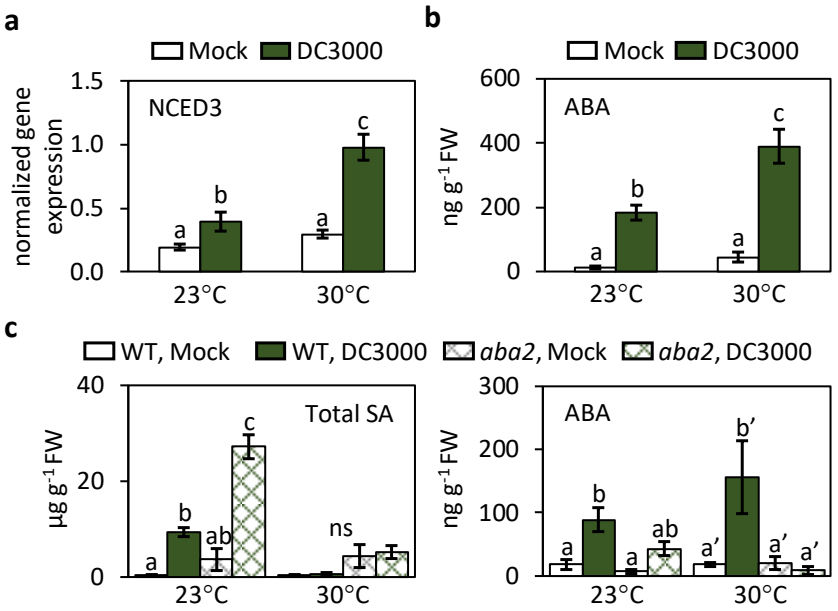

Figure 23. DC3000-induction of ABA involves *ABA2* but does not account for loss of SA. ABA marker gene expression (**a**) and SA and ABA metabolite quantification (**c - d**) in temperature acclimated WT and *aba2* mutant plants 24 h after vacuum infiltration with mock or *Pst* DC3000. (**a**) qPCR was used for *NCED3* gene expression analysis (n = 3), which was normalized to the expression of *PP2AA3*. (**b, c**) metabolites were quantified using LCMS (n = 4). An SA standard curve was used to determine the SA and SAG metabolite concentrations (nM) within each sample, which were then combined to get Total SA. An ABA standard curve was used to determine ABA concentrations. All sample concentrations were then converted to ng and normalized by sample fresh weight (FW) mass (g). Data are representative of two (**a**), three (**b**) and one (**c**) independent experiments, respectively. Data are presented as the mean ± standard error of the mean (SEM) with n = number of biological replicates. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis (P<0.05); samples sharing letters are not significantly different. Data in (**c**) were analyzed in two groups based on temperature as indicated by the prime symbol (').

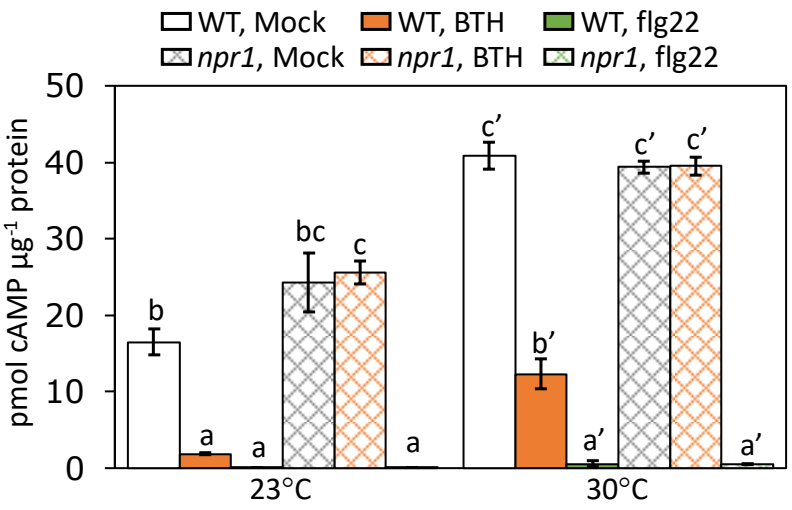

Figure 24. BTH-mediated reduction of translocation of bacterial T3E proteins occurs at 30°C and requires *NPR1*. WT and *npr1* mutant plants were temperature acclimated 24 h prior to syringe infiltration with mock, BTH or flg22 (500 nM). Plants were syringe infiltrated with *Pst* DC3000 P_{nptII}*::avrPto-cyaA* (6.7×10^7 cfu ml^{-1}) 24 h after chemical treatment. Tissue for cAMP quantification was collected 6 hpi. cAMP levels were normalized by total protein, and higher levels of cAMP indicate more translocation of bacterial effectors. Data are representative of two independent experiments, and are presented as the mean (n = 3) ± SEM. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis (P<0.05); samples sharing letters are not significantly different. Data were analyzed in two groups based on temperature as indicated by the prime symbol (').

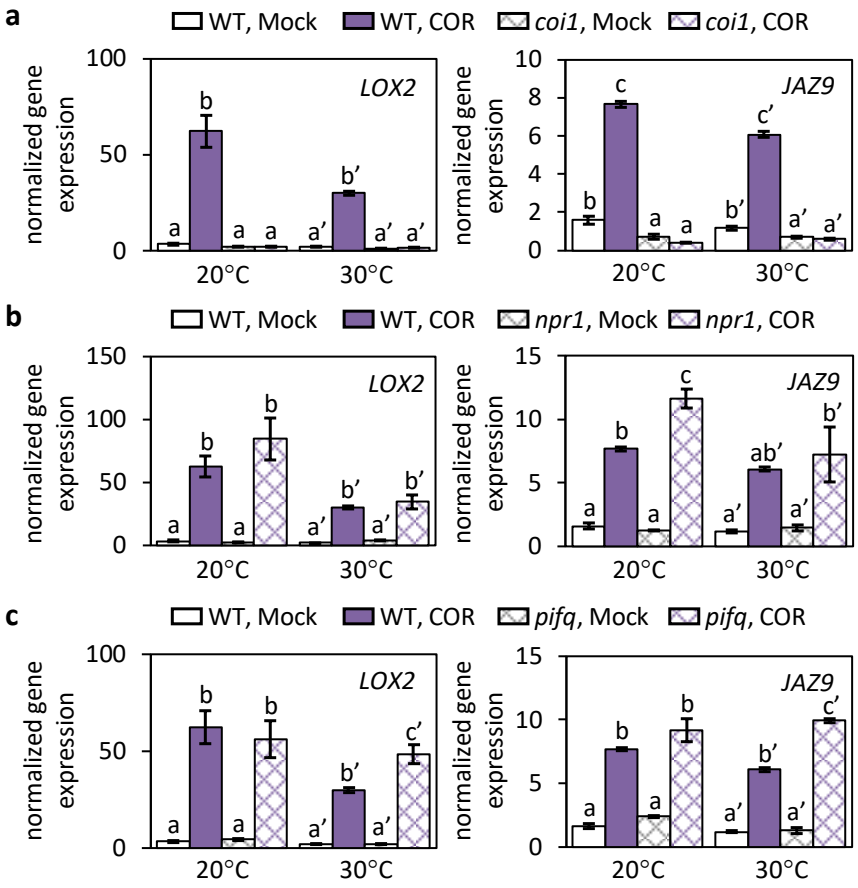

Figure 25. Effect of elevated temperature on JA marker gene induction in WT, *coi1*, *npr1* and *pifq* mutant plants. JA marker gene expression in WT vs. *coi1* (**a**) *npr1* (**b**) and *pifq* (**c**) mutant plants. Plants were grown at 20°C and temperature acclimated to test chambers at either 20°C or 30°C for 24 h, then sprayed with mock or COR. Tissue for RNA extraction was collected 24 h after chemical treatment. qPCR was used for gene expression analysis, with expression of *LOX2* and *JAZ9* normalized to the expression of *PP2AA3*. Data are representative of two independent experiments, and are presented as the mean (n = 3) ± SEM. Letters indicate statistical significance based on a two-factor ANOVA with Tukey HSD post hoc analysis ($P<0.05$); samples sharing letters are not significantly different. Data in (**b**) were analyzed in two groups based on temperature as indicated by the prime symbol (').

APPENDIX B

Growth-defense tradeoffs in plants: a balancing act to optimize fitness

This was published as an invited review article:

Huot B**,** Yao J, Montgomery BL and He SY (2014) Growth-defense tradeoffs in plants: a balancing act to optimize fitness. *Molecular Plant* **7(8)**: 1267-1287. ***Editor's Choice***

ABSTRACT

Growth-defense tradeoffs are thought to occur in plants due to resource restrictions, which demand prioritization towards either growth or defense depending on external and internal factors. These tradeoffs have profound implications in agriculture and natural ecosystems, as both processes are vital for plant survival, reproduction and, ultimately, plant fitness. While many of the molecular mechanisms underlying growth and defense tradeoffs remain to be elucidated, hormone crosstalk has emerged as a major player in regulating tradeoffs needed to achieve a balance. In this review, we cover recent advances in understanding growth-defense tradeoffs in plants as well as what is known regarding the underlying molecular mechanisms. Specifically, we address evidence supporting the growth-defense tradeoff concept, as well as known interactions between defense signaling and growth signaling. Understanding the molecular basis of these tradeoffs in plants should provide a foundation for the development of breeding strategies that optimize the growth-defense balance to maximize crop yield to meet rising global food and biofuel demands.

Keywords: plant immunity; plant hormone; salicylic acid; jasmonate; PAMP; plant growth

INTRODUCTION

While the deployment of defense mechanisms is imperative for plant survival, defense activation generally comes at the expense of plant growth (Fig. 20). The "growth-defense tradeoff" phenomenon was first observed in forestry studies of plant-insect interactions, and is based on the assumption that plants possess a limited pool of resources that can be invested either in growth or in defense[240-242]. As plants must both grow and defend in order to survive and reproduce, growth-defense tradeoffs have important ecological, agricultural and economic consequences. In nature plants live in diverse and complex environments in which they constantly encounter a variety of pathogens and insect herbivores with a wide array of life styles and infection strategies. In adaptation to such natural conditions, plants have evolved sophisticated mechanisms to balance growth and defense[242-244]. However, in agricultural settings, crops have been bred for centuries to maximize growth-related traits resulting in a loss of genetic diversity that often compromises defense[1]. Understanding the molecular mechanisms used by plants to balance growth and defense can enrich plant breeding and engineering strategies for selection of elite genetic traits that will maximize plant fitness.

In this review, we discuss the evidence supporting the concept of growth-defense tradeoffs in plants as well as the recent advances in deciphering the molecular mechanisms underlying their occurrence. As numerous studies have implicated hormone crosstalk as having a fundamental role in fine-tuning the growth-defense process, we provide brief descriptions of each defense and growth signaling pathway to introduce key players, and then discuss relevant hormone-crosstalk. Due to space constraints, we focus our discussion on tradeoffs between defenses mediated by pathogen-associated-molecular-pattern (PAMP)-triggered immunity (PTI), salicylic acid (SA) and jasmonate (JA) vs. growth mediated by auxin, brassinosteroids (BR) and gibberellins (GA), for which most progress has been made (Fig. 20). Readers are referred to several recent reviews related to this topic, including discussions of the roles of ethylene (ET) and cytokinins[120, 123, 231, 245-248]. We conclude with a summary of concepts that may be drawn from current knowledge as well as several key areas where further research is needed.

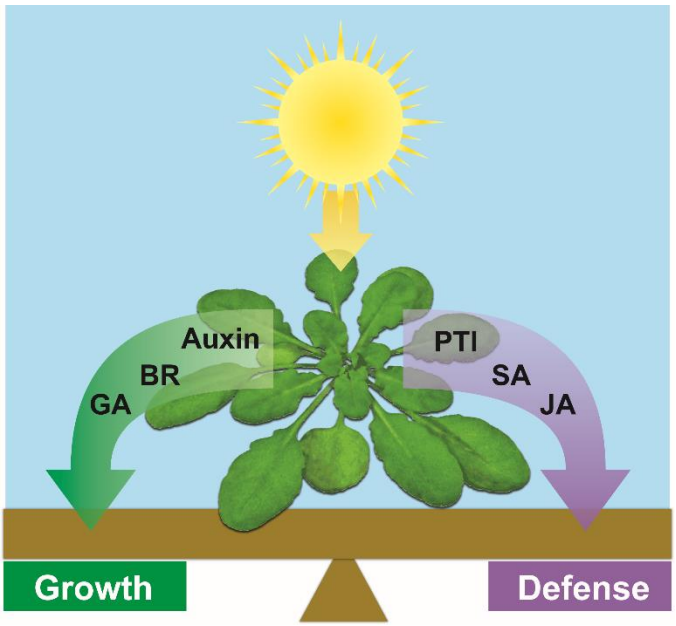

Figure 26. Plant growth-defense trade-offs. Plants use photosynthesis to convert light energy into chemical energy in the form of carbohydrates. These resources are then allocated towards growth or defense depending on the presence/absence of specific stresses. This process is facilitated by hormone crosstalk and is referred to as the growth-defense tradeoff. BR, brassinosteroid; GA, gibberellin; PTI, pathogen-associated-molecular-pattern-triggered immunity; SA, salicylic acid; JA, jasmonates.

DEFENSE SIGNALING

The ability to perceive and mount a rapid response to pathogen attack is critical for plant survival. Plants have evolved a sophisticated immune system that is initiated upon detection of highly conserved PAMPs by membrane-associated pattern recognition receptors (PRRs), which leads to activation of PTI[249, 250]. While PTI is believed to provide sufficient defense against non-pathogenic microbes, pathogens have developed the ability to secrete virulence effectors into the plant cell to suppress PTI and promote disease[10, 48, 251]. Plants have evolved resistance (*R*) genes to recognize these effectors and activate a much stronger immune response, effector-triggered immunity (ETI), which often results in a type of programmed cell death response known as the hypersensitive response (HR) in pathogen-infected tissue[5, 7, 40, 252]. ETI may also trigger secondary immune responses in distal, uninfected tissues and lead to so-called systemic acquired resistance (SAR)[13, 253].

Plant hormones are small organic molecules that are required by plants in low concentrations and regulate growth, development, reproduction and immune responses. Changes in environmental signals—both abiotic and biotic—induce changes in the quantity and composition of these signal molecules to facilitate appropriate plant responses[120, 123, 254, 255]. Plant defense hormones such as SA, JA and ET play important roles in the precise regulation of plant immune responses both locally and systemically to co-ordinate plant defense against different types of pathogens and in different parts of the plant[256-258]. SA signaling is primarily induced by and used to effectively defend against biotrophic pathogens, whereas JA signaling is primarily induced by and used to effectively defend against insect herbivores and, in conjunction with Et, against necrotrophic pathogens[168, 259]. SA and JA signaling pathways are generally antagonistic to each other[256]. For example, elevated SA signaling in response to biotrophic pathogens is often correlated with reduced JA signaling and decreased resistance to necrotrophic pathogens[260]. The following sections provide brief summaries of PTI, SA and JA signaling pathways relevant to this review.

PAMP-Triggered Immunity

As mentioned above, PTI is triggered following detection of PAMPs by PRRs[249]. The best characterized PRRs are leucine-rich repeat receptor kinases (LRR-RKs) consisting of an extracellular LRR domain, which can vary in the number of repeats and is directly involved in ligand perception, a transmembrane domain and an intracellular kinase domain[261]. FLAGELLIN SENSING 2 (FLS2) and ELONGATION FACTOR-TU RECEPTOR (EFR) are LRR-RKs that recognize bacterial flagellin and bacterial EF-Tu, respectively[262-265]. Upon ligand perception, both FLS2 and EFR rapidly recruit a LRR-RK, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), resulting in their transphosphorylation[266-269]. Treatment with flg22, a bioactive 22-amino acid peptide derived from bacterial flagellin, activates the FLS2/BAK1 co-receptor complex and triggers a phosphorylation cascade, including the phosphorylation and displacement of BOTRYTIS-INDUCED KINASE 1 (BIK1) from the FLS2 complex to promote the immune response[270, 271]. Initial PTI responses occur within minutes to hours following PAMP perception, and include elevation of reactive oxygen species (ROS), calcium influx, activation of calcium/calmodulin-dependent kinases and of mitogen-activated protein kinase signaling cascades and

transcriptional reprogramming[6, 250]. PTI-associated transcriptional reprogramming is facilitated in part by the WRKY family of transcription factors, members of which are involved in both positive and negative regulation of PTI[119, 135, 272]. Later responses attributed to PTI activation include deposition of callose at the cell wall near the site of pathogen infection and seedling growth inhibition[250, 273].

Salicylic Acid

SA is a phenolic hormone shown to affect many plant processes including growth, development, senescence and stress responses[12, 274]. It is primarily recognized for its role in local defense induced against biotrophic and hemi-biotrophic pathogens and in the establishment of SAR[13]. After years of searching, two recent studies have proposed NONEXPRESSOR OF PATHOGENESIS RELATED PROTEINS 1 (NPR1) and its paralogs, NPR3 and NPR4, to act as SA receptors[111-113]. Multiple genetic screens led to the identification of NPR1, which is a key regulator of SA signaling[103-106]. Under non-induced conditions, NPR1 proteins oligomerize in the cytoplasm[107]. SA accumulation in response to pathogen detection triggers the release of NPR1 monomers, which then translocate to the nucleus and activate defense gene expression[107-109]. NPR1 regulates gene expression through physical interaction with TGA transcription factors, which bind to promoters of *PATHOGENESIS RELATED* (*PR*) genes to activate expression in the presence of SA and repress expression in the absence of SA[13, 115]. *PR* genes encode small proteins, some of which have been shown to possess antimicrobial or antifungal properties *in vitro*[116]. Of the many *PR* genes identified, *PR1*, *PR2* and *PR5* have been shown to be induced by SA and have long been used as markers of SA signaling[13]. Other genes identified as direct targets of NPR1 include WRKY transcription factors and components required for the synthesis and secretion of PR proteins[117]. WRKYs are involved in both NPR1-dependent and NPR1-independent SA signaling and, as in the case of PTI, include both positive and negative regulators of SA-mediated defense[13, 117-119].

Jasmonate

JAs are a group of lipid-derived hormones that regulate plant defense against necrotrophic pathogens and insect herbivores[256] and also affect several other physiological processes including abiotic stress responses, reproductive development, and primary and secondary metabolism[275, 276]. JA-isoleucine (Ile) is perceived by a co-receptor complex formed with the F-box protein CORONATINE INESENSTIVE 1 (COI1) and the JASMONATE ZIM-DOMAIN (JAZ) family of transcription repressors[139]. COI1 is required for almost all known JA-dependent responses[143, 277, 278]. The JAZ-family proteins repress JA signaling by directly binding to the MYC family of transcription factors required for the expression of JA-responsive genes[140-142]. Under normal growth conditions where JA-Ile levels are low, JAZ proteins recruit co-repressors, TOPLESS (TPL) or TPL-related proteins, either directly through their ETHYLENE RESPONSE FACTOR-ASSOCIATED AMPHIFILIC REPRESSION (EAR) motifs, or indirectly through NOVEL INTERACTOR of JAZ (NINJA) protein to suppress MYC activities[279, 280]. It was recently shown that physical association of JAZ proteins with MYC2 is required for the nuclear localization of JAZ repressors[281]; however, the mechanism for JAZ repression of MYC activity is not clearly understood. Upon wounding or

pathogen attack, JA-Ile is rapidly synthesized in both local and distal tissues[282, 283]. An increasing concentration of JA-Ile promotes physical interaction between COI1 and JAZ proteins, which leads to ubiquitination and subsequent degradation of JAZs through the 26S proteasome, thereby relieving the repression on MYC transcription factors and initiating the expression of JA-responsive genes[140, 141, 143].

GROWTH PROMOTING HORMONE SIGNALING

Plant growth and development is coordinately regulated by a complement of hormones in order to optimize growth and reproduction[284]. Growth hormones implicated in growth-defense tradeoffs are auxin, BRs, GAs and cytokinins. As excellent reviews have been written on each of these hormones[74, 255, 285], we will briefly describe what is known regarding the main signaling components for the three growth hormones relevant to this review.

Auxin

Auxins regulate many fundamental aspects of plant growth and development including stem and petiole elongation and root architecture in response to light, temperature and gravity[255, 286-288]. Biosynthesis of indole-3-acetic acid (IAA), one of the primary auxins studied, occurs primarily in young leaves via multiple biosynthetic pathways and IAA is transported throughout the plant[289, 290]. Once synthesized, accumulation of free IAA is regulated by GH3 proteins, which conjugate IAA with amino acids to yield metabolites for storage (IAA-alanine and IAA-leucine) or oxidation and degradation (IAA-aspartate and IAA-glutamic acid)[291-293]. When auxin levels are low, auxin response genes are actively repressed by heterodimerization of the AUX/IAA family of transcriptional repressor proteins with the AUXIN RESPONSIVE FACTORS (ARF) family of transcription factors[294-297]. The F-box proteins, TRANSPORT INHIBITOR RESISTANT 1 (TIR1) and AUXIN SIGNALING F-BOX (AFB), are substrate-recognition components of an SKP-Cullin-F box (SCF) E3 ubiquitin ligase complex, $SCF^{TIR1/AFB}$ [298, 299]. When auxin concentration reaches a threshold in the cell, auxin directly facilitates $SCF^{TIR1/AFB}$ binding to AUX/IAA proteins, resulting in the ubiquitination and degradation of AUX/IAA repressors via the 26S proteasome thereby de-repressing ARF-dependent transcription of auxin-regulated genes[300-302]. Auxin-regulated genes include the *AUX/IAA* and *GH3* gene families[303, 304], expression of which forms part of a feedback mechanism to re-set auxin signaling homeostasis.

Brassinosteroids

BRs are polyhydroxylated steroid phytohormones that influence diverse developmental processes from seed germination to plant senescence[305-307]. Plants that are insensitive to or deficient in BR signaling have severely stunted growth and are male infertile, whereas exogenous application of BR has a positive impact on the quality and quantity of crop yield[305-308]. In the absence of BR, the glycogen-synthase-kinase-3-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) phosphorylates two nuclear-localized transcription factors, BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1), to block activation of BR-responsive genes[309-314]. The presence of BR stabilizes the BRASSINOSTEROID INSENSITIVE 1

(BRI1)/BAK1 co-receptor complex, causing activation of their respective kinase domains and subsequent transphosphorylation[315-320]. The resulting cascade of phosphorylation events leads to the phosphorylation and inactivation of BIN2 kinase and the dephosphorylation and activation of BES1 and BZR1 to promote the expression of BR-regulated genes[321, 322].

Gibberellins

GAs are tetracyclic diterpene acids that control seed development and germination, vegetative growth, and flower initiation and development[74]. GA induces gene expression by relieving the repression of a family of transcriptional repressors known as DELLA proteins[323, 324]. In the absence of bioactive GAs, DELLAs bind to and inactivate PHYTOCHROME INTERACTING FACTORS (PIFs), a group of bHLH-family transcription factors[74]. The presence of a growth signal stimulates the biosynthesis of GA, which is perceived by GA INSENSTIVE DWARF 1 (GID1) through direct binding. This leads to a conformational change of GID1, facilitating its binding to DELLA proteins[325]. The formation of the GID1-DELLA complex enhances the interaction between DELLA and the F-box protein SLEEPY 1 in the SCF^{SLY1} E3 ubiquitin ligase complex, which results in DELLA ubiquitination and degradation that relieves PIF repression and promotes GA-mediated gene expression and growth[326-329].

IN DEFENSE OF THE GROWTH TRADEOFF

Implementation of defense imposes a substantial demand for resources, which has been suggested to reduce growth. This negative impact on growth could result from diminished photosynthesis, which would decrease the overall pool of energy reserves, and/or from a diversion of resources away from growth and towards defense. As deficiencies in defense capabilities can result in pathogen-induced decimation of a plant population, a balance must be achieved between growth and defense to optimize plant fitness.

Finding Balance to Optimize Fitness

Fitness costs associated with defense have been clearly demonstrated[25, 330-334]. For example, silencing components in JA-mediated defense signaling was shown to alleviate fitness costs observed in wild type plants[334]. In the case of constitutive defense responses, reduced fitness may be due in part to unnecessary diversion of energy reserves away from growth in the absence of stress. Benzothiadiazole (BTH) is a synthetic analog of SA used commercially to enhance disease resistance by inducing SAR in crops[22, 194]. Application of BTH to wheat was observed to negatively impact fitness in the absence of pathogens[335] and to increase fitness in the presence of powdery mildew[22]. Another fitness cost attributed to constitutive defense is the inability of the plant to respond appropriately to environmental conditions that limit energy production. In support of this, enhanced susceptibility to the hemi-biotrophic pathogen, *Pseudomonas syringae (P. syringae)*, and the necrotrophic pathogen, *Botrytis cinerea*, is observed in shade-grown plants attributed to the need to prioritize growth under these light-restrictive conditions[336-338].

While it is easy to understand the costs associated with constitutive defense, fitness is also compromised in the absence of defense. Loss of NPR1-dependent, SA-mediated defense was shown to reduce the fitness of field-grown plants[25], whereas overexpression of *NPR1* was shown to enhance resistance to biotrophic and hemi-biotrophic pathogens without adversely affecting growth or fitness[25, 339]. This is most likely due to the fact that SA signaling is not constitutively active but rather primed for quicker response to pathogen detection in these plants[339]. Together these studies indicate that approaches used to achieve an enhanced primed state can ameliorate the fitness costs associated with constitutive defense, while optimizing the fitness benefits of rapid defense induction upon pathogen detection. They also emphasize the point that increased growth is not equivalent to enhanced fitness. Rather, plant fitness is optimized when growth and defense are appropriately prioritized in response to both environmental and developmental cues[25, 340].

Impacts on Photosynthesis

Pathogen/herbivore activity that results in damage to photosynthetic machinery, loss of photosynthetic tissue and/or disruption of the vasculature affecting water and sugar transport has been shown to negatively impact photosynthesis[341-345]. In addition, pathogen/herbivore attack has been shown to suppress components of photosynthesis at both the level of gene expression and of protein abundance[344, 346-354]. The negative impact of defense on photosynthesis has been best demonstrated in response to JA treatment, which results in a reduction of components essential for light harvesting and carbon fixation[346, 347, 355, 356] as well as a substantial decrease in photosynthetic activities and chlorophyll contents in *Arabidopsis*[357]. Conversely, RuBPCase activase (RCA), which has a critical role in carbon fixation, has been shown to diminish JA-mediated defenses by promoting JA-Ile metabolism[358].

However, down-regulation of photosynthetic genes following defense activation does not always correlate with changes in protein profiles[350], leading to the hypothesis that the stability of most photosynthetic proteins allows for a temporary halt at the transcriptional level without a significant impact on photosynthesis itself. This appears to be supported by some studies using chlorophyll fluorescence to measure photosynthetic rates following infection with biotrophic, hemi-biotrophic or necrotrophic pathogens. A similar spatial pattern has been reported for each pathogen type where inhibition of photosynthesis is confined to infected cells and is offset by elevated photosynthesis in the surrounding cells whereas no impact is observed in distal, uninfected tissues[359-362]. Also, proteomic and biochemical analyses of resistant and susceptible plants have shown that the ability to maintain photosynthesis during infection is a vital element of defense[341, 363]. Together, these studies indicate that the ability to appropriately maintain photosynthesis is crucial for defense. Whether or not the observed effects on photosynthesis are a programmed part of the defense response or merely a by-product remains to be determined.

Resource Diversion

In support of the growth-defense tradeoff theory, diversion of plant resources has been shown to occur at all levels, including machinery involved in transcription, translation and

protein secretion from cells as well as prioritization of carbon and nitrogen towards production of defense compounds. Transcriptomic and proteomic studies have demonstrated transcriptional reprogramming and altered protein profiles upon pathogen/herbivore detection to promote defense at the expense of growth[117, 346-353]. Production and secretion of proteins with specific defensive properties, such as PR proteins, place a significant demand on the protein folding and secretory systems, which have also been shown to be required for defense[364-366]. Allocation of resources involved in protein folding and secretion towards defense has been proposed to be regulated in part by *TL1* BINDING TRANSCRIPTION FACTOR 1 (TBF1)[366]. Many TBF1-regulated genes encode ER resident proteins involved in protein folding and secretion, and loss of *TBF1* was shown to compromise the unfolded protein response as well as to impair PTI and SAR[366]. Furthermore, *tbf1* knockout mutants were shown to exhibit partial suppression of growth inhibition associated with defense activation, and transcriptional profiling of these mutants showed a general promotion of growth-related genes and repression of defense-related genes[366].

Studies using radiolabeled carbon or nitrogen have shown that pathogen/herbivore detection alters the normal metabolic flux to enable the incorporation of these resources into defense-related compounds[367, 368]. Reallocation of labeled nitrogen from ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) into nicotine and phenolamide compounds following simulated herbivory was shown to rely on a functional JA pathway[368]. Carbon availability has been shown to be important for SA-regulated defense, as starch-free mutants, which have a general reduction in carbohydrates compared with wild type plants, showed a delayed production of SA-regulated defense compounds resulting in increased susceptibility to the hemi-biotrophic pathogen *Colletotrichum higginsianum*[367]. Carbohydrates are produced in photosynthetic "source" tissues and transported in the form of sucrose to non-photosynthetic "sink" tissues[369]. Upon pathogen infection in the leaves, this process is disrupted by up-regulation of cell wall invertases, which cleave sucrose into glucose and fructose thereby preventing sucrose export from infected cells[369-372]. Transgenic suppression of cell wall invertase activity results in elevated sucrose to hexose ratios accompanied by reduced and delayed callose deposition and inhibition of *PR* gene expression following pathogen infection[371, 373], whereas ectopic expression of a yeast cell wall invertase has been shown to activate defense responses in tobacco[374]. Comparison of resistant and susceptible barley interactions with the biotrophic fungal pathogen *Blumeria graminis* revealed a more robust activation of cell wall invertase in the resistant interaction resulting in accumulation of hexose sugars localized to regions of actively defending cells[370]. In addition, a recent study has shown cell wall invertase activity to be a possible virulence target of the biotrophic pathogen, *Xanthomonas campestris* pv. *vesicatoria,* to promote disease in pepper[375], providing further evidence supporting a role for cell wall invertases in redirecting carbon resources to enable plant defense.

Together these studies begin to reveal some of the regulatory mechanisms underlying resource reallocation to mediate the growth-defense tradeoff in plants. Along with the co-opting of energy reserves and cellular machinery to produce compounds necessary for defense, transcriptional reprogramming induced by defense activation is often accompanied by repression of growth hormone signaling as a fundamental aspect of growth-defense tradeoffs. In the following sections, we discuss current knowledge regarding crosstalk between defense signaling and growth hormone.

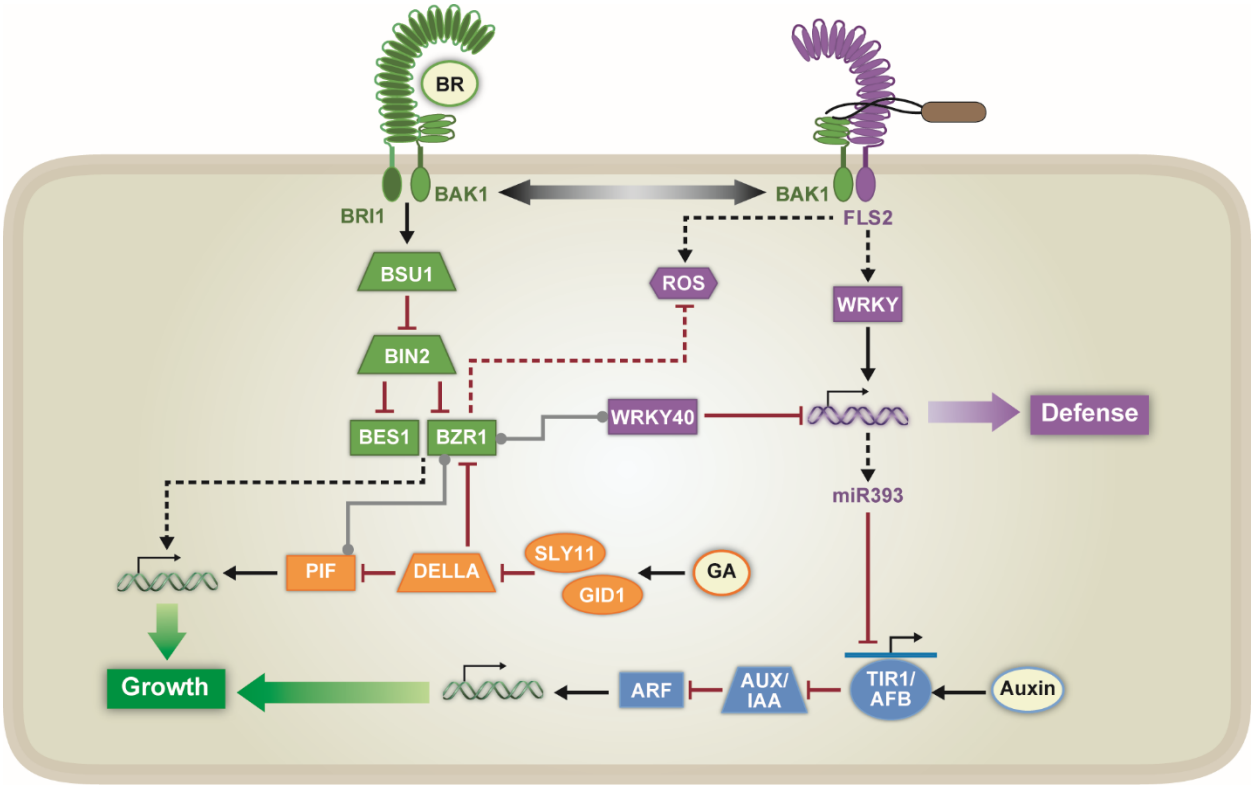

Figure 27. Known signaling contributing to growth-defense tradeoffs between PTI-mediated defense and auxin-, brassinosteroid (BR)- and gibberellin (GA)-mediated growth. Black arrows and red, blunted lines represent positive and negative regulation, respectively. Double helices with arrows represent global transcriptional reprogramming, and gray lines with dots at both ends indicate protein-protein interactions. Solid lines indicate a known connection between two components, whereas dashed lines indicate unknown connections or missing steps between two components. The solid blue line with an arrow represents *TIR1/AFB* genes, which are targeted by miR393. FLS2, FLAGELLIN SENSING 2; ROS, reactive oxygen species; WRKY, WRKY DNA-BINDING PROTEIN; miR393, microRNA 393; TIR1, TRANSPORT INHIBITOR RESPONSE 1; AFB, AUXIN SIGNALING F-BOX; AUX/IAA, AUXIN-INDUCIBLE/INDOLE-3-ACETIC ACID INDUCIBLE; ARF, AUXIN RESPONSE FACTOR; BAK1, BRI1-ASSOCIATED RECEPTOR KINASE 1; BRI1, BRASSINOSTEROID INSENSITIVE 1; BSU1, BRI1 SUPPRESSOR 1; BIN2, BRASSINOSTEROID-INSENSITIVE 2; BES1, BRI1-EMS-SUPPRESSOR 1; BZR1, BRASSINAZOLE-RESISTANT 1; SLY1, SLEEPY 1; GID1, GA INSENSITIVE DWARF 1A; DELLA, repressor protein; PIF, PHYTOCHROME INTERACTING FACTOR.

PAMP-TRIGGERED IMMUNITY-MEDIATED DEFENSE VS. GROWTH

One of the most noticeable physiological consequences of prolonged or constitutively active PTI is growth inhibition, which is observed upon treatment of a plant with a PAMP[264, 273]. As discussed in the previous section, there is mounting evidence to support a mechanism whereby resources normally allocated towards growth are diverted to support defense and, as discussed below, hormonal crosstalk appears to play a major role in regulating the tradeoff between growth and PTI-mediated defense.

PAMP-Triggered Immunity Crosstalk with Auxin

Auxin has long been implicated in suppressing plant defense due to the fact that many pathogens, including *P. syringae* and *Agrobacterium tumefasciens*, can directly synthesize auxin or manipulate auxin synthesis and signaling in plants to promote disease[132-134, 136, 376]. Microorganisms primarily synthesize IAA from tryptophan, and in some cases the genes encoding the enzymes required for this process are located on a pathogen virulence plasmid[133]. Analysis of plant transcriptional reprogramming following some pathogen infections has shown a general de-repression of the auxin pathway including promotion of auxin biosynthetic genes and repression of *AUX/IAA* genes resulting in enhanced plant susceptibility[134, 135]. Furthermore, virulence of the bacterial hemi-biotrophic pathogen *P. syringae* pv. *tomato* DC3000 (*Pto* DC3000) can be enhanced by treatment with synthetic auxins prior to pathogen inoculation[136, 137].

To combat the effects of pathogen produced or induced auxin to promote disease, plants actively suppress auxin signaling during defense[377]. Following flg22-treatment, wild type *Arabidopsis* plants show a reduction in both transcript and protein levels of the auxin F-box receptors, resulting in stabilization of AUX/IAA proteins and repression of auxin-responsive genes[137]. This suppression is partially due to the activity of the microRNA miR393 (Fig. 21), which is induced by flg22 and directly targets and cleaves *TIR1*, *AFB2* and *AFB3* transcripts[137, 378, 379]. However, additional mechanisms such as transcriptional repression must also contribute to PTI inhibition of auxin signaling, as partial reduction in transcript levels is still observed in the *DICER LIKE 1* (*DCL1*) mutant, *dcl1-9,* which is required for miR393 function[137]. Also, the *AFB1* transcript is partially resistant to miR393 activity, and shows reduced transcript levels in both wild type and *dcl1-9* mutant plants[137].

Suppression of auxin signaling has been shown to be biologically relevant to PTI, as overexpression of *miR393* enhances resistance to virulent pathogens and overexpression of *AFB1* increases susceptibility relative to that observed in wild type plants, as measured by bacterial growth[137]. One study has shown that pathogen manipulation of auxin metabolism to generate higher levels of IAA-aspartate (IAA-Asp) promotes disease by positively regulating the expression of bacterial virulence genes rather than by directly suppressing PTI[380]. This was shown to require the GH3.2 enzyme, as *gh3.2* knockout plants exhibited reduced susceptibility to *Pto* DC3000[380]. However, Mutka et al., (2013) were unable to reproduce these results, making the role of GH3.2 in this process unclear. If GH3.2 is involved, it cannot fully account for auxin-induced susceptibility because *gh3.2* knockout plants crossed with plants overexpressing the auxin biosynthetic gene, *YUCCA 1*, retained enhanced susceptibility[129]. Therefore, while

there is much evidence to implicate auxin in promoting plant disease, the exact mechanism underlying this phenomenon remains unclear.

PAMP-Triggered Immunity Crosstalk with Brassinosteroids and Gibberellins

Unlike the mutually antagonistic interactions observed between PTI and auxin-mediated growth, negative crosstalk between PTI and BR-mediated growth is unidirectional[381, 382]. Elevation of BR signaling in *Arabidopsis* using either transgenic modifications[382, 383] or exogenous application of BR[381] results in inhibition of flg22-mediated protection against *Pto* DC3000. Conversely, treatment with brassinazole, which inhibits BR biosynthesis, elevates ROS production in response to PAMP treatment[384], indicating that endogenous levels of BR are sufficient to suppress PTI. Due to the association of BAK1 with both FLS2 and BRI1 receptors, it was hypothesized that FLS2 and BRI1 competition for BAK1 might facilitate BR-mediated suppression of PTI-mediated defense (Fig. 21). However, while overexpression of BRI1 was shown to inhibit PTI responses in a BAK1-dependent manner[382], neither exogenous BR nor expression of a hyperactive form of BRI1, BRI1^{sud1}, were shown to affect FLS2-BAK1 complex formation, transphosphorylation or phosphorylation of downstream targets[381, 384].

A recent study by Lozano-Duran et al. (2013) has shown that constitutively active BZR1, but not BES1, is sufficient to block PAMP-triggered ROS burst, gene expression and seedling growth inhibition (Fig. 21). BZR1 inhibition of PTI appears to be mediated through its downstream targets, which include transcription factors known to promote BR responses and/or block defense[384, 385]. For example, a group of WRKY transcription factors known to negatively regulate PTI were identified as BR-induced BZR1 targets[384]. Of these, WRKY40 was shown to have a role in suppression of PAMP-induced ROS production and seedling growth inhibition. It is possible that BZR1 and WRKY40 act together to suppress PTI, as co-immunoprecipitation experiments indicated that these two proteins physically interact (Fig. 21), and analysis of publicly available gene expression data revealed that all WRKY40 regulated genes are also targets of BZR1[384]. Another example of a BZR1 target involved in PTI suppression is *HBI1*, which encodes a bHLH transcription factor shown to promote BR-regulated cell elongation by inducing the expression of expansin genes[386]. In addition to enhanced growth phenotypes, overexpression of *HBI1* was shown to suppress PAMP-induced ROS and seedling growth inhibition downstream of FLS2-BAK1 complex formation[385]. While the mechanism for HBI1-mediated suppression of PAMP-responses is not known, identification of specific defense gene targets of BZR1-regulated transcription factors, including the WRKYs identified by Lozano-Duran et al. (2013) begin to shed light on the molecular mechanism behind BR suppression of PTI-mediated defense.

GA suppression of PAMP-induced seedling growth inhibition most likely occurs through promotion of BR signaling. BR- and GA-mediated signaling pathways work additively or synergistically to promote growth in response to environmental and developmental cues[387, 388]. This cooperative relationship is facilitated in part by the formation of a BZR1/PIF4 heterodimer (Fig. 21), which binds to the promoters of some 2,000 shared target genes to promote growth[75]. DELLA proteins have been shown to inhibit both BZR1 and PIF4 proteins and may also target the BZR1/PIF4 heterodimer[389-391]. While exogenous application of GA did not affect PAMP-induced seedling growth inhibition, chemical inhibition of GA synthesis completely

blocked the effect of BR on seedling growth inhibition, and GA treatment in combination with BR resulted in an additive effect on PAMP-induced seedling growth inhibition[384]. This is most likely due to the effect of GA on DELLA stabilization. In the absence of GA, increased DELLA stabilization would result in BZR1 inhibition and loss of BR-mediated seedling growth inhibition (Fig. 21). In support of this, flg22-induced stabilization of DELLA proteins has been shown to be a mechanism for PTI inhibition of GA-mediated growth[392].

SALICYLIC ACID-MEDIATED DEFENSE VS. GROWTH

Suppression of growth by SA is best illustrated by constitutive defense mutants, which typically have a dwarf plant phenotype due in part to elevated SA accumulation or signaling[124, 125]; however, as these mutants may be perturbed in cellular processes other than SA defense, it is difficult to ascertain how SA itself is directly contributing to growth suppression in these plants. To demonstrate the effect of SA on plant growth, experiments employing chemical inducers or genetic manipulation to alter SA accumulation or perception have been used. Cold temperature-induced growth reduction in *Arabidopsis* has been shown to be due to endogenous elevation of SA as it was lost in plants compromised in SA accumulation[83]. Also, repeated application of BTH reduced plant biomass in a reproducible and dose-dependent manner that was correlated with induction of SA-mediated defense responses[126]. Mutants isolated in a screen based on resistance to BTH-induced growth inhibition were compromised in SA-mediated disease resistance and were primarily identified as non-functional alleles of *NPR1*[393]. The mechanisms for SA-induced suppression of growth are most likely mediated by crosstalk with growth hormone signaling pathways, as discussed in the following sections.

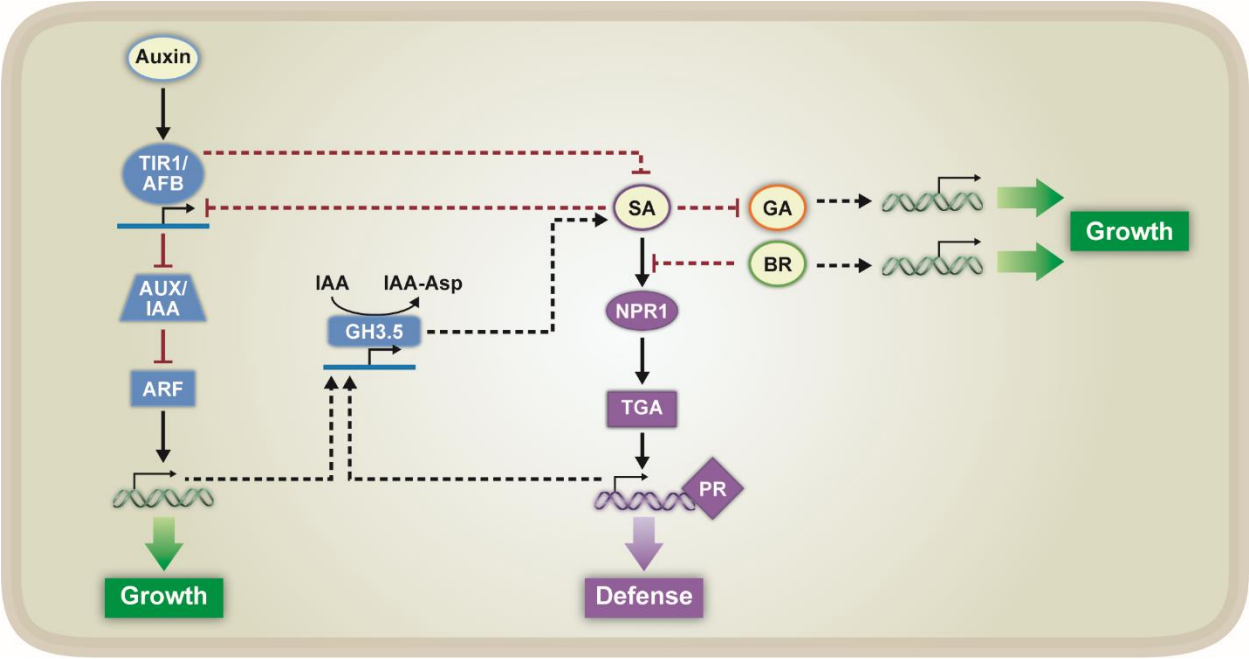

Figure 28. Known signaling contributing to growth-defense tradeoffs between salicylic acid (SA)-mediated defense and auxin-, brassinosteroid (BR)- and gibberellin (GA)-mediated growth. As in Figure 2, black arrows and red, blunted lines represent positive and negative regulation, respectively. Double helices with arrows represent global transcriptional reprogramming, and solid lines associated with arrows represent specific genes and indicate an effect on gene expression. Solid lines indicate a known connection between two components, whereas dashed lines indicate unknown connections or missing steps in between two components. NPR1, NONEXPRESSOR OF PR GENES 1; TGA,TGACG SEQUENCE-SPECIFIC BINDING PROTEIN; PR,PATHOGENESIS RELATED; IAA, INDOLE-3-ACETIC ACID; Asp, aspartate; TIR1, TRANSPORT INHIBITOR RESPONSE 1; AFB, AUXIN SIGNALING F-BOX; AUX/IAA, AUXIN-INDUCIBLE/IAA INDUCIBLE; ARF, AUXIN RESPONSE FACTOR.

Salicylic Acid Crosstalk with Auxin

One of the primary ways SA has been shown to inhibit growth is by suppression of auxin signaling (Fig. 22). A microarray study revealed that a number of auxin-responsive genes were affected by BTH treatment, i.e., twenty-one genes encoding proteins involved in auxin reception, import and export and signaling were down-regulated and two genes encoding GH3 enzymes were up-regulated[117, 394]. As GH3 enzymes are responsible for regulating auxin homeostasis by conjugating IAA with different amino acids[293], the transcriptional profile indicates a general BTH-dependent repression of auxin homeostasis and signaling. A follow-up study confirmed this by investigating the effect of SA on auxin levels, uptake, sensitivity and signaling[394]. It was shown that SA does not affect auxin synthesis, but instead represses the expression of the *TIR1/ABF* F-box genes (Fig. 22), resulting in stabilization of AUX/IAA repressor proteins to decrease auxin signaling[394].

One of the two *GH3* genes identified in the microarray study encodes GH3.5[117, 394], which conjugates IAA with Asp[293]. The *gh3.5* knockout mutants were shown to be compromised in SAR while overexpression lines exhibited a dwarf phenotype, accumulated higher levels of SA, had elevated expression of *PR1*, and increased resistance to *Pto* DC3000[395-397]. IAA-Asp is an inactive form of auxin that is targeted for catabolic metabolism[291, 398]; therefore, it would seem logical to infer that GH3.5 directly facilitates the growth-defense tradeoff between SA and auxin by simultaneously elevating SA levels and reducing active IAA levels. However, the dwarf phenotype observed in several GH3.5 overexpression lines did not always correlate with a reduction in free IAA[395, 396]. As *GH3.5* expression is also induced by IAA to regulate its homeostasis[304, 399], it is possible for GH3.5 to inhibit the auxin pathway directly by conjugating IAA and also indirectly by promoting SA biosynthesis and signaling, which then acts to block auxin responses (Fig. 22).

SA-mediated defense has also been shown to be affected by auxin, as transgenic overexpression of the *AFB1* gene, which enhances auxin signaling, led to a reduction in pathogen-induced SA biosynthesis relative to wild type plants (Fig. 22)[128]. However, transgenic overexpression of the *YUCCA 1* gene showed that elevation of auxin levels alone can promote plant disease without affecting SA levels or signaling[129]. Auxin positively regulates expansins, which are involved in cell wall loosening, to promote growth[130, 131], and the ability of *Xanthomonas oryzae* pv *oryzae* to induce expansins in rice was shown to be important in determining the outcome of the plant-pathogen interaction[131]. Together these studies indicate a dual function for auxin in direct interference with SA-mediated defense and in positive regulation of physiological changes that aid pathogen proliferation in the plant.

Salicylic Acid Crosstalk with Brassinosteroids and Gibberellins

There is much less known regarding the relationships between SA-mediated defense and BR- and GA-mediated growth. BR treatment was shown to block BTH-mediated resistance in rice, indicating suppression of SA signaling[400]. Based on analysis of mutant plants affected in SA production or NPR1-mediated signaling it was concluded that this antagonism occurs downstream of SA biosynthesis and upstream of NPR1 signaling (Fig. 22), but the mechanism for this suppression is unknown[400]. A recent study showed that down-regulation of the gene

encoding the hydroxycinnamoyl CoA (HCT) enzyme resulted in stunted plant growth that was directly correlated with lignin reduction and endogenous SA elevation[401]. These same plants were also shown to be impaired in both GA accumulation and perception[401, 402]. Crosses between *HCT* RNAi plants and plants defective in SA biosynthesis, accumulation, or perception by NPR1 revealed that loss of SA production and accumulation, but not NPR1-dependent SA perception, was responsible for growth suppression in these plants[401]. Loss of SA accumulation was also shown to restore gene induction and growth enhancement in response to exogenous GA, implicating SA in repression of GA-signaling and growth[401]. As mentioned previously, BZR1 is directly targeted and suppressed by the DELLA family of growth-suppressing proteins[390]. SA-mediated suppression of GA would most likely result in increased DELLA stability, which may lead to suppression of BR-mediated signaling. Further studies are needed to both establish a molecular mechanism for SA-inhibition of GA signaling and to determine whether this suppression of GA results in loss or reduction in BR signaling.

JASMONATE-MEDIATED DEFENSE VS. GROWTH

It has long been known that activation of JA signaling by applying JA into the growth medium results in growth inhibition[403]. Correlated with growth inhibition, JA suppresses mitosis, arrests the cell cycle in G1 prior to the S transition, and delays the switch from the mitotic cell cycle to the endoreduplication cycle[404, 405]. Transcriptomic analysis further confirmed that JA activates several critical regulators of endoreduplication and affects the expression of key determinants of DNA replication[404]. As in the case of PTI and SA-mediated defense, the effects of JA on growth appear to be mediated by crosstalk with growth hormone signaling.

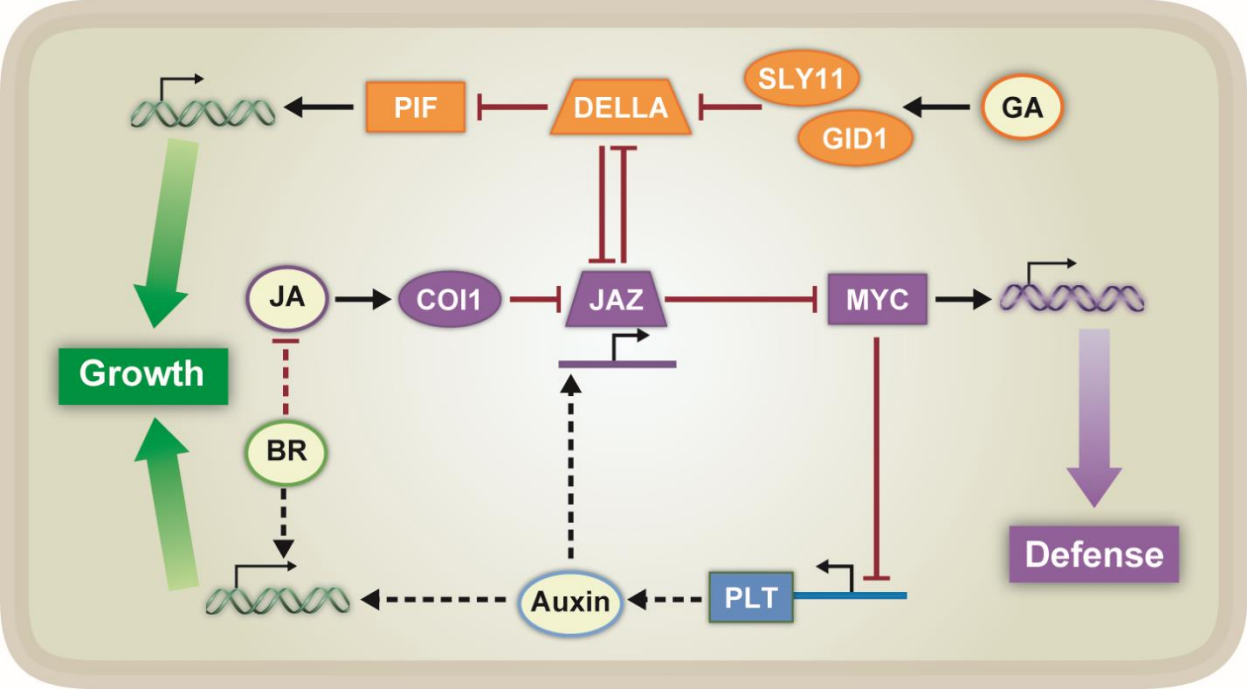

Figure 29. Known signaling contributing to growth-defense tradeoffs between jasmonate (JA)-mediated defense and auxin-, brassinosteroid (BR)- and gibberellin (GA)-mediated growth. Black arrows and red, blunted lines represent positive and negative regulation, respectively. Double helices with arrows represent global transcriptional reprogramming, and solid lines with arrows represent specific genes and indicate an effect on gene expression. Solid lines indicate a known connection between two components, whereas dashed lines indicate unknown connections or missing steps in between two components. COI1, CORONATINE INSENSITIVE 1; JAZ, JASMONATE ZIM DOMAIN; MYC, transcription factor; SLY1, SLEEPY 1; GID1, GA INSENSITIVE DWARF 1A; DELLA, repressor protein; PIF, PHYTOCHROME INTERACTING FACTOR; PLT, PLETHORA.

Jasmonate Crosstalk with Auxin and Brassinosteroids

The auxin signaling pathway has been implicated in JA-induced growth inhibition in *Arabidopsis* (Fig. 23)[406]. JA not only suppresses the expression of the auxin efflux carrier *PINFORMED 2* (*PIN2*) but also inhibits PIN2 endocytosis and membrane accumulation[329]. Consequently, the normal auxin distribution in roots is disrupted after JA treatment[329]. Moreover, MYC2 has been shown to negatively regulate the expression of *PLETHORA* (*PLT1* and *PLT2*) transcription factors[407], which are important regulators of auxin-mediated root stem cell development and auxin biosynthesis in roots (Fig. 23)[408]. Taken together, it is postulated that JA changes the spatial and temporal distribution pattern of auxin in plants to suppress normal plant growth mediated by auxin. However, JA was also shown to increase auxin biosynthesis by inducing *ANTHRANILATE SYNTHASE* (*ASA1* and *ASB1*) and *YUCCA* (*YUC8* and *YUC9*) gene expression in certain plant tissues[409, 410], and JA-induced auxin biosynthesis and lateral root formation were impaired in *yuc* knockout mutants[410]. Conversely, auxin has been shown to induce expression of *JAZ1*, suggesting that auxin may suppress JA signaling through JAZ1 (Fig. 23)[411]. These latter studies illustrate the complexity of the interaction between JA and auxin signaling pathways.

BR signaling has also been implicated in antagonizing JA-induced growth suppression (Fig. 23)[257]. The first indication of a connection between JA-mediated defense and BR-mediated growth was the identification of a *partially suppressing coi1* (*psc1*) mutant, which carries a mutation in a key enzyme involved in BR biosynthesis, DWARF 4 (DWF4)[412]. In *Arabidopsis* the *psc1* mutation partially suppresses the loss of JA-induced growth inhibition in the *coi1* mutant background[412] and displays increased JA-induced growth inhibition in the wild-type background[413]. The negative impact of BR signaling on JA signaling has also been demonstrated in tomato, where BR was shown to antagonize several JA-dependent traits including trichome density and allelochemical content[414]. Unlike in *Arabidopsis*, BR appears to act upstream of COI1 in tomato since loss of BR synthesis cannot suppress the tomato *coi1* mutation (Fig. 23)[414]. However, BR has also been shown to have positive effects on some JA-mediated traits, as JA-induced anthocyanin accumulation is reduced both in BR-biosynthetic mutants and a BR signaling mutant[415, 416]. Thus, as in the case of the JA-auxin interaction, the crosstalk between JA and BR appears to be complicated.

Jasmonate Crosstalk with Gibberellins

A wave of recent studies has shown an important role for JA-GA signaling crosstalk in regulating the growth-defense tradeoff (Fig. 23)[185, 417-419]. In *Nicotiana attenuata*, elevated JA has a negative effect on GA biosynthesis in stems resulting in growth inhibition[419]. In several *Arabidopsis* mutants in which the DELLA transcriptional repressors are stabilized, MYC2-dependent JA-responsive genes are hypersensitive to JA treatment resulting in increased growth inhibition[417]. In addition, overexpression of a DELLA protein, RGA LIKE 3 (RGL3), which reduces GA-mediated growth, increases MYC2-dependent gene expression; whereas *rgl3* mutation reduces MYC2-dependent gene expression[418]. MYC2 has also been shown to positively regulate *RGL3* by directly binding to the promoter of this gene, creating a positive feedback loop in JA signaling[418]. Consistent with GA antagonism of JA signaling, DELLA

repressor proteins have been shown to be positive regulators of JA-mediated disease resistance against necrotrophic pathogens, as JA-mediated defense is compromised in DELLA loss-of-function mutants and is enhanced by overexpression of *RGL3*[392, 418].

Direct physical interaction between JAZ and DELLA repressor proteins turns out to be crucial for the JA-GA crosstalk in regulating growth and defense (Fig. 23)[185, 417, 418]. JAZ proteins interact with the GRAS domain of DELLA proteins, which is important for the interaction between DELLAs and growth-promoting PIF transcription factors[391]. JAZ binding to DELLA proteins was shown to block the interaction between DELLAs and PIFs, thereby relieving the inhibition of DELLAs on PIFs and promoting GA-dependent growth in *Arabidopsis*[185]. Accordingly, *Arabidopsis coi1* mutants, *JAZ* overexpression lines and *COI1*-silenced rice plants show enhanced growth; whereas *Arabidopsis della* mutants and *PIF* overexpression lines are compromised in JA-induced growth inhibition[185]. These results suggest that in response to pathogen or herbivore attack, degradation of JAZ proteins makes more DELLA proteins available for interaction with and inhibition of PIF transcription factors as part of a mechanism to inhibit growth (Fig. 23)[147, 185]. Conversely, GA has also been demonstrated to have a positive effect on some JA-mediated traits[420]. The RGA DELLA protein can interact with and repress MYC2 activity resulting in inhibition of JA-mediated terpene biosynthesis; in this case, GA-mediated degradation of DELLAs promotes a specific JA-mediated trait[420]. Together, these findings suggest that interactions between JA and GA signaling pathways can occur at multiple levels and in different directions, illustrating the dynamic nature of JA-GA crosstalk in regulating the growth-defense tradeoff.

CONCLUSIONS AND FUTURE PERSPECTIVES

Pathogen and herbivore-induced damage is known to reduce plant yield, causing substantial economic losses[2, 421]. However, simply breeding plants to have constitutively active defense is not a viable solution as there are known fitness costs associated with the induction of defense responses[25, 330-332], as well as conditions under which growth must be prioritized in spite of pathogen or herbivore attack[336, 384]. Plants have evolved mechanisms, such as hormone crosstalk, to optimize fitness in response to the dynamic environments in which they live. A critical step in harnessing this process for the improvement of crop performance is the identification of molecular targets responsible for implementing resource reallocation to facilitate prioritization of growth or defense.

Studies reviewed here and elsewhere have revealed a web of interconnected hormone signaling networks that enable fine-tuning of plant responses to environmental and developmental cues[120, 123, 231, 245-248, 306]. However, it can be challenging to compare and integrate data collected using different experimental parameters— i.e. plant growth conditions and/or age. Untangling this web is also constrained by the tools and methods available. For instance, while "omic" methods have enabled global visualization of changes in gene expression and protein profiles to some extent, the snapshots they provide are incapable of capturing the full range of dynamic temporal and spatial processes of growth-defense interactions. Also, tools currently available to isolate or amplify certain effects, such as the use of exogenous application of elicitors/hormones and stable genetic manipulation, may result in the identification of interactions that do not exist in nature or fail to identify those that do[330].

Other issues include the limitations of using whole seedlings or tissues to investigate changes occurring on a sub-organismal scale, and the relatively few studies conducted to investigate the effects of multiple or variable stresses on growth-defense interactions.

Therefore, while the use of simple laboratory conditions are essential for establishing foundational knowledge of individual signaling pathways, it will also be necessary in the future to design experiments that more accurately reflect natural environments—fluctuating conditions, exposure to multiple stresses and field studies—to identify network interactions and to test putative molecular mechanisms. As technology advances, the ability to observe plant growth and plant-pathogen/herbivore interactions at a cellular level and in a spatiotemporal manner will provide valuable insight towards elucidating the timing and sub-cellular localization of molecular interactions as well as to distinguish between local and global effects on plant growth and defense. Understanding the specific molecular interactions that facilitate these tradeoffs will provide powerful tools to genetically tailor plants that optimize this balance to maximize crop yield in fluctuating environmental conditions.

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