DISSERTATION

CHARACTERIZATION AND INSIGHTS INTO THE MOLECULAR MECHANISM OF CYTOKININ-INDUCED PRIMING OF PLANT DEFENSES

Submitted by

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ABSTRACT

CHARACTERIZATION AND INSIGHTS INTO THE MOLECULAR MECHANISM OF CYTOKININ-INDUCED PRIMING OF PLANT DEFENSES

Plants have developed several mechanisms to cope with pathogenic challenges. One of these mechanisms, known as defense priming can be effective at reducing susceptibility to pathogens. Compared to unprimed plants, the immune response from primed plants, upon pathogen attack, is much stronger. This mechanism of induced disease resistance can be initiated by biological and chemical agents. The major benefit of priming is the induction of a high level of protection with considerably low fitness costs making it an attractive disease management strategy to preserve agricultural output. Recent research has demonstrated that the plant hormone cytokinin (CK) has a priming effect against biotrophic pathogens, a phenomenon referred to here as cytokinin-induced priming (CIP). This dissertation aims to gain further understanding of CIP against the hemibiotrophic bacterial pathogens *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Pseudomonas syringae* pv. *maculicola* (*Psm*) in *Arabidopsis thaliana* (Arabidopsis) and *Brassica napus,* respectively as well as the necrotrophic fungal pathogen *Botrytis cinerea* in Arabidopsis.

Chapter 2 focuses on characterizing CIP as a true priming agent by investigating the timeframe in which CIP is most effective at reducing susceptibility to *Pst* and *Psm* in both Arabidopsis and its closely related relative, *B. napus* and the impacts on plant growth due to CIP in these pathosystems. Moreover, we discovered that other known priming agents depend on endogenous CK signaling suggesting CK-mediated processes are involved in the priming of defense responses.

The role of CK in primed defenses against *B. cinerea* is explored in chapter 3 where CIP is demonstrated to reduce necrotic lesion size caused by *B. cinerea* in a manner dependent on the JA-mediated defenses and partially on SA-mediated defenses. Transcriptome analysis revealed that during the priming stage, CK prepares the plants for pathogenic challenge through the accumulation of cellular components needed for translation and metabolites utilized for energy production and defense. Following *B. cinerea* inoculation, CIP suppresses defense while increasing photosynthetic-related processes.

In the final chapter, molecular mechanisms are explored during CIP against *Pst.* Through transcriptome changes, priming by CK potentiates gene expression associated with systemic induction of defense, also known as systemic acquired resistance (SAR), following *Pst* challenge. Using this information, it is demonstrated that CK treatment can also induce SAR and that the known SAR inducer, L-pipecolic acid, is dependent on endogenous CK signaling. Due to the previously identified relationship between CK and source-sink relationships, amino acid transport was demonstrated to have a role in both CIP and CK-induced SAR.

New agricultural practices that mitigate crop loss due to plant diseases are beneficial in terms of sustainability and economic costs. The use of CK as a priming agent offers an avenue for a new disease management strategy in that CIP protects plants against a broad range of pathogens with minimal effects on plant growth. The molecular mechanisms underlying CIP discovered here offers new insights into the relationship between plant metabolism and defense, where its exploitation could be used to create disease protection strategies.

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CHAPTER 1:

CYTOKININ REGULATION OF SOURCE-SINK RELATIONSHIPS IN PLANT GROWTH AND PLANT-PATHOGEN INTERACTIONS[1]

1.1 SUMMARY

Cytokinins are plant hormones known for their role in mediating plant growth. First discovered for their ability to promote cell division, this class of hormones is now associated with many other cellular and physiological functions. One of these functions is the regulation of source-sink relationships, a tightly controlled process that is essential for proper plant growth and development. As discovered more recently, cytokinins are also important for the interaction of plants with pathogens, beneficial microbes, and insects. Here, we review the importance of cytokinins in source-sink relationships in plants, with relation to both carbohydrates and amino acids, and highlight a possible function for this regulation in the context of plant biotic interactions.

1.2 INTRODUCTION

Cytokinins are a group of plant hormones derived from adenine, classified by the presence of an isoprenoid or an aromatic chain at the N^6 position of their adenine moieties (Mok and Mok, 2001). Although different compounds with cytokinin activity have been shown to regulate various physiological processes in plants, cytokinins are broadly described as growth-promoting plant hormones. The first cytokinin discovered by Miller and Skoog in the 1950s,

kinetin, was defined as a plant-derived chemical that could promote cell division (Miller et al., 1956). In a following study, it was demonstrated that kinetin, in combination with auxin, was responsible for promoting cell division and organ development from undifferentiated cells in culture (Skoog and Miller, 1957). While the study of cytokinins began in the middle of the 1900s, they are in fact an ancient hormone, being one of the first four hormones to emerge in photosynthetically capable organisms (Wang et al., 2015). Evolutionary studies indicate that the common ancestor of all land plants, charophytes, contains the genetic sequences of orthologs to known members of the cytokinin signaling pathway (Wang et al., 2015). These data suggest that cytokinins had a role in plants as early as 450 million years ago. Today, cytokinins are known for their broad role in plant growth (Kieber and Schaller, 2018), and also roles in preventing senescence, as well as regulation of biotic and abiotic stress tolerance (Argueso et al., 2009; Cortleven et al., 2019).

One important physiological response that has been classically associated with cytokinins is the regulation of source-sink relationships and nutrient allocation in plants. Shortly after cytokinins were discovered to have a role in cell division, a study in 1961 in *Nicotiana rustica* demonstrated that exogenous application of kinetin to leaves led to increased accumulation of the amino acid glycine to the area of hormone application (Mothes and Engelbrecht, 1961). Similarly, kinetin application to leaves of fava bean plants that had been unrooted was also shown to correlate with the movement of the amino acid alanine to the site of hormone application, which the researchers termed “mobilization” (Mothes and Engelbrecht, 1963). These early reports indicated that cytokinins could have a pivotal role in the allocation of amino acids in plants, with important consequences for plant growth.

In this review, we start by providing readers with an overview of the process of source-sink relationships in plants, and then proceed to highlight the evidence for a regulatory role for cytokinins in this important physiological process, starting with their first initial association and finishing with the most recent evidence. We conclude by pointing out some emerging evidence of the importance of this plant hormone as a regulator of nutrient availability in plant biotic interactions during disease susceptibility and promotion of plant immunity.

1.3 OVERVIEW OF SOURCE-SINK RELATIONSHIPS IN PLANTS

Photosynthesis leads to the production of reduced carbon products, also known as photoassimilates. Photoassimilates generated in the mesophyll cells, such as sucrose, oligosaccharides, and amino acids, are transported to other parts of the plant to maintain plant growth. Generally, the rate of photosynthetic activity and the accumulation of photoassimilates can be used to classify organs as sinks or sources. Sources are defined as photosynthetically active leaves that export photoassimilates to heterotrophic sink tissues that are dependent on imported sugars and amino acids for growth and development. Fully mature source leaves export as much as 80% of photoassimilate to sink tissues (Kalt-Torres et al., 1987). Sinks are defined as the opposite: an organ that is dependent on sugar and amino acid import to support growth and development. Sinks include young leaves, reproductive organs, and roots. Photosynthetic activity changes during the course of leaf development. Young leaves are sink organs that need to import photoassimilates from mature leaves to support growth and development (Geiger and Sheigh, 1993). As the immature leaf grows, it becomes photosynthetically active and eventually becomes an exporter of photoassimilates, through a process known as the sink-source transition (Turgeon, 1989). The relationship between source and sink organs has been the focus of intensive research

because of its impact on plant growth and yield (White et al., 2016) and its potential for using transgenic approaches for modifying yield and/or nutritional quality (Yadav et al., 2015). Sucrose is the end product of photosynthesis, and the primary sugar transported within plants. In source leaves, sucrose produced, from photosynthesis during the day or starch degradation occurring at night, is loaded into the phloem for transport to sinks (Figure 1.1 A). Although this review focuses mostly on sucrose, as it is present in the phloem sap of all plant species, it should be noted that the phloem sap of some plant species also contains sugar alcohols and/or oligosaccharides from the raffinose family (Zimmermann and Ziegler, 1975; Noiraud et al., 2001). Depending on the anatomical connections of the plant species, the loading of sucrose into the phloem can be achieved by three different loading mechanisms: symplastic, apoplastic, and polymer trapping (Braun et al., 2014). For the purposes of this review, we will focus on the apoplastic loading pathway, which is the predominant pathway used in most plant species, including the model plant species *Arabidopsis thaliana* (hereafter, Arabidopsis).

Apoplastic phloem loading in the leaf is mediated by a proton-sucrose symporter (Bush, 1993). Sucrose is transported out of mesophyll cells into the intercellular space by sucrose transporters known as SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS (SWEETs) (Chen et al., 2012). Once in the intercellular space, sucrose is then actively loaded into the phloem cells against a significant concentration gradient by proton-sucrose symporters, named SUCROSE TRANSPORTERS/CARRIERS (SUTs/SUCs) (Reinders et al., 2012; Zhang and Turgeon, 2018). Sucrose accumulates to molar levels in the leaf phloem thereby creating a high osmotic potential that draws in water. Since the phloem cells are surrounded by an inelastic cell wall, this creates high hydrostatic pressure that drives the mass flow of solution to sink tissues where sucrose is released and used for growth, development, or carbohydrate storage

(Bush, 2020). There are two main mechanisms by which sucrose is moved into sink cells (Braun et al., 2014): (i) it is released into the intracellular space by SWEETs and then transported into the sink tissue by SUTs/ SUCs (Weber et al., 1997) or (ii) it is released into intracellular space by SWEETs and then hydrolyzed into glucose and fructose by extracellular invertases (Ruan et al., 2010) followed by import into sink cells by proton/hexose symporters (HXTs) (Zhang et al., 2006; Hayes et al., 2007). Due to sucrose being the major form of carbon being translocated from source to sink (Fife et al., 1962; Turgeon, 1989), and starch being the main storage form of sucrose, the homeostasis of these two carbohydrates is essential for the regulation of their metabolism and allocation in plants (Smith and Stitt, 2007).

The production, storage, and movement of amino acids can also define organs as sinks or sources (Figure 1.1 A) (Bush, 1999). Roots are the site of uptake of inorganic nitrogen (N) from the soil, in the form of nitrate or ammonium, a process regulated by transporters located in root epidermal hairs and root cortical and endodermal cells (Tegeder, 2014). However, some plant species are also able to take up organic N in the form of amino acids, depending on environmental and soil conditions (Nasholm et al., 2009; Tegeder and Rentsch, 2010; Bloom, 2015). The location of N assimilation, or the conversion of inorganic N into amino acids, varies among plant species (Masclaux-Daubresse et al., 2010). Nitrate taken up by the roots is primarily transported to the shoot before assimilation, while ammonium, due to its toxic nature, is assimilated after uptake in the roots (Tegeder and Masclaux-Daubresse, 2018).

Transport of amino acids from the roots to the above-ground areas of the plant occurs through the xylem, while translocation between source and sink organs occurs via the phloem (Tegeder and Masclaux-Daubresse, 2018) (Figure 1.1 A). Once formed in source leaves or roots, amino acids are loaded into the phloem and then unloaded into sinks tissues by amino acid

transporters (Okumoto et al., 2004; Tegeder and Hammes, 2018). Also relevant to amino acid allocation is the process of plant senescence. During senescence, leaf proteins are degraded, providing a large quantity of amino acids that are used for growth in other organs, a process known as amino acid remobilization. Amino acid remobilization also occurs through the action of amino acid transporters. In both Arabidopsis and *Brassica napus*, it has been shown through ^{15}N tracing that senescing leaves are the primary source of N provided to sink tissues during the late vegetative phase or to flowers and seeds during the reproductive phase (Malagoli et al., 2005; Diaz et al., 2008; Lemaitre et al., 2008).

1.4 CYTOKININ REGULATION OF SOURCE-SINK RELATIONSHIPS: EFFECT ON PHOTOSYNTHESIS AND SUCROSE TRANSPORT

In many plant species, cytokinins positively affect photosynthetic rates (reviewed in Cherniad'ev, 2000). This effect is associated with increases in stomatal conductance and gas exchange, leading to higher photosynthetic rates and sucrose production (Ahanger et al., 2018, 2020). Cytokinins have also long been associated with an increase in chloroplast number per cell (Boasson and Laetsch, 1969), a process that has been coupled to the anti-senescence activity of this hormone. In Arabidopsis, this increase in chloroplast number per cell is facilitated by the transcriptional regulation of components of the chloroplast division machinery, which is mediated by the cytokinin-regulated transcription factor CYTOKININ RESPONSE FACTOR 2 (CRF2) (Okazaki et al., 2009). Because the number of chloroplasts within a cell can affect overall photosynthetic rates (Austin II and Webber, 2005; Xiong et al., 2017), cytokinin regulation of chloroplast number and their development may have a role in regulating source activity and strength and the availability of photoassimilates.

The photosynthetic activity of source tissues also changes, depending on the demand for photoassimilates by sinks (Paul and Foyer, 2001; Sonnewald and Fernie, 2018). When source leaves are shaded or removed by defoliation, the remaining source leaves display an increase in their rate of photosynthesis, compensating for the removed/shaded source leaves, and responding to the rate of utilization of carbohydrates in sinks (Thorne and Koller, 1974; Peet and Kramer, 1980; McCormick et al., 2006). A study in tomatoes provided evidence that endogenous cytokinin levels could be responsible for altering the response of source leaves following defoliation. After defoliation, the increased photosynthesis levels observed in the remaining source leaves were positively correlated not only to increased levels of the cytokinin *trans*-zeatin riboside, but also to increased leaf expansion and decreased levels of sugar export. This study suggests that the increased cytokinin concentration in the source leaves caused higher photosynthetic activity, resulting in sugar production that was used for leaf expansion, instead of transport to sinks, which ultimately increased the strength of the source tissue (Glanz-Idan et al., 2020).

As previously mentioned, one method of sugar uptake in sinks following unloading from the phloem is through the activity of extracellular invertases, located within the cell wall. Extracellular invertases catalyze the hydrolysis of sucrose into glucose and fructose (Ruan, 2014), a process that can influence the sink strength (Ho, 1988; Herbers and Sonnewald, 1998; Sturm and Tang, 1999; Lemoine et al., 2013). This process seems to be regulated by cytokinins: Exogenous application of the cytokinin *trans*-zeatin can increase the expression of extracellular invertases (Godt and Roitsch, 1997; Lara et al., 2004) and in a detached leaf assay, cytokinins can prevent senescence and maintain sink strength through regulation of the activity of extracellular invertases (Lara et al., 2004).

Cytokinins may also have a role in sucrose transport from source to sink organs by regulating the expression of SWEET and SUT/SUC transporters (Table 1.1). In potatoes, expression of the sucrose transporter StSUT1 was shown to be induced in mature leaves following exogenous treatment with the cytokinin benzyladenine (BA) (Harms et al., 1994) and in *Brassica napus* expression of *BnSUTs* and *BnSWEETs* was increased after exogenous application of BA to leaves (Jian et al., 2016). Endogenous levels of cytokinins also regulate *SUC/SUT/SWEET* expression. In peas, the content of several cytokinin species in source leaves is correlated with the increase in expression of genes encoding SWEETs and SUTs (Ninan et al., 2019). Developing fruits and seeds are considered major sink tissues. As seeds develop, the walls of siliques in *B. napus* show an increase in expression of *BnSUTs*, which is also correlated with an increased expression in genes responsible for cytokinin biosynthesis (Song et al., 2015). Although the correlation in the expression of sugar transporters and cytokinin content is not necessarily causative, evidence exists for a functional role of this regulation in sugar transport: Application of the cytokinins BA, kinetin, or *trans*-zeatin to *Chenopodium rubrum* suspension cells did not only lead to increased expression of the hexose transporter genes *CST2* and *CST3*, but also increased uptake of ^{14}C-labeled glucose from the cell suspension media as compared to untreated cells (Ehness and Roitsch, 1997).

1.5 CYTOKININ REGULATION OF SOURCE-SINK RELATIONSHIPS: EFFECT ON AMINO ACID TRANSPORT

The first evidence of a potential role for cytokinins in source-sink relationships came from studies on the movement of amino acids in response to kinetin application to plants. Mothes and Engelbrecht showed that when kinetin was applied to detached leaves of *Nicotiana rustica*,

^{14}C-labeled glycine migrated to the site of kinetin application (Mothes and Engelbrecht, 1961). A similar experiment in unrooted seedlings of fava beans also showed translocation of ^{14}C-labeled alanine to sites of kinetin application. However, if plants were rooted, ^{14}C-labeled alanine migration to sites of cytokinin application was diminished, with more ^{14}C-labeled alanine being mobilized to roots (Mothes and Engelbrecht, 1963). Given that roots are sites of cytokinin biosynthesis (Miyawaki et al., 2004), these experiments showed a direct link between amino acid mobilization and cytokinin content. A similar effect of cytokinin on amino acid mobilization was shown in other plant species, including monocot species, such as oats (Gunning and Barkley, 1963), as well as beans and maize plants (Leopold and Kawase, 1964). Importantly, non-proteinogenic amino acids, such as α-aminoisobutyric acid, are also mobilized to sites of cytokinin application, indicating that the effect of cytokinin is not due to an increased need for amino acids for protein synthesis, but on amino acid translocation *per se* (Mothes and Engelbrecht, 1961).

The relationship between cytokinin and amino transporters has been examined mostly at the level of regulation of gene expression of amino acid transporter genes, such as those from the family amino acid permeases (AAP), lysine and histidine transporters (LHT), and cationic amino acid transporters (CAT). Application of cytokinin increases the expression of *AAP3* (Brenner et al., 2005; Kiba et al., 2005), *CAT1* (Kiba et al., 2005), and *CAT6* (Brenner et al., 2005; Kiba et al., 2005; Yokoyama et al., 2007), and decreases expression of *AAP2*, *AAP5*, and *LHT1* (Brenner et al., 2005; Kiba et al., 2011; Figure 1.1 B and Table 1.1). Further, transgenic plants with reduced cytokinin signaling display decreased expression of *CAT1* and *AAP3* (Lee et al., 2007).

The different effects that cytokinins have on the levels of expression of genes encoding amino acids transporters are likely explained by the differences in the ability of these transporters

in facilitating the movement of specific amino acids, as well as their distinct expression patterns in different tissues within plants. In general, those that are upregulated by cytokinin tend to be expressed in sink organs, such as roots and flowers (Okumoto et al., 2004; Su et al., 2004; Tegeder et al., 2011), and some of them, such as *CAT6*, which is expressed in root tips, has been shown genetically to function in supplying sink cells with amino acids (Hammes et al., 2006). *AAP2*, *AAP5*, and *LHT1*, on the other hand, are downregulated by cytokinins, and their function and expression patterns seem to be associated with phloem loading in sources. *aap2* mutants display reduced amino acid content in the phloem, thus suggesting a function in phloem loading (Zhang et al., 2010). *AAP5* expression is observed in source leaves, but not in sink leaves (Fischer et al., 1995, 2002). Similarly, *LHT1* expression is observed mostly in source organs and is likely involved in the transport of amino acids between mesophyll cells and the xylem (Ehness and Roitsch, 1997; Hirner et al., 2006). Experiments outside of the model plant species Arabidopsis have also provided evidence of the association between cytokinins and regulation of the expression of genes involved in amino acid transport (Song et al., 2015; Ninan et al., 2019; Zhu et al., 2020). In addition to these observed changes in expression of amino acid transporter genes in response to cytokinins, corresponding changes in amino acid translocation are also observed. A study in wheat showed that application of the cytokinin BA to source leaves dramatically decreases the content of amino acids present in the phloem, thus suggesting a function in decreasing phloem loading (Criado et al., 2009).

Finally, amino acid and sugar metabolism are connected in several ways, including through the non-proteinogenic amino acid γ-amino butyric acid (GABA). GABA is synthesized through the GABA shunt pathway, named as such because it bypasses two steps of the tricarboxylic acid (TCA) cycle that is essential to the catabolism of sugars for cellular respiration

(Bouche and Fromm, 2004). GABA production through the GABA shunt results from the decarboxylation of the amino acid glutamate, and GABA catabolism leads to the production of succinate that then enters the TCA cycle. Thus, GABA connects amino acid production and sugar utilization. Cytokinins have not been directly associated with GABA production, but plants with increased levels of the cytokinin *trans*-zeatin accumulate GABA at higher levels, and that are correlated to increased drought tolerance (Merewitz et al., 2012).

1.6 CYTOKININS AND SOURCE-SINK RELATIONSHIPS IN THE OUTCOME OF PLANT BIOTIC INTERACTIONS

Although cytokinins are broadly known as plant hormones involved in the regulation of plant growth, in the last few decades, their involvement in plant-pathogen interactions has become evident (reviewed in Albrecht and Argueso 2017; Akhtar et al., 2020). Similarly, a growing body of evidence has accumulated that indicates an important role for nutrient partitioning in creating metabolic conditions that favor or restrict pathogen growth in plant hosts. In the paragraphs that follow, we highlight a role for source-sink relationships in plant biotic interactions, with emphasis on plant-pathogen interactions, and suggest a function for the plant hormone cytokinin in the regulation of this process.

1.7 MAY I OFFER YOU SOMETHING TO EAT? CYTOKININS AND SOURCE-SINK RELATIONSHIPS IN DISEASE SUSCEPTIBILITY

After the successful invasion of the host, plant pathogens use effectors (secreted proteins, secondary metabolites, or nucleic acids of pathogen origin) to colonize the host and create host metabolic conditions that are favorable for pathogens, leading to plant susceptibility. Such

metabolic conditions include the manipulation of plant metabolism to feed the growing number of pathogens that starts to multiply on the infected plant tissue. While some examples exist of studies on the importance of source-sink relationships in the association of plants with necrotrophic pathogens (Lemonnier et al., 2014; Veillet et al., 2016), which are those that kill plant host cells for their nutrition, the majority of studies have focused on the association of plants with biotrophic pathogens, given the dependency of such pathogens on living plant cells as their source of nutrients.

The role of cytokinins in increasing plant susceptibility to pathogen attack has been well documented. This effect is most commonly seen when lower concentrations of cytokinins are applied to plants before pathogen infection (Babosha, 2009; Argueso et al., 2012; Hann et al., 2014). However, in addition to plants, several other organisms can also produce cytokinins or manipulate cytokinin metabolism and/or signaling in plants, including parasitic plants, insects, and plant-associated microbes (reviewed in Spallek et al., 2018). Such microbes include plant pathogenic microbes, able to cause disease on plants, and also beneficial ones, whose association with plants results in enhanced plant growth and protection from disease. By manipulating cytokinin metabolism and/or signaling in plants, such organisms can also potentially regulate plant susceptibility, through manipulation of host physiology.

For the most part, the majority of interactions involving cytokinin production or manipulation by pathogens involve the creation of sink tissues for pathogen nutrition, accompanied by plant developmental changes, such as galls, tumors, and knots, which are usually noted as disease symptoms. Such developmental changes are associated with one of the primary functions of cytokinins, namely, cell division. However, as a secondary effect, these regions of cell proliferation and growth create new sink tissues and thus alter the balance of

source-sink relationships within the plant. A classic example of a plant pathogen that utilizes biosynthesis of cytokinins to create new sink tissues is *Agrobacterium tumefaciens*, the causal agent of crown gall disease. Agrobacterium cells carry a *Tumor- inducing* (*Ti*) plasmid containing the cytokinin biosynthesis gene *trans-zeatin synthesizing* (*tzs*), which is inserted into the plant genome to lead to cytokinin biosynthesis in plant cells (Liu and Kado, 1979; Akiyoshi et al., 1984, 1987; Kutáček and Rovenská, 1991; Lee et al., 2009; Hwang et al., 2010). Along with bacterial-induced auxin biosynthesis, the induction of cytokinin biosynthesis by Agrobacterium results in cell proliferation and the formation of galls. Metabolites needed for gall tumor growth are then rerouted from the host plant source leaves to the crown gall tumor, which becomes a strong sink (reviewed in Gohlke and Deeken, 2014).

Another root gall-forming plant pathogen, the obligate biotroph *Plasmodiophora brassicae*, causes clubroot disease in cruciferous plants. The genome of *P. brassicae* contains two cytokinin biosynthesis genes (Schwelm et al., 2015) that were shown to contribute, albeit in a small manner, to the overall cytokinin content in infected tissue (Malinowski et al., 2016). Infection of Arabidopsis by *P. brassicae* alters the carbohydrate metabolism of the host, resulting in increased sugar and starch content at the site of infection (Williams et al., 1968; Evans and Scholes, 1995; Brodmann et al., 2002). This carbohydrate mobilization was suggested to be due to high localized concentrations of cytokinins, which create a carbohydrate sink (Dekhuijzen, 1980) mediated by the sugar transporters SWEET11 and SWEET12 (Walerowski et al., 2018). However, decreased disease symptoms were seen after *P. brassicae* infection of the cytokinin biosynthesis mutant ipt1;3;5;7 indicating that the pathogen-derived cytokinins are not sufficient to create a sink (Malinowski et al., 2016). The gall-forming bacteria, *Rhodococcus fascians*, is also known to produce cytokinins as part of its virulence strategy (Stes et al., 2013). Pea plants

infected with *R. fascians* show an increase in chlorophyll content, bacterial-produced cytokinins, and endogenous plant-derived cytokinins in infected cotyledons (Depuydt et al., 2008; Dhandapani et al., 2017). Moreover, this is accompanied by an increase in expression of *PsCWINV*, *PsSUT*, and *PsSW* (SWEET) sugar transporter genes (Dhandapani et al., 2017), suggesting that during infection cytokinins may play a role in creating and maintaining infection sites as sinks tissues. A similar relationship is seen between Arabidopsis and the cyst nematode *Heterodera schachtii*. Upon invading plant roots, this species of nematode induces the formation of specialized structures named syncytia. *H. schachtii* was shown to produce and secrete cytokinins during infection of plant cells, and silencing of the *HsIPT* gene encoding the nematode cytokinin biosynthetic enzyme led to decreased syncytia size and decreased nematode size (Siddique et al., 2015). Given that syncytia are essential sites for juvenile feeding, these results implicate cytokinin as a nematode factor that is necessary to establish nematode feeding sites as sinks, promoting pathogen growth. Further, Arabidopsis amino acid transporters AAP3 and AAP6, which belong to a class of amino acid transporters known to be transcriptionally regulated by cytokinins (Brenner et al., 2005; Kiba et al., 2005; Lee et al., 2007), are necessary for infection of Arabidopsis plants by the root-knot nematode *Meloidogyne incognita*, indicating that successful colonization is dependent on amino acid transport to the sites of infection (Marella et al., 2013), in a process that may be mediated by cytokinins.

Plant-pathogen associations involving cytokinins can also contribute to changes in source-sink relationships without the activation of cell division to create sinks. Such an effect of cytokinins can be seen in the formation of green islands, small areas of live and green leaf tissue surrounded by yellow, senescing tissue, in plants infected with biotrophic fungi (Bushnell, 1967). Green islands have an increased cytokinin content within the green areas (López-Carbonell et al.,

1998), which also display increased rates of photosynthesis in comparison with the surrounding senescing tissue (Walters et al., 2008), as well as increased levels of amino acids, sugars, and starch (Raggi, 1974, 1976; Angra and Mandahar, 1991; Angra-Sharma and Mandahar, 1993). These physiological changes in green islands are reminiscent of cytokinin-mediated changes in source-sink relationships mediated by cell wall invertases (Lara et al., 2004), and likely function to maintain these sites as sinks suitable for biotrophic pathogen growth. *Magnaporthe oryzae*, the rice blast fungus, also can produce cytokinin (Chanclud et al., 2016). *M. oryzae* mutants in the cytokinin biosynthetic gene *CKS1* have reduced virulence and are impaired in their ability to multiply in planta, but not in vitro, implicating pathogen nutrition through host-derived mechanisms in the reduced virulence phenotype of the mutant (Chanclud et al., 2016). This cytokinin-dependent virulence was associated with the allocation of sugars and amino acids (namely, aspartate and glutamate) to the sites of infection (Chanclud et al., 2016), thus suggesting a function for cytokinin in acting to change source-sink relationships and nutrient allocation in sites of infection, promoting conditions for pathogen multiplication (Figure 1.2).

On a final note, it is important to mention that beneficial microbes also utilize cytokinins in their association with plants to manipulate source-sink relationships and plant growth. In one of the most well-studied examples, cytokinins are essential for nodule formation during the interaction between Rhizobia bacteria and legume plants. In such interactions, plants redirect photoassimilates, mainly in the form of sucrose, to the bacteria, in exchange for organic nitrogen (Kennedy, 1966; Bergersen and Turner, 1967; Kouchi and Yoneyama, 1984). Root nodules can then be classified as sink organs, which require cytokinin for their formation. In *Medicago truncatula*, this requirement for cytokinins is mediated by the ABC transporter ABCG56, which functions as a cytokinin exporter and is required for nodule formation (Jarzyniak et al., 2021). In

addition, plant-derived cytokinins are also needed for the activity of certain volatile organic compounds produced by beneficial rhizobacteria, which induce plant growth. This cytokinin-dependent, rhizobacteria-mediated plant growth is associated with increased photosynthesis and nutrient acquisition, thus linking it to source-sink relationships (Ryu et al., 2003; Zhang et al., 2008; Gutiérez-Luna et al., 2010; Vacheron et al., 2013; Ditengou et al., 2015; Cordovez et al., 2018).

1.8 STARVING THE ATTACKER AND SOUNDING THE ALARM: CYTOKININS AND SOURCE-SINK RELATIONSHIPS IN DEFENSE RESPONSES

In addition to a role in creating sink tissues for pathogen nutrition, accumulating evidence also exists for the role of source-sink relationships in defense responses as well. In Arabidopsis, regulation of the sugar transporter STP13 leads to altered susceptibility to pathogens (Yamada et al., 2016). *STP13* is expressed in leaf tissues after infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. Its transport activity was shown to be suppressed via phosphorylation by a protein complex composed of the extracellular immune receptor FLS2 (FLAGELLIN SENSITIVE 2) and its associated kinase BIK1 (BRASSINOSTEROID INSENSITIVE 1). Thus, upon the perception of pathogen presence by the FLS2/BIK1 complex, plants diminish STP13 activity, effectively halting sugar transport to the apoplast and preventing pathogen feeding and multiplication (Yamada et al., 2016). STP13 is also important for resistance to the necrotrophic pathogen *Botrytis cinerea*, although it is unknown whether the regulatory mechanisms cited above also apply (Lemonnier et al., 2014).

Other examples of source-sink relationships being modified for defense responses to pathogens, rather than pathogen feeding, include the genes encoding proteinaceous invertase

inhibitors. Proteinaceous invertase inhibitors are endogenous plant signals for invertase regulation in plants. In response to *Pseudomonas syringae* pv. *tomato* DC3000 infection, the expression of genes encoding these invertase inhibitors in Arabidopsis is downregulated, a fact that has always been interpreted as manipulation of plant metabolism by the pathogen to increase glucose and fructose availability for pathogen nutrition (Bonfig et al., 2006). However, the activity of these invertase inhibitors has been shown to in fact increase in infected resistant plants, thus functioning as a defense mechanism to prevent the pathogen from cleaving sucrose for its nutritional needs (Bonfig et al., 2010). Finally, the sugar transporters SUT1 and SUT2 in tomatoes have also been connected to defense responses in plants. *SUT1* and *SUT2* expression are downregulated during the infection of tomato plants with *Candidatus Phytoplasma solani*, an obligate biotrophic bacterial pathogen that inhabits host phloem cells (De Marco et al., 2021). Antisense analyses of *SUT1* and *SUT2* genes in tomatoes showed that the absence of SUT1 and SUT2 function decreases the susceptibility of tomato plants to this pathogen, without compromising plant growth, and at the same time increasing the expression of defense genes (De Marco et al., 2021), thus connecting source-sink relationships to defense activation. While a function for cytokinins in the control of source-sink relationships for pathogen nutritional deprivation has not yet been demonstrated, the general importance of this plant hormone in the physiological processes cited above makes it a likely candidate for such regulatory action.

In further agreement with a general role for source-sink relationships in defense is the fact that not only changes in sugar allocation but also changes in amino acid allocation, lead to altered susceptibility to pathogens. Mutations or overexpression of genes encoding amino acid transporters can also lead to decreased susceptibility to pathogens. This is the case, for example, of the cytokinin-regulated amino acid transporter gene *LHT1*. *lth1* mutants display decreased

susceptibility to *Pseudomonas syringae* p.v. *tomato*, as well as the hemibiotrophic fungus *Colletotrichum higginsianum* and the biotrophic fungus *Golovinomyces cichoracearum* (Liu et al., 2010). Overexpression of the gene encoding the amino acid transporter *CAT1*, whose expression is also transcriptionally regulated by cytokinins (Kiba et al., 2005), leads to a decrease in susceptibility to *Pseudomonas syringae* p.v. *tomato* (Yang et al., 2014). Most recently, a mutation in the gene encoding the amino acid transporter USUALLY MULTIPLE ACIDS MOVE IN AND OUT 36 (UMAMIT 36) was shown to confer resistance to the oomycete *Phytophthora parasitica* (Pan et al., 2016), and overexpression of *UMAMIT 14* was shown to decrease susceptibility of Arabidopsis to another oomycete, *Hyaloperonospora arabidopsidis* (Besnard et al., 2021). What is interesting about the examples cited above is that the decreased susceptibility phenotypes of the lines with altered amino acid transporter genes are also accompanied by an increase in the endogenous levels of the defense hormone salicylic acid (SA), and elevated basal levels of the known SA defense marker gene *PATHOGENESIS-RELATED-1* (*PR-1*). Thus, the decrease in pathogen susceptibility is likely not due to altered amino acid transport leading to nutritional deprivation but is in fact due to activation of plant defense pathways and responses.

Amino acids are directly linked to the production of secondary metabolites with important roles in defense, such as glucosinolates and camalexins (derived from tryptophan), SA biosynthesis (derived from phenylalanine), and the biosynthesis of a primer of defense responses, pipecolic acid (derived from lysine). Therefore, it would be reasonable to conclude that these genetic alterations on amino acid transporter genes lead to changes in the cellular amino acid pool, with consequences to the biosynthesis of defense compounds and defense activation. Counterarguments to this amino acid pool hypothesis are several: (i) the fact that the amino acid

transporter genes linked to altered pathogen responses are not directly linked to the transport of the particular amino acids necessary for the corresponding defense compounds; (ii) that the resistance observed seems to be broad spectrum, and not associated with the effect of a particular defense compound; (iii) that increased levels of GABA, the non-proteinogenic amino acid involved in connecting N and C metabolism, are also associated with abiotic stress tolerance and resistance to pests (Seifikalhor et al., 2019; Tarkowiski et al., 2020); and (iv) and most importantly, that alterations in sugar transport and signaling also seem to activate defense responses similarly to changes in amino acid homeostasis (Gebauer et al., 2017; De Marco et al., 2021). Such counterarguments favor another hypothesis, where cellular metabolic alterations may lead to the activation of defense responses, through a mechanism similar to metabolic priming. Priming is an activated state where plants can deploy stronger and faster defenses, resulting in enhanced pathogen protection (reviewed in Mauch-Mani et al., 2017), and the idea of metabolic priming for defense responses has recently been further investigated (Liu et al., 2010; Schwachtje et al., 2018, 2019).

The concept of metabolic priming shares remarkable similarities with the effect of cytokinins on plants. When applied in high concentrations to plants cytokinins can also lead to reduced susceptibility to a broad spectrum of pathogens (reviewed in Akhtar et al., 2020). This is accompanied by the increased production of antimicrobial compounds, such as phytoalexins (Ko et al., 2010; Grosskinsky et al., 2011), and also the production of reactive oxygen species (ROS) and increased defense gene expression, in a manner that is dependent on the defense hormones SA (Choi et al., 2010; Argueso et al., 2012; Naseem et al., 2012) and jasmonic acid (Gupta et al., 2020). Of note, similarly to what happens in defense priming, these responses to cytokinin only

happen after pathogen detection. Therefore, cytokinins do not directly activate responses; rather, they trigger physiological conditions that potentiate defense.

The two hypotheses mentioned above, namely, changes in photoassimilate availability altering the production of defense compounds or changes in photoassimilate availability altering cellular metabolic stress leading to priming, are not mutually exclusive. Both hypotheses could be parts of an integrated plant defense response involving the regulation of source-sink relationships, coordinated by the plant growth hormone cytokinin (Figure 1.2). In this context, cytokinin levels, through their general effect on source-sink relationships, would serve as a signal for changes in cellular and organismal metabolism that would activate defense. Such a mechanism would likely be beneficial to plants, as it would provide a way to connect defense activation to photoassimilate production, depending on fluctuating environmental conditions.

1.9 CONCLUSIONS AND PERSPECTIVES

Because plants are sessile organisms, their ability to effectively respond to environmental change is vital to their survival. To maintain proper growth and development, plants have adapted response mechanisms to regulate photosynthetic ability and photoassimilate partitioning, depending on environmental conditions, such as light intensity, temperature, and water availability. Just like other plant hormones that act on the regulation of cell expansion or cell division, cytokinins have long been associated with the promotion of plant growth. In the case of cytokinins, the ability to promote greening and increasing photosynthesis rates is likely also involved in its stimulation of plant growth and yield, as this is centrally linked to the generation of more photoassimilates for plant growth. Further, how these photoassimilates are distributed in the plant are just as important for plant growth and yield, and it is in this aspect that the

regulation by cytokinins of source-sink relationships plays a significant role, so much so that genes involved in aspects of cytokinin metabolism and signaling have been a frequent target of crop breeding programs centered on yield improvement (White et al., 2016). Because breeding programs target increased yields in different parts of the plant (seed, fruits, and vegetative organs) depending on the crop, the role of cytokinins in regulating sugar and amino acid transporters with tissue-specific patterns of expression may be of particular interest and importance.

Similarly, response to pathogen attack also requires complex responses by plants. To do so, plants have evolved sophisticated perception and signaling strategies, often mediated by plant hormones, including cytokinins. Timing and degree of defense activation must be tightly controlled, as insufficient defense responses could lead to host death, whereas excessive defense may result in inhibition of plant growth (reviewed in Albrecht and Argueso, 2017). The maintenance of balanced source-sink relationships is therefore vital to sustain growth while ensuring proper defense response against the pathogen. Evidence for the importance of this balanced response comes from the fact that pathogens have developed mechanisms of manipulation of source-sink relationships, to obtain nutrients for growth and multiplication. As it is common in the always-evolving arms race between plants and pathogens, plants have also evolved ways to manipulate these source-sink relationships for defense purposes, and there is evidence that both processes may be partly regulated by cytokinins. Given the negative effect of plant pathogens on plant growth and yield, and the importance of photoassimilate partitioning to plant susceptibility and resistance, investigating the role of cytokinin-mediated source-sink relationships in the context of plant-pathogen interactions may provide new avenues not only for yield improvement, but also for pathogen resistance.

1.10 AUTHOR CONTRIBUTIONS

KM wrote the manuscript and prepared figures. CA wrote and edited the manuscript and figures. DB edited the manuscript. All authors contributed to the article and approved the submitted version.

1.11 FIGURES

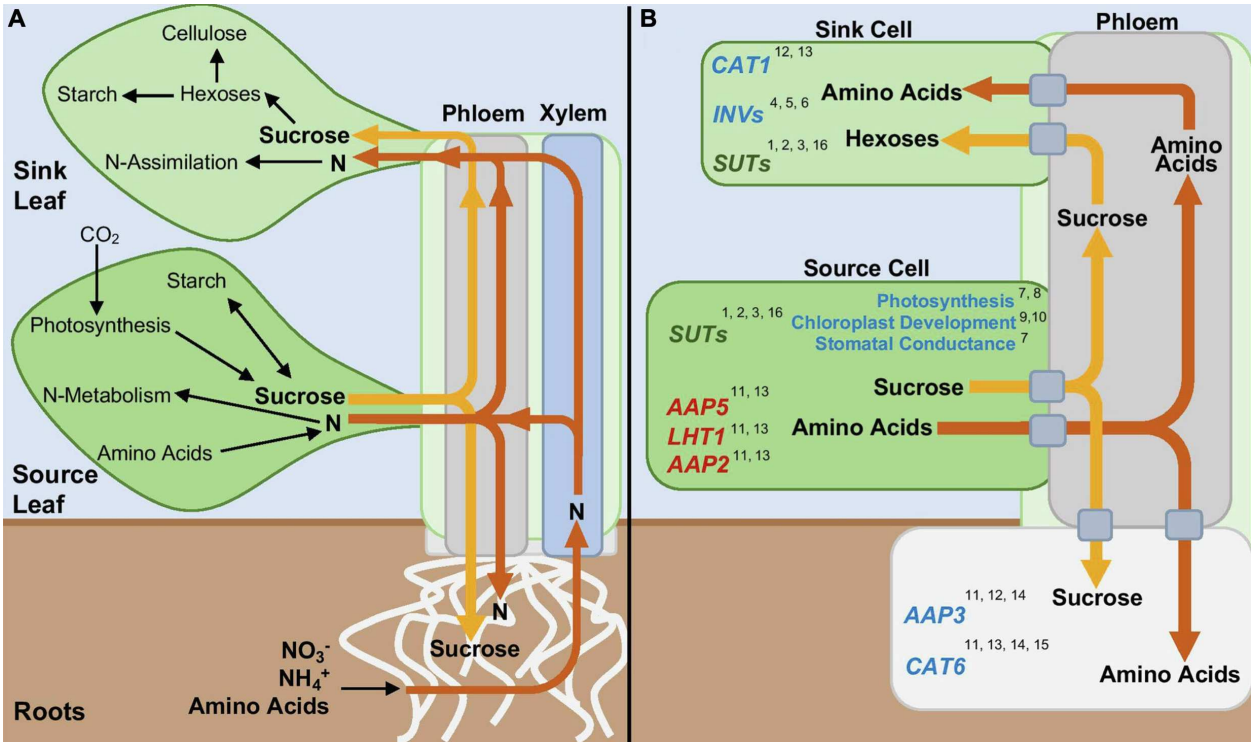

Figure 1.1 | Source-Sink Relationships in Plants. **(A)** Whole plant movement of nitrogen containing compounds (N, orange arrows) and sugars (yellow arrows) between source and sink tissues. **(B)** Regulation by cytokinin of specific enzymes, transporters, and processes involved in source-sink relationships. Blue and red symbolize positive or negative regulation by cytokinin, respectively. Green symbolizes both a positive and negative regulation by cytokinin. Numbers correspond to references. **References:** 1. Harms et al., 1994; 2. Ninan et al., 2019; 3. Song et al., 2015; 4. Ehness and Roitsch, 1997; 5. Godt and Roitsch, 1997; 6. Yang et al., 2014; 7. Chernyad'ev, 2000; 8. Ahanger et al., 2018; 9. Boasson and Laetsch, 1969; 10. Okazaki et al., 2009; 11. Brenner et al., 2005; 12. Lee et al., 2007; 13. Kiba et al., 2011; 14. Kiba et al., 2005; 15. Yokoyama et al., 2007; and 16. Jian et al., 2016.

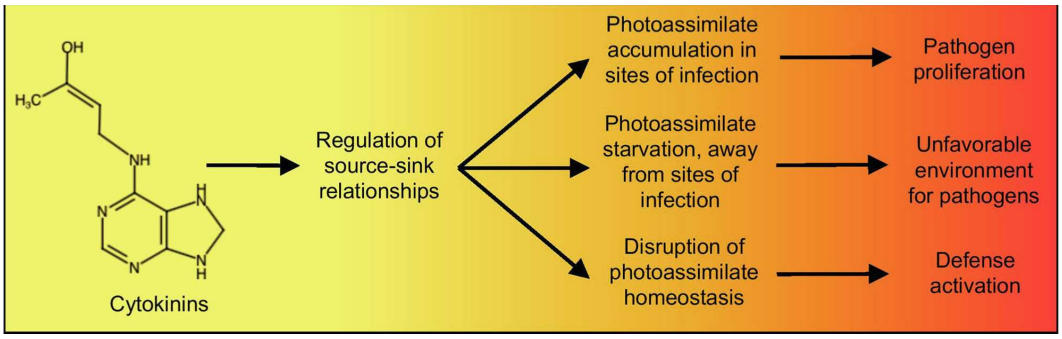

Figure 1.2 | Summary of the proposed effect of cytokinins, through their role in the regulation of source-sink relationships, on the outcome plant-pathogen interactions. Chemical structure created using http://chem-space.com.

1.12 TABLE

Table 1.1 | Transcriptional regulation by cytokinins of genes encoding invertases, sugar, and amino acid transporters in various plant species.

Species	Gene	Gene description	CK regulation
Carbohydrate-related			
Zea mays	*IVR1*	Vacuolar invertase	+
	IVR2	Vacuolar invertase	+
Chenopodium rubrum	*CIN1*	Extracellular invertase	+
Solanum lycopersicum	*LIN6*	Extracellular invertase	+
Sucrose transport			
Arabidopsis thaliana	*CST2*	Hexose transporter	+
	CST3	Hexose transporter	+
Pisum sativum	*PsSW12*	Sucrose transporter	–
	PsSUT1	Sucrose transporter	–
	PsSUT2	Sucrose transporter	–
Solanum tuberosum	*StSUT1*	Sucrose transporter	+
Brassica napus	*BnSUT1*	Sucrose transporter	+
	BnSUT2	Sucrose transporter	+
	BnSUT3	Sucrose transporter	+
	BnSUT4	Sucrose transporter	+
Amino acid transporter			
Arabidopsis thaliana	*AAP2*	Amino acid transporter	–
	AAP3	Amino acid transporter	+
	AAP5	Amino acid transporter	–
	CAT1	Amino acid transporter	+
	CAT6	Amino acid transporter	+
	LHT1	Amino acid transporter	–
Brassica napus	*BnAAP1*	Amino acid transporter	–
	BnAAP2	Amino acid transporter	–
	BnAAP4	Amino acid transporter	–
	BnAAP5	Amino acid transporter	–
	BnAAP6	Amino acid transporter	–
	BnAAP7	Amino acid transporter	–
	BnAAP8	Amino acid transporter	–
Pisum sativum	*PsAAP3*	Amino acid transporter	–
	PsAAP6a	Amino acid transporter	–
	PsAAP7b	Amino acid transporter	–
Oryza sativa	*OsAAP1*	Amino acid transporter	+
	OsLHT1	Amino acid transporter	+

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CHAPTER 2:

CHARACTERIZATION OF CYTOKININ-INDUCED PRIMING

2.1 SUMMARY

Priming of plant defenses is a plant response in which, following the application of certain chemical or biological agents, a primed plant responds by displaying a faster and more robust defense response to pathogen challenge than an unprimed plant. The plant hormone cytokinin (CK), mostly recognized as a regulator of plant development, has also been shown to have a role in plant defense. CK has been used as a priming agent for plant defense, however, a detailed characterization of its priming role is lacking. Using *Arabidopsis thaliana* as a model species, we uncover the timing of CK-induced priming against a bacterial pathogen, *Pseudomonas syringae*, in which this response may be transgenerational, and that other known priming agents require endogenous CK signaling for their priming function. Finally, we show that CK-induced priming is a conserved process that can be elicited in a related species, *Brassica napus*.

2.2 INTRODUCTION

Vascular plants have existed for millions of years as sessile organisms, and thus have evolved complex mechanisms to sense and respond to their changing environment, including the presence of pathogens. To respond appropriately to pathogenic organisms, plants have developed several defense strategies, which include direct or indirect recognition of the presence of pathogens leading to defense activation, or mechanisms that prepare the plant for future

pathogenic challenges, without strong activation of defense responses. The first strategy involves the recognition of Pathogen-Associated Molecular Patterns (PAMPs) by cell-surface receptors leading to Pattern-Triggered Immunity (PTI), or recognition of pathogenic effectors by mostly intracellular receptors, known as Effector-Triggered Immunity (ETI). Activation of either PTI or ETI initiates signaling cascades that lead to defense responses that contain the pathogen infection. The second strategy is a process known as priming, where exposure to specific chemical or biological agents prepares the plant for a future pathogen challenge. Recognition of the priming stimulus leads to a low level of defense activation, which is amplified upon pathogen attack, resulting in faster and more robust defense responses in primed plants as compared to unprimed plants (Figure 1.1) (Conrath et al., 2015, Mauch-Mani et al., 2017). Furthermore, direct and constitutive plant defense activation often leads to a detrimental effect on plant growth (Tian et al., 2003, Kempel et al., 2011, Denance et al., 2013), whereas priming has been shown to have a minimal negative effect (van Hulten et al., 2006, Worrall et al., 2012, Takatsuji 2014). Well-characterized chemical priming agents include the non-proteogenic amino acids β-aminobutyric acid (BABA) and L-pipecolic acid (Pip), as well as benzothiadiazole (BTH), which can be applied to plants by either soil drench, foliar spray application, or direct injection into leaves (Lawton et al., 1996, van Hulten et al., 2006, Navarova et al., 2012), often resulting in a reduction in susceptibility to a broad spectrum of pathogens (Zhou and Wang 2018, Zhang et al., 2020). Studies exploring the impact of BABA and BTH priming treatments on plant growth have shown a negative impact on seed yield (Heil et al., 2000, Jakab et al., 2001, van Hulten et al., 2006). However, when either priming treatment is applied at lower concentrations that do not activate defense responses the overall seed yield is similar to control-treated Arabidopsis plants (van Hulten et al., 2006). Research on the impact of Pip-induced priming on plant growth is

severely limited, yet one study of Arabidopsis seedlings grown on media containing Pip showed an inhibitory effect on root growth (Wang et al., 2018). Although these few studies show that priming of plant defense has a marginal effect on plant growth, phenotypic characterization in greater detail is needed.

Studies of well-known priming agents have revealed that most of them activate defense responses regulated by the plant hormone salicylic acid (SA), such as increased expression of *PATHOGENESIS-RELATED-1* (*PR1*) (Lawton et al., 1996, Ton and Mauch-Mani 2004, Navarova et al., 2012, Floryszak-Wieczorek et al., 2015). SA is well-recognized as being a master regulator of plant defense, mainly against hemibiotrophic or biotrophic plant pathogens (Thomma 1998, Ding and Ding 2020). However, priming agents can also induce resistance against pathogens of necrotrophic lifestyle, indicating that other plant hormones are likely involved in the process of priming plant defenses. The plant hormone cytokinin (CK), is involved in plant growth and development (Kieber and Schaller 2018), but more recently the function of CK has extended beyond plant development to include a role in plant-pathogen interactions. Plants lacking two of the three CK receptors are more susceptible to various pathogens, including the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) (Choi et al., 2010), the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) (Argueso et al., 2012), and the necrotrophic fungus *Botrytis cinerea* (Li et al., 2021), suggesting that CK may have a broad role in plant defense.

In addition to the role of endogenous CK in plant defense, exogenous application of CK to plants also alters defense responses. For example, the application of micromolar concentrations of CK to Arabidopsis (Choi et al., 2010, Argueso et al., 2012), tomato (Gupta et al., 2020b), rice (Zhang et al., 2022), and tobacco (Grosskinsky et al., 2011) decreases

susceptibility to *Pst*, *B. cinerea*, *Nilaparvata lugens*, and *Pseudomonas syringae* pv. *tabaci*, respectively. The decrease in pathogen susceptibility seen in CK-treated plants was not through direct activation of plant defenses, indicating that CK functions as a priming agent, a process we have named CK-induced priming (CIP). Although examples of CIP have been documented, a detailed characterization of the process leading to CIP is lacking. For example, most studies have shown that CIP is effective at reducing susceptibly 24 to 48 hours (H) after CK application, but it is unknown whether the priming effect of CK is extended beyond 48H. Further, there have been no studies to determine any effects on plant growth due to CIP, which is necessary to determine if CIP can be used effectively in agricultural settings. Moreover, even though CIP has been demonstrated in several pathosystems, the effectiveness of CIP in different plant species is difficult to determine, due to the variable experimental and environmental conditions used between studies.

Here, we aimed to gain more understanding of CIP by first determining the timing of its effectiveness after CK application, whether other priming agents require endogenous CK signaling, and if CIP has transgenerational effects. To determine possible plant growth effects induced by CIP, we measured various plant growth parameters following single or multiple CK applications. We then compared these CIP responses in Arabidopsis to CIP elicited in another member of the Brassicaceae family, *Brassica napus,* to determine whether CIP is a conserved plant process.

2.3 METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0, wild type) and transgenic seeds in Col-0 background were stratified for 2-4 days at 4ºC before being placed on soil. The mutant line

used in the study was *ahk2-7 ahk3-2* (*ahk2,3*) (Argyros et al., 2008). All plants were grown in soil in either a Conviron growth chamber (Model# ATC60) or an Environmental Growth Chamber (Model #M25 R-401A) at Colorado State University Plant Growth Facility. Plants were grown in either Pro-mix HP Mycorrhizae or Sunshine Mix #4 soil for 5-7 weeks under a 10:14 H day:night light regime, at 160 ± 20 μmol m-2s-1, at 21ºC, 50% relative humidity (RH) in the day and 19ºC, 60% RH at night.

Cytokinin-Induced Priming Treatments

A stock solution of 100mM cytokinin (CK) was made by dissolving 6-benzylaminopurine (BA) (Sigma Aldrich) into dimethyl sulfoxide (DMSO) and stored at -20°C for up to three months. 100μM BA priming solutions were prepared by diluting the 100mM BA stock into water, and adding 0.002% Silwet L-77 (Lehle Seeds). Control plants were sprayed until run-off with an aqueous solution containing corresponding amounts of DMSO, plus 0.002% Silwet L-77. Plants were placed into different flats based on treatment and genotype. Following spray treatments, transparent plastic domes were placed over plants for 24 H to prevent rapid evaporation. Plants were inoculated with pathogens 48 H after priming or mock treatment, unless otherwise noted.

β-aminobutyric acid and L-pipecolic acid Priming Treatments

20mg/mL β-aminobutyric acid (BABA) (Sigma Aldrich) and 1 mM L-pipecolic acid (Pip) (Sigma Aldrich) solutions were made by dissolving the powdered chemicals directly in diH_2O. 5- to 6-week-old plants were grown in individual disposable pots and separated into different flats based on treatment and genotype. To induce BABA- or Pip-induced priming,

20mL of diH_2O, 20mg/mL BABA, or 1mM Pip were supplied directly to the soil of plants avoiding contact with shoot tissue. Transparent plastic domes were placed over plants for 24 H to prevent rapid evaporation. Plants were inoculated with pathogens 48 H after priming or mock treatment, unless otherwise noted.

Bacterial Disease Assays in Arabidopsis thaliana

Arabidopsis leaves were infiltrated with a bacterial suspension as described by (Tornero and Dangl 2001) with the following noted changes. *Pseudomonas syringae* pv. *tomato* DC3000 containing an empty vector with kanamycin resistance marker (*Pst*) (Tornero and Dangl 2001) were grown on King's B media supplemented with rifampicin (50mg/mL) and kanamycin (50mg/mL). Bacteria were resuspended in 10mM $MgCl_2$ for a bacterial concentration of $1x10^5$ colony forming units (CFU)/mL. Plants were inoculated by leaf infiltration with a needleless syringe on four fully developed leaves per plant. After bacterial infiltration, plants were covered with a lightly sprayed dome for 24 H. The amount of *in planta* bacterial growth was quantified at 1 H post-inoculation (0 dpi) and at 3 days post-inoculation (3 dpi). One leaf disc was collected from four different plants for each time point and ground together in 10mM $MgCl_2$. Serial dilutions were plated onto KB media containing kanamycin (50mg/mL) and rifampicin (50mg/mL) at 1 hpi, or rifampicin (50mg/mL) and cycloheximide (100mg/mL) at 3 dpi, and used to determine the CFU per cm^2 of leaf tissue.

Bacterial Disease Assays in Brassica napus

Pseudomonas syringae pv. *maculicola* ES4326 (*Psm*) was grown on KB media plates supplemented with rifampicin (50mg/mL). On the day of inoculation, bacteria were resuspended

in 10 mM $MgCl_2$ for a concentration of $1x10^5$ CFU/mL. One fully developed leaf (number 2 or 3) per plant was inoculated by infiltration with a needleless syringe, totaling eight inoculated plants per treatment. The amount of *in planta* bacterial growth was quantified at 1 H post inoculation (0 dpi) and at 4 days post inoculation (4 dpi). Leaf discs from four plants were pooled for one sample, four samples were collected for each treatment at each time point. Leaf discs were ground in 10mM $MgCl_2$ and serial dilutions plated onto KB media containing rifampicin (50mg/mL) to determine the number of CFU per cm^2 of leaf tissue.

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted using RNeasy Plant kit (QIAGEN), following manufacturer's instructions. The quality and integrity of RNA was assessed by A_{260}/A_{280} and A_{260}/A_{230} ratios having a value of greater than 1.7. RNA samples of good quality underwent DNase Treatment using TURBO DNase-Free (Invitrogen) as per the manufacturer's instructions and were checked for the absence of genomic DNA by qRT-PCR using primers for AT5G66770 (For 5'-GGTTTGGTTTGGTTATCGCCAGGA-3', Rev 5'-TGGCTTCATCTCTTTGGCCTGGA-3'). cDNA was synthesized using Qscript Supermix (QuantaBio) and checked for full-length cDNA synthesis through qRT-PCR using primers for *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE GAPDH* (AT1G13320). Primers used were: *GAPDH-1* (For 5'-TAGATCGCTCGGAACTTGGAAA-3', Rev 5'-CCTCACCAAAACTCAAATCACTCC-3'); *GAPDH-3* (For 5'-AACTAGGACGGATCTGGTGCCT-3', Rev 5'-GCTATCCGA ACTTCTGCCTCATTAT-3'), and *GAPDH-5* (For 5'-AAATTTAAC GTGGCCAAAATGATGC-3', Rev 5'-GTTCTCCACAACCGCTTGGT-3'). qRT-PCR reactions were performed with PerfeCTa SYBR Green (QuantaBio) on a CFX Connect Real-Time System

(BioRad). cDNAs with Ct/Cq differences between each GAPDH primer of less than 1.5 were considered fully extended and of good quality. AT4G05320 *UBIQUITIN10* (*UBQ10)* was used as a housekeeping gene in all reactions (For 5'-CGTTAAGACGTTGACTGGGAAAACT-3', Rev 5'-GCTTTCACGTTATCAATGGTGTCA-3'). Gene specific primers used are listed in Table 2.1. At least three biological replicates of each experiment were obtained unless otherwise stated.

Transgenerational Effects of Cytokinin-Induced Priming

Col-0 plants were grown in long-day conditions to promote flowering with a light regime of 16:8 H day:night at 160 ± 20 μmol m-2s-1, at 21ºC and 60% relative humidity (RH) in the day and 19ºC, 70% RH at night. Three-week old plants were spray-treated until run-off with either a mock control solution or a solution of 100μM CK, every other day for one week, for a total of 3 treatments with 100μM CK or mock solution just prior to bolting. Individual plants were kept separate by the use of Aracons (plastic cylinders surrounding the stems and reproductive structures) to prevent any loss of seed during seed set. Seeds were collected from individual parental plants and progeny seeds were used to test for susceptibility against *Pst* following 6 weeks of growth in short-day conditions as described above.

Vegetative Growth Experiment in Arabidopsis thaliana

Three-week-old plants were sprayed with DMSO mock solution or 100μM CK solutions, one time or once every three days. Plants were grown for a total of six weeks before being photographed and harvested for measurements. Rosette area was measured using the Fiji software (Schindelin et al., 2012). Fresh weight was measured with a Sartorius scale (Model

ENTRIS822-1S). Following fresh weight measurements, the entire shoot and root tissue were placed in a paper bag in an oven at 65ºC to dry for 4 days. Dry weight was measured with Ohaus Scout® scale (Model SPX222). A total of 18 plants were measured for each treatment group.

Vegetative Growth Experiment in Brassica napus

Two weeks after seeds were sowed, after the emergence of true leaves, *B. napus* plants were sprayed with DMSO mock solution or 100μM CK solutions one time or every four days, totaling eight spray treatments until plants were 8-weeks-old. Fresh weight was measured with a Sartorius scale (Model ENTRIS822-1S). Shoot and root tissue were placed in a paper bag and placed in an oven at 65ºC to dry for 4 days. Dry weight was measured with Ohaus Scout® scale (Model SPX222). A total of 20-22 plants were measured for each treatment group.

Statistical Analysis:

For experiments with two comparisons, a two-way ANOVA with TUKEY HSD *p*-value correction was used and for experiments with one comparison a Student's T-test or one-way ANOVA was used to evaluate statistical significance. The significance of these tests was based on a p-value ≤ 0.05.

2.4 RESULTS

Cytokinin-induced priming is a late physiological response

Previous studies on cytokinin-induced priming (CIP) have shown that one application of CK to plants 24 or 48 H before pathogen inoculation is sufficient to activate plant defenses and reduce pathogen multiplication (Choi et al., 2010, Argueso et al., 2012). To better understand the

process of CIP, we decided to determine how long one application of CK is effective in reducing susceptibility to pathogens.

Five-week-old Arabidopsis plants were treated with one spray application of 100μM CK or a mock solution. Plants were inoculated with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) by syringe infiltration at 10^5 CFU/ml at 3 H, 24 H, 48 H, 72 H, 1, and 2 weeks after a single priming treatment. For all CK priming treatments, *in planta* bacterial multiplication was assessed at 3 days post-inoculation (dpi) with *Pst* (Figure 2.2). With three hours of CK priming, a measurable although non-significant reduction in *Pst* multiplication was observed in CK-primed plants. On the other hand, 24 and 48 H after priming resulted in a strong reduction of *in planta* bacterial multiplication in CK-treated plants, supporting previously published results (Argueso et al., 2009, Choi et al., 2010, Gupta et al., 2021). No significant priming effect was observed in plants inoculated at 72 H, 1 or 2 weeks after CK priming. These results demonstrate that CIP does not take place immediately after hormone application, peaking at 24-48 H after CK application, and likely encompasses late changes in plant defense responses.

CK signaling occurs shortly after exogenous CK application, peaking at 45 minutes after CK exposure (D'Agostino et al., 2000). To address the correlation between cytokinin signaling and the timeframe when CIP is most effective at reducing susceptibility to *Pst*, we measured the expression over time of known CK marker genes following CK application. Five-week-old Arabidopsis plants were treated with one spray application of 100μM CK, or mock solution, and tissue was harvested from individual plants at 1 H, 2 H, 24 H, 48 H, and 1 week after treatment. The expression of the cytokinin marker genes *ARABIDOPSIS RESPONSE REGULATOR 5* (*ARR5*), *CYTOKININ RESPONSE FACTOR 2* (*CRF2*), and *EXPANSIN 1* (*EXP1*), known to be primary targets of type-B ARR transcription factors that mediate CK signaling (Argyros et al.,

2008, Zubo and Schaller 2020), was determined by qRT-PCR. The results in Figure 2.3 show that CK signaling is up-regulated beginning at 1 H after CK treatment, peaks at 3 H after treatment, and then decreases by 24 H, being mostly gone by 72 H after CK treatment. These results indicate that CIP results from physiological changes downstream from CK primary signaling.

Cytokinin is the basis of priming activity

Although priming agents like BABA and Pip have differences in their respective molecular mechanisms that result in primed plant defenses, they share a common outcome in that the application of these priming chemicals induces expression of *PR1* to a higher level following pathogen challenge than their respective control-treated plants (Zimmerli et al., 2000, Bernsdorff et al., 2016, Yildiz et al., 2021). Likewise, priming by CK has also been shown to potentiate the expression of *PR1* following a challenge by *Pst* and *Hpa* (Choi et al., 2010, Argueso et al., 2012). Due to this commonality between priming agents, we tested the dependence of BABA- and Pip-induced priming on functional CK signaling. Five-week-old wild type and a CK signaling mutant harboring mutations in two of the three genes encoding CK receptors (*ahk2,3*) were primed by a soil drench application of aqueous solutions of 20mg/mL BABA or 1mM Pip, or a water control, 48 H before *Pst* infiltration (Figure 2.4). As expected, BABA and Pip-treated Col plants displayed a decrease in *in planta* bacterial growth as compared to the water-treated plants. However, *ahk2,3* plants did not show priming, with no observed difference in bacterial concentrations between *ahk2,3* plants treated with water or the two priming agents. These results suggest BABA and Pip require endogenous CK signaling to prime plants against *Pst* pathogen

challenge. Therefore, it is possible that CK signaling is a general requirement for priming of plant defenses.

Cytokinin-induced priming displays a transgenerational effect in progeny

Some priming agents can increase defense in the progeny of primed plants, a term coined transgenerational priming (Slaughter et al., 2012). For example, the treatment of parental plants with BABA led to decreased susceptibility of their progeny to *Pst* and *Hpa* in Arabidopsis (Slaughter et al., 2012) and to *Pseudomonas syringae* pv. *phaseolicola* in beans (Ramirez-Carrasco et al., 2017). The fact that the priming agents BABA and Pip both depend on CK signaling and that exogenous application of CK results in priming, raises the possibility that, similarly to these priming agents, CIP could also be transgenerational.

To test this hypothesis, wild type plants were grown in long-day conditions (16:8 H day:night light regime) to promote flowering. At 3-weeks-old, plants were treated every other day with 100μM CK or mock solution, totaling three treatments before bolting (Figure 2.5 A). Seeds were collected from individual plants at the end of their life cycle, approximately 4 weeks later. The progenies of individual CK- or mock-treated plants were then grown in short-day conditions, and at 4 weeks old were inoculated with *Pst* by infiltration, as described above. This experiment was replicated independently 3 times.

As seen in Figure 2.5 B, the CK-primed progeny from biological replicates 1 and 3 showed a statistically lower bacterial concentration at 3 dpi when compared to those progenies from mock-treated plants. A similar trend was seen in biological replicate 2, however, no statistical significance was observed between treatment groups. The table in Figure 2.5 C displays the difference at 3 dpi of *in planta* bacterial levels between the individual progenies of mock-treated parents or CK-treated parents in each biological replicate and the resulting *p*-value.

Pooling of the results from the three biological replicates revealed a difference between the progeny of mock- and CK-treated parents (Figure 2.5 D). These results indicate a trend for CIP to exert a transgenerational effect, however, this transgenerational effect of CK treatments is variable.

Cytokinin-induced priming negatively affects Arabidopsis growth

The model of priming of plant defense asserts that, after priming treatment (priming phase) plants undergo a low level of defense activation, and then upon pathogen challenge defenses are significantly increased (post-priming phase), resulting in decreased pathogen susceptibility (Figure 2.1). The model suggests that due to a low level of defense activation during the priming phase, the balance of plant growth and defense is not disrupted, thus the priming treatment alone should not have a substantial effect on plant growth (van Hulten et al., 2006, Cooper and Ton 2022). In contrast, plants that have constitutively activated immune responses often display negative impacts on growth and development, resulting in dwarfed phenotypes (Kempel et al., 2011, Denance et al., 2013, Albrecht and Argueso 2017). This is due to the inverse relationship between plant growth and plant defense, also known as the growth-defense-tradeoff (He et al., 2022).

To evaluate whether priming by CK alters plant growth, wild type plants grown in short-day conditions (10:14 H day:night light regime) were sprayed one time with solutions of 100μM CK or a mock solution at 3 weeks old, and shoot tissue collected 3 weeks later. Figure 2.6 A-D shows that one CK application decreases rosette area and fresh and dry weight, as compared to mock-treated plants. Given that CIP is effective at reducing susceptibility to *Pst* up to 72 H after CK application (Figure 2.2), we tested whether the growth effects due to CK application would

be exacerbated by maintaining plants in a CK-primed state for an extended period. To test this hypothesis, 3 week old plants grown under short-day conditions were treated 3 times with 100μM CK or a mock solution every 3 days, and shoot tissue was collected for measurements 3 weeks later. Figure 2.6 E-H shows that multiple CK treatments further decrease rosette area and fresh and dry shoot weight as compared to a single CK treatment.

We then addressed whether CK priming also had an effect on reproductive tissues. Seed yield from each of the three biological replicates of the transgenerational priming experiment (Figure 2.5) was measured, from both CK- and mock-treated plants (Figure 2.7). CK treatment decreased seed yield, indicating that the increase in defense caused by the multiple CK treatments alters the overall plant growth. Taken together, these results suggest that activation of defense by application of CK does contribute to the growth-defense trade-off, with CIP activating defense, and also decreasing plant growth.

Cytokinin-induced priming is effective in Brassica napus

To date, exogenous CK application has been shown to work as a priming agent against a variety of pathogens in tomato (Gupta et al., 2020b), tobacco (Grosskinsky et al., 2011), and rice (Zhang et al., 2022). Treatment of wheat (Babosha 2009b) and poplar (Dervinis et al., 2010) with CKs have also been shown to reduce susceptibility to powdery mildew and gypsy harvest moth insect larvae, respectively. However, these studies vary in CK application methods to either attached or detached leaves, different incubation times before pathogen challenge, and different forms of CKs used, making it difficult to characterize CK as a generally effective priming agent in plants. To determine if CIP is a conserved process we tested its effectiveness in a closely related plant species to Arabidopsis, *Brassica napus*, and whether it affected these species similarly.

Five-week-old *B. napus* plants were treated with spray solutions of 100μM CK or a mock solution, followed by syringe infiltration with *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326 48 H later. *In planta* bacterial multiplication 4 days post infiltration determined that CIP is also effective at reducing bacterial concentrations in *B. napus* (Figure 2.8). Moreover, we addressed if, like in Arabidopsis, there is a specific timeframe of reduced susceptibility in the *Psm-B. napus* pathosystem after priming by CK. Figure 9 shows that CIP is effective at reducing bacterial growth in *B. napus* within the timeframe of 48 to 72 H after CK treatment, which is 24 H later than CIP in Arabidopsis (Figure 2.2). Thus, CK application decreases plant susceptibility to two different *Pseudomonas* species in Arabidopsis and *B. napus*, however, this timeframe of decreased bacterial growth in *B. napus* begins 24 H later than in Arabidopsis.

Due to similar CIP results seen in Arabidopsis and *B. napus* (Figures 2.2, 2.8 and 2.9), we hypothesized that there would be similar growth effects due to CIP in *B. napus* plants. Following the emergence of true leaves, *B. napus* plants were sprayed with 100μM CK or a mock solution, and shoot and root tissue were collected for measurements after 8 weeks of growth. The results from these experiments indicate that CIP has little effect on plant growth in *B. napus* (Figure 2.10). There were no differences between treatments on the average fresh or dry weight, even when looking at the weights of shoots and roots separately. A single application of CK to shoots did lead to an increase in petiole length (Figure 2.10 G) and shoot height (Figure 2.10 F), although this effect did not alter plant weight. These results are in contrast to the effect of the single CK treatment on Arabidopsis plants (Figure 2.7 A-C), suggesting that Arabidopsis may be more sensitive to 100μM CK than *B. napus.*

Because the effectiveness of CIP in reducing susceptibility diminished after 72 H following cytokinin treatment (Figures 2.2 and 2.9), it is possible that implementation of CIP in

agricultural settings would require multiple CK treatments to ensure a state of sustained, increased plant defense. In order to determine if multiple CK treatments would alter plant growth we aimed to simulate a constant CK-primed state where *B. napus* plants were treated with 100μM CK or a mock solution every 4 days. During 8 weeks of growth, the plants underwent a total of 8 treatments before tissue was collected for measurements (Figure 2.11). CK treatments increased shoot weight and decreased root weight, resulting in no difference in overall plant fresh or dry weight between CK and mock treatments. Petiole length and shoot height increased in CK-treated plants, but overall primary root length showed little difference between treatments indicating that another phenotypic trait other than root length could be responsible for the differences in root weight. Figures 2.11 J and K show the similarity in primary root lengths between treatments. Although unmeasured, the overall number and/or thickness of the roots was visibly lower in the CK-treated plants, most likely due to the known detrimental effect CK has on lateral root development (Werner et al., 2003, Li et al., 2006). These results indicate that *B. napus* plants subjected to a constant CK-primed state undergo changes in growth where CK had a positive effect on above-ground tissues at the site of application but an opposite effect on below-ground tissues.

2.5 DISCUSSION

Several studies have provided evidence that the role of the CK in plant biology goes beyond that of a regulator of plant growth, extending to a role in plant defense (Albrecht and Argueso 2017, Akhtar et al., 2020, McIntyre et al., 2021). The first study to implicate that CK could be used as an inducer of plant defense was in 2000 (Clarke et al., 2000), however, it was another ten years before CK was associated with the term priming (Choi et al., 2010, Argueso et

al., 2012). Although there are several studies that show that the application of CKs can reduce disease susceptibility, CK is still not widely known as a priming agent, possibly due to the minimal characterization of CIP. Therefore, in this study, we aimed to characterize CK as a priming agent.

Most studies utilizing exogenous CK to induce plant defense have shown evidence of priming activity 24 to 48 H after CK application. Therefore, we determined how long Arabidopsis is primed for decreased susceptibility to *Pst* following one spray application of CK. Three H following CK application, plants show a decrease in bacterial growth, however this is not significant until 24 or 48 H after priming. Increasing the time between CK treatment and *Pst* inoculation to 72 hours or longer leads to loss of the priming effect and no differences in *in planta* bacteria multiplication between CK-treated and mock-treated plants. Additionally, the characterization of other priming agents like BTH, BABA, and Pip have mainly utilized this same timeframe in their studies. A few studies have tested a longer period of reduced susceptibility after priming. Pip was tested up to 4 days post-priming in tomato, but 24 H post Pip application was the only time point where reduced susceptibility to *Pst* was clearly observed (Zhang et al., 2020). On the other hand, there are two studies that have shown that BABA-induced priming retains Arabidopsis and potato plants in a primed state for 28 days through heightened *PR1* expression following pathogen inoculation and reduced susceptibility to *Hpa* and *Phytophthora infestans*, respectively (Luna et al., 2014, Floryszak-Wieczorek et al., 2015).

Following the exogenous application of CK, genes encoding components of the CK signaling pathway showed an almost immediate upregulation in their expression (Brenner et al., 2005). Therefore, if CIP is due to direct activation of CK signaling, a robust decrease in susceptibility 3 H after CK application should be observed. To test this hypothesis, we analyzed

the expression of three known CK-regulated genes. *ARR5* is a CK signaling gene and primary transcriptional target of CK perception within the CK signaling machinery (Hwang and Sheen 2001) and has been shown to be highly expressed 15 minutes after CK application (D'Agostino et al., 2000, Brenner et al., 2005). CRF2 has been shown to interact with members of the CK signaling pathway (Cutcliffe et al., 2011), and *CRF2* expression is rapidly increased following CK application (Kiba et al., 2005, Rashotte et al., 2006). *EXP1*, a CK primary response gene (Taniguchi et al., 2007), was shown to be up-regulated 15 minutes after CK treatment (Brenner et al., 2005). These genes showed peak expression within the first 3 H after CK application, followed by a trend of diminished expression, suggesting that CIP is due to a process downstream of CK signaling.

Even though BABA- and Pip-induced priming do not share identical molecular mechanisms (Hartmann and Zeier 2019, Yildiz et al., 2021, Cooper and Ton 2022), they do share similar characteristics. For instance, both BABA and Pip are non-proteinogenic amino acids resulting from the catabolism of thymine and lysine, respectively. Both also lead to the activation of salicylic acid (SA)-dependent defense responses, such as increased expression of *PR1* after pathogen challenge, SA accumulation, and require the master regulator of SA signaling, NPR1 (Zimmerli et al., 2000, Navarova et al., 2012, Luna et al., 2014, Bernsdorff et al., 2016, Yildiz et al., 2021). Interestingly, CIP also shares the same responses (Choi et al., 2010, Argueso et al., 2012), therefore, we tested BABA and Pip-induced priming against *Pst* using the CK signaling mutant *ahk2,3*. Without endogenous CK signaling, mutant plants did not show a reduction in susceptibility to *Pst* following treatment with either priming agent. These results suggest that there is an additional common feature between BABA and Pip, which is that both priming agents reduce susceptibility to *Pst* by a CK-mediated process.

Induced resistance, or IR, is a term that can also be used to describe priming but broadens the category of stimuli to include plant pathogens, volatile organic compounds, and physical stress (De Kesel et al., 2021). An interesting area of study of IR is its effects on disease resistance in the progeny of treated plants, known as transgenerational priming. For example, the treatment of plants with pathogenic microbes or herbivory leads to decreased susceptibility to pathogens in the progeny of the treated plants (Luna et al., 2012, Rasmann et al., 2012, Lopez Sanchez et al., 2021). Moreover, BABA-IR has also been shown to have similar effects in the progeny of treated plants in Arabidopsis (Luna et al., 2012, Slaughter et al., 2012), potato (Floryszak-Wieczorek et al., 2015), and the common bean (Ramirez-Carrasco et al., 2017). Due to the common characteristics between CIP and BABA-induced priming, the transgenerational effects of CIP were tested against *Pst* challenge in the progeny of CK- or mock-treated plants. The overall effect of CIP on the progeny showed a decrease in susceptibility in plants whose parents were treated with CK, however, it is important to note that this effect was not seen consistently in all offspring. This variation could be due to the experimental design where the rosette of parental plants was treated only before bolting, meaning that there were approximately 2 months between CK treatments and when seeds were mature enough to harvest, thus far outside the timeframe of observed reduced susceptibility to *Pst* due to CIP. These results suggest the possibility that CIP induces epigenetic or metabolic changes within the seeds, as described in other transgenerational studies (Luna et al., 2012, Cooper and Ton 2022), however further investigation is needed to address this.

Constitutively activated defense mutants or auto-immune mutants who exhibit decreased susceptibility to pathogens and elevated basal defense gene expression often display a common suppression of growth (Bowling et al., 1994, Clarke et al., 1998). This dwarfed phenotype is

suggested to be the result of increased metabolic resources syphoned towards biosynthesis of defensive compounds and gene expression, at the cost of metabolic resources being used for normal growth and development (Kempel et al., 2011, Denance et al., 2013, Albrecht and Argueso 2017). As opposed to constant activation of plant defenses, priming limits a heightened defense response until a pathogen is perceived, thus providing a less metabolically costly alternative. However, our results suggest that, under the conditions of this study, CIP does have negative effects on plant growth after just one treatment, and that these negative effects are exacerbated if plants are kept in a primed state for a longer period, with a decrease in overall seed yield and biomass. Interestingly, similar results have been described following priming by BABA, BTH, and other inducers of resistance (Walters and Heil 2007). BABA and BTH were shown to decrease seed yield in Arabidopsis (Jakab et al., 2001, van Hulten et al., 2006), and BTH was also shown to decrease seed yield and overall growth in wheat (Heil et al., 2000).

Priming of plant defenses has been shown to be effective in many different plant species. For instance, BABA has been shown to be an operative priming agent in over 40 plant species (Cohen et al., 2016), BTH can provide protection in 120 different pathosystems (Zhou and Wang 2018), and, more recently explored, Pip-induced priming is effective in Arabidopsis (Navarova et al., 2012), tomato (Zhang et al., 2020), and tobacco (Vogel-Adghough et al., 2013). Due to several common features CIP has with these other priming agents, we were interested in exploring if CIP is a conserved defense strategy in a related plant species, *B. napus*. We determined that treating *B. napus* plants with 100μM CK does decrease susceptibility to *Psm* 48 H after treatment. Thus, the effect of CK as a priming agent expands to other members of the Brassicaceae family. In addition to showing that CIP is effective in *B. napus,* we also observed that the effective timeframe of CIP is between 48 and 72 H after CK treatment. Although this

two-day timeframe of priming is similar to Arabidopsis (Figure 2.2), decreased susceptibility begins one day later in *B. napus* suggesting the 100μM CK application may have a different effect in *B. napus*. Further evidence of different responses between the two species was seen in growth effects following CK application to *B. napus.* In contrast to Arabidopsis, one application of CK to *B. napus* has minor effects on plant growth and promotes a slight increase in petiole length and shoot height. Comparing the effects of keeping the two plant species in a constant state of priming through multiple CK applications, we also see different impacts on plant growth, where *B. napus* plants show no differences in overall plant biomass with CK applications, but there is an increase in shoot biomass and conversely decrease root biomass, implicating areas of the plant that had direct contact with CK grew more than areas below ground. These results highlight the widely known role of CK on plant growth, promoting shoot development (Argueso et al., 2009, Schaller et al., 2014, Kieber and Schaller 2018) possibly through a reallocation of nutrients away from below-ground tissues (Argueso et al., 2009, McIntyre et al., 2021), and resulting in decreased root development.

The different responses to CIP between these plant species may be due to different concentrations of CK having a different effect on various plant species. This has been documented in other cases. For example, in rice, the most effective concentration of applied CK to reduce susceptibility to brown plant hoppers was between 0.1 μM and 10μM CK, whereas higher concentrations had no effect on resistance (Zhang et al., 2022). Additionally, CIP in a Poplar hybrid was most effective at decreasing susceptibility to insect larvae when applied at concentrations of 100nM CK (Dervinis et al., 2010). The variations in plant responses to CK may be attributed to differences in CK signaling levels. Various CK receptors have distinct binding affinities for different CK forms, which, upon binding, activate CK signaling to varying

degrees (Spichal et al., 2004, Romanov et al., 2006, Stolz et al., 2011). Consequently, while Arabidopsis and *B. napus* both exhibit similar susceptibility effects from 100 μM CK, the CK signaling outcomes in these two plant species may differ, influencing their respective growth responses.

Our results suggest that CIP has a detrimental effect on plant growth and thus, follows the paradigm of the growth-defense trade-off, where plant growth and defense must be in a coordinated balance if there are to be no detrimental effects in either area (Kliebenstein 2016). Moreover, these results support the role for CKs in the regulation of the growth-defense tradeoff (Albrecht and Argueso 2017). A study conducted in tomato showed that two of the three tested priming agents, both biological, *Trichoderma harzianum* and *Bacillus megaterium*, can promote plant growth and decrease susceptibility to the necrotrophic fungal pathogen *Botrytis cinerea* in a CK-dependent manner. The third priming agent tested, BTH, a chemical priming agent, also reduced susceptibility to *B. cinerea*, however, its use negatively impacted plant growth while not requiring endogenous CK signaling activation (Leibman-Markus et al., 2023). Although these results are contradictory to the growth results presented in this study, they suggest that CK could have a role in balancing the growth defense trade-off in different plant species.

However, this generalized statement does not take into account the concentration of the priming agents applied to the plants, as it may result in a different effect on the balance of the growth-defense trade-off. For instance, the studies on Arabidopsis showed that when BABA is applied at low concentration there is a decreased detrimental effect on seed yield as compared to plants treated with higher concentrations of BABA (Jakab et al., 2001, van Hulten et al., 2006). Similarly, when pearl millet seeds were treated with lower concentrations of BABA, there was an increase in plant shoot and root length that was associated with a decrease in susceptibility to

downy mildew (Shailasree et al., 2001). Therefore, the negative impacts of CIP on plant growth may also be able to be fine-tuned to promote both plant growth and disease protection.

Plant loss due to disease has been previously estimated to be up to 15% of crops produced (Oerke 2005). A more recent survey of 67 nations combined with national yield statistics stated that within the top five food crops (wheat, rice, maize, soybean, and potato) crop losses are between 17-30 % (Savary et al., 2019) thus, new strategies to combat plant disease are essential for food security. CKs represent a new type of priming agent that can be used to combat disease in a variety of plant species. Further optimization of CIP would be required to reduce its negative effect on plant growth if CK were to be used as a priming agent in agricultural settings.

2.6 CONCLUSIONS

In this research, we characterized the use of the plant hormone, CK, as a priming agent to increase plant defense against the bacterial hemibiotroph *Pseudomonas* in Arabidopsis and *B. napus*. The timing of reduced susceptibility to *Pst* seen following CIP occurs within a 48 H time period with the most significant effects observed 24 to 48 H in Arabidopsis and 48 to 72 H in *B. napus* after a single CK application—extended time periods after these intervals result in a loss of the priming effect. Analysis of the expression of CK-regulated genes suggests that CIP operates downstream of CK signaling rather than through direct CK activation due to the decreasing expression levels just 3 H after induction by CK.

Application of CK has been demonstrated to have common characteristics with known priming agents like BABA and Pip in that CK application induces SA-dependent defense responses like *PR1* potentiation after pathogen challenge and the requirement for NPR1 for increased defense. Aiming to understand the role of CK signaling in other chemically-induced

priming responses show that both BABA- and Pip-induced priming against *Pst* require endogenous CK-signaling to reduce susceptibility. Moreover, like other priming agents, CIP displays transgenerational effects on the progeny of primed plants, indicating the potential involvement of epigenetic changes in offspring. However, our results were variable suggesting further studies and optimization is warranted.

Characterizing a new priming agent merits an understanding of the effect on plant growth following the priming treatment. The concept of a growth-defense tradeoff is supported by our results in Arabidopsis as CIP negatively impacts plant growth, a phenomenon observed with other priming agents. However, in *B. napus* these effects on plant growth were not as pronounced unless plants were kept in a constantly primed state for two months. The difference in growth responses to CK between plant species is supported in the literature where different species show varied responses to CK treatments, possibly due to differences in CK signaling levels and concentration effects.

Ultimately these results have promising implications for food security, as CKs could serve as an effective priming agent for enhancing disease resistance in various plant species. However, optimizing CK application is necessary to minimize the negative impact on plant growth, particularly in agricultural settings.

2.7 FIGURES

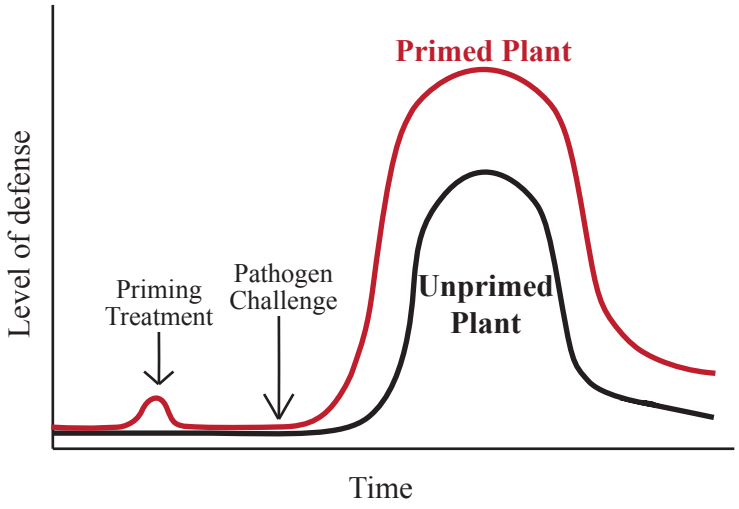


Figure 2.1 | The model of priming of plant defenses: Primed plants experience a low level of defense activation following a priming treatment, however after experiencing a pathogen challenge, a primed plant has a quicker, more robust activation of defenses, as compared to an unprimed plant.

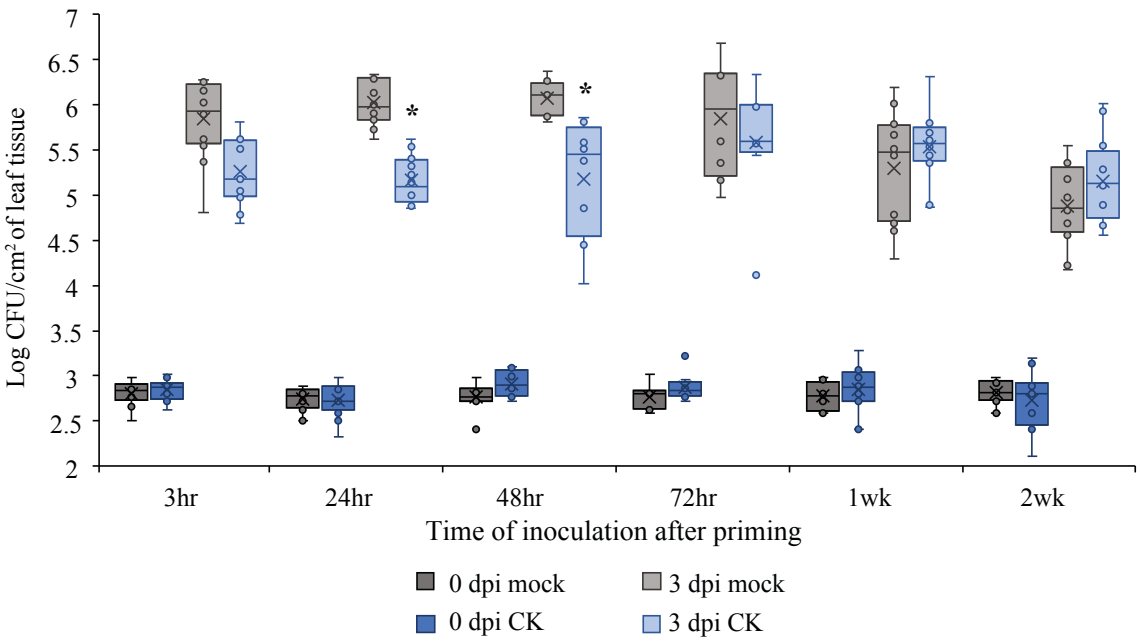


Figure 2.2 | One application of 100µM of cytokinin (CK) to Arabidopsis reduces susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst)* 24 to 48 hours (hr) after application: Plants were sprayed with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO, and then inoculated with *Pst* at either 3hr, 24hr, 48hr, 72hr, 1 week, or 2 weeks after priming. Leaf discs were collected at 1 hr post inoculation (0 dpi) and 3 days post inoculation (3 dpi) to determine *in planta* bacterial levels. Data pooled from 3 biological replicates. Asterisks indicate statistical difference with a p-value < 0.05 in treatments at the specified time point by one-way ANOVA with TUKEY HSD correction.

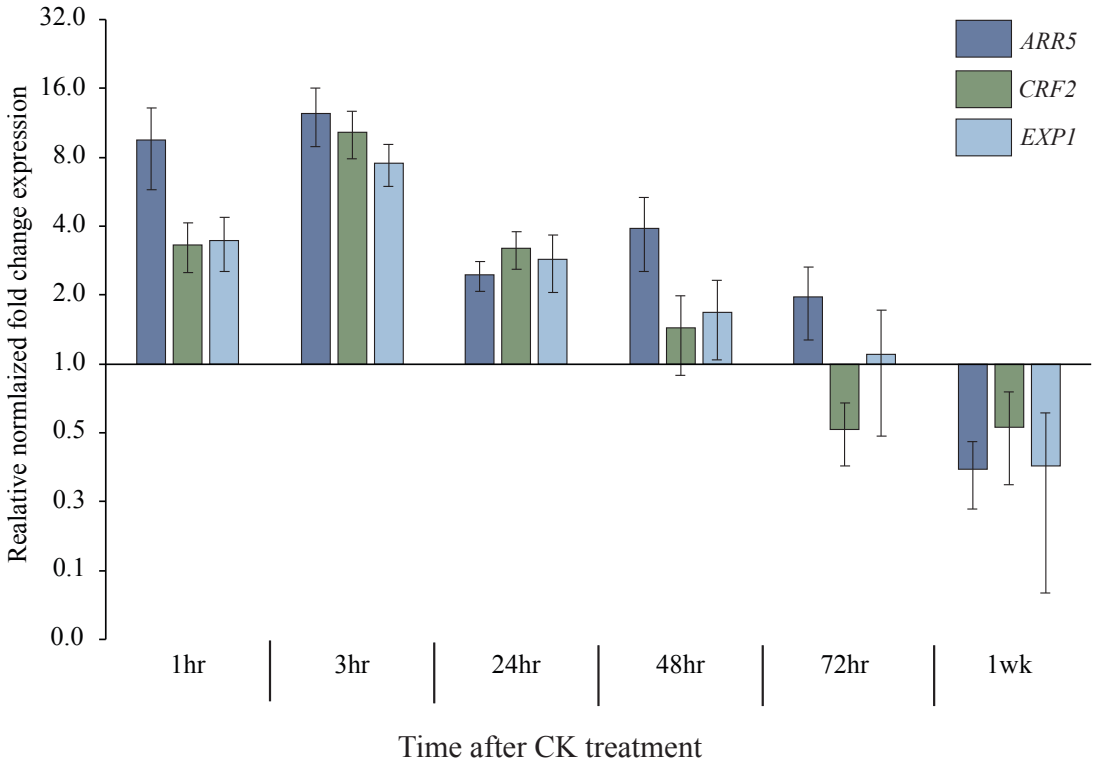


Figure 2.3 | Cytokinin (CK)-mediated gene expression peaks at 3 hours (hr) after treatment with 100μM CK: Plants were sprayed with either 0.01% DMSO mock solution or 100μM of the CK benzylaminopurine in 0.01% DMSO, followed by tissue collection for RNA extraction at the times listed. The expression of *ARR5*, *CRF2*, and *EXP1* was assessed by qRT-PCR and reported as a fold change of 100μM CK/mock. Data is pooled from two biological replicates.

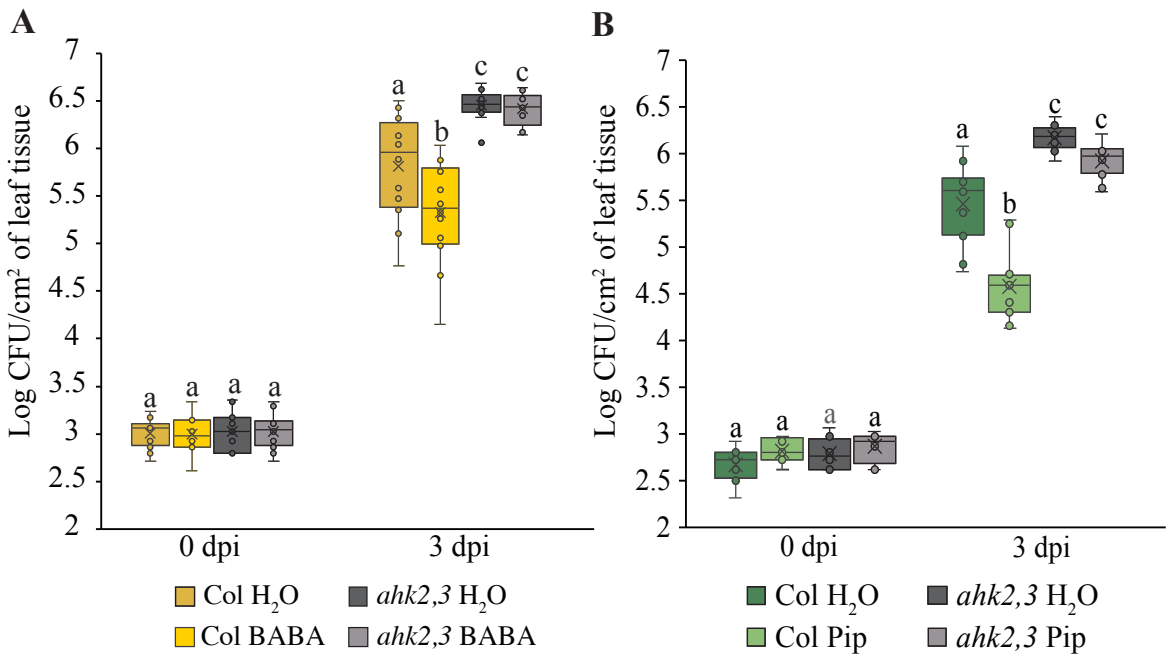
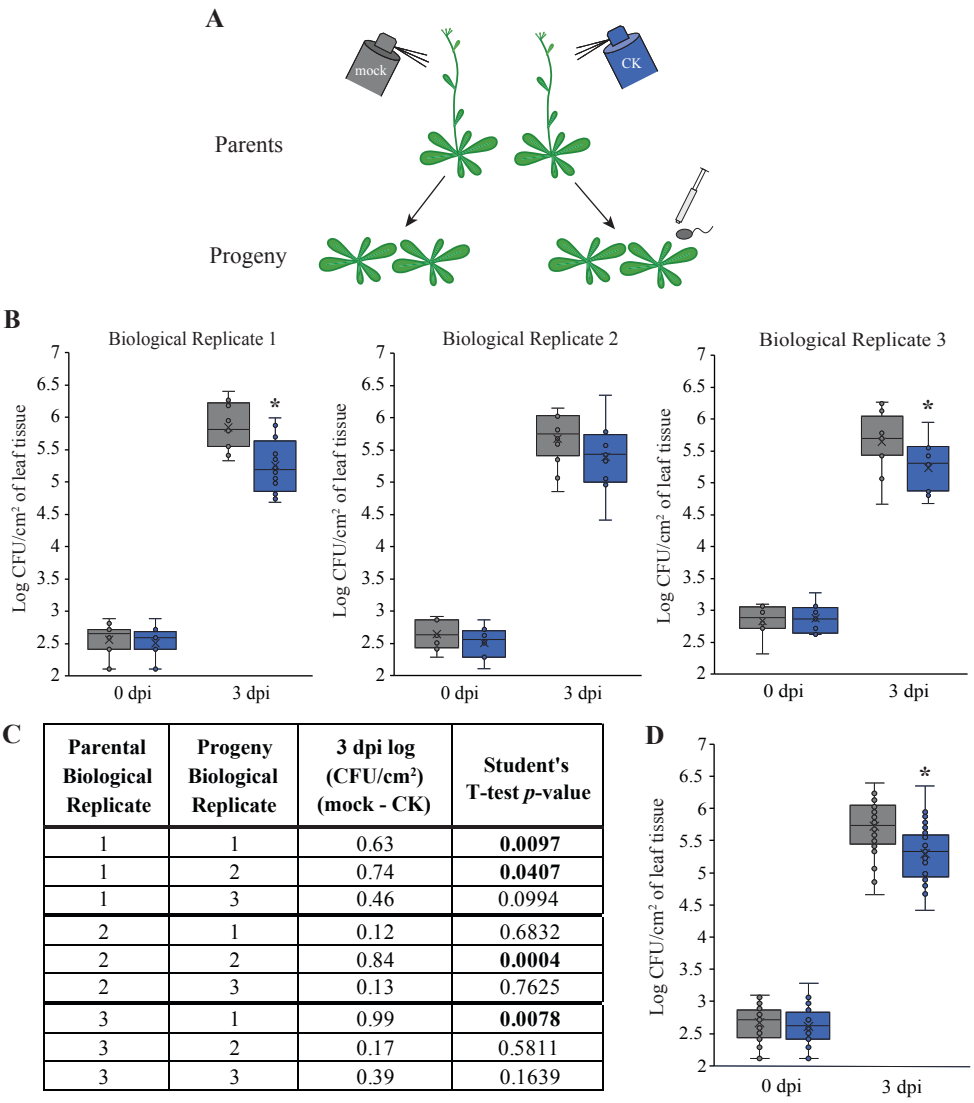


Figure 2.4 | Endogenous cytokinin signaling is required for the priming activity of β-aminobutyric acid (BABA) and L-pipecolic acid (Pip): Five to six-week-old wild type Col and CK signaling mutant (*ahk2,*3) plants were soil drenched with 20mg/mL BABA (**A**) or 1mM Pip (**B**) 48 hours prior to infiltration with *Pseudomonas syringae* pv. *tomato* DC3000. Leaf discs were collected at 1 hour post inoculation (0 dpi) and 3 days post inoculation (3 dpi) to determine *in planta* bacterial levels. Data pooled from 3 biological replicates. Different letters indicate statistical differences with a *p*-value < 0.05 at the specified time point by two-way ANOVA with TUKEY HSD correction.



C

Parental Biological Replicate	Progeny Biological Replicate	3 dpi log (CFU/cm²) (mock - CK)	Student's T-test *p*-value
1	1	0.63	**0.0097**
1	2	0.74	**0.0407**
1	3	0.46	0.0994
2	1	0.12	0.6832
2	2	0.84	**0.0004**
2	3	0.13	0.7625
3	1	0.99	**0.0078**
3	2	0.17	0.5811
3	3	0.39	0.1639

Figure 2.5 | Priming plants with cytokinin (CK) before bolting results in decreased, but variable, susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) in progeny: (**A**) For each biological replicate, 3 parental plants were treated with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO, three times prior to bolting. Seeds collected from individual plants were used to grow progeny plants. Untreated progeny plants were grown for 6 weeks before being infiltrated with *Pst* to determine a transgenerational priming effect by measuring *in planta* growth 3 days post-inoculation (dpi). (**B**) Results of *Pst in planta* multiplication of each of the 3 biological replicates of progeny from CK- or mock-treated parentals. (**C**) shows the individual results from testing the susceptibly of the progeny that include the biological replicate origin of the parents and progeny, the difference in bacterial growth at 3 days post-inoculation between progeny plants whose parents were treated with CK or mock solution, and the *p*-value resulting from a Student's T-test to determine if the *in planta* growth was statistically different in the progeny of treated parents. Graph (**D**) represents the results of pooling all of the individual replicates from table (**C**). Asterisks indicate the statistical difference with a p-value < 0.05 at the specified time point by one-way ANOVA with TUKEY HSD correction.

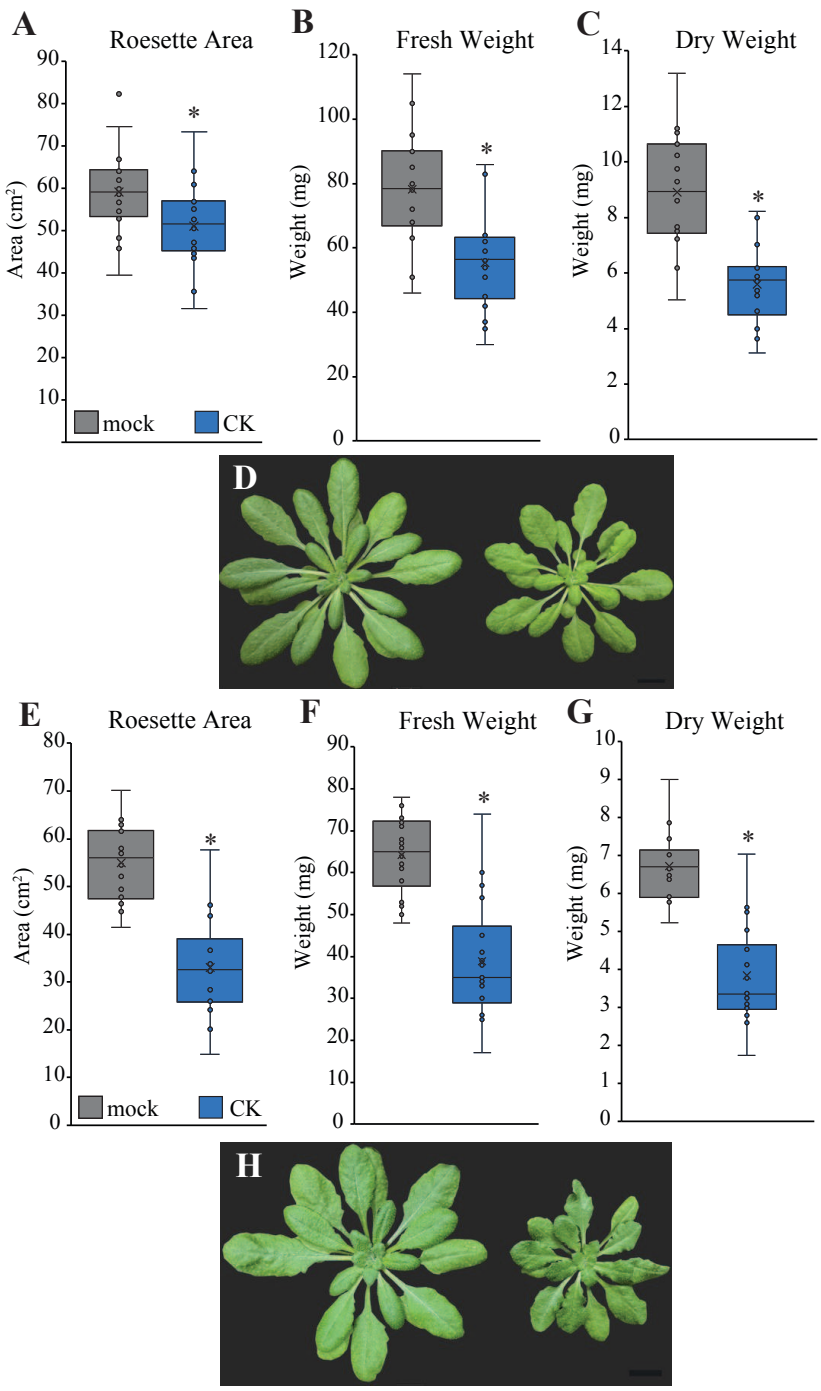


Figure 2.6 | Cytokinin (CK) application decreases plant shoot growth in Arabidopsis: Three-week-old plants were treated one time (**A-D**) or three times (**E-H**) every three days with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO. After six weeks of growth, plants were harvested and measured for rosette area (**A** and **E**), fresh weight (**B** and **F**), and dry weight (**C** and **G**). Asterisks indicate statistical differences as determined by Student's T-test. Eighteen plants were measured per treatment.

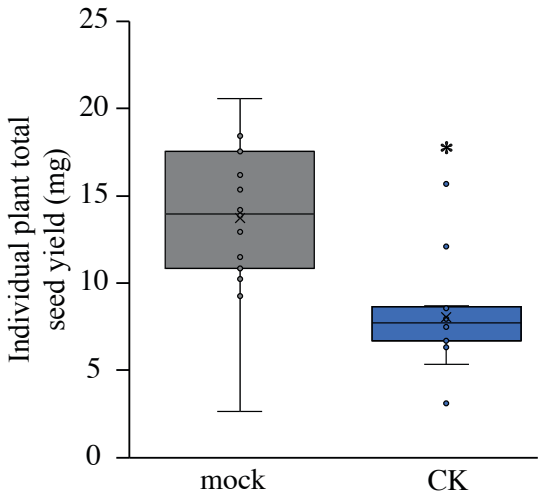


Figure 2.7 | Treatment of plants with cytokinin (CK) three times before bolting decreases overall seed yield: Four-week-old wild type plants were sprayed with 0.01% DMSO mock solution or 100μM of the CK benzylaminopurine in 0.01% DMSO three times prior to bolting. Total seeds from individual plants were collected after 12 weeks and weighed. Data is representative of three biological replicates. Asterisk indicates a statistical difference between treatments by Student's T-test with a *p*-value < 0.05.

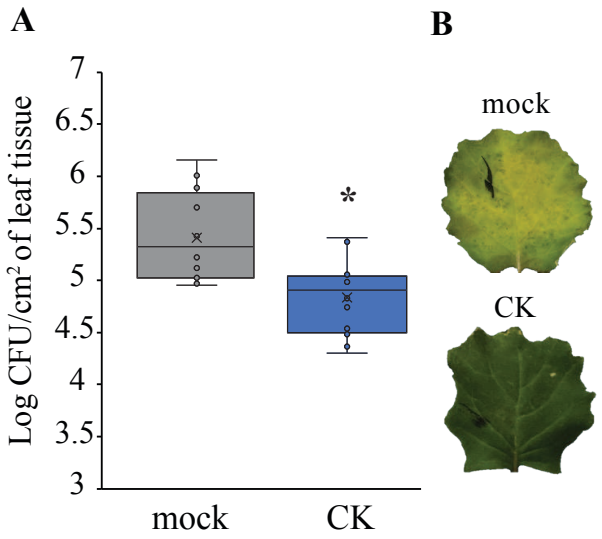


Figure 2.8 | Treatment of *Brassica napus* plants with cytokinin reduces susceptibility to *Pseudomonas syringae* pv. *Maculicola* (*Psm*)*: B. napus* plants were treated with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO for 48 hours prior to infiltration with *Psm.* Leaf discs were collected at four days post inoculation to determine *in planta* bacterial levels (**A**). (**B**) displays representative pictures of disease symptoms. Data was pooled from three biological replicates. The asterisk indicates *p*-value < 0.05 by Student's T-test.

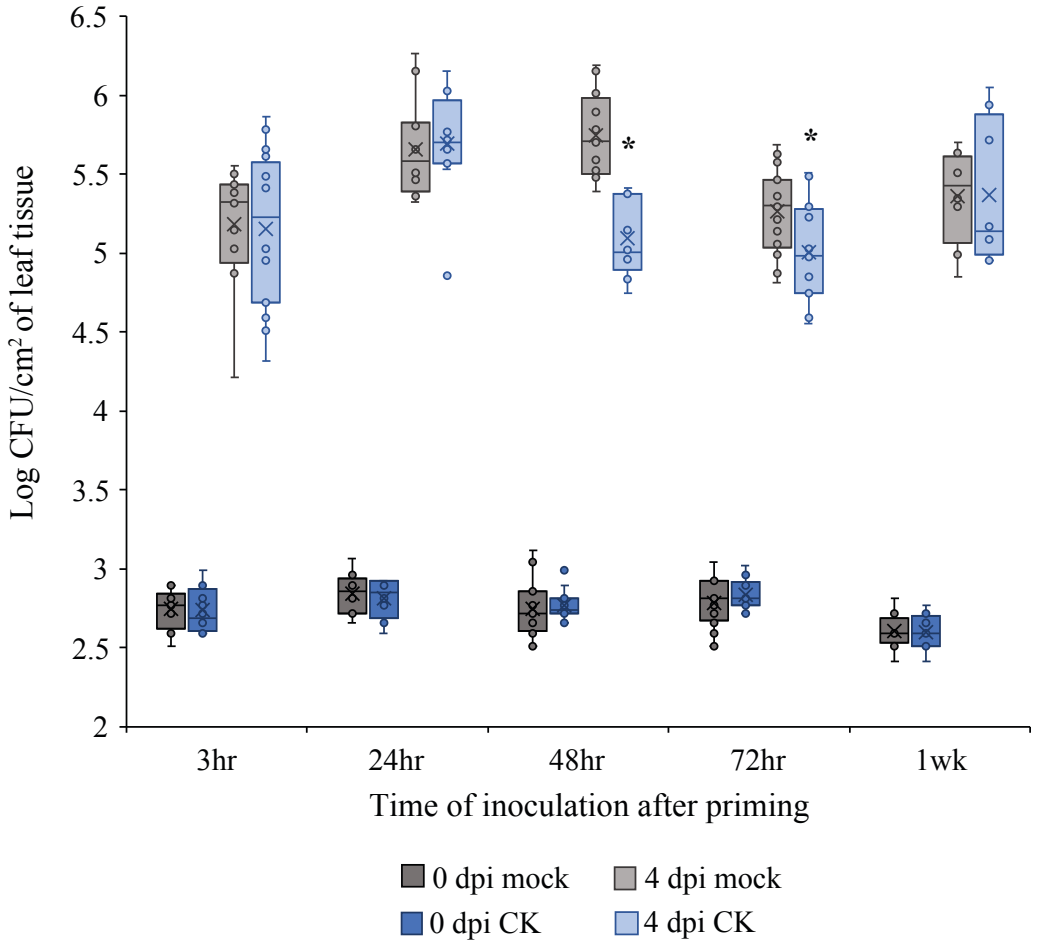


Figure 2.9 | Application of cytokinin (CK) to *Brassica napus* causes a reduction in susceptibility to *Pseudomonas syringae* pv. *maculicola* (*Psm*) after 48 to 72 hours (hr)*:* Plants were treated with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO followed by infiltration of *Psm* 3hr, 24hr, 48hr, 72hr, and 1 week after treatment. Leaf discs were collected at one hr post inoculation (0 dpi) days and four days post inoculation (4 dpi) to determine *in planta* bacterial levels. Data was pooled from multiple experiments with each time point having 2 to 3 biological replicates. Asterisks indicate a *p*-value of < 0.05 between treatments at the specified time point by one-way ANOVA with TUKEY HSD correction.

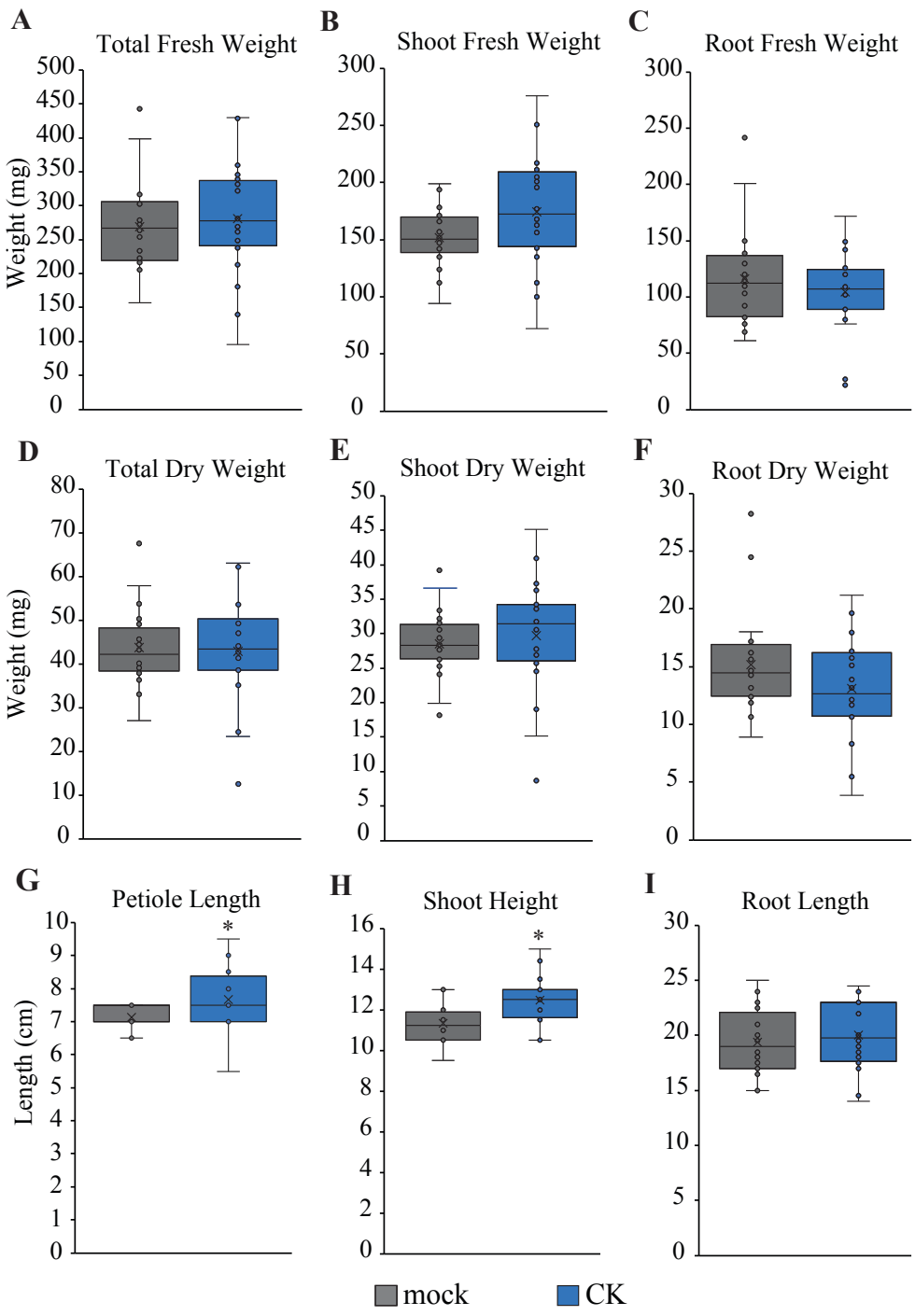


Figure 2.10 | One application of cytokinin (CK) to *Brassica napus* has minimal effect on plant growth: Plants were treated once with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO before tissue collection following six weeks of growth. Total fresh (**A**) and dry (**D**) weight, fresh (**B**) and dry (**E**) shoot weight, fresh (**C**) and dry (**F**) root weight, petiole length (**G**), shoot height (**H**), and root length (**I**) were measured. Asterisks indicate statistical differences as determined by Student's T-test. Eighteen plants were measured per treatment.

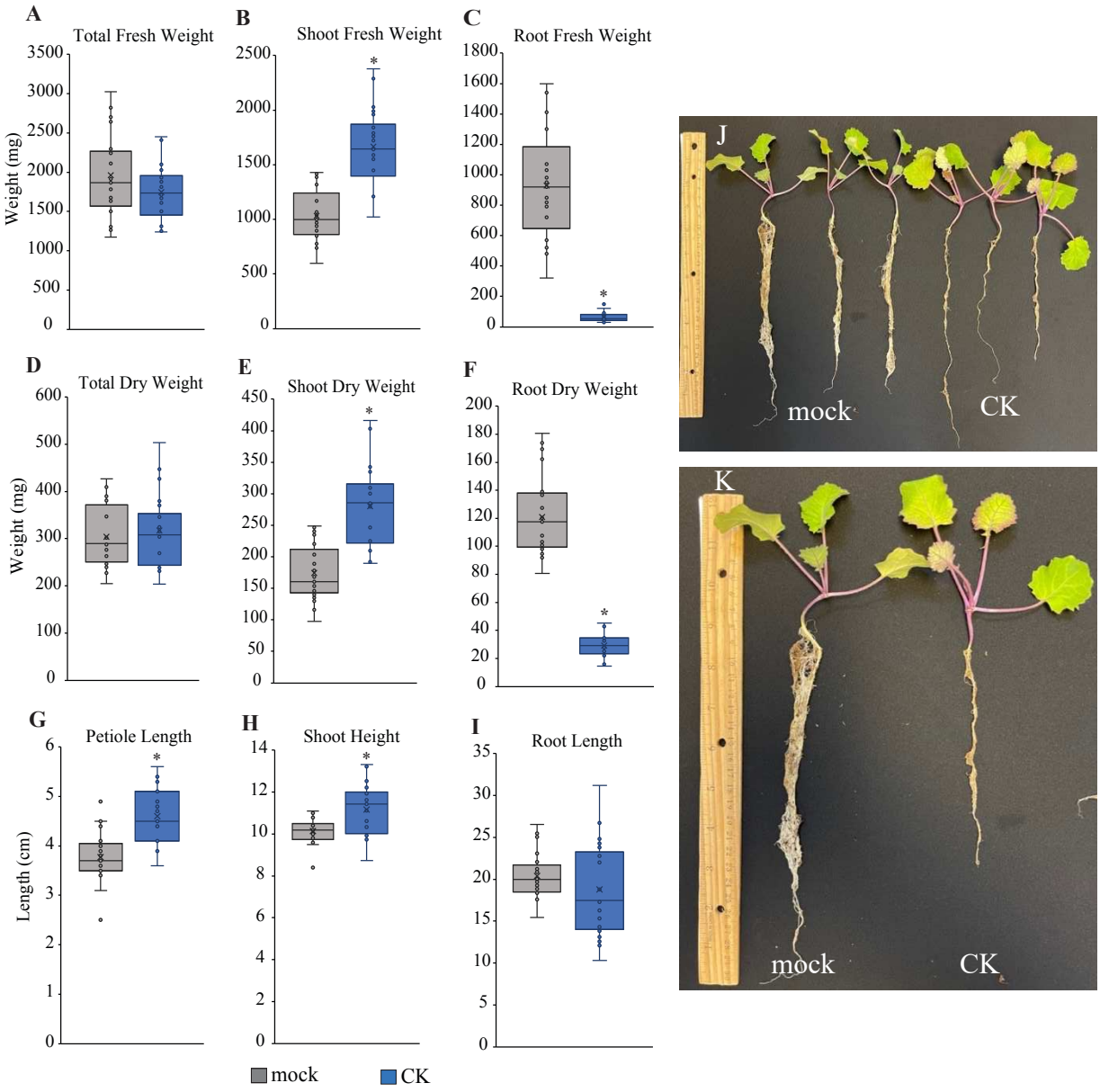


Figure 2.11 | Constitutive activation of cytokinin (CK)-induced priming (CIP) in *Brassica napus* increases shoot growth but decreases root development: Plants were treated every three days with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO before tissue collection following six weeks of growth. Total fresh (**A**) and dry (**D**) weight, fresh (**B**) and dry (**E**) shoot weight, fresh (**C**) and dry (**F**) root weight, petiole length (**G**), shoot height (**H**), and root length (**I**) were measured. Asterisks indicate *p*-value < 0.05 as determined by Student's T-Test. Eighteen plants were measured per treatment. (**J**) and (**K**) are representative pictures of consistent activation of CIP in *B. napus.*

2.8 TABLE

Table 2.1 | RT-qPCR primers used in this study: The table shows the primer sequences used within this study for RT-qPCR for gene expression analysis. All sequences are written in the 5' to 3' direction.

Gene	Forward Primer Sequence	Reverse Primer Sequence
ARR5	TCTGAAGATTAATTTGATAATGACGG	TCACAGGCTTCAATAAGAAATCTTCA
CRF2	CAGTGACGACGAAGAAGAAGA	AGCACCGGAATCGAGATAGAC
EXP1	CAACGCATCGCTCAATACAG	CTCCGACGTTAGTGATCAGAAC
UBQ-10	CGTTAAGACGTTGACTGGGAAAACT	GCTTTCACGTTATCAATGGTGTCA

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CHAPTER 3:

PHYTOHORMONAL NETWORKS IN CYTOKININ-INDUCED PRIMING AGAINST *BOTRYTIS CINEREA*

3.1 SUMMARY

The plant hormone cytokinin (CK), well-known for its role in plant growth, also has a role in plant defense against pathogens. Mainly demonstrated in defense responses mediated by salicylic acid, exogenous application of CK, also known as CK-induced priming (CIP), can effectively reduce susceptibility to hemi- or biotrophic pathogens. Recently CIP has been demonstrated as an effective strategy for protection against necrotrophic pathogens, however, insights into how this hormone can mediate defense against pathogens of different lifestyles are lacking. In this study, using Arabidopsis as a model, we describe the hormone signaling requirements for CIP against the necrotrophic pathogen *Botrytis cinerea*. Ultimately, we show through transcriptome analysis that CIP against *B. cinerea* involves changes in expression of genes associated with defense and photosynthetic-related processes.

3.2. INTRODUCTION

Plants have evolved to perceive and respond to environmental cues, adjusting plant growth and developmental programs accordingly. In the process known as priming, an initial response to an environmental challenge prepares the plant to respond to future challenges more effectively. Priming can be associated with both abiotic (Gamir et al., 2014) and biotic stress

responses, and can be induced by chemical, physical, and biological agents (Conrath et al., 2015, Mauch-Mani et al., 2017).

In response to pathogen challenges, plants must effectively recognize the type of pathogen involved, initiate specific signaling mechanisms, and mount a proper defense to induce resistance or mitigate the damage for survival. In terms of phytohormone regulation, defense against pathogens is mostly mediated by two plant hormones: salicylic acid (SA) and jasmonic acid (JA). SA mediates defense responses against biotrophic and hemibiotrophic pathogens, which are those that require living cells to obtain their nutrients (Gaffney 1993, Wildermuth 2001, Adam et al., 2018), whereas JA mediates defense responses against necrotrophic pathogens, which kill host cells to obtain nutrients and survive (Thomma 1998, Thomma 1999, Pieterse et al., 2012). The SA and JA defense pathways have a mostly antagonistic relationship in the mediation of plant defense, where activation of one resistance pathway leads to decreased resistance to pathogens of the other pathway (Spoel et al., 2007).

In addition to SA and JA, CKs also participate in plant defense. Exogenous application of CKs is effective at reducing susceptibility of plants to a variety of pathogens, through priming of immune responses (Choi et al., 2010, Grosskinsky et al., 2011, Argueso et al., 2012, Gupta et al., 2020, Zhang et al., 2022). Cytokinin-induced priming (CIP) has been demonstrated mostly against biotrophic pathogens. For instance, the application of the CKs 6-benzylaminopurine (BA) or *trans*-zeatin decreases susceptibility of Arabidopsis to the oomycete *Hyaloperonospora arabidopsidis* (Argueso et al., 2012) and the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Choi et al., 2010), respectively. Studies in tomato have also shown that either spray application or soil drenching with BA, *trans*-zeatin, or the CK kinetin, decrease susceptibility to the fungus *Oidium neolycopersici,* and the bacterial pathogens *Xanthomonas campestris* and

Pseudomonas syringae pv. *syringae*, respectively (Gupta et al., 2020, Gupta et al., 2021b). This decreased susceptibility is accompanied by the potentiation of various plant immune responses, such as defense gene expression, accumulation of reactive oxygen species (ROS), ethylene and phytoalexin production, as well as callose deposition, which increase to higher levels in CK-treated plants than in mock-treated plants, and therefore can be considered a priming response (Choi et al., 2010, Grosskinsky et al., 2011, Argueso et al., 2012, Gupta et al., 2020, Zhang et al., 2022). Importantly, the priming activity of CK against the biotrophic pathogens mentioned above was genetically determined to require SA (Choi et al., 2010, Grosskinsky et al., 2011, Argueso et al., 2012, Gupta et al., 2020, Zhang et al., 2022).

Botrytis cinerea, the causal agent of grey mold, infects over 200 plant species (Filleur 2016). Recent studies have shown CIP is effective at reducing susceptibility of tomato to *B. cinerea* (Gupta et al., 2020, Gupta et al., 2021b), but these studies lack insights into how CK acts in relation to the JA and SA defense pathways and into potential molecular mechanisms at play. Here, the role of CK as a priming agent against *B. cinerea* in Arabidopsis was investigated. Priming of plants with different concentrations of CK altered susceptibility to *B. cinerea*, a result phenocopied by CK signaling mutants. Using mutants in the SA and JA plant hormone signaling pathways, we demonstrate that JA and SA biosynthesis is required for effective priming, while SA-mediated signaling has a minor role. Analysis of the effect of CIP on the Arabidopsis transcriptome shows that the effect of CK goes decreased defense gene expression, leading to alterations in expression of genes involved in photosynthesis, primary metabolism, and RNA processing required for cell survival.

3.3 METHODS

Plant Materials and Growth Conditions:

Arabidopsis thaliana ecotype Columbia-0 (Col, wild type) and transgenic seeds in Col background were stratified for 2-4 days at 4ºC before being placed on soil. The mutant lines used in the study were *ahk2-7 ahk3-2* (*ahk2,3*) (Argyros et al., 2008), *arr3 arr4 arr5 arr6 arr8 arr9* (*arr3,4,5,6,8,9*) (To et al., 2004) , *eds16* (Dewdney et al., 2000), *dde2-2* (von Malek et al., 2002), *npr1-2* (Cao et al., 1997). All plants were soil grown in either a Conviron growth chamber (Model# ATC60) or an Environmental Growth Chamber (Model #M25 R-401A) at Colorado State University Plant Growth Facility. Plants were grown in either Pro-mix HP Mycorrhizae or Sunshine Mix #4 soil for 5-7 weeks under a 10:14 H day:night light regime, at 160 ± 20 µmol m-2s-1, at 21ºC, 50% relative humidity (RH) in the day and 19ºC, 60% RH at night.

Cytokinin-Induced Priming Treatments

A stock solution of 100mM cytokinin (CK) was made by dissolving 6-benzylaminopurine (BA) (Sigma) into dimethyl sulfoxide (DMSO) and stored at -20ºC for up to three months. 100µM BA priming solutions were prepared by diluting the 100mM BA stock 1:1000 into water, plus 0.002% Silwet L-77 (Lehle Seeds). Control plants were sprayed until run-off with an aqueous solution containing corresponding amounts of DMSO, plus 0.002% Silwet L-77. Plants were placed into different flats based on treatment and genotype. Following spray treatments, transparent plastic domes were placed over plants for 24 H to prevent rapid evaporation. Plants were inoculated with pathogens 48 H after priming or mock treatment, unless otherwise noted.

Fungal Disease Assays

Botrytis cinerea B10.5 sclerotia obtained from Dan Kliebenstein at the University of California, Davis was placed directly on sterile organic apricots slices on 0.5X potato dextrose agar (PDA) (Becton-Dickinson DIFCO). The resulting mycelia and conidia were placed on new 0.5X PDA plates to generate glycerol stocks stored in -80^{o}C freezer. 5 to 7 days prior to inoculation, 500μL of the *B. cincera* glycerol stock was placed on 0.5X PDA plates. *B. cinerea* spores were harvested by placing a 0.05% Tween-20 solution into the 0.5X PDA plate. Using a rubber policeman, spores and mycelia were scraped off the agar into the solution and filtered through miracloth to harvest the spores. The *B. cinerea* spore solution was diluted to a specific concentration quantified by a hemacytometer.

For lesion expansion experiments, 4 μL droplets of a solution of $0.5x10^4$ spores/mL in ½ strength organic grape juice (R.W. Knudson Family Organic Juice, Just Concord) containing 0.05% Tween-20 were placed on 4 leaves per plant. Inoculated plants were separated by treatment (if applicable) and genotype and placed in a flat under a water-sprayed plastic dome to maintain a high humidity environment. Photos of plants were taken at the times indicated and necrotic lesions were measured using Fiji photo analysis software (formally known as ImageJ) (Schindelin et al., 2012).

For analysis of gene expression, the spore solution described above was diluted to $30x10^5$ spores/mL in half-strength organic grape juice containing 0.05% Tween-20. Plants were sprayed with either a half-strength organic grape juice containing 0.05% Tween-20 as a control or the $30x10^5$ spores/mL in half-strength organic grape juice containing 0.05% Tween-20. Plants were separated by treatment (if applicable) and genotype and placed in a flat under a plastic dome to

maintain a high-humidity environment before tissue was harvested and flash-frozen in liquid nitrogen for RNA extraction.

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted using RNeasy Plant kit (QIAGEN), following manufacturer's instructions. The quality and integrity of RNA was assessed by A_{260}/A_{280} and A_{260}/A_{230} ratios having a value of greater than 1.7. RNA samples of good quality underwent DNase Treatment using TURBO DNase-Free (Invitrogen) as per the manufacturer's instructions and were checked for the absence of genomic DNA by qRT-PCR using primers for AT5G66770 (For 5'-GGTTTGGTTTGGTTATCGCCAGGA-3', Rev 5'-TGGCTTCATCTCTTTGGCCTGGA-3'). cDNA was synthesized using Qscript Supermix (QuantaBio) and checked for full-length cDNA synthesis through qRT-PCR using primers for *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE GAPDH* (AT1G13320). Primers used were: *GAPDH-1* (For 5'-TAGATCGCTCGGAACTTGGAAA-3', Rev 5'-CCTCACCAAAACTCAAATCACTCC-3'); *GAPDH-3* (For 5'-AACTAGGACGGATCTGGTGCCT-3', Rev 5'-GCTATCCGA ACTTCTGCCTCATTAT-3'), and *GAPDH-5* (For 5'-AAATTTAAC GTGGCCAAAATGATGC-3', Rev 5'-GTTCTCCACAACCGCTTGGT-3'). qRT-PCR reactions were performed with PerfeCTa SYBR Green (QuantaBio) on a CFX Connect Real-Time System (BioRad). cDNAs with Ct/Cq differences between each GAPDH primer of less than 1.5 were considered fully extended and of good quality. AT4G05320 *UBIQUITIN10* (*UBQ10)* was used as a housekeeping gene in all reactions (For 5'-CGTTAAGACGTTGACTGGGAAAACT-3', Rev 5'-GCTTTCACGTTATCAATGGTGTCA-3'). Gene specific primers used are listed in

Table 3.1. At least three biological replicates of each experiment were obtained unless otherwise stated.

RNA-seq for Whole Transcriptome Analysis

Total RNA was extracted using RNeasy Plant kit (QIAGEN), following the manufacturer's instructions. The quality and integrity of RNA were assessed by A_{260}/A_{280} and A_{260}/A_{230} ratios having a value of greater than 1.7. Further RNA quality and integrity were assessed using a TapeStation 2200 (Agilent) with High Sensitivity RNA materials (Agilent Screentape 5067-5579, Agilent Sample Buffer 5067-5580). An RNA gel was also run to confirm RNA quality before being submitted for sequencing. Paired-end, 150bp reads were sequenced by Novogene (Sacramento, CA, USA) at a depth of 40 million reads. Novogene used an Illumina based library construction kit (NEB Next Ultra 2). Sequence files underwent a quality control check by FastQC V0.12.0 (Wingett and Andrews 2018) and adapters were removed by Trimmomatic V0.39 (Bolger et al., 2014). Trimmed sequences were aligned to the *Arabidopsis thaliana* genome TAIR 10 (Berardini et al., 2015) using STAR 2.7.0a (Dobin et al., 2013) allowing a maximum of 20 multiple alignments allowed per read. FeatureCounts (Liao et al., 2014) was used to count how many reads aligned to genes and differential gene expression was assessed using DESeq2 R package (Love et al., 2014).

Statistical Analysis

For experiments with two comparisons, a two-way ANOVA with TUKEY HSD *p*-value correction was used and for experiments with one comparison a Student's T-test or one-way

ANOVA was used to evaluate statistical significance. The significance of these tests was based on a p-value ≤ 0.05.

3.4 RESULTS

Cytokinin-induced priming is effective at reducing susceptibility to Botrytis cinerea

Previous research demonstrated that CK can prime plants for disease protection against many pathogens, most of which have biotrophic or hemibiotrophic lifestyles. To address whether CK could be effective at priming plants of different lifestyles, we treated Arabidopsis plants with different concentrations of CK and tested their susceptibility to the necrotrophic pathogen *B. cinerea*. Five- to six-week-old wild type, Col-0 (Col), plants were spray-treated with mock solution, 100nM, or 100μM of CK 48 H prior to droplet inoculation of *B. cinerea* spores to leaves. At 48 and 72 H post-inoculation (hpi), necrotic lesions caused by *B. cinerea* inoculation were measured using the image analysis software Fiji (Schindelin et al., 2012). Plants treated with 100nM CK showed an increase in lesion size, although not significantly different than control-treated plants, whereas plants treated with 100μM CK had a decrease in lesion size, as compared to mock-treated plants, conferring a primed state (Figure 3.1). These results demonstrate that CK treatment can alter susceptibility to a necrotrophic fungal pathogen possibly in a concentration-dependent manner, with higher CK concentrations decreasing susceptibility.

To determine whether the application of exogenous CK alters susceptibility to *B. cinerea* in a manner dependent on the activation of endogenous cytokinin signaling, two CK signaling mutants were inoculated with *B. cinerea* spores and the necrotic lesion size was measured over time (Figures 3.2 A and 3.2 C). The *ahk2,3* mutant lacks two of the three CK receptors resulting in a minimal endogenous CK signaling (Riefler et al., 2006). In opposition, the sextuple mutant,

arr3,4,5,6,8,9, lacks six of the ten type-A ARABIDOPSIS RESPONSE REGULATORS (ARRs) that function as negative regulators of CK signaling, resulting in an increased endogenous CK signal (To et al., 2004). As compared to Col, *ahk2,3* was more susceptible to *B. cinerea*, as shown by an increased average lesion size. Conversely, *arr3,4,5,6,8,9* had, on average, smaller necrotic lesions as compared to wild type plants. Therefore, altering endogenous CK signaling results in changes in susceptibility to *B. cinerea,* with CK signaling having a positive effect on protection against this necrotrophic pathogen.

Cytokinin-induced priming against Botrytis cinerea requires both jasmonic acid- and salicylic acid-mediated responses

Because JA regulates defenses against necrotrophic pathogens, we decided to address whether CIP alters susceptibility via JA-dependent processes. The Arabidopsis mutant *dde2* (DELAYED DEHISCENCE 2), impaired in JA biosynthesis due to a mutation in the *ALLENE OXIDASE SYNTHASE* gene (von Malek et al., 2002), underwent CIP following by *B. cinerea* (Figure 3.3 A). As expected, mock-treated *dde2* plants showed increased susceptibility to *B. cinerea* in relation to mock-treated Col plants, highlighting the importance of JA accumulation to resistance against necrotrophic pathogens. More importantly, in contrast to CK-primed Col plants, which showed less susceptibility *B. cinerea*, *dde2* plants had an average lesion size similar to the mock-treated plants. These results indicate that CIP against *B. cinerea* requires JA.

Past studies have shown that CIP against biotrophic pathogens involves a crosstalk between CK and SA, with CK potentiating defense responses (Choi et al., 2010, Argueso et al., 2012, Jiang et al., 2013, Arnaud et al., 2017, Gupta et al., 2021b). Even though *B. cinerea* is a necrotroph, a partial role for SA in resistance to *B. cinerea* has also been demonstrated (Ferrari et al., 2003). However, whether SA is needed in CIP remains unknown. The Arabidopsis *eds16*

(*ENHANCED SUSCEPTIBILITY 16*) mutant harbors a mutation in the gene encoding the enzyme ISOCHORISMATE SYNTHASE 1 (ICS1) (Wildermuth 2001), responsible for the biosynthesis of SA via the chorismate pathway, the most prominent of the two SA biosynthetic pathways in Arabidopsis (Rekhter et al., 2019, Torrens-Spence et al., 2019). *eds16* plants have minimal SA production, basally and after pathogen infection (Wildermuth 2001). To address whether SA is important to CIP against *B. cinerea*, lesion sizes were measured in inoculated *eds16* following priming treatments (Figure 3.3 B). Mock-treated *eds16* plants displayed a smaller lesion size in comparison to mock-treated Col plants. These results are likely to reflect a change in the SA-JA antagonism, where the lack of SA biosynthesis due to the *eds16* mutation alleviates the repression of the JA pathway, reducing susceptibility to necrotrophic pathogens (Figure 3.3 B). CK-treated *eds16* plants showed a small, but statistically significant reduction in lesion area. In comparison to CK priming of Col plants, which reduced lesion area by an average of 28.7%, *eds16* plants only showed a 12.9 % average reduction in lesion area (Figure 3.3 B, inset table). These results indicate that SA is only partially required for CIP against *B. cinerea*.

NONEXPRESSOR OF PATHOGENESIS RELATED 1 (NPR1) is a major regulator of SA signaling. NPR1 binds SA (Liu et al., 2020) and is then translocated to the nucleus, where it initiates SA-mediated defense responses through protein-protein interactions with TGA transcription factors, acting as a transcriptional co-activator (Fan and Dong 2002). To further explore the role of SA in CIP, we addressed the requirement of SA signaling, by performing CIP experiments on *npr1* mutant plants. Under mock control conditions, *npr1* mutant plants showed susceptibility to *B. cinerea* that was comparable to mock-treated Col plants, indicating that NPR1 does not have as prominent of a role in the basal defense against this pathogen (Figure 3.3 C). After CK priming, both Col and *npr1* plants showed a decrease in the *B. cinerea* lesion size

as compared to pre-treatment with the mock solution. The average suppression of lesion size by CK was larger in Col plants (33.6 %) than *npr1* plants (23.4 %), suggesting that, like SA, NPR1 is only partially required for CIP against *B. cinerea* (Figure 3.3 C, inset table).

A notable characteristic of priming against biotic stress is the subtle activation of defense gene expression upon application of the priming agent. Then, after the primed plant experiences a pathogen challenge, defense genes are more rapidly and more robustly expressed than in unprimed, pathogen-challenged plants (Conrath et al., 2015, Mauch-Mani et al., 2017). The requirement for JA in CIP led us to test whether CIP reduces susceptibility to *B. cinerea* by priming the expression of the defense gene *PLANT DEFENSIN 1.2* (*PDF1.2*), associated with defense against necrotrophic pathogens (Penninckx et al., 1996). Due to the mostly antagonistic relationship between JA and SA, if CIP primes the JA-mediated defense pathway, increased expression of *PDF1.2* would be expected, accompanied by a decrease in the expression of the SA-regulated defense response gene *PATHOGENESIS RELATED 1* (*PR1*). To test this hypothesis, 4 and 24 H after priming and post-pathogen challenge, leaf tissue was collected for gene expression analysis. Figure 3.4 A shows that *PDF1.2* was down-regulated 4 H after CK application as compared to mock-treated plants, and by 24 H, CK application slightly increased the expression of *PDF1.2*. These results are in line with the known expression pattern of *PDF1.2* in response to JA, which is up-regulated around 24 to 48 H after JA treatment (Penninckx et al., 1996). Surprisingly, following *B. cinerea* inoculation, CIP led to decreased *PDF1.2* expression at both the early and later tissue collection time points, indicating that CIP does not decrease susceptibility of Arabidopsis to *B. cinerea* via JA-mediated defense pathway. While these results are in opposition to the CIP requirement for JA biosynthesis, determined by the use of the mutant *dde2* (Figure 3.3 A), it is possible that genes in the JA pathway other than *PDF1.2* may be

participating in CIP, or that the time points investigated may not capture the timing of expression of *PDF1.2* after pathogen inoculation, which has been shown to occur 1 to 3 days after infection by necrotrophic pathogens (Penninckx et al., 1996).

Interestingly, CIP against *B. cinerea* did prime the SA-mediated defense pathway, as shown by the expression of *PR1* during CIP (Figure 3.4 B). Following CK application we saw a subtle increase in *PR1* expression, followed by a fast, heightened up-regulation of *PR1* expression in response to the inoculation of *B. cinerea* spores in the post-pathogen challenge phase. Thus, it is possible that CIP mediates defense against *B. cinerea* through the known positive relationship CK has on the SA-mediated defense pathway (Choi et al., 2010, Argueso et al., 2012).

The Transcriptional Landscape of Cytokinin-Induced Priming

The results from our defense gene expression analysis prompted us to pursue an in-depth understanding of how CIP reduces susceptibility to *B. cinerea* through RNA-seq, analyzing both the priming phase and the post-pathogen challenge phase for transcriptome changes. Wild type plants were primed 48 H before spray inoculation of a half-strength grape juice solution (as a control) or grape juice solutions containing *B. cinerea* spores. Leaf tissue was collected for RNA extraction at 4 and 24 H, in both the priming phase and the post-pathogen challenge phase for transcriptome analysis (Figure 3.5 A). Sequencing was performed using Illumina technology, with 150bp paired-end reads, and a depth of approximately 40 million reads per sample. Reads were mapped to the Arabidopsis genome version TAIR.10 (Berardini et al., 2015), and differentially expressed genes (DEGs) between the two sample groups were determined by DeSEQ2 (Love et al., 2014) meeting the criteria of a $\log_2$ fold change greater or less than 1 and a

p-value less than 0.05 as determined by Wald's test with Bonferroni correction. For analyses, CK- and/or *B. cinere*a-treated samples were compared to specific controls, explained in Figure 3.5 B.

Transcriptional Responses of the Priming Phase

We analyzed the transcriptional responses of the priming phase using a principal component analysis (PCA). Figure 3.6 A shows a PCA plot of the normalized gene counts from the aligned transcripts of the six samples collected during the priming phase, representing three biological replicates per time point and treatment. The PCA plot shows that samples taken at 4 H or 24 H after priming grouped distinctly apart, indicating that transcripts at these time points after priming are not similar. Moreover, the individual biological replicates at 4 H are not grouped together as closely as compared to the samples at 24 H suggesting that priming by CK has a larger effect on the transcriptome at an earlier time following application.

Between the two time points of the priming phase (4 and 24 H after mock or CK application), there were more DEGs between the control samples and CK-treated samples at 4 H after application (Figure 3.6 B). There were 118 genes that were differentially expressed due to CK at both time points (Figure 3.6 C). Gene Ontology (GO) analysis was completed on the 118 genes using Panther 17.0 (Thomas et al., 2022) to determine the fold enrichment of biological processes greater than 1 and an FDR corrected *p*-value less than 0.05 (Figure 3.6 C). The greatest fold enriched term was for diaminopimelate (DAP) biosynthetic process. DAP is a precursor of lysine, an important amino acid in plant defense. Catabolism of lysine produces the hydroxylated form of L-pipecolic acid known for being the mobile signal in systemic defense (Hartmann et al., 2018, Holmes et al., 2019, Yildiz et al., 2021). Other enriched GO terms include biological

processes of protein translation and protein structure. Of note, no GO categories associated with defense or plant immunity were identified, in agreement with CK acting as a priming agent and not a direct defense activator.

Using the normalized average transcript counts for the samples in the priming phase, a hierarchal clustering heatmap was created using Pheatmap (RRID:SCR_016418) to determine how related the sample groups are to one another (Figure 3.7 A). As expected, the samples collected 24 H after priming are more similar than the samples collected at 4 H. Another distinction between the two time points is the high degree of opposition in clustered genes, as indicated by the colors, between the 4-H samples as compared to the 24-H samples, indicating that priming has a greater impact on gene expression earlier after treatment, likely reflecting the fast response to CK perception after application (D'Agostino et al., 2000).

Given that most changes in gene expression occurred at the earlier time point of 4 H after priming, a GO analysis was performed using the genes that were up-regulated (Figure 3.7 B) or down-regulated by CK treatment as compared to mock-treated plants during this time point (Figure 3.7 B and C). As a validation that the spray treatment of CK induced known CK responses, CK catabolic process was a highly enriched term within the list of up-regulated DEGs (Figure 3.7 B). Most other highly enriched terms were those that related to translation and RNA processing. From the down-regulated DEGs, the GO terms that were highly enriched were related to metabolic processes including biosynthesis or catabolism of amino acids, sugars, and starch (Figure 3.7 C). These GO results suggest that CIP increases processes related to translation, while decreasing primary metabolic processes.

Transcriptional responses of the post-priming phase

To analyze the impact that CIP has on the post-pathogen challenge phase, a hierarchal cluster heatmap was also used to compare all the treatments and time points of the collected samples (Figure 3.8). The primed and unprimed samples that were collected 4 H following the treatment with either half-strength juice as a control or *B. cinerea* spores in half-strength juice show little difference in the degree of expression of the clusters of genes indicating a high degree of similarity between samples, suggesting at this time point, the transcriptome has yet to be affected by the pathogen inoculation. Twenty-four H after inoculation with either juice as a control or *B. cinerea* (Figure 3.8) shows an increase in the intensity of differential expression, indicating at this later time point, responses to *B. cinerea* can be seen and differentiated from the response of the juice control treatments. This is in agreement with other studies, which have shown transcriptional responses to necrotrophic pathogens happening 24 H or later after pathogen inoculation (Penninckx et al., 1998). Based on these results, further analysis of the post-pathogen challenge phase focused on the samples collected 24 H after inoculation.

One of the first responses of the post-pathogen challenge phase we analyzed was the effect of *B. cinerea* alone on the Arabidopsis transcriptome, without priming. These analyses involved samples that were treated with a mock solution, and then 24 H later treated with either grape juice, or with *B. cinerea* spores diluted in grape juice. GO term enrichment was used to analyze either up- (Figure 3.9 A) or down-regulated DEGs (Figure 3.9 C). DEGs that were up-regulated due to *B. cinerea* showed a GO enrichment for plant defense terms known to have a role in defense against necrotrophic fungal pathogens (Ferrari et al., 2003, Ferrari et al., 2007, Sham et al., 2017, Zhang et al., 2017, Chen et al., 2022), including biosynthesis of camalexin, a

phytoalexin associated with defense to *B. cinerea*. Other biological processes that were enriched include many terms related to DNA replication. In response to *B. cinerea*, DEGs that were down-regulated showed enrichment for GO terms relating to photosynthetic machinery and carbon fixation (Figure 3.9 B).

Following these analyses, we then focused on genes that could have been primed by CK treatment, and whose expression was potentiated upon *B. cinerea* inoculation. Hierarchical clustering analyses showed samples that were primed with CK did not display a high degree of difference from samples that were treated with the mock solution 24 H after inoculation (Figure 3.8), indicating that the effect of CIP on the transcriptome could possibly not be different than unprimed plants, or that a small, select subset of genes regulate CIP defense responses. However, given that priming agents can potentiate defense gene expression, it is possible that the same genes are impacted in unprimed and primed plants following pathogen challenges, and that they differ only in their degree of expression. Therefore, we compared the lists of DEGs of primed and unprimed samples that were inoculated with *B. cinerea* to their respective juice control inoculation (Figure 3.10 A) and determined that there were 6429 similar DEGs between samples the mock-treated, *B. cinerea* inoculated samples (unprimed and challenged) and the CK-treated, *B. cinerea* inoculated samples (primed and challenged). If CIP acts by potentiating the expression of similar DEGs, these genes would show an increase in the degree of expression. To test this hypothesis, we divided the DEGs into up- or down-regulated lists. Out of the 6429 DEGs, 2007 were up-regulated and 4064 were down-regulated in both primed and unprimed *B. cinerea* samples as compared to their respective juice-inoculated control samples, indicating that potentiated gene expression was possible. To determine if there was a difference in gene expression between primed or unprimed *B. cinerea*-inoculated samples (Figure 3.10 B), the two

lists of DEGs between either priming treatment as compared to its juice-inoculated control were combined, filtered for duplicated genes, and used to determine DEGs (Figure 3.5 B). Using this subset of DEGs, GO term enrichment analysis was completed on either up- (Figure 3.10 C) or down-regulated (Figure 3.10 D) DEGs in CK-treated samples as compared to mock-treated samples. The results show that CIP increases the expression of genes associated with the synthesis or maintenance of photosynthetic machinery or primary metabolism while decreasing the expression of genes relating to defense, DNA replication, and processes related to protein translation. In comparison to the response of unprimed plants to *B. cinerea* (Figure 3.9), CIP has the opposite effect, where defense is decreased while increasing the processes involved in carbon fixation.

These results from the transcriptome experiments did not elucidate any information as to the role of CIP on either SA- or JA-mediated processes. Therefore, using the gene expression data from the RNA-seq results, we identified the expression of known JA signaling genes and the SA biosynthetic gene *ICS1*, either 4 H after priming or 24 H *B. cinerea* pathogen challenge (Figure 3.11). *ICS1* had a lower expression due to CK treatment in both phases of CIP (Figure 3.11 B). Downstream of JA biosynthesis, there are two branches of JA-mediated signaling: one controlled by the transcription factor MYC2, and another controlled by the transcription factors ERF1 and ERF59 (Kazan and Manners 2008). Activation of the MYC or the ERF branch of JA signaling is marked by the expression of *VSP2* (Lorenzo et al., 2004, Kazan and Manners 2008, Verhage et al., 2011) or *PDF1.2* (Berrocal-Lobo et al., 2002, Lorenzo et al., 2003), respectively. In both the priming phase and post-challenge phase, the expression of *VSP2* is up-regulated whereas *PDF1.2* is down-regulated, indicating that CIP results in promoting the activation of the MYC branch over the ERF branch of JA-mediated signaling (Figure 3.11 B).

3.5 DISCUSSION

This study aimed to gain insight into how the plant growth-promoting hormone CK can act as a priming agent against the necrotrophic fungal pathogen *B. cinerea*. The effect of exogenously applied CK on pathogen susceptibility depends on the concentration of CK applied. For example, the application of less than 100μM CK to Arabidopsis increases susceptibility to *Hpa* infection (Argueso et al., 2012), and only greater than 1μM CK decreases susceptibility to *B. cinerea* in tomato (Gupta et al., 2020). To understand if there is also a concentration-dependent effect of applied CK on susceptibility to *B. cinerea* in Arabidopsis, we primed wild type plants with both 100nM CK and 100μM CK 48 H before inoculation with pathogen spores. Our results showed that, as other studies have seen, different concentrations of applied CK differentially affect susceptibility, where lower concentrations of applied CK increase lesion size and higher concentrations of CK limit the lesion size.

A previous study demonstrated that application of the CK kinetin, prior to *B. cinerea* infection altered susceptibility to the pathogen (Li et al., 2021). Further, it was shown that fungal growth, including *B. cinerea*, in media containing high CK concentration, inhibits spore germination and germ tube growth ultimately reducing overall fungal growth (Gupta et al., 2021a). Similar results were seen in a separate study in canola where CK inhibited colony growth of two other necrotrophic pathogens, *Leptosphaeria maculans* and *Alternaria brassicae*, and also restricted *in vitro* mycelial growth (Sharma et al., 2010). Therefore, it was possible that the application of CK to the surface of leaves was leading to negative effects of *B. cinerea* spores and not CK-mediated plant defense responses resulting in reduced necrotic fungal lesions sizes. Utilizing two CK signaling mutants that either increased or decreased endogenous CK signaling, we showed that genetic alterations in endogenous CK signaling and susceptibility to *B. cinerea*

were correlated, thus suggesting that exogenous application of CK activates endogenous CK signaling to ultimately alter pathogen susceptibility.

As previously mentioned, CIP has been shown to be an effective priming agent against a broad range of pathogens with different lifestyles. However, until now it has been unclear whether CIP can prime both SA-mediated defense and JA-mediated defense pathways. These two defense pathways are known to be antagonistic to one another, however, this binary activation of only one hormone-mediated defense pathway at a time has been demonstrated to have more nuance (Zhang et al., 2018). For instance, during effector-triggered immunity against *Pseudomonas syringae* pv. *maculicola avrRpt2* both SA and JA accumulate and JA signaling is activated by the SA receptors NPR 3 and NPR4 (Liu et al., 2016). Interestingly, reduced susceptibility to *B. cinerea* in tomatoes treated with CK was demonstrated not to be JA-dependent, but rather dependent on SA-mediated defenses and another plant hormone known to contribute to JA-mediated defenses, ethylene (Gupta et al., 2020, Gupta et al., 2021b). Our results showed that CIP had no effect on *dde2* plants, indicating that priming by CK requires biosynthesis of JA in order to reduce susceptibility. On the other hand, using SA signaling and biosynthesis mutants we demonstrated that these processes are partially required for CIP.

While these results provided evidence of the requirement of JA and SA for CIP, they provided no insights into how CIP works at the molecular level and how these hormones mediate CIP. To address if the application of CK primes both JA- and SA-mediated defense, gene expression of *PDF1.2* and *PR1*, markers of the JA and SA defense pathways, respectively, were determined during the priming phase and the post-priming pathogen challenge phase. Surprisingly, our results show that CIP does not potentiate the expression of the JA marker *PDF1.2*, and potentiates *PR1*. In contrast, CIP in tomatoes increased the expression of

pathogenesis-related genes *PR1a* and *PR1b* following priming (Gupta et al., 2020). However, after *B. cinerea* inoculation, both genes were down-regulated in CK-primed plants, suggesting CK-induced defense may utilize different hormonal pathways in different plant species. Another study conducted in Arabidopsis showed that *PDF1.2* did demonstrate potentiated expression levels following *B. cinerea* infection while *PR1* had similar up-regulated expression levels as untreated plants (Li et al., 2021). In contrast to our methodology, the authors treated plants for three consecutive days prior to the pathogen challenge and thus would not be considered priming due to the plants being in an extended state of heightened defense mediated by multiple CK applications (refer to results in Chapter 2). An explanation for our results can be attributed to the JA-mediated defense signaling has two separate branches: MYC and ERF. The MYC branch, named after the MYC transcription factors involved in JA signaling, is known for mediating defense against physical wounding like herbivory, and its activation is marked by the expression of *VEGETATIVE STORAGE PROTEIN 2* (*VSP2)* gene (Lorenzo et al., 2004, Kazan and Manners 2008, Verhage et al., 2011). The ERF branch mediated synergistically by JA and ethylene, is activated in response to a necrotrophic pathogen attack and its activation is marked by increased expression of *PDF1.2*, controlled by ETHYLENE RESPONSE FACTOR 1 (ERF1) and ERF59 (Berrocal-Lobo et al., 2002, Lorenzo et al., 2003). Together with our RNA-seq results showing that *VSP2* is induced in CIP, our results demonstrate that CK may act to increase the MYC branch of JA signaling over the ERF branch, even though the ERF branch has been demonstrated to have a larger role in responses to necrotrophic pathogen. The partial requirement for SA and NPR1 explain the potentiation of *PR1* observed in CIP, and are in line with previous observations that demonstrated that infection of *npr1* with different *B. cinerea* isolates are able to increase *PR1* expression without a functional NPR1 (Zhang et al., 2017). These results highlight

the complex contribution of phytohormonal networks to CIP reflecting the quantitative nature of genetic resistance against *B. cinerea* in Arabidopsis and other plant species, and likely to be dependent on the contribution of several plant hormone pathways (Caseys et al., 2021).

Transcriptome analysis of CIP against *B. cinerea* was performed using tissue collected at the same time points during the priming phase and post-priming pathogen, at 4 and 24 H after either priming and pathogen challenge. Our analyses showed that there is a greater change in the transcriptome early after CK application, confirming previous results (Chapter 2). GO term enrichment analysis to determine genes overrepresented during CIP. One interesting aspect uncovered by our analyses is the fact that during the priming phase, the term with the highest fold enrichment was the biosynthesis of DAP, a precursor molecule to lysine. Lysine is an important compound in defense, that when catabolized forms the non-proteinogenic amino acid L-pipecolic acid or Pip (Navarova et al., 2012, Ding et al., 2016). Pip is a known priming agent that accumulates upon pathogen infection (Navarova et al., 2012) and can be hydrolyzed to form N-hydroxypipecolic acid (NHP) (Hartmann et al., 2018, Holmes et al., 2019). NHP has been recently proven to be the molecule responsible for the mobile signal in Systemic Acquired Resistance (SAR) (Yildiz et al., 2021). Therefore, CIP could initiate the accumulation of the starting molecules needed to quickly synthesize the compound responsible for increasing plant defense distally when challenged with a pathogen. Other biological processes enriched were those pertaining to RNA processing, modification, and ribosomal biogenesis. These results suggest that early responses to priming by CK begin to prepare the plant for potentially faster or more efficient protein translation (Yao et al., 2008), a process that has been recently associated with activation of plant immunity and regulation of cell death (Yoo et al., 2020).

Within the post-pathogen challenge phase, we observed that the majority of transcriptional changes occurred later after pathogen inoculation. GO term enrichment for up- and down-regulated DEGs after inoculation of unprimed plants with *B. cinerea* showed an up-regulation of genes relating to defense responses, and down-regulation of genes that contribute to photosynthetic-related processes, a transcriptional signature similar to previous studies of *B. cinerea* infection in Arabidopsis (Berger et al., 2004, Rossi et al., 2017). Other studies in strawberry (Badmi et al., 2022), cucumber (Kong et al., 2015), and grape (Agudelo-Romero et al., 2015) have also shown a down-regulation in photosynthesis in response to *B. cinerea* infection indicating a conserved defense strategy induced by the host plant aimed to limit nutrient availability to the pathogen.

For the analyses of the DEGs from CK primed and unprimed plants subjected to *B. cinerea* infection, the resulting DEGs were separated into up- and down-regulated lists before performing GO term enrichment analysis. Interestingly, results from GO analyses shows similar biological processes enriched during CIP with *B. cinerea*, as compared to *B. cinerea* infection alone, however, priming by CK has the opposite effect on the regulation. Contrary to what was seen for infection with *B. cinerea* alone, CIP samples showed up-regulation of genes associated with the photosynthetic machinery and lower expression for defense responses and DNA replication.

While puzzling, the results above may in fact reflect the necrotrophic nature of *B. cinerea*, and its virulence strategies. When *B. cinerea* infects plant cells it utilizes toxins to induce cell death in order obtain nutrients for its survival and reproduction. This resulting cell death is likely reflected in our transcriptome analyses by the down-regulation of genes involved in photosynthesis. Similarly, the up-regulation of genes associated with defense activation is

often accompanied by the activation of plant cell death for activation of the hypersensitive response. Thus, both transcriptional responses align well with what is expected from dying cells during infection with a necrotrophic pathogen. Conversely, the opposite transcriptional response observed in CIP, with increased expression of genes associated with photosynthesis and down-regulation of defense genes, may reflect a plant defense strategy to suppress defense resulting in inhibition of cell death while promoting cell survival through activation of photosynthesis, thus keeping the necrotrophic pathogen at bay. Of interest, the role of NPR1 in promoting cell death and cell survival has been recently elucidated (Zavaliev et al., 2020), providing a possible mechanistic link to the molecular mechanisms of CIP. More specifically this study demonstrated that in response to SA, NPR1 will not only monomerize, enter the nucleus, and initiate SA-dependent responses (including cell death) but also form cytoplasmic condensates that target substrates for degradation leading to cell survival. This dual role of NPR1 is thought it be in response to varying endogenous concentrations of SA (Zavaliev et al., 2020), where low concentrations of SA trigger the degradation of NPR1, preventing it from initiation of SA-transcriptional response. High SA concentrations also lead to NPR1 degradation but in a manner that inhibits the repression of programmed cell death (Fu et al., 2012). Interestingly, after infection with *B. cinerea*, hypersensitive-like cell death occurs in a SA-dependent manner (Shlezinger et al., 2011), thus combined with our results indicating a role for NPR1 in CIP, it is possible that the CIP inhibits defense responses by regulating the role of SA-mediated defenses. Future research should focus on how CIP balances cell death and survival in plants and how this could be manipulated to create new strategies of disease protection.

3.6 CONCLUSIONS:

In this study, the versatile role of the plant growth-promoting hormone CK as a priming agent against the necrotrophic fungal pathogen *B. cinerea* was investigated. Notably, the effect of exogenously applied CK on susceptibility to *B. cinerea* was found to be concentration-dependent. While lower concentrations of applied CK increased lesion size, higher concentrations limited lesion development, a result consistent with previous findings on using CK as a priming against *B. cinerea* in other plant-pathogen interactions. Genetic alterations of endogenous CK signaling correlated with altered susceptibility to *B. cinerea*, validating our assumption that the exogenous application of CK activates endogenous CK signaling to influence pathogen susceptibility rather than modifying *B. cinerea* spore development.

With the aim of furthering our understanding of how CIP can reduce susceptibility to both hemi/biotrophic and necrotrophic pathogens, we aimed to identify the role SA- and JA-mediated defense mechanisms induced during CIP against *B. cinerea*. The results unveiled a complex interplay, where CK-induced priming required JA biosynthesis but partially relied on SA-mediated pathways, providing insights into the nuanced regulation of defense mechanisms needed in response to *B. cinerea*. Transcriptome analysis during the priming phase and post-priming pathogen challenge of CIP furthered our understanding of CK-mediated defenses. Early responses to CK application showed an up-regulation of genes involved in the biosynthesis of precursor molecules for defense compounds, possibly used for quick initiation of plant defense mechanisms after pathogen challenge. During the post-pathogen challenge phase, significant transcriptional changes were observed in response to *B. cinerea* infection in unprimed plants. Genes associated with defense responses were up-regulated, while those related to photosynthesis were down-regulated, aligning with previous studies on plant responses to

necrotrophic pathogens. Notably, the study uncovered that CIP led to an opposite transcriptional response when compared to *B. cinerea* infection alone where primed plants exhibited an up-regulation of photosynthesis-related genes and down-regulation of defense genes. These results led us to hypothesize that CIP suppresses defense responses, like the hypersensitive response resulting in cell death, while promoting cell survival during pathogen attack. The transcriptome analysis further indicated the role of CK on JA defense pathways, demonstrating a more prominent role for the wound-response branch of JA signaling over the pathogen-response branch.

In conclusion, this research has provided valuable insights into the intricate mechanisms of CIP against *B. cinerea*. The findings have significant implications for the development of innovative strategies to enhance plant resistance and optimize crop protection against devastating pathogens like *B. cinerea*. Future research endeavors should delve deeper into the mechanistic details of CIP, including the balance between cell death and survival in plants, offering new avenues for agricultural disease management strategies.

3.7 FIGURES:

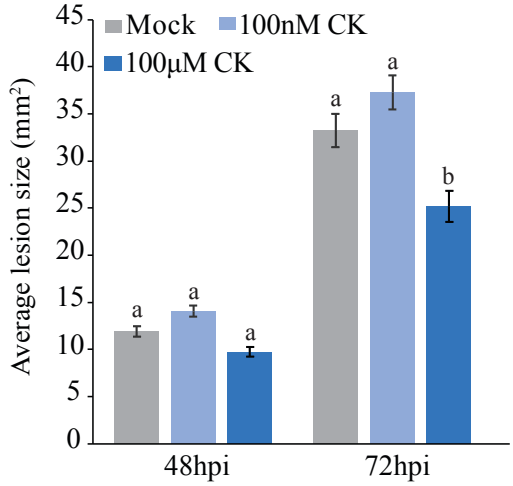


Figure 3.1 | Spray application of different concentrations of cytokinin (CK) to Arabidopsis alters susceptibility to *Botrytis cinerea* 48 hours after application: Five-week-old wild type plants were sprayed with 0.01% DMSO mock solution or different concentrations of the CK benzylaminopurine (BA) in 0.01% DMSO 48 hours before inoculation with *B. cinerea* spores. Necrotic lesions were measured 48 and 72 hours post inoculation (hpi). Data is pooled from 3 biological replicates. Different letters indicate statistical differences between treatments with a *p*-value < 0.05 at the specified time point by two-way ANOVA with TUKEY HSD correction.

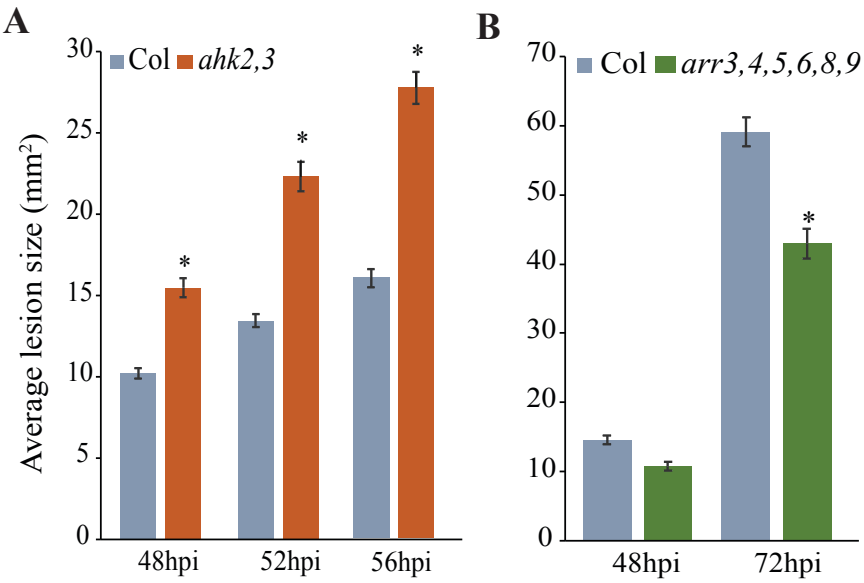


Figure 3.2 | Perturbation in endogenous cytokinin signaling alters basal defense against *Botrytis cinerea:* Six-week-old Col, *ahk2,3* (**A**), and *arr3,4,5,6,8,9* (**B**) plants were inoculated with *B. cinerea* spores. Necrotic lesion size was measured at the specified hours post-inoculation (hpi). Data pooled from three biological replicates. Asterisks indicate a statistical difference with a *p*-value < 0.05 as determined by one-way ANOVA with TUKEY HSD correction per time point.

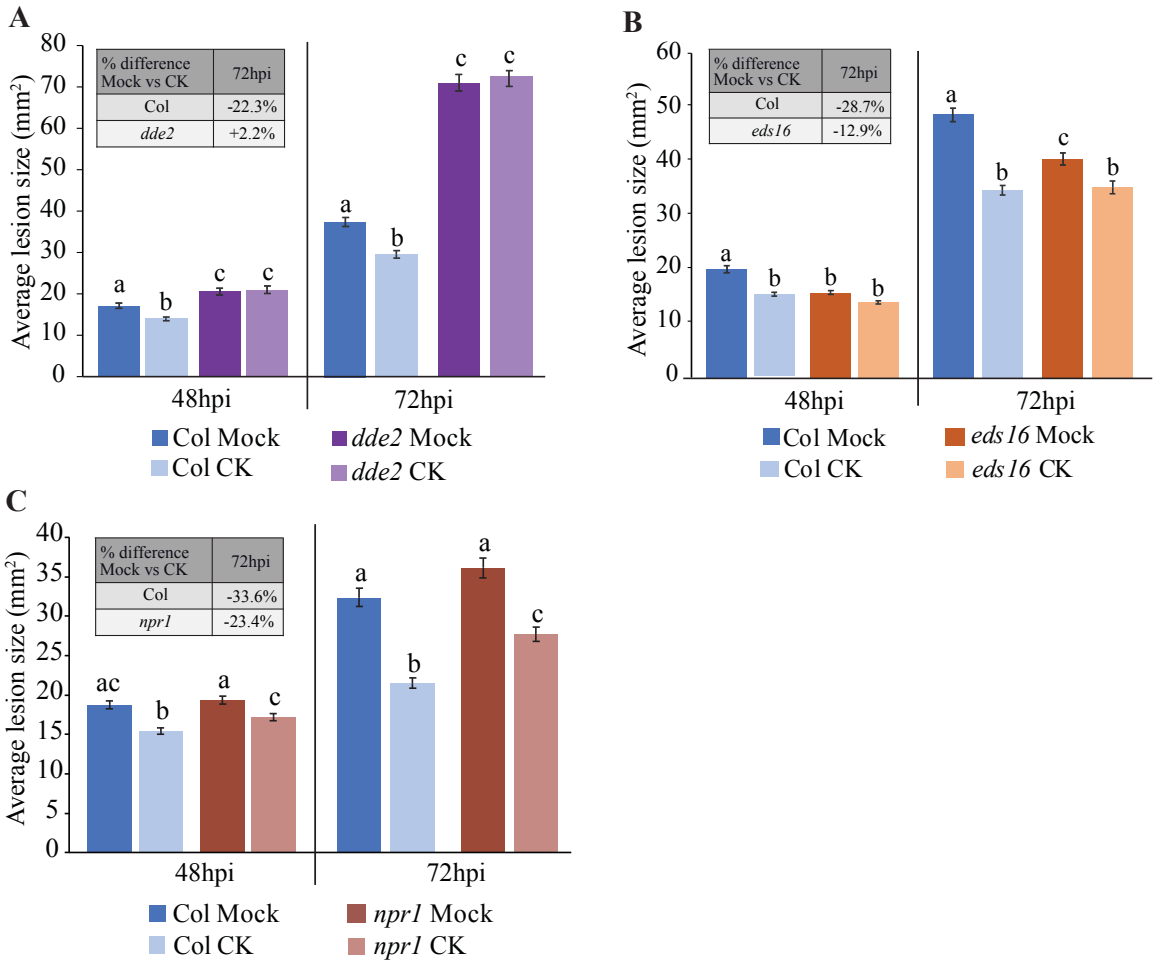


Figure 3.3 | Cytokinin-induced priming against *Botrytis cinerea* requires jasmonic acid biosynthesis but only partially requires salicylic acid biosynthesis and signaling: Five-week-old Col, *dde2* (**A**), *eds16* (**B**), and *npr1* (**C**) plants were sprayed with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours prior to inoculation with *B. cinerea* spores. Necrotic lesions were measured 48 and 72 hours post-inoculation (hpi). Grey boxes display the percent difference in average lesion size between CK- and mock-treated plants at 72 hpi. Data is pooled from three biological replicates. Different letters indicate statistical differences with a *p*-value < 0.05 at the specified time point by two-way ANOVA with TUKEY HSD correction.

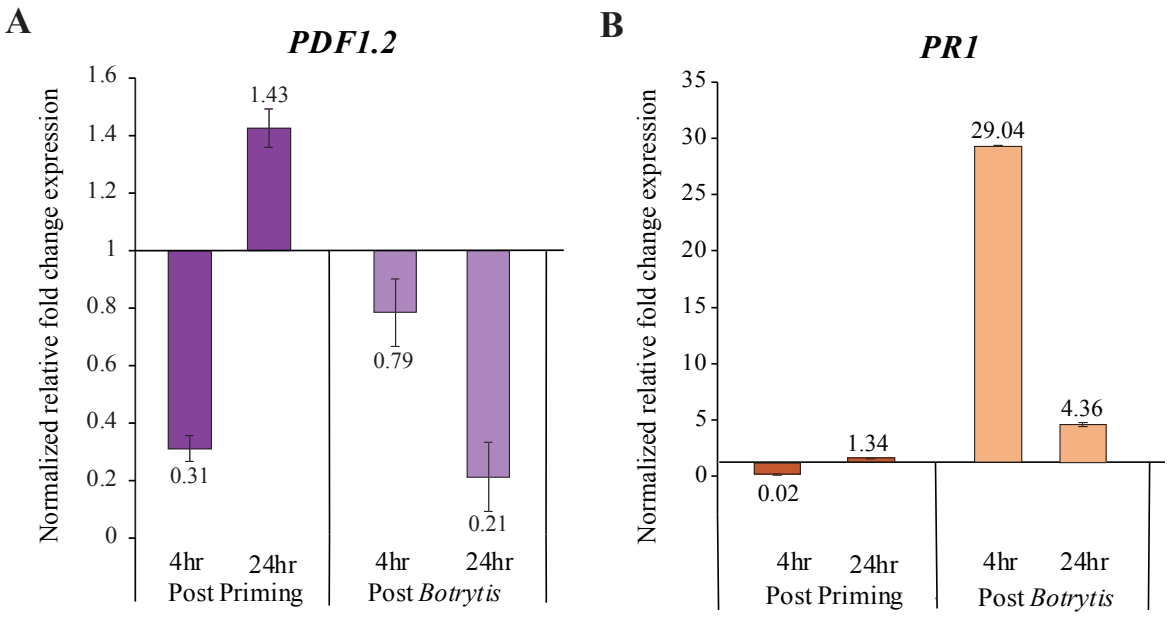
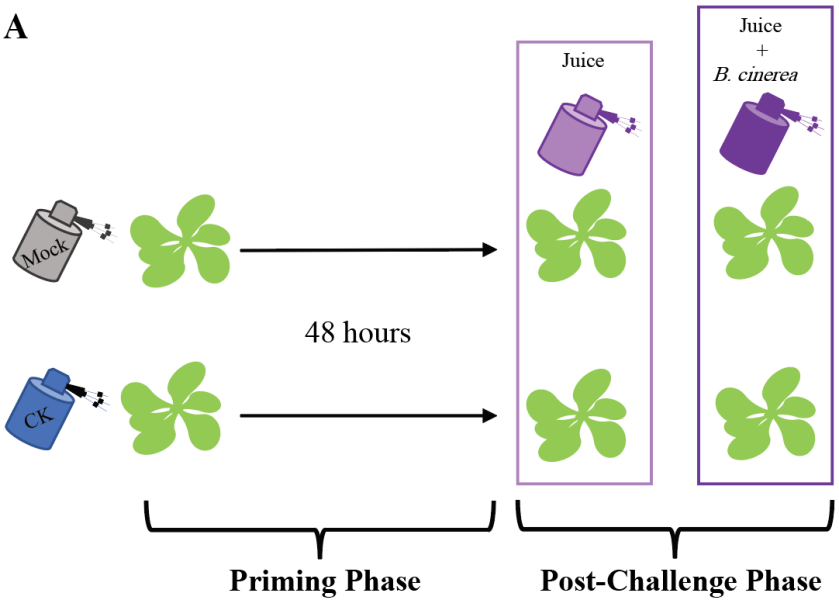


Figure 3.4 | Cytokinin-induced priming against *Botrytis cinerea* potentiates the expression of *PR1* but does not potentiate the expression of *PDF1.2:* Five-week-old wild type plants were primed with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO prior to spray-inoculation with *B. cinerea* spores. Leaf tissue was collected 4 and 24 hours after priming treatments and pathogen inoculation. Four leaves from three plants of similar treatment were pooled prior to RNA extraction for *PDF1.2* (**A**) and *PR1* (**B**) expression analysis by qRT-PCR. Values are indicative of the fold change normalized expression fold change of 100µM CK/Mock relative to *UBQ*. Data is representative of three biological replicates.

A



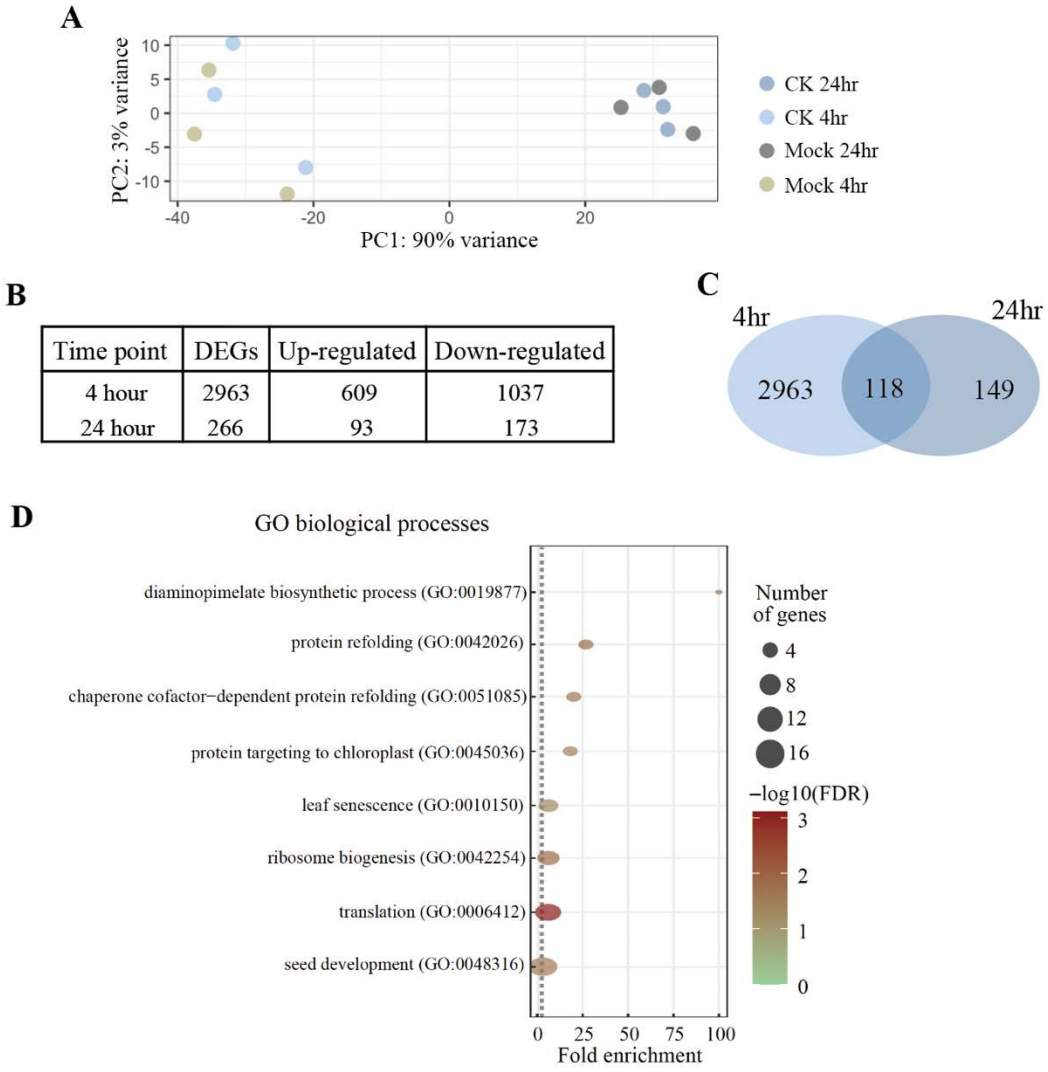
Stimuli	Timepoint
Mock	4hr
Mock	24hr
CK	4hr
CK	24hr

Stimuli	Inoculation	Timepoint
Mock	Juice	4hr
Mock	Juice	24hr
Mock	Juice + *B. cinerea*	4hr
Mock	Juice + *B. cinerea*	24hr
CK	Juice	4hr
CK	Juice	24hr
CK	Juice + *B. cinerea*	4hr
CK	Juice + *B. cinerea*	24hr

B

Timepoint	Comparison	Outcome	# DEGs
4 hour	Mock vs CK	The effect of CK in the priming phase	2963
24 hour	Mock vs CK		266
4 hour	Mock Juice vs Mock *B. cinerea*	The effect of *B. cinerea* while controlling for the effect of the application of juice	9
24 hour	Mock Juice vs Mock *B. cinerea*		7825
4 hour	CK Juice vs CK *B. cinerea*	The effect of *B. cinerea* in CK-primed plants in the post-challenge phase while controlling for the effect of the application of juice	3
24 hour	CK Juice vs CK *B. cinerea*		7948
24 hour	Mock *B. cinerea* vs CK *B. cinerea*	The effect of *B. cinerea* in CK-primed plants in the post-challenge phase	8147

Figure 3.5 | Transcriptome analysis experimental design of cytokinin (CIP)-induced priming against *Botrytis cinerea*: (**A**) Diagram of the experimental design for CIP against *B. cinerea* showing the applied stimuli, inoculations, and time points of tissue collection for RNA-seq analysis during the priming and post-challenge phase. Leaf tissue was collected for RNA-seq analysis 4 and 24 hours after treatment of five-week-old plants with either with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO or 4 and 24 hours after subsequent inoculation with grape juice control or *B. cinerea* spore in grape juice. Table **B** shows the comparisons of samples and the outcome of the comparison with the number of overall differentially expressed genes (DEGs) as determined by a $\log_2$ fold change of +/- 1 and a *p*-value < 0.05 as determined by Wald test using DESeq2 (Love et al., 2014).



Time point	DEGs	Up-regulated	Down-regulated
4 hour	2963	609	1037
24 hour	266	93	173

Figure 3.6 | Priming by cytokinin (CK) alters the transcriptome early but also has a sustained effect on gene expression: Leaf tissue was collected for RNA-seq analysis 4 and 24 hours after treatment of five-week-old plants with either with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO. (**A**) PCA plot of the individual biological replicates following priming. (**B**) The number of differentially expressed genes (DEGs) with $\log_2$ fold change of +/- 1 and a *p*-value < 0.05 as determined by Wald test using DESeq2 (Love et al., 2014). (**C**) A comparison of the overall DEGs for each time point following priming shows 118 genes have sustained differential expression mediated by 100µM BA treatment. (**D**) Gene Ontology (GO) term enrichment of the common 118 genes (**C**) was determined by Panther 17.0 (Thomas et al., 2022) with fold enrichment > 1 and an FDR corrected *p*-value < 0.05 are shown.

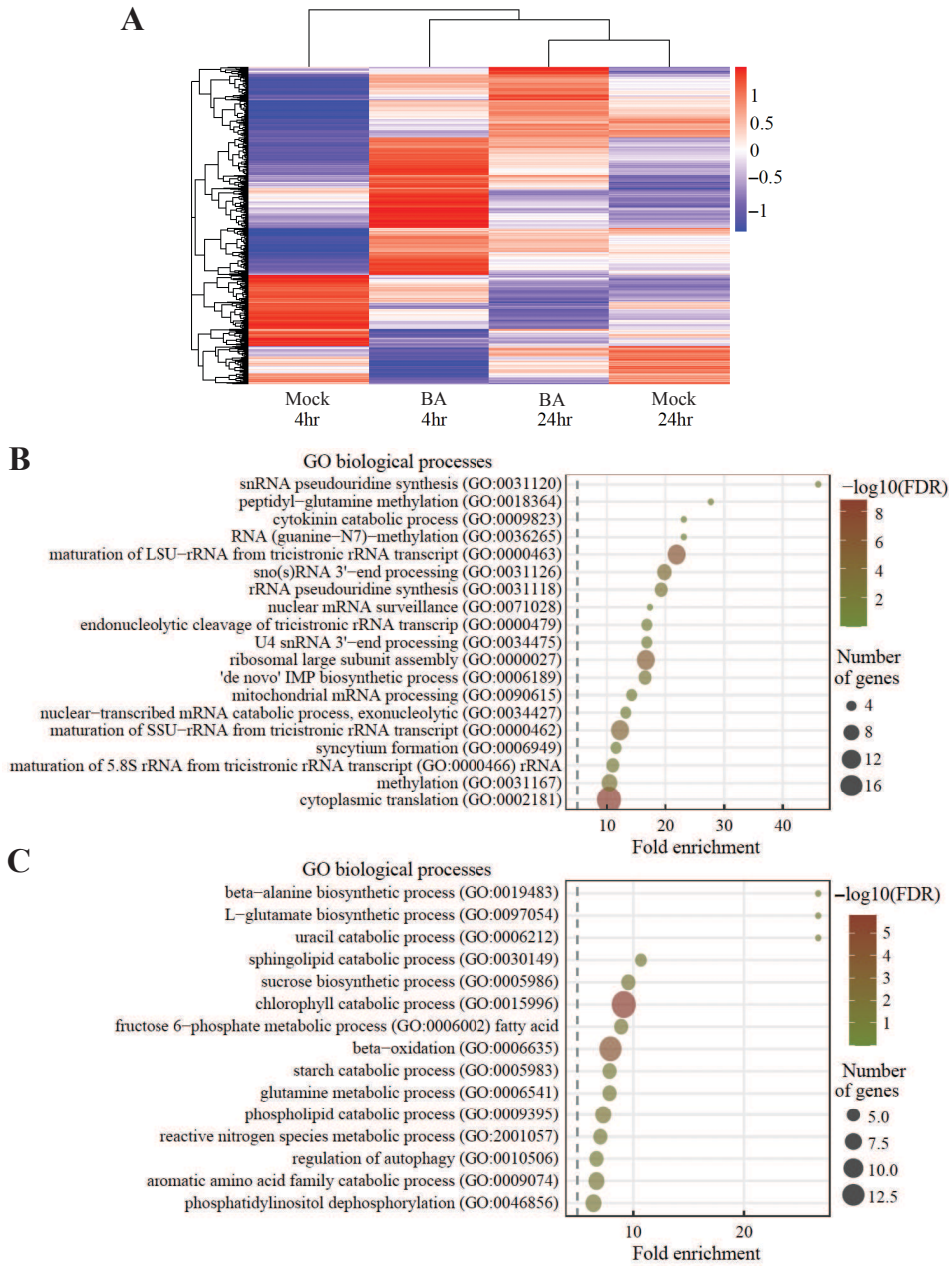


Figure 3.7 | Priming by cytokinin (CK) has a greater impact on the transcriptome early after application: Leaf tissue was collected for RNA-seq analysis 4 and 24 hours after treatment of five-week-old plants with either with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO. Hierarchal clustering heatmap (**A**) compared the average normalized gene counts of the spray treatments at 4 and 24 hours after application. Four hours after priming, differentially expressed genes (DEGs) with a $\log_2$ fold change of +/- 1 and a *p*-value < 0.05 as determined by the Wald test using DeSeq2 (Love et al., 2014), were used to determine Gene Ontology (GO) term enrichment (**B**) and (**C**) using Panther 17.0 (Thomas et al., 2022). (**B, C**) GO term enrichment for the up-regulated DEGs with a fold enrichment >10 (**B**) and for the down-regulated DEGs with a fold enrichment >5 (**C**) and a FDR corrected *p*-value >0.05 are shown.

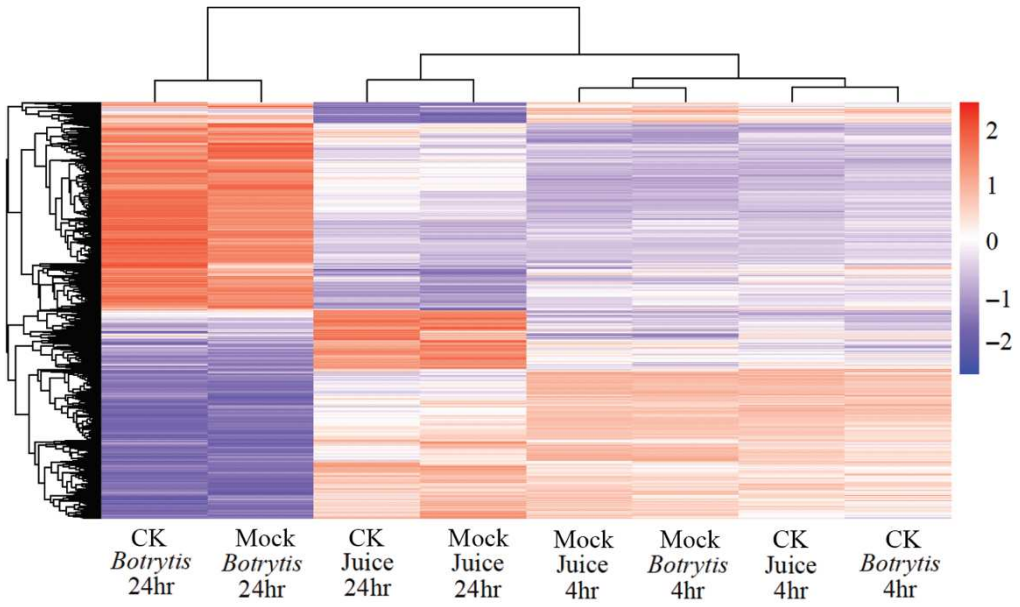
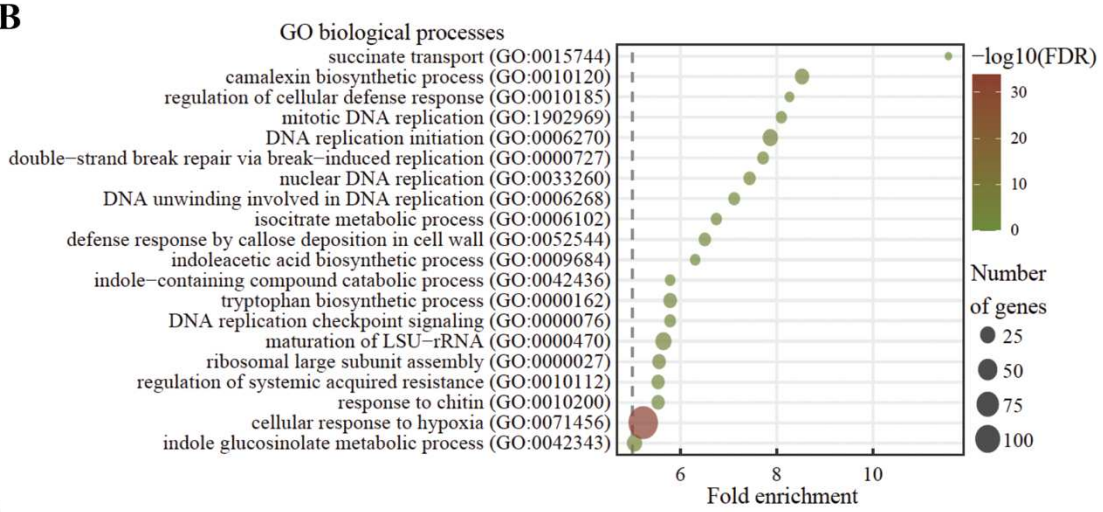


Figure 3.8 | In the post-challenge phase of cytokinin (CK)-induced priming, *Botrytis cinerea* alters the plant transcriptome 24 hours after inoculation: Five-week-old plants treated with either 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours before inoculation with juice control solution or *B. cinerea* spores in juice solution. Leaf tissue was collected for RNA-seq analysis 4 and 24 hours after inoculation. Hierarchal clustering heatmap compared the average normalized gene counts of the post-pathogen challenge samples at 4 and 24 hours after application of either a juice control solution or *B. cinerea* spores in juice solution.

A

TimePoint	Comparison	Overall DEGs	Up-regulated DEGs	Down-regulated DEGs
24 hour	Mock Juice vs Mock *B. cinerea*	**7825**	2421	5404

B



C

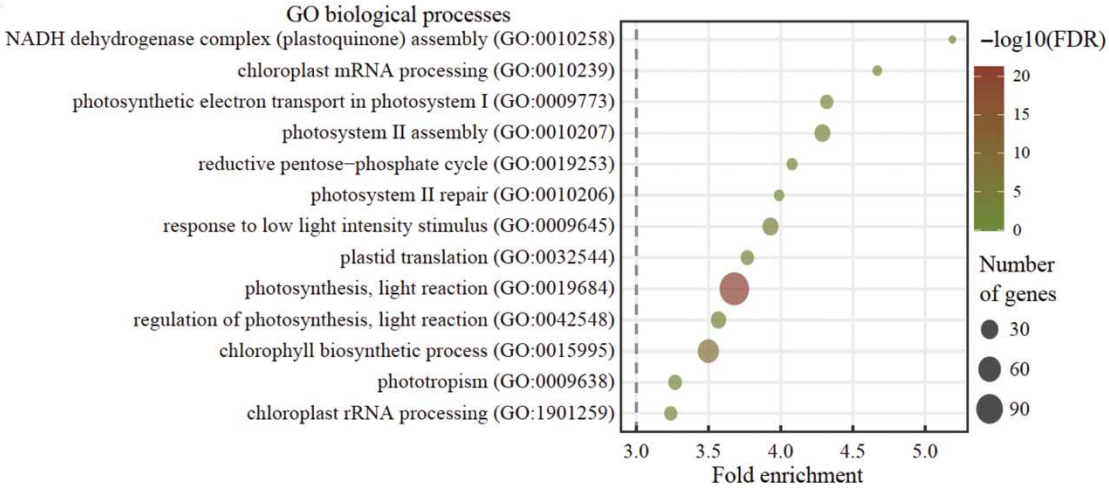


Figure 3.9 | The effect of *Botrytis cinerea* challenge on the plant transcriptome shows altered expression of genes associated with defenses and photosynthesis: Five-week-old plants treated with either 0.01% DMSO mock solution 48 hours before inoculation with a juice control solution or *B. cinerea* spores in a juice solution. Leaf tissue was collected for RNA-seq 24 hours after inoculation. (**A**) Differentially expressed genes (DEGs) with a $\log_2$ fold change of +/- 1 and a *p*-value < 0.05 as determined by the Wald test using DeSeq2 (Love et al., 2014) were used to determine Gene Ontology (GO) term enrichment using Panther 17.0 (Thomas et al., 2022). GO term enrichment for the up-regulated DEGs with a fold enrichment > 4 (**B**) and for the down-regulated DEGs with a fold enrichment >5 (**C**) and a FDR corrected *p*-value < 0.05 are shown.

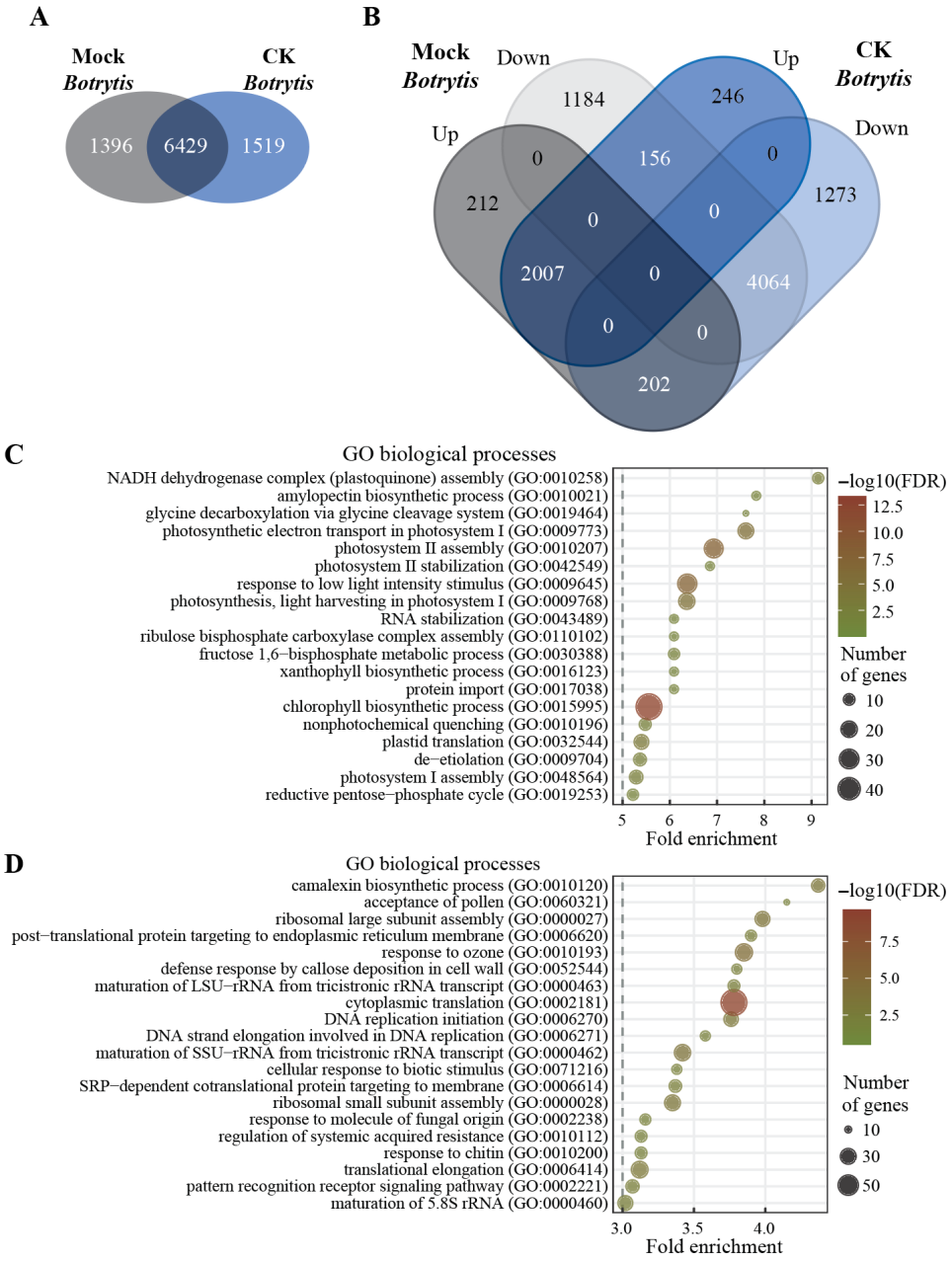


Figure 3.10 | Cytokinin (CK)-induced priming against *Botrytis cinerea* increases carbon fixation while decreasing defense responses in the post-challenge phase: Five-week-old plants treated with either 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours before inoculation with a juice control solution or *B. cinerea* spores in a juice solution. Leaf tissue was collected for RNA-seq analysis 24 hours after inoculation. (**A**) Venn diagram of all differentially expressed genes (DEGs) with a $\log_2$ fold change of +/- 1 and a *p*-value < 0.05 as determined by the Wald test using DeSeq2 (Love et al., 2014) between mock- or CK-treated samples sprayed with *B. cinerea* spores as compared to their respective juice inoculation show 6429 similar DEGs. To identify if the 6429 DEGs were similarity regulated between priming treatments, lists of DEGs were separated into up-and down-regulated genes as

compared to their respective juice-inoculated control (**B**). To understand the difference between mock- and CK-treated samples challenged with *B. cinerea*, a subset of genes that were differentially expressed as compared to the respective juice inoculation control was used to determine DEGs between the *B. cinerea* inoculated primed and unprimed treatments. These DEGs were used for Gene Ontology (GO) term enrichment (**C**) and (**D**) using Panther 17.0 (Thomas et al., 2022). (**C, D**) GO term enrichment for the up-regulated DEGs with a fold enrichment > 5 (**C**) and for the down-regulated DEGs with a fold enrichment >4 (**D**) and a FDR corrected *p*-value < 0.05 are shown.

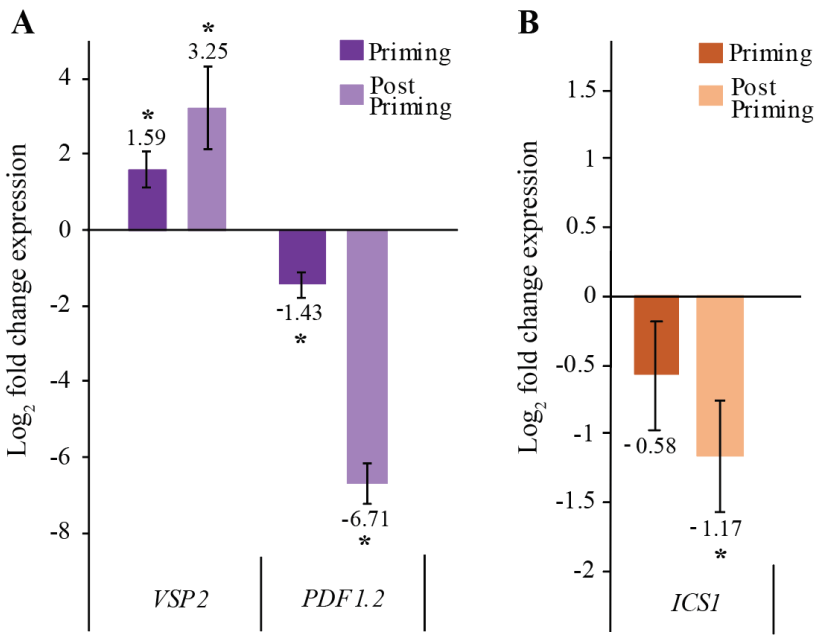


Figure 3.11 | Cytokinin (CK)-induced priming against *Botrytis cinerea* up-regulates a specific branch of jasmonic acid (JA) signaling while down-regulating salicylic acid (SA) biosynthesis: Five-week-old plants treated with either 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours before inoculation with *B. cinerea* spores. Leaf tissue was collected for RNA-seq analysis 4 hours after priming and 24 hours after pathogen inoculation. Select genes from the RNA-seq analysis are displayed as a $\log_2$ fold change in expression of CK/mock treatments from the priming phase and (CK + *B. cinerea*)/(mock + *B. cinerea*) from the post-challenge phase as described in Figure 3.10. (**A**) displays JA signaling genes *VSP2* and *PDF1.2* and (**B**) displays SA biosynthesis gene *ICS1*. Asterisks indicate a significant difference as determined by a $\log_2$ fold change of +/- 1 and a *p*-value < 0.05 as determined by the Wald test using DeSeq2 (Love et al., 2014).

3.8 TABLE

Table 3.1 | qRT-PCR primers used in this study: The table shows the primer sequences used within this study for qRT-PCR for gene expression analysis. All sequences are written in the 5' to 3' direction.

Gene	Forward Primer Sequence	Reverse Primer Sequence
PDF1.2	GCTTCCATCATCACCCTTATCTTC	ACATGGGACGTAACAGATACACTTGT
PR1	ACACGTGCAATGGAGTTTGTGGTC	TACACCTCACTTTGGCACATCCGA
UBQ-10	CGTTAAGACGTTGACTGGGAAAACT	GCTTTCACGTTATCAATGGTGTCA

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CHAPTER 4:

THE MOLECULAR MECHANISM OF CYTOKININ-INDUCED PRIMING IN *ARABIDOPSIS THALIANA*

4.1 SUMMARY

Cytokinins (CK) are plant hormones known for the regulation of plant growth and physiology. When applied to plants, CK leads to a state of induced resistance known as defense priming. CK-induced priming (CIP) leads to a subtle activation of defense outputs, which is rapidly increased upon pathogen perception, resulting in decreased pathogen susceptibility. In this study, we aimed to provide insights into the molecular mechanisms of CIP. We demonstrate that CIP can induce defense responses that are similar to Systemic Acquired Resistance (SAR) at the transcriptional level and that these two responses share biochemical and genetic requirements. Furthermore, our metabolomics and genetic analyses suggest that the physiological changes induced by CIP require the function of amino acid transporters, suggesting that CKs connect the regulation of primary metabolism to defense activation.

4.2 INTRODUCTION

Induced resistance (IR) refers to physiological states in which plants display decreased susceptibility to pests and pathogens after an initial exposure to certain biological or chemical stimuli. The most well-known forms of IR are induced systemic resistance (ISR) and systemic acquired resistance (SAR). These two types of IR can be differentiated from one another based on the inducer stimulus and the hormonal pathways activated following the induction. Non-

pathogenic plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF) induce a jasmonic acid (JA) and ethylene-mediated defense responses resulting in ISR (Pieterse et al., 2014), whereas necrotizing biotrophic pathogens trigger a salicylic acid (SA)-mediated defense response that results in SAR (Shine et al., 2019). A defining characteristic of ISR and SAR is their systemic nature, in which a local stimulus increases defense in distal parts of the plant that had no direct exposure to the stimulus applied, as well as the genetic requirement for the signaling protein NONEXPRESSOR OF PATHOGENESIS RELATED 1 (NPR1) (Shah and Zeier 2013, Mauch-Mani et al., 2017).

When the IR stimulus does not directly activate high levels of defense following the induction, the term “priming” can be used. Priming refers to an induced state, where following the priming stimulus, a low level of defense is activated. Primed plants are then able to induce a heightened defense response to a subsequent pathogen challenge, through a faster and more effective defense activation, as compared to unprimed plants. Depending on the nature and dose of the priming stimulus, priming can also lead to better plant growth and fitness than immediate defense activation, which often results in decreased growth (Heil et al., 2000, Jakab et al., 2001, van Hulten et al., 2006, Walters and Heil 2007). Thus, understanding priming responses can lead to strategies of disease protection without yield loss, and can be important to agriculture.

Several biological, chemical and physical agents can activate priming, being effective against a variety of pathogens and pests (Conrath et al., 2015). Although priming does not directly activate defense responses, there are noted differences in the transcriptome and metabolome between primed and unprimed plants. A study aimed to determine the transcriptional fingerprint of the priming phase using four different priming stimuli on four different plant species identified only 44 differentially expressed genes that were common

between samples (Baccelli et al., 2020), highlighting that transcriptome changes due to priming are largely stimulus- and plant species-dependent, and can vary with time following the stimulus. On the other hand, comparisons of transcriptome changes after priming with similar stimuli reveals more similar transcriptional responses: induction of SAR with a necrotizing bacterial pathogen or chemical inducers in Arabidopsis leads to a 43% overlap of similarly regulated genes between these stimuli (Bernsdorff et al., 2016, Hartmann et al., 2018, Yildiz et al., 2021). At the metabolome level, studies have compared changes in metabolites following multiple priming stimuli (Gamir et al., 2014, Pastor et al., 2014, Balmer et al., 2015) and common trends can be seen. For instance, priming with the priming agent β-aminobutyric acid (BABA) and the avirulent pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pst* avrRpt2) led to a common increase in the biosynthesis of amino acids (cystine, methionine, and tyrosine), salicylic acid, and indole-3-acetic acid (IAA) (Pastor et al., 2014). Other studies show similar changes to primed plants where alterations in primary metabolism and amino acid content were observed (Brotman et al., 2012, Gao et al., 2020, Cai and Aharoni 2022, Cooper and Ton 2022). These changes in the metabolome have been proposed to contribute to defense through the biosynthesis of secondary compounds with a role in defense, or by providing energy for other defense processes, or limit the nutrients available for the pathogen.

The plant hormone, cytokinin (CK) is known for its role on the regulation plant development, (Kieber and Schaller 2018, Hudecek et al., 2022), however CK also has a role in plant defense (Choi et al., 2010, Argueso et al., 2012, Akhtar et al., 2020). Exogenous application of high concentrations of CK to leaves decreases susceptibility (Babosha 2009, Argueso et al., 2012) whereas, when applied at lower concentrations, CK increases susceptibility (Argueso et al., 2012, Gupta et al., 2020a, Zhang et al., 2022). Moreover, the application of CK

has also been shown to activate the JA-and the SA-mediated defense pathways in the presence of pathogens (Gupta et al., 2020b, Gupta et al., 2021), demonstrating that CK has a broad role in plant defense. Previous studies have shown that CK acts on defense by potentiating immune responses, rather than directly activating defenses, and thus it can be considered a priming agent (Choi et al., 2010, Argueso et al., 2012). However, the molecular mechanisms underlying the role CK has in plant defense are largely unknown.

This study aimed to determine molecular and physiological mechanisms mediating CK-Induced Priming (CIP) of Arabidopsis plants against the hemibiotrophic bacterial pathogen, *Pseudomonas syringae* pv. *tomato* (*Pst*). Transcriptomics analyses of CIP against *Pst* uncovered transcriptional similarities to SAR, and follow-up genetic analyses confirmed that CIP is dependent of the SAR regulator NPR1. Moreover, CIP requires the SAR inducer L-pipecolic acid (Pip). Metabolomic analysis revealed no changes in free amino acid levels however genetic analyses determined that amino acid transporters have a necessary role in CIP and CK-induced SAR suggesting CK-induced amino acid allocation alters plant defense through localized disruption of amino acid homeostasis.

4.3 METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col, wild type) and transgenic seeds in Col background were stratified for 2-4 days at 4ºC before being placed on soil. All lines used for experiments were homozygous. Lines used in the study include *ahk2-7 ahk3-2* (*ahk2,3*) (Argyros et al., 2008), *npr1-2* (Cao et al., 1997)*, fmo1-1* (Mishina and Zeier 2006)*, cat1* (Yang et al., 2014)*, cat6* (Alonso et al., 2003, Hammes et al., 2006)*, lht1*-4 (Hirner et al., 2006)*,* and *aap2-1*

(Zhang et al., 2010). All plants were soil grown in a Conviron growth chamber (Model# ATC60) or an Environmental Growth Chamber (Model #M25 R-401A) at Colorado State University Plant Growth Facility. Plants were grown in either Pro-mix HP Mycorrhizae or Sunshine Mix #4 soil for 5-7 weeks under a 10:14 hour (H) day:night light regime at 160 ± 20 µmol m-2s-1 at 21ºC, 50% relative humidity (RH) in the day and 19ºC, 60% RH at night.

Cytokinin-Induced Priming Treatments

A stock solution of 100mM cytokinin (CK) was made by dissolving 6-benzylaminopurine (BA) (Sigma Aldrich) into dimethyl sulfoxide (DMSO) and stored at -20ºC for up to three months. 100µM BA priming solutions were prepared by diluting the 100mM BA stock into water, and adding 0.002% Silwet L-77 (Lehle Seeds). Control plants were sprayed until run-off with an aqueous solution containing corresponding amounts of DMSO, plus 0.002% Silwet L-77. Plants were placed into different flats based on treatment and genotype. Following spray treatments, transparent plastic domes were placed over plants for 24 H to prevent rapid evaporation. Plants were inoculated with pathogens 48 H after priming or mock treatment, unless otherwise noted.

L-pipecolic acid infiltration and soil drench treatments

1 mM L-pipecolic acid (Pip) (Sigma Aldrich) solution was made by dissolving the powdered chemical directly in diH_2O. Five- to six-week-old plants were grown in individual disposable pots and separated into different flats based on treatment and genotype. For soil treatments to induce Pip-induced priming, 20mL of diH_2O or 1mM Pip were supplied directly to the soil of plants being careful to avoid contact with shoot tissue. Transparent plastic domes were

placed over plants for 24 H to prevent rapid evaporation. For Pip-induced SAR, diH_2O or 1mM Pip were directly injected, using a needleless syringe, into 3 leaves. Plants were infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000 containing an empty vector 48 H after soil drench.

Bacterial Disease Assays in Arabidopsis:

Arabidopsis leaves were infiltrated with a bacterial suspension as described by (Tornero and Dangl 2001) with the following noted changes. *Pseudomonas syringae* pv. *tomato* DC3000 containing an empty vector with kanamycin resistance marker (*Pst*) (Tornero and Dangl 2001) were grown on King's B media supplemented with rifampicin (50mg/mL) and kanamycin (50mg/mL). Bacteria were resuspended in 10mM $MgCl_2$ for a bacterial concentration of $1x10^5$ Colony Forming Units or CFU/mL. Plants were inoculated by leaf infiltration with a needleless syringe on four fully developed leaves per plant. After bacterial infiltration, plants were covered with a lightly sprayed dome for 24 H. The amount of *in planta* bacterial growth was quantified at 1 H post-inoculation (0 dpi) and at 3 days post-inoculation (3 dpi). One leaf disc was collected from four different plants for each time point and ground together in 10mM $MgCl_2$. Serial dilutions were plated onto KB media containing kanamycin (50mg/mL) and rifampicin (50mg/mL) at 0 dpi, or rifampicin (50mg/mL) and cycloheximide (100mg/mL) at 3 dpi, and used to determine the CFU per cm^2 of leaf tissue.

RNA Extraction and qRT-PCR

Total RNA was extracted using RNeasy Plant kit (QIAGEN), following manufacturer's instructions. The quality and integrity of RNA was assessed by A_{260}/A_{280} and A_{260}/A_{230} ratios having a value of greater than 1.7. RNA samples of good quality underwent DNase Treatment

using TURBO DNase-Free (Invitrogen) as per the manufacturer's instructions and were checked for the absence of genomic DNA by qRT-PCR using primers for AT5G66770 (For 5'-GGTTTGGTTTGGTTATCGCCAGGA-3', Rev 5'-TGGCTTCATCTCTTTGGCCTGGA-3'). cDNA was synthesized using Qscript Supermix (QuantaBio) and checked for full-length cDNA synthesis through qRT-PCR using primers for *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE GAPDH* (AT1G13320). Primers used were: *GAPDH-1* (For 5'-TAGATCGCTCGGAACTTGGAAA-3', Rev 5'-CCTCACCAAAACTCAAATCACTCC-3'); *GAPDH-3* (For 5'-AACTAGGACGGATCTGGTGCCT-3', Rev 5'-GCTATCCGA ACTTCTGCCTCATTAT-3'), and *GAPDH-5* (For 5'-AAATTTAAC GTGGCCAAAATGATGC-3', Rev 5'-GTTCTCCACAACCGCTTGGT-3'). qRT-PCR reactions were performed with PerfeCTa SYBR Green (QuantaBio) on a CFX Connect Real-Time System (BioRad). cDNAs with Ct/Cq differences between each GAPDH primer of less than 1.5 were considered fully extended and of good quality. AT4G05320 *UBIQUITIN10* (*UBQ10)* was used as a housekeeping gene in all reactions (For 5'-CGTTAAGACGTTGACTGGGAAAACT-3', Rev 5'-GCTTTCACGTTATCAATGGTGTCA-3'). Gene specific primers used are listed in Table 4.1. At least three biological replicates of each experiment were obtained unless otherwise stated.

Cytokinin-Induced Priming RNA-seq and Metabolomics Assay

Five- to six-week-old plants were treated with a mock solution or 100μM CK as stated above. Plants were covered by a clear, plastic dome to reduce spray solution evaporation for 24 H. 48 H after spray treatment tissue was collected. 48 H after priming treatments, the remaining plants were infiltrated with *Pst* or $MgCl_2$ as described above except no tissue was collected for

assessment of *in planta* bacterial growth. 24 H after *Pst* infiltration, tissue was collected from *Pst*- or MgCl2-infiltrated leaves. Two leaves were collected per plant per treatment. Three biological replicates were collected for RNA extraction and further RNA-seq analysis.

RNA-seq for Whole Transcriptome Analysis

Total RNA was extracted using RNeasy Plant kit (QIAGEN), following the manufacturer's instructions. The quality and integrity of RNA were assessed by A_{260}/A_{280} and A_{260}/A_{230} ratios having a value of greater than 1.7. Further RNA quality and integrity were assessed using a TapeStation 2200 (Agilent) with High Sensitivity RNA materials (Agilent Screentape 5067-5579, Agilent Sample Buffer 5067-5580). An RNA gel was also run to confirm RNA quality before being submitted for sequencing. Paired-end, 150bp reads were sequenced by Novogene (Sacramento, CA, USA) at a depth of 40 million reads. Novogene used an Illumina based library construction kit (NEB Next Ultra 2). Sequence files underwent a quality control check by FastQC V0.12.0 (Wingett and Andrews 2018) and adapters were removed by Trimmomatic V0.39 (Bolger et al., 2014). Trimmed sequences were aligned to the *Arabidopsis thaliana* genome TAIR 10 (Berardini et al., 2015) using STAR 2.7.0a (Dobin et al., 2013) allowing a maximum of 20 multiple alignments allowed per read. FeatureCounts (Liao et al., 2014) was used to count how many reads aligned to genes and differential gene expression was assessed using DESeq2 R package (Love et al., 2014).

Metabolomics Sample Preparation

Samples were lyophilized, and ground using the "Bullet Blender" (Next Advance) and transferred to the extraction vials containing 1 mL of a solvent mix of MTBE/methanol/water

(6:3:1, by vol) for biphasic extraction. Samples were mixed for 2 H at 4°C, followed by sonication for 20 min. Then 0.35 mL of water was added and samples were mixed for 30 min at 4°C. After centrifugation at 2,000 g x 15 min and 4°C, the upper organic layers were removed. 0.6 mL of MTBE was added to the lower layer, and after a brief vortexing, the samples were centrifuged at the same condition as above. The upper layers were removed, and the lower aqueous layers were recovered, and dried under nitrogen. For untargeted metabolomics analysis, small polar metabolites were analyzed by Gas Chromatography-Mass Spectrometry (GCMS). For amino acid targeted analysis, samples were resuspended in 0.5mL of 50% MeOH and stored at -80^{o}C until analysis by Liquid Chromatography-Mass Spectrometry (LCMS).

Liquid Chromatography-Mass Spectrometry Analysis

LC-MS/MS was performed on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on a SeQuant ZIC-pHILIC column (2.1 x 20 mm, 5 µm) column. Mobile phases were water with 10 mM ammonium bicarbonate (pH 9.6) (A) and acetonitrile (B). The analytical gradient was as follows: time = 0 min, 90% B; time = 0.50 min, 90% B; time = 3.10 min, 10% B; time = 4.10 min, 10% B; time= 4.20 min, 90% B; time =6 min, 90% B. Flow rate was 270 µL/min. Samples were held at 6°C in the autosampler, and the column was operated at 50°C. The injection volume was 1 µL. The capillary voltage of MS detector was set to 0.6 kV MS in positive ionization mode. Inter-channel delay was set to 3 msec. The source temperature was 150°C and the desolvation temperature 450°C. Desolvation gas flow was 1000 L/hr, cone gas flow (nitrogen) was 150 L/hr, and collision gas flow (argon) was 0.15 mL/min. NebµLizers pressure (nitrogen)

was set to 7 Bar. Autodwell feature was set for the collection of 12 points-across-peak. The MRM transitions can be found in Table 4.2.

Liquid Chromatography-Mass Spectrometry Data Analysis

All raw data files were imported into the Skyline (MacLean et al., 2010) open-source software package for data processing. Because internal standards were not used, raw peak areas were used for relative quantification. Statistical analysis for the determination of differences between samples was completed by two-way ANOVA with TUKEY HSD *p*-value correction.

Systemic Acquired Resistance Bacterial Assays

To induce systemic acquired resistance (SAR), 5 to 6-week-old plants were treated with solutions of 1mM L-pipecolic acid (diH_2O as control) or 100µM CK (DMSO as control) either by brushing leaves with a small paint brush or by infiltration with a needleless syringe. Brushing solutions contained 0.002% Silwett as a surfactant. Each plant had three fully developed leaves connected parastichy to another treated to induce SAR unless otherwise stated. These treated leaves were denoted as "local" leaves whereas other non-SAR-induced leaves were denoted as "distal" leaves. Forty-eight H following SAR induction, three distal leaves were infiltrated with a needleless syringe with *Pst* to leaves, connected parastichy to another, that had not been treated with the SAR inducer. The amount of *in planta* bacteria was quantified 1 H after inoculation (0 dpi) and 3 days post inoculation (3 dpi). Leaf discs were pooled for one sample from one leaf from four separate plants for each genotype per treatment at each time point. Leaf discs were ground in 10mM $MgCl_2$ and serial dilutions of ground tissue were used to determine the CFU per cm^2 of leaf disc tissue. Day 0 dilutions were plated on $KB_{rif,kan}$ plates and day 3 dilutions were

plated on $KB_{rif,chx}$ (cycloheximide) plates, both were incubated at 28ºC for 24-48 H before counting CFUs.

Systemic Acquired Resistance Assays for Gene Expression

Five to six-week-old plants had three local leaves infiltrated, using a needleless syringe, with 1mM L-pipecolic acid (diH_2O as control) or 100μM CK (DMSO as control) 48 H after SAR induction, three distal leaves per plant were infiltrated with *Pst* as previously described. A water-sprayed, clear plastic dome was placed over pathogen-infiltrated plants for 3-4 H before removing. Tissue samples for gene expression analysis were collected at the indicated times by removing three local (chemically treated) or three distal (*Pst* infiltrated) leaves from four plants, pooled, and placed in liquid nitrogen. Total pooled tissue was macerated by mortar and pestle and cooled by liquid nitrogen before 50-80mg of the pooled sample was aliquoted into individual microcentrifuge tubes prior to RNA extraction and qRT-PCR analysis as previously described.

Statistical Analysis

For experiments with two comparisons, a two-way ANOVA with TUKEY HSD *p*-value correction was used and for experiments with one comparison, a Student's T-test or one-way ANOVA was used to evaluate statistical significance. The significance of these tests was based on a $p\text{-value} \leq 0.05$.

4.4 RESULTS

Cytokinin-induced priming induces transcriptional changes in defense-related genes

CIP has been proven effective in reducing disease in a number of plant species, but the molecular mechanisms at play are largely unknown. Therefore, we determined the genome-wide transcriptional signatures induced by CIP against the hemibiotrophic bacterial pathogen, *Pseudomonas syringae* pv. *tomato* (*Pst*). Wild type plants were sprayed with either a mock solution or 100μM CK. Two days later, plants were infiltrated with *Pst* or $MgCl_2$ as a wounding control. Leaf tissue was collected 48 H after priming (priming phase) to determine if, at the time point right before being challenged by a pathogen, there were any differentially expressed genes mediated by the priming treatment. Leaf tissue was also collected 24 H following pathogen or $MgCl_2$ infiltration, to determine gene expression during the pathogen challenge phase. Sequencing was performed using Illumina technology, with 150bp paired-end reads, and a depth of approximately 40 million reads per sample. Reads were mapped to the Arabidopsis genome version TAIR.10 (Berardini et al., 2015), and differentially expressed genes (DEGs) between the two samples groups were determined by DeSEQ2 (Love et al., 2014) meeting the criteria of a $\log_2$ fold change greater or less than 1 and a *p*-value less than 0.05 as determined by Wald's test with Bonferroni correction. A graphical summary of the experimental design is shown in Figure 4.1 A. For analyses, CK and/or *Pst*-treated samples were compared to specific controls, explained in Figure 4.1 B.

Analyses of the priming phase transcriptome were performed by comparing CK-treated plants to mock-treated plants at 48 H after treatment. Very few genes were differentially expressed (21 genes), suggesting that at this late time point most of the transcriptional response to CK had subsided with no clear signature of CK-regulated genes observed. To determine the

differences in gene expression in the post-pathogen challenge phase, we first compared mock- or CK-treated plants infiltrated with $MgCl_2$ to those similarly treated and infiltrated with *Pst*, thus minimizing changes in gene expression due to the wounding effect. Our results show that mock treatment followed by *Pst* infiltration resulted in a total of 4954 DEGs, and CK treated followed by *Pst* infiltration resulted in 5497 DEGs (Figure 4.1 B).

The priming effect is often categorized by the priming agent inducing a potentiation of gene expression, where after the primed plant experiences a pathogen challenge, similar genes will be up-regulated between the non-primed and primed plant, with primed transcriptome showing a stronger regulation (Conrath et al., 2015, Mauch-Mani et al., 2017). We then looked for signatures of potentiation between the lists of up-regulated DEGs between mock-treated and 100μM CK-treated, post-inoculation plants (Figure 4.2 A). Of the 2555 DEGs induced by *Pst* in mock-treated plants and 2816 DEGs induced by *Pst* in CK-treated plants, 1332 genes were shared between these two gene lists. To determine if these genes have potentiated expression due to CK priming, the difference in expression levels between the two gene lists was calculated, and after applying a gene expression fold cut-off of greater than or equal to 1.1 used, 411 genes were identified as having potentiated expression. Gene Ontology (GO) analysis was performed using Panther (Thomas et al., 2022) with Fisher's test and FDR *p*-value correction to determine term enrichment for biological function. Of the 411 potentiated genes, 316 were able to be categorized by biological function and then further grouped into 9 more general categories (Figure 4.2 B). By grouping similar GO terms into general categories, we saw that almost 1/3 of the genes with potentiated expression due to CK are related to defense (Figure 4.2 C), which suggests that the decreased susceptibility seen after CIP may be due to differential expression of defense genes, as

reported before (Choi et al., 2010, Grosskinsky et al., 2011, Argueso et al., 2012, Gupta et al., 2020b, Gupta et al., 2021).

Further inspection of the genes within the generalized defense-related GO category showed that many of them were associated with Systemic Acquired Resistance (SAR) or Induced Systemic Resistance (ISR) (Figure 4.2 C). A comparison of the DEGs list of 411 potentiated genes to genes known to be regulated by pathogen-induced-SAR genes (Bernsdorff et al., 2016), showed that 66% of them were also regulated in SAR (Figure 4.3 A). A similar comparison with genes regulated by ISR induced by beneficial microbes (Desrut et al., 2020) showed that only 18.7% of these genes were regulated in ISR (Figure 4.3 B). These results led us to conclude that the molecular mechanism behind CIP is more similar to SAR than ISR defense responses.

In addition to pathogens, SAR can also be induced by chemicals, such as L-pipecolic acid (Pip), a lysine catabolite (Navarova et al., 2012, Bernsdorff et al., 2016, Hartmann et al., 2018). Comparison of CIP-potentiated DEGs to genes known to be positively regulated by Pip showed that only 29.1% were also regulated by Pip (Yildiz et al., 2021) (Figure 4.3 C). However, recent research has determined that while the application of Pip can induce SAR, the hydroxylated version of Pip, N-hydroxy-pipecolic acid (NHP), is the de facto mobile signal for SAR (Hartmann and Zeier 2019, Yildiz et al., 2021, Shields et al., 2022). A comparison to NHP positively-regulated genes (Yildiz et al., 2021) revealed that 41.7% of CIP-potentiated DEGs were also induced by NHP application (Figure 4.3 D). Taken together, these results suggest that the molecular mechanism of CIP is similar to biologically- and chemically-induced SAR.

The application of cytokinin can induce a systemic defense response

The results from the transcriptome analysis of CIP lead to the hypothesis that CK could induce systemic resistance. To test this, wild type Col plant and the CK signaling mutant *ahk2,*3, lacking two of the three CK receptors ARABIDOPSIS HISTIDINE KINASE 2 (AHK2) and ARABIDOPSIS HISTIDINE KINASE 3 (AHK3), were tested to determine whether local application of CK to leaf tissue would decrease susceptibility to *Pst* in distal leaf tissues. Wild type or *ahk2,3* plants had one or two leaves brushed with either a mock solution or 100μM CK and 48 H later, three distal, non-pretreated leaves were infiltrated with *Pst* (Figure 4.4). *In planta* bacterial growth determined in distal leaves shows that in comparison to mock-treated samples, brushing 1 local leaf with 100μM CK leads to a reduction in bacterial multiplication in wild type plants, while the same is not observed in *ahk2,3* plants lacking CK signaling (Figure 4.4 A). These results were even more pronounced when two leaves were primed, rather than only one, resulting in a decrease in bacterial growth in distal tissues by almost 5 times (Figure 4.4 B). These results suggest that CIP is able to induce a systemic defense and that this is dependent on endogenous CK signaling.

One of the most well-known markers of SAR is the induction of the *PATHOGEN-RELATED-1* (*PR1*) gene in untreated, distal tissues following SAR stimulus to local tissues (Yalpani et al., 1991, Gaffney 1993, Wildermuth 2001, Bernsdorff et al., 2016, Yildiz et al., 2021). CIP has been shown to potentiate *PR1* expression (Choi et al., 2010, Argueso et al., 2012) however, these studies only analyzed gene expression in local tissues. Therefore, to understand if CK can be categorized as an inducer of SAR, the expression of *PR1* was determined in local and distal tissues. To ensure CK entered the leaf tissue, mock solutions or 100μM CK were infiltrated, not brushed, into two local leaves of Col and *ahk2,3* plants followed by *Pst*

inoculation of distal, untreated leaves 48 H after induction. Gene expression was analyzed in local and distal leaf tissues 3 and 24 H after induction and in distal leaves 24 H after pathogen challenge (Figure 4.5). To validate that CK signaling was induced after the hormone infiltration, the expression of the CK-inducible gene *ARABIDOPSIS RESPONSE REGULATOR 5* (*ARR5*), involved in CK signaling, was also determined. Figure 4.5 A shows that in Col, 100μM CK induced *ARR5* in local tissues at both 3 and 24 H after treatment, whereas *ahk2,3* plants showed a low level of induction, confirming that CK signaling was properly activated. No expression of *ARR5* was seen in distal tissues at 24 H post-treatment, in both Col or *ahk2,3* plants, indicating that CIP does not require distal induction of CK signaling. Local Col tissues showed induction of *PR1* expression 3 H post-CK infiltration and further increased expression 24 H later, while little to no expression was seen in the CK signaling mutant (Figure 4.5 B), describing a known positive relationship between CK and *PR1* expression (Siemens et al., 2006, Choi et al., 2010, Argueso et al., 2012). Interestingly, treatment of Col local tissues with CK also caused induction of *PR1* in distal tissues 24 H after hormone induction and after subsequent *Pst* infiltration, further suggesting that CK can induce SAR.

Another defining characteristic of SAR is the genetic requirement for NONEXPRESSOR OF PR GENES 1 (NPR1), a major regulator of SA signaling (Cao et al., 1994, Cao et al., 1997, Bernsdorff et al., 2016, Hartmann et al., 2018). To understand if CK-induced SAR also requires NPR1, Col and *npr1* mutants underwent CK-induced SAR as previously described. Figure 4.5 shows that Col distal leaves have a reduction of *in planta* bacterial concentrations following local CK-infiltration as compared to local leaves infiltrated with the control as previously seen (Figure 4.4). In comparison, *npr1* mutants did not show a reduction in bacteria levels in distal leaves after local treatment with CK, providing another piece of data that CK can induce SAR.

Cytokinin and L-pipecolic acid-induced systemic acquired resistance show endogenous interdependence on another

As mentioned above, Pip is a known inducer of SAR. Treatment of plants with Pip potentiates the expression of *PR1* following pathogen challenge (Bernsdorff et al., 2016, Zhang et al., 2020). To understand if endogenous CK signaling is involved in this potentiation of defense gene expression, Pip was administered by soil-drench in Col and the CK signaling mutant *ahk2,3* and inoculated with *Pst* 48 H after. Leaf tissue was collected 24 H after Pip application and pathogen challenge to determine the expression of *PR1* (Figure 4.7 A). Similar to other published studies (Navarova et al., 2012, Bernsdorff et al., 2016), wild type plants displayed defense gene *PR1* potentiation following soil drench with Pip, and following pathogen challenge the expression level of *PR1* more than doubled as compared the control treatment. In contrast, an opposite effect was seen in *ahk2,3* plants, where Pip treatment decreased *PR1* expression following pathogen challenge, indicating that CK signaling mediates the potentiated expression of *PR1* by Pip. Interestingly, 24 H after Pip treatment, *PR1* expression was ~6-fold higher than H_2O treatment in the CK signaling mutant suggesting that CK may act to mediate the suppression of defense response until there is an actual threat of pathogen present.

We next addressed whether CK was also needed for CK in Pip-induced SAR measured by bacterial multiplication. To do so, Col and *ahk2,3* leaves underwent Pip-induced SAR and *in planta* bacterial growth was determined in untreated, distal tissues (Figure 4.7 B). As expected, Pip-induced SAR decreased bacterial concentrations in wild type plants as compared to the control treatment. In *ahk2,3* plants, there was no significant difference in bacterial concentrations in distal tissues of plants infiltrated locally with H_2O or Pip, signifying a dependence on endogenous CK signaling for Pip-induced SAR.

Although Pip application can induce SAR, NHP, hydroxylated form of Pip, is the mobile signal that is translocated from the site of induction to distal parts of the plant during SAR (Yildiz et al., 2021). The hydroxylation of Pip into NPH is catalyzed by the enzyme FLAVIN-DEPENDENT MONOOXYGENASE 1 or FMO1 (Hartmann et al., 2018). Given the requirement for endogenous CK signaling in Pip-induced SAR (Figure 4.7 B) and that CK-induced SAR does not elicit CK-signaling in distal tissues (Figure 4.5 A), we hypothesized that CK-induced SAR may require NPH made by FMO1. To test this hypothesis Col and *fmo1* mutants, which are unable to convert Pip to NHP, were used in CK-SAR pathogen susceptibility assays, conducted as described previously (Figure 4.8). The results show that *fmo1* plants infiltrated locally with 100μM CK have similar distal bacterial concentrations to *fmo1* plants infiltrated with the mock solution, whereas Col distal leaves display reduced susceptibility to *Pst* following 100μM CK local infiltration. Without the conversion of Pip to NHP, CK-induced SAR is not observed, implying that CK-induced SAR depends on the conversion of Pip to NHP, providing more data on the intertwined relationship between CK, SA, Pip, and SAR.

Cytokinin-induced priming does not alter free amino acid levels

Given the role of the non-proteogenic amino acid Pip in CK-induced SAR, and the importance of CK to plant metabolism, we decided to address the effect of CIP on the plant amino acid levels. Total wild type rosettes were primed by CK followed by *Pst* infiltration 48 H later. Plant leaf tissue was collected for targeted metabolomics using liquid chromatography-mass spectrometry (LC-MS) of samples harvested 48 H after the priming treatment and 24 H after pathogen inoculation. From this analysis, 16 of the 21 proteinogenic amino acids and Pip were identified (Figure 4.9). Of the 16 identified amino acids there were few significant

differences in the amino acid relative signal intensities between mock treatment and 100μM CK treatment. Pathogen challenge by *Pst* altered levels of alanine, methionine, proline, tryptophan, valine, and Pip, as compared to non-pathogen-treated samples. However, the only difference in the relative normalized abundances of amino acids seen between CK-primed or non-primed, pathogen-challenged samples was that primed samples had a decrease in alanine and an increase in methionine. These results show that priming by CK does not result in significant alterations in free amino acid levels.

Cytokinin-mediated defense depends on the movement of amino acids

The role of CK separate from plant defense is mainly associated with plant growth (Kieber and Schaller 2018). One of the processes of plant growth regulated by CK is source-sink relationships, through the regulation of the photosynthesis (Ahanger et al., 2018, Ahanger et al., 2020, Hudecek et al., 2022) and the allocation of photoassimilates (Harms et al., 1994, Ehness and Roitsch 1997, Lara et al., 2004, Jian et al., 2016, Ninan et al., 2019) including amino acids (Mothes and Engelbrecht 1961, Brenner et al., 2005, Kiba et al., 2005, Lee et al., 2007, Yokoyama et al., 2007). Sugars and amino acids contribute to plant-pathogen interactions (Moormann et al., 2022), either by being precursors to defense compounds (Erb and Kliebenstein 2020, Cai and Aharoni 2022) or as sources of pathogen nutrition (Chen et al., 2010, Anderson et al., 2014, Borer et al., 2023).

The absence of a strong effect of CIP on amino acid levels throughout the plant led us to investigate whether amino acid transport could be involved in CIP. The expression of several genes encoding amino acid transporters is regulated by CK, amongst them the genes encoding CATIONIC AMINO ACID TRANSPORTERS, *CAT1* and *CAT6* and *AMINO ACID*

PERMEASE 2 (*AAP2*) and *LYSINE/HISTIDINE TRANSPORTER 1* (*LHT1*) (Brenner et al., 2005, Kiba et al., 2005, Lee et al., 2007, Yokoyama et al., 2007, Kiba et al., 2011), which have been shown to be responsible for amino acid translocation in and out of sink and source tissues (Fischer et al., 1995, Ehness and Roitsch 1997, Okumoto et al., 2004, Su et al., 2004, Hammes et al., 2006, Hirner et al., 2006). To understand if CIP treatment depends on these CK-regulated transporters, *cat1*, *cat6*, *aap2* and *lht1* amino acid transporter mutants were tested alongside wild type plants, for their requirement for CIP reduction of infiltrated bacterial concentrations. As seen in Figure 4.10, differently from wild type plants, all amino acid transporter mutants tested showed failed to induce CIP, indicating that CIP depends on amino acid translocation. Because these amino acid transporters have been shown to transport various amino acids (Fischer et al., 2002), the decreased susceptibility seen after CK treatment may be due to a broad alteration in amino acid transport, rather than the specific promotion or inhibition of the translocation of a specific amino acid.

Amongst all four tested amino acid transporter mutants *cat1* and *lht1* plants showed the least difference in average bacterial concentrations between treatments (Figure 4.10 A and D) suggesting that these amino acid transporters may have a more prominent role in amino acid translocation as a response to CIP. To test this hypothesis, analysis of gene expression was performed in wild type Col and *ahk2,3* plants 4 and 24 H following mock or CK spray treatments and sequential *Pst* infiltration (Figure 4.11). As expected, following CK spray treatment *ARR5* showed an increase in expression only prior to pathogen challenge, and that was diminished in the *ahk2,3* signaling mutant (Figure 4.11 A). The expression of *PR1* was minimal until after *Pst* infiltration, and was also diminished in *ahk2,3* plants (Figure 11 B). *LHT1* and *CAT1* both showed an increase in expression 24 H following CK treatment (Figure 4.11 C and D)

demonstrating that both genes are regulated by CK, although not immediately after CK treatment. Both *CAT1* and *LTH1* showed differential CK-mediated expression following pathogen infiltration in the post-pathogen challenge phase, and again this response was less pronounced in the absence of CK signaling, supporting the involvement of both amino acid transporters in CIP.

In order to understand if these amino acid transporters are also involved in CK-mediated SAR, *cat1* and *cat6* distal leaves were tested for altered bacterial growth 48 H after local leaves were infiltrated with a mock solution or 100μM CK. Although the gene expression data suggested a role for *LHT1* in CK-mediated defense (Figure 4.11 C)*, lht1* mutants are dwarfed and experience premature senescence of older leaves (Svennerstam et al., 2007) making the results of a SAR experiment more difficult to interpret, thus not included. Both *cat1* and *cat6* mutants were used in this experiment to test if there was a broad or specific role in CK-mediated defense for multiple members of the CATIONIC AMINO ACID TRANSPORTER family of proteins. As compared to Col both *cat1* and *cat6* did not show a reduction in *Pst in planta* growth in distal leaves between plants that had local leaves treated with either a mock solution or 100μM CK (Figure 4.12). There are differences between the two amino acid transporter mutants where *cat6* plants (Figure 4.12 B) showed a higher difference in bacterial growth between the two treatments as compared to *cat1* (Figure 4.12 B). Taken together, these results (Figures 4.10, 4.11, and 4.12) not only show that part of the underlying molecular mechanism behind CIP against *Pst* involves amino acid transporters, but they also suggest that the systemic nature of the said defense is possibly due to the movement of amino acids.

4.5 DISCUSSION

Priming of plant defenses is a state of induced plant resistance that can be induced by several biological, chemical and physical agents, that is effective against a broad range of pathogens and has less detrimental impacts on plant growth (van Hulten et al., 2006, Worrall et al., 2012, Takatsuji 2014, Conrath et al., 2015, Zhou and Wang 2018). Although different priming inducers result in decreasing plant susceptibility to pathogens, the molecular mechanisms involved are mostly unknown. For example, the application of the SA analog benzothiadiazole (BTH) increases the expression of the defense gene *PR1* and is NPR1-dependent in Arabidopsis (Lawton et al., 1996, van Hulten et al., 2006), but is independent of the NPR1 ortholog in rice, where increased defense is dependent on the transcription factor OsWRKY45 (Shimono et al., 2007). Another common priming stimulus, β-aminobutyric acid (BABA), also mediates the expression of *PR1* (Zimmerli et al., 2000, Slaughter et al., 2012, Floryszak-Wieczorek et al., 2015) through mechanisms that can be dependent or independent on SA-mediated defenses in Arabidopsis (Zimmerli et al., 2000, Ton and Mauch-Mani 2004). Furthermore, in tomato, BABA-induced defense has been shown to be mediated by JA (Janotik et al., 2022). These results indicate that similar in that different stimuli can induce priming, different molecular mechanisms may be involved in the priming response, resulting in plant defense.

One proposed molecular mechanism for priming of plant defense is through changes in the plant metabolome. Early studies showed that metabolites with anti-microbial function, such as indole-3-carboxaldehyde (Gamir et al., 2014) and camalexin (Navarova et al., 2012, Gamir et al., 2014, Balmer et al., 2015, Bernsdorff et al., 2016), accumulate during priming. The same has been observed for metabolites that do not have a direct antimicrobial function, but that are

known to induce IR, such as Pip (Navarova et al., 2012) and azelaic acid (Jung et al., 2009). Moreover, studies have also shown that following IR alterations in the levels of primary metabolites, including sugars and amino acids, can also occur (Balmer et al., 2013, Gamir et al., 2014, Gao et al., 2020, Luna et al., 2020).

Widely known for its role in plant growth and development, CK also has a role in plant defenses, where the application of high concentrations of CKs prior to pathogen infection results in reduced disease susceptibility (Choi et al., 2010, Argueso et al., 2012, Gupta et al., 2020b), through priming of plant immune responses (Choi et al., 2010, Argueso et al., 2012). Cytokinin-Induced Priming (CIP) is also effective in different pathosystems (Dervinis et al., 2010, Grosskinsky et al., 2011, Gupta et al., 2021, Zhang et al., 2022). To gain insights into the molecular mechanism involved in CIP, we addressed transcriptomic responses to the application of CK to plants (priming phase), known to result in priming, as well as changes induced by the presence of the *Pst* pathogen in primed and unprimed plants (post-priming challenge phase). Our first observations were that by 48 H after CK application most of the response to this plant hormone had already subsided, as seen by the small numbers of DEGs identified, and the absence of CK-regulated genes. This is in line with other studies that have shown that CK-induced gene expression is mainly observed immediately after induction, peaking at 1 H after CK application (D'Agostino et al., 2000, Brenner et al., 2005, Kiba et al., 2005). Thus, the transcriptional changes associated with CIP happen early and are manifested in later physiological processes downstream of CK primary transcriptional targets.

Priming of plant defenses has a common characteristic where following a challenge by a pathogen, plant defense gene expression shows higher expression in primed plants as compared to unprimed plants. We determined that a portion of CIP up-regulated genes also displays this

characteristic genome-wide and using GO analysis showed the majority of the genes displaying potentiated expression involved plant defenses. Further analysis of these genes showed a high degree of genes responsive to pathogen-induced SAR (66%), less so to non-pathogenic-induced ISR (18.7%), and also showed common genes with chemically induced SAR induced by Pip (29.1%) and NHP (41.7%). Therefore, CIP transcriptomic responses are more similar to SAR responses that are initiated by SA-mediated responses.

The results of our transcriptomics studies led us to determine that CIP could in fact induce a response similar to SAR, in which local application of CK induced a distal defense response resulting in less pathogen multiplication and increased *PR1* expression in distal tissue. Previously other studies have shown that CIP functions locally (Choi et al., 2010, Grosskinsky et al., 2011, Argueso et al., 2012) thus, our results comprise the first report that CIP can affect susceptibility at a distal, non-treated site after local CK treatment. Furthermore, we observed that in CK-induced SAR there is local induction of the CK-signaling, as measured by *ARR5* expression, following CK treatment to local leaves. However, CK signaling expression was minimal in distal tissues, indicating that CK is not responsible for the induction of defense in distal tissues even though it can initiate distal defenses when applied locally.

CIP has been shown to aid in SA-mediated local defenses that depend on NPR1 (Choi et al., 2010, Liu et al., 2020). Our genetic studies showed that CK-induced SAR is also dependent on a functional NPR1. Given that Pip and NHP-induced SAR are also dependent on NPR1 (Yildiz et al., 2021), using a CK signaling mutant we showed that endogenous CK signaling is necessary to induce SAR by Pip application. As previously stated, Pip can induce SAR but must first be converted to NHP by FMO1, as NHP is the mobile signal moving from local tissues to distal tissues to increase defense in systemic areas of the plant (Mishina and Zeier 2006,

Hartmann et al., 2018). We show that CK-induced SAR also requires NHP, because *fmo1* mutants fail to show distal defense in response to CIP. CK-induced SAR displays similar characteristics to either biological or chemically-induced SAR through the requirement of NPR1 and the conversion of Pip to NHP by FMO1.

Through the exploration of transcriptome analysis, we were able to determine that CK can be used as an initiator of systemic-induced defenses. Although we were unable to determine early genetic responses altered by the CK treatment, potentiated expression of defense genes were more common to SAR responses as compared to ISR leading, indicating the CK-induced SAR we observe is mediated by SA-mediated defenses, no JA-mediated defenses. Markers of SAR are SA accumulation and increased *PR1* expression at the local and distal site of initiation, and the requirement of both NPR1 and the mobile signal compound, NHP, between the local and distal sites. Our results show that CK-induced SAR displays all the markers of SAR although SA accumulation was not analyzed.

Given the results above, one possible mechanism by which CK could induce a systemic defense similar to SAR is by regulating FMO1, at the level of gene expression or enzymatic activity. In such scenario, local regulation of *FMO1* would lead to more NHP that could be translocated distally, establishing SAR. Without CK to induce *FMO1*, Pip would not be able to induce SAR, as we have observed. Furthermore, without FMO1, CK would not be able to induce SAR on its own, which we also observed. However, at least at the transcriptional level, our data does not support this hypothesis, as *FMO1* is not potentiated by CIP (data not shown). Further experiments would have to be performed to test whether *FMO1* activity or protein levels are changed after CIP to be considered causal.

In search of another possibility to explain how CK can induce a systemic response similar to SAR we performed a targeted metabolomic analysis for amino acids in CIP, using LC-MS. The only identified amino acid that showed a statistically different abundance after priming alone was valine, whose concentration increased as compared to the control treatment. Comparison of the priming phase to the post-priming pathogen phase shows that levels of alanine, methionine, proline, tryptophan, valine, and Pip change in response to the pathogen challenge, but treatment with CK did not lead to statistically significant differences in their content. Thus, it is possible to say that CIP does not alter free amino acid content.

CK has been shown to mediate the movement of photoassimilates (reviewed by (McIntyre et al., 2021)) that includes amino acids (Mothes and Engelbrecht 1961). Following the results from the metabolomics experiments and our results showing CK can induce defense distally, we hypothesized that CIP mediates amino acid transport that in turn would increase plant defense. CK-mediates the expression of six amino acid transporters in Arabidopsis including *AAP2, AAP3, AAP5, CAT1, CAT6,* and *LHT1* (Brenner et al., 2005, Kiba et al., 2005, Lee et al., 2007, Yokoyama et al., 2007, Kiba et al., 2011). Of these amino acid transporters, CAT1 is located in leaf sink tissues (Hammes et al., 2006), and CAT6 and AAP3 are located in root sink tissues (Hammes et al., 2006) where CK-mediates an increase in their gene expression. AAP2, AAP5, and LHT1 are located in the source leaf tissue (Fischer et al., 1995, Fischer et al., 2002, Hirner et al., 2006) where CK mediates a decrease or repression of their gene expression. Mutant plants in four amino acid transporters displayed an inability to decrease susceptibility to *Pst* following CK treatment suggesting a role of these transporters in CK-induced defense. These amino acid transporters function to move multiple different types of amino acids in and out of cells (Fischer et al., 2002) suggesting that general amino acid transport is involved in CIP against

Pst. Observation of gene expression following CIP showed that expression of *LHT1* was induced by CK application alone, early after *Pst* infiltration, but expression was repressed at later stage of the pathogen challenge. LHT1 has been previously linked to altering plant defense in that knockout mutants display decreased susceptibility to *Pst*, in a manner dependent on SA-mediated defenses (Liu et al., 2010). Interestingly, this study also determined that LHT1 suppresses the expression of *PR1* and *FMO1.* Comparison of these results to our gene expression analysis, suggests that CIP-mediated *LHT1* expression may be fine-tuning the SAR-associated defense gene expression to mount a more effective defense response at the appropriate time. The expression of *CAT1* showed a minimal increase in expression after priming by CK but showed a similar trend in expression to *LHT1* following pathogen challenge. These results show that while CIP involves the general movement of amino acids, CK does not mediate the level nor the timing of the expression of amino acid transporters in similar ways. *CAT1* was shown to be of many amino acid transporters up-regulated during infection with *Pst* (Toufighi et al., 2005) and the overexpression of *CAT1* has been shown to decrease susceptibility (Yang et al., 2014). Our expression of *CAT1* was characteristic of priming through the quicker, more robust expression following pathogen challenge thus, leading us to focus on CAT1 and the other cationic amino acid transporter in relation to CK-induced SAR. Using these amino acid transporter mutants we showed that CK-induced SAR was lost in both *cat1* and *cat6* indicating a dependence on these two transporters for the distal defense induced by CK. Interestingly, these two amino acid transporters are located in sink tissues (Hammes et al., 2006) where at least CAT1 functions to move amino acids to and from the apoplast into the cytoplasm (Yang et al., 2014), suggesting that CK functions to alter amino acid movement away from the area of *Pst* colonization resulting

in decreased susceptibility. Further, the overexpression of *CAT1* increased SA accumulation and SA treatment increased the expression of *CAT1* (Yang et al., 2014).

Taken together with our results, the above study raises the question of whether CK-induced defenses are altering the nutrient environment for pathogens in a way that limits their food source, preventing their proper colonization. During plant infection, *Pst* colonizes in the apoplast. Interestingly, this bacterial pathogen lacks the ability to assimilate the amino acids that are present at low levels in the apoplast but can utilize 4 of 6 abundant amino acids in the apoplast (Mithani et al., 2011). A study in tomato demonstrated more specifically that *Pst* can utilize certain amino acids like asparagine, aspartate, glutamate, and glutamine present in the apoplast for carbon sources but only arginine for its source of nitrogen (Rico and Preston 2007). Additionally, it has been demonstrated that specific amino acids within the apoplast alter plant susceptibility by the level of amino acids present will act as a signal for the pathogen in their deployment of bacterial effectors through the type III secretion system (Anderson et al., 2014).

Another possibility linking amino acid movement and plant defense is that an altered amino acid homeostasis is a signal for defense activation. As previously stated, LHT1 was shown to mediate SA-mediated defense against *Pst.* Similar to our results, their analysis of free amino acid content during *lht1* defense response was shown to only significantly alter the content of three amino acids. However, the authors attributed the increased defense to an overall alteration of amino acid levels creating a cellular environment that altered redox levels ultimately inducing SA-mediated defense responses (Liu et al., 2010). Although more research needs to be done to determine which amino acids are being translocated in or out of the apoplast or to and from local and distal tissues during *Pst* infection following CK application, this study provides a framework

linking CK signaling, amino acid movement, and possible alteration in amino acid homeostasis signaling for an increase in plant defense.

4.6 CONCLUSIONS

This study explores the molecular mechanisms of CK in an area of IR known as priming of plant defenses and as an inducer of systemic resistance. With the use of transcriptome analysis during CIP against *Pst,* we discovered that priming by CK potentiates the expression of defense genes commonly associated with pathogen-induced or chemically-induced SAR. Using these results, we discovered that local application of CK can decrease susceptibility to *Pst* infiltrated nontreated, distal leaves through a mechanism requiring a known SAR regulator, NPR1, and the mobile SAR signal NHP. CK-indued SAR also shares another common characteristic of SAR in that distal expression of *PR1* is up-regulated following local CK application. However, this expression is not dependent on distal endogenous CK signaling indicating the mobile SAR signal does not initiate CK signaling in distal tissues. The need for NHP, a derivative of Pip, in CK-induced SAR revealed that Pip-induced SAR relies on endogenous CK signaling for decreased susceptibility to *Pst* and the enhanced expression of *PR1* in distant, untreated plant tissues.

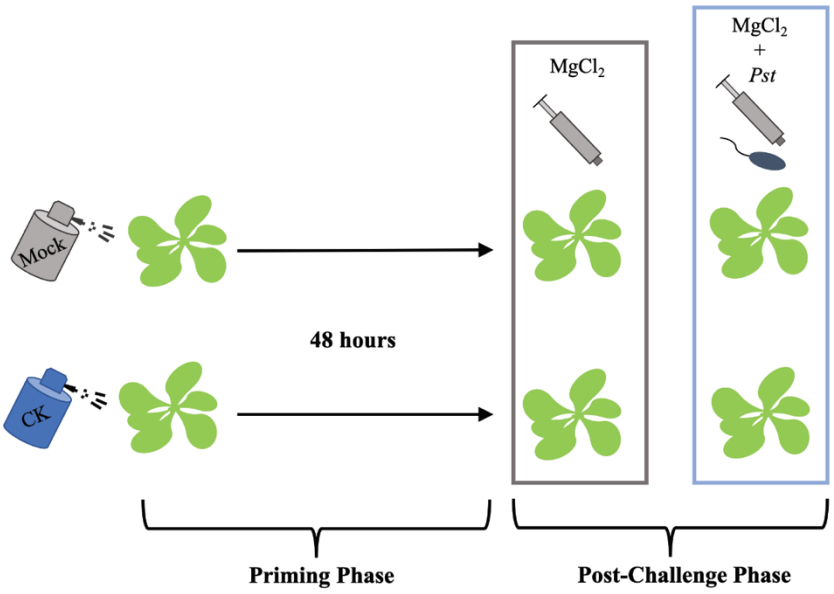
This study included targeted metabolomic analysis of free amino acids during CIP. The results indicated that the abundance of most amino acids remained relatively unchanged following CK treatment, suggesting that CIP may not significantly alter free amino acid content. However, it was observed that four general amino acid transporters, known to be regulated by CK, are required for reduced susceptibility during CIP against *Pst*. The expression of *CAT1* as compared to *LHT1* was robust and rapid in CK-primed plants following pathogen challenge, indicative of a primed defense gene although the expression of both genes were mediated by CK.

The two cationic amino acid transporters discovered to be required for CIP, are also required for CK-induced SAR. The role of amino acid transporters in CIP raises the possibility that altering the nutritional environment for *Pst* may suppress bacterial multiplication. This nutrient-driven alteration may act as a strategy to limit the pathogen's access to essential amino acids, thereby hindering its growth and colonization. Another possibility is that the CK-regulation of amino acid transporters alters amino acid homeostasis, serving as a trigger for defense activation. These theories offer new avenues for research in understanding the intricate defense mechanisms mediated by CK.

In summary, this study contributes to the field of plant defense priming, providing novel insights into the molecular framework of CIP, and the role of amino acids as potential signals of plant defense. These findings expand our understanding of plant-pathogen interactions during IR, opening doors to innovative strategies for enhanced disease protection in agriculture. Future research endeavors should delve deeper into the mechanistic details of amino acid signaling, ultimately leading to the development of sustainable and effective approaches for crop protection.

4.7 FIGURES

A



Stimuli	Timepoint
Mock	48 hr
CK	48 hr

Stimuli	Inoculation	Timepoint
Mock	$MgCl_2$	48 hr
Mock	$MgCl_2$ + *Pst*	24 hr
CK	$MgCl_2$	48 hr
CK	$MgCl_2$ + *Pst*	24 hr

B

Timepoint	Comparison	Outcome	# DEGs
48 hour	Mock vs CK	The effect of CK prior to pathogen challenge	21
24 hour	Mock $MgCl_2$ vs Mock *Pst*	The effect of *Pst* while controlling for the effect of the $MgCl_2$ infiltration	4954
24 hour	CK $MgCl_2$ vs CK *Pst*	The effect of *Pst* in CK-primed plants in the post-challenge phase while controlling for the effect of the $MgCl_2$ infiltration	5497
24 hour	(Mock $MgCl_2$ vs Mock *Pst*) vs (CK $MgCl_2$ vs CK *Pst*)	Similarly regulated genes between CK and *Pst* in the post-challenge phase while controlling for the effect of the $MgCl_2$ infiltration	1332

Figure 4.1 | Transcriptome analysis experimental design of cytokinin (CIP)-induced priming against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*): (**A**) Diagram of the experimental design for CIP against *Pst* showing the applied stimuli, inoculations, and timepoints of tissue collection for RNA-seq analysis during the priming and post-challenge phase. Leaf tissue was collected for RNA-seq analysis 48 hours after five-week-old plants with either 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO priming treatment and 24 hours after subsequent inoculation with $MgCl_2$ control or *Pst* in $MgCl_2$. Table **B** shows the comparisons of samples and the outcome of the comparison with the number of overall differentially expressed genes (DEGs) as determined by a $\log_2$ fold change of +/- 1 and a *p*-value < 0.05 as determined by Wald test using DESeq2 (Love et al., 2014).

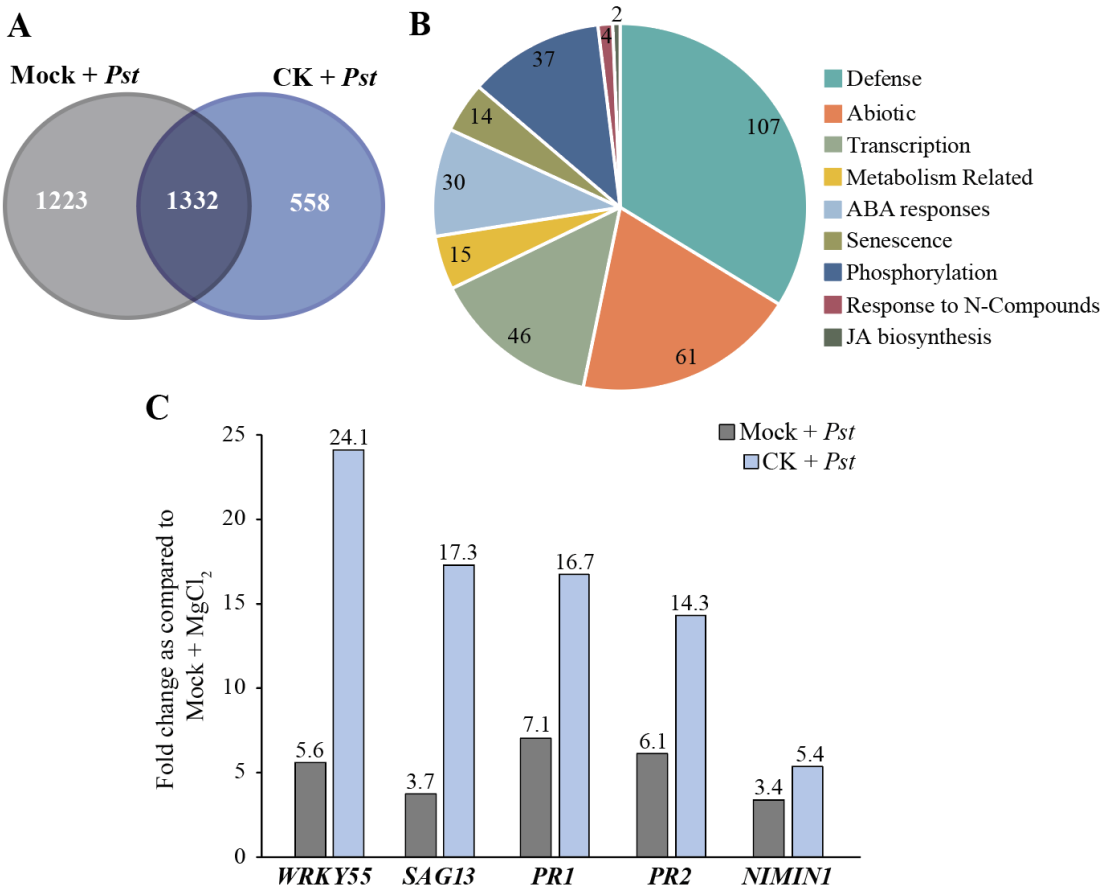


Figure 4.2 | Transcriptome analysis reveals that cytokinin (CK)-induced priming potentiates gene expression induced by *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*): Six-week-old wild type plants were sprayed with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours prior to infiltration with $MgCl_2$ or *Pst*. Tissue was collected for transcriptome analysis 24 hours after infiltration. **(A)** Comparison of differentially expressed genes (DEGs) between Mock + *Pst* and CK + *Pst* has 1332 similar genes that are up-regulated as compared to $MgCl_2$ control samples. Of the 1332 similar genes, 465 genes had a higher expression in CK samples as compared to mock samples. **(B)** Gene ontology analysis of the 465 genes showed that 107 of these genes function in plant defense. **(C)** displays a subset of common defense genes that have a potentiated expression in *Pst*-inoculated samples that were pretreated with CK. DEGs were determined fold change of ≥ 1.5 and a *p*-value of < 0.05 as determined by Wald test through Deseq2.

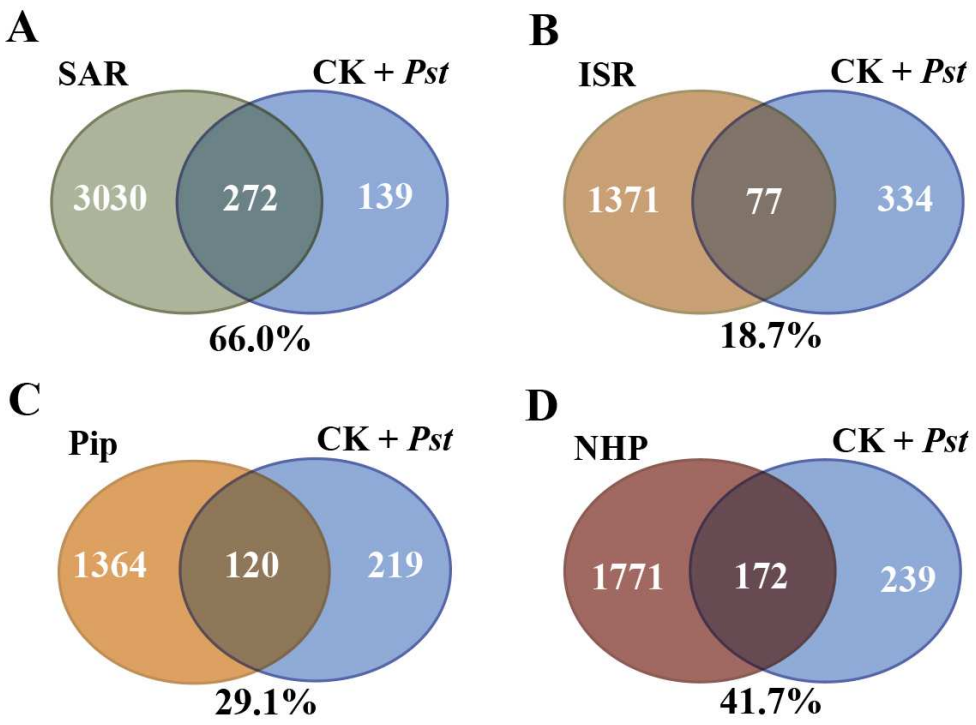


Figure 4.3 | Genes showing potentiated expression after pathogen challenge due to cytokinin (CK)-induced priming are similar to known systemic acquired resistance genes: Comparison of the 412 genes that show potentiated expression in CK-primed, infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) are similar to **A)** 66% of systemic acquired resistance (SAR) (Bernsdorff et al., 2016), **B)** 18.7% of induced systemic resistance (ISR) (Desrut et al., 2020), **C)** 29.1% of L-pipecolic acid (Pip) (Yildiz et al., 2021), and **D)** 41.7% of N-hydroxy-pipecolic acid (NHP) (Yildiz et al., 2021) positively regulated genes.

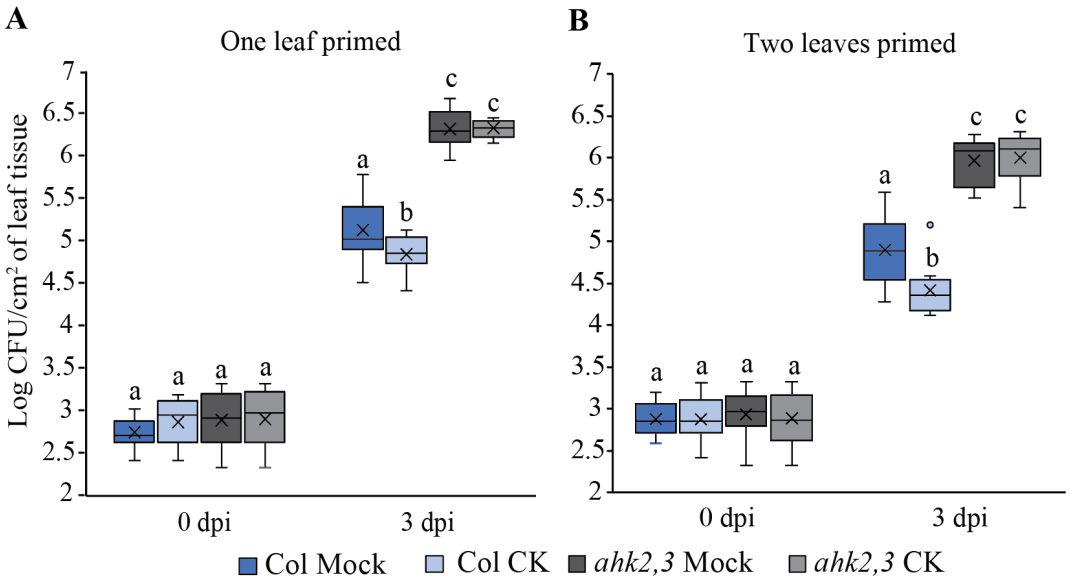


Figure 4.4 | Application of 100µM cytokinin (CK) to one or two leaves reduces *in planta* bacterial growth in systemic tissues: Five- to six-week-old plants treated with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO by brushing 1 **(A)** or 2 **(B)** leaves 48 hours before non-treated or systemic leaves were infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000. Leaf discs were collected at 1 hour post inoculation (0 dpi) and 3 days post inoculation (3 dpi) to determine *in planta* bacterial levels. Data are pooled from three biological replicates. Different letters indicate statistical differences with a *p*-value < 0.05 in treatments at the specified time point by two-way ANOVA with TUKEY HSD correction.

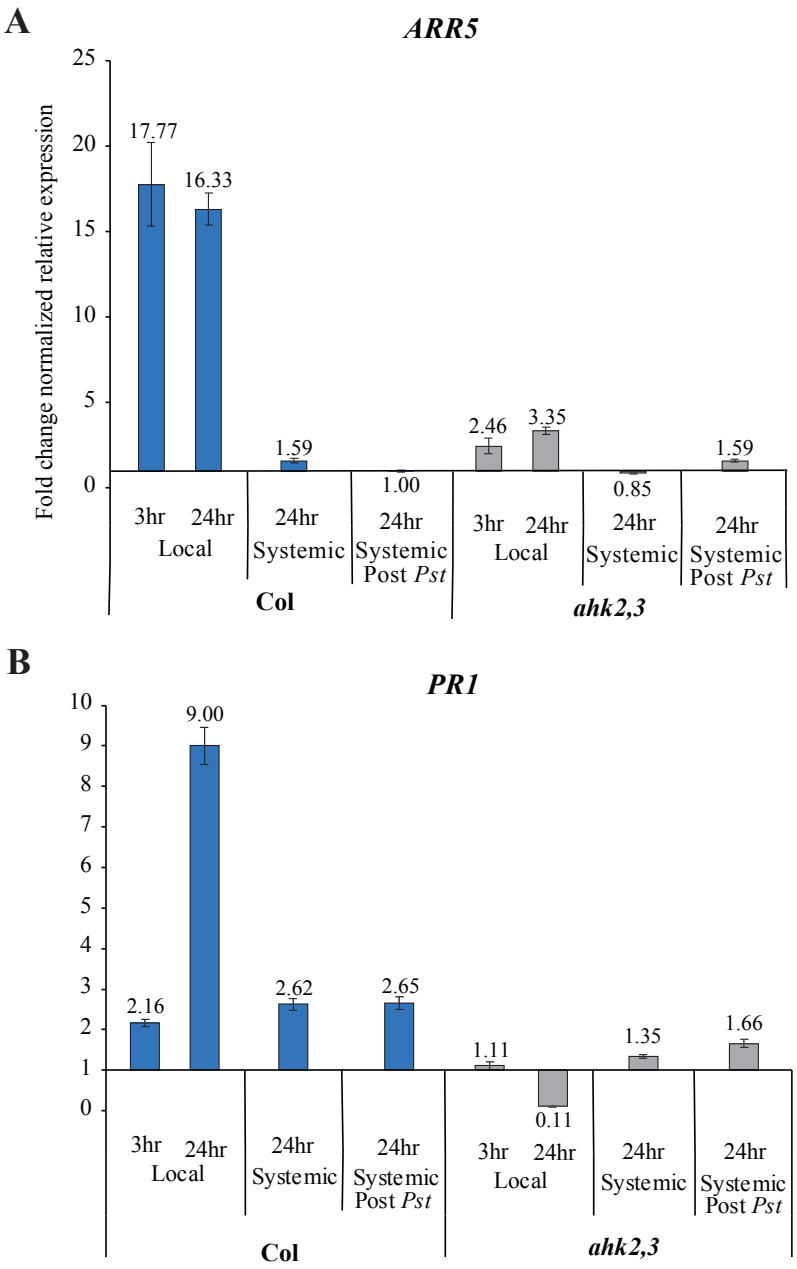


Figure 4.5 | Endogenous cytokinin (CK) signaling is required for the induction of *PR1* expression in systemic tissues following cytokinin infiltration locally: Six-week-old plants infiltrated leaves with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours before systemic leaves were infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). Tissue was collected from local tissues 3 and 24 hours post priming, systemic tissues 24 hours after priming, or systemic tissues 24 hours after *Pst* infiltration. Three leaves were collected from four plants and pooled prior to RNA extraction. Expression of **A)** *ARR5* and **B)** *PR1* was analyzed by qRT-PCR. Data is representative of three biological replicates and graphed as normalized fold change of priming treatment/control treatment relative to *UBQ*.

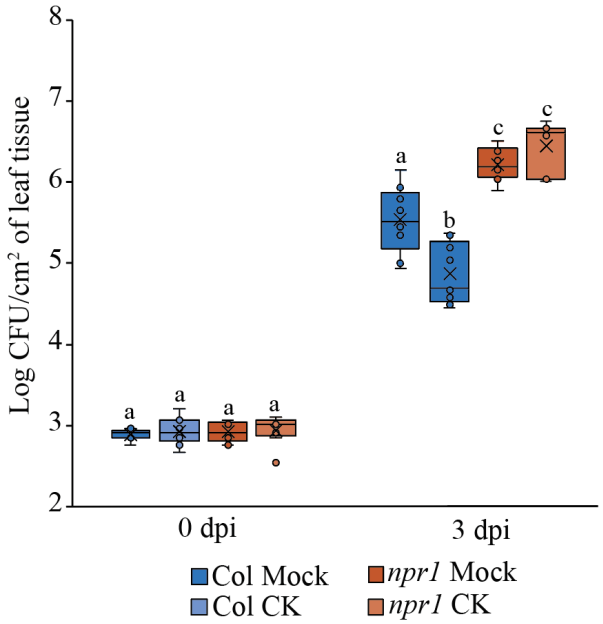


Figure 4.6 | Cytokinin (CK)-induced systemic acquired resistance is dependent on NPR1 to reduce susceptibly to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) in systemic tissues: Five-week-old plants had three local leaves infiltrated with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours prior to the infiltration of three systemic leaves with *Pst.* Leaf discs were collected at 1 hour post inoculation (0 dpi) and 3 days post inoculation (3 dpi) to determine *in planta* bacterial levels. Data are pooled from three biological replicates. Different letters indicate statistical differences with a p-value < 0.05 in treatments at the specified time point by two-way ANOVA with TUKEY HSD correction.

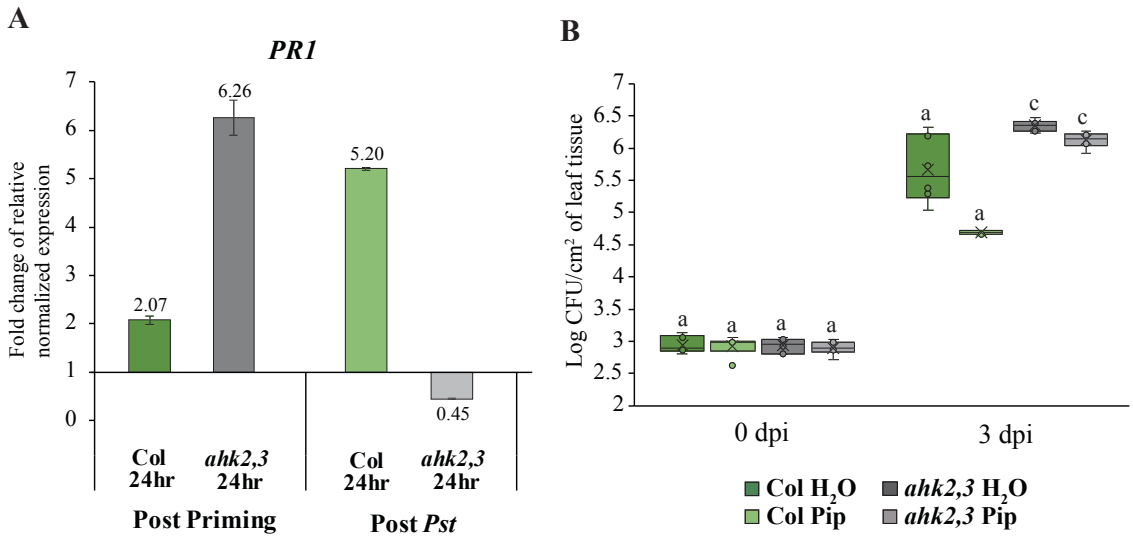


Figure 4.7 | Endogenous cytokinin signaling alters defense in L-pipecolic acid (Pip)-induced priming and Pip-induced systemic acquired resistance (SAR): Five- to six-week-old plants treated with H_2O or 1mM Pip by soil drench 48 hours prior to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) inoculation **(A)**. Leaf tissue was from two plants collected 24 hours after priming and after pathogen infiltration and pooled prior to RNA extraction. The expression of *PR1* was analyzed by qRT-PCR. Data is representative of three biological replicates and graphed as normalized fold change of priming treatment/control treatment relative to *UBQ*. **(B)** Infiltration of local leaves with H_2O or 1mM Pip 48 hours before non-treated, systemic leaves were infiltrated with *Pst*. Leaf discs were collected at 1 hour post inoculation (0 dpi) and 3 days post inoculation (3 dpi) to determine *in planta* bacterial levels. Data are pooled from two biological replicates. Different letters indicate statistical differences with a p-value < 0.05 at the specified time point by two-way ANOVA with TUKEY HSD correction.

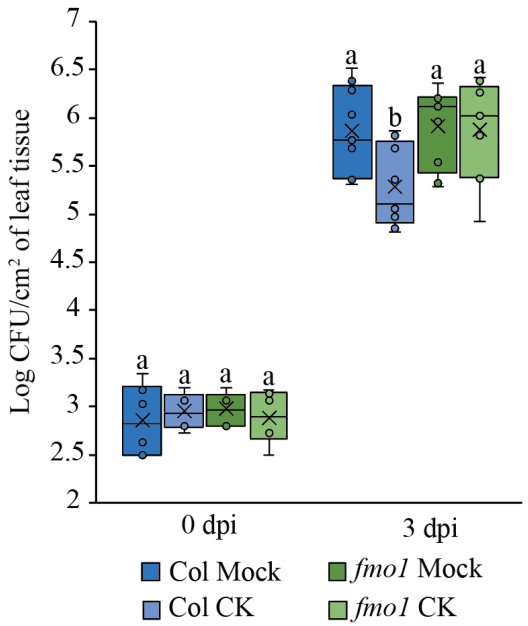


Figure 4.8 | Cytokinin (CK)-induced systemic acquired resistance (SAR) is dependent on FMO1 to reduce susceptibly to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) in systemic tissues: Five-week-old plants had three local leaves infiltrated with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours prior to infiltration of three systemic leaves with *Pst.* Leaf discs were collected at 1 hour post inoculation (0 dpi) and 3 days post inoculation (3 dpi) to determine *in planta* bacterial levels. Data are pooled from three biological replicates. Different letters indicate statistical differences with a p-value < 0.05 at the specified time point by two-way ANOVA with TUKEY HSD correction.

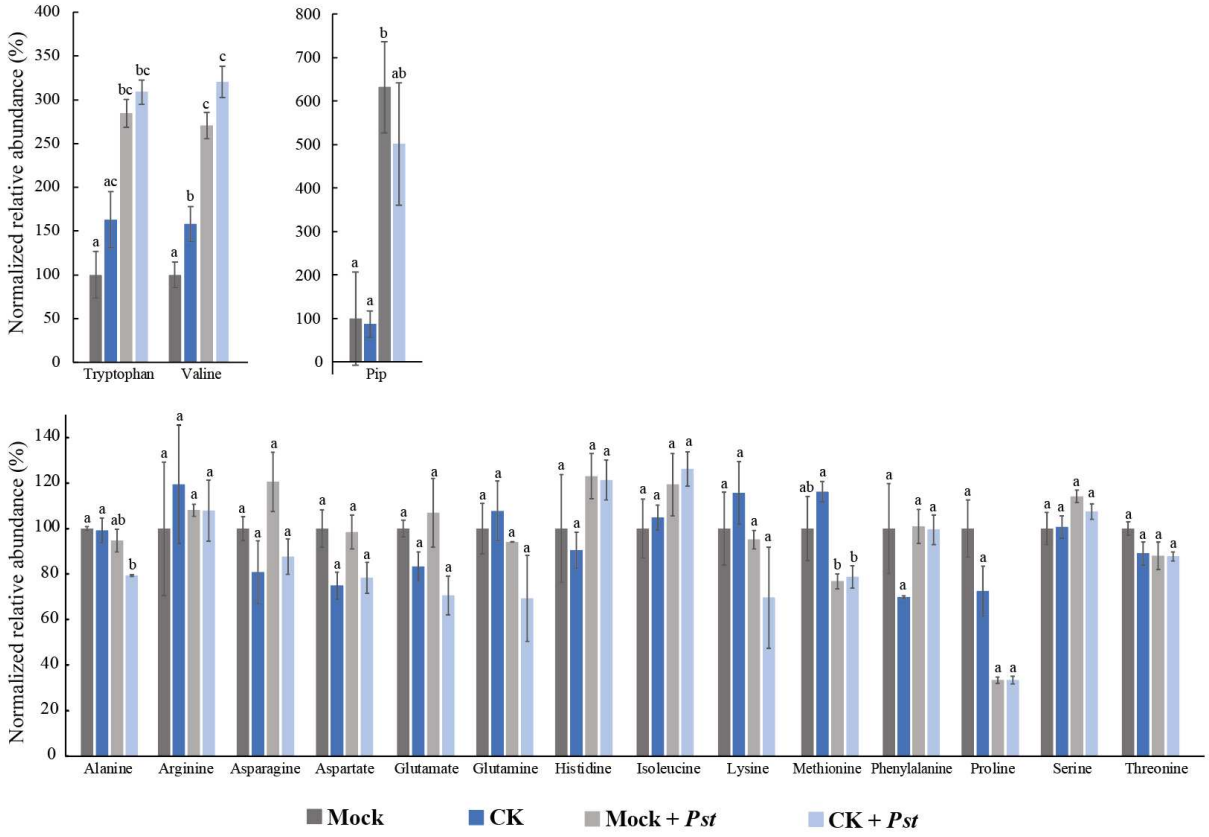


Figure 4.9 | Targeted amino acid metabolomics analysis following cytokinin (CK)-induced priming shows few alterations in amino acid relative abundances: Five to six-week-old plants were subjected to spray application of 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO followed by inoculation of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst)* 48 hours after. Leaf tissue was collected from primed plants 48 hours after spray treatment and 24 hours after the pathogen challenge for analysis by liquid chromatography-mass spectrometry. Different letters indicate a statistical difference with a *p*-value < 0.05 as determined by two-way ANOVA test with TUKEY HSD correction.

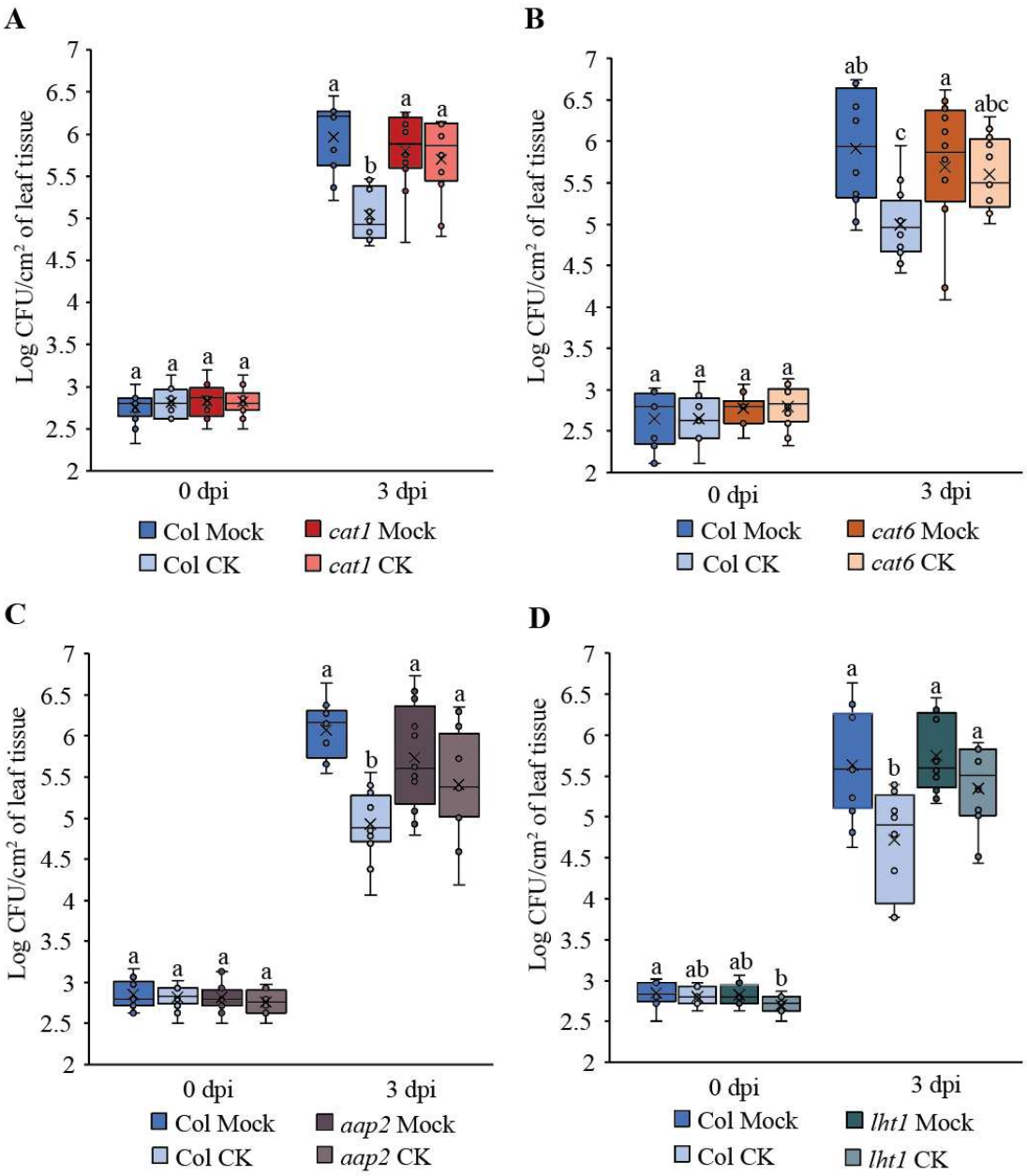


Figure 4.10 | Cytokinin-induced priming requires general amino acid transporters to reduce susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*): Five-week-old wild type Col, **A)** *cat1*, **B)** *cat6*, **C)** *aap2*, and **D)** *lht1* were sprayed with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours prior to infiltration with *Pst.* Leaf discs were collected at 1 hour post inoculation (0 dpi) and 3 days post inoculation (3 dpi) to determine *in planta* bacterial levels. Data are pooled from three biological replicates. Different letters indicate statistical differences with a *p*-value < 0.05 at the specified time point by two-way ANOVA with TUKEY HSD correction.

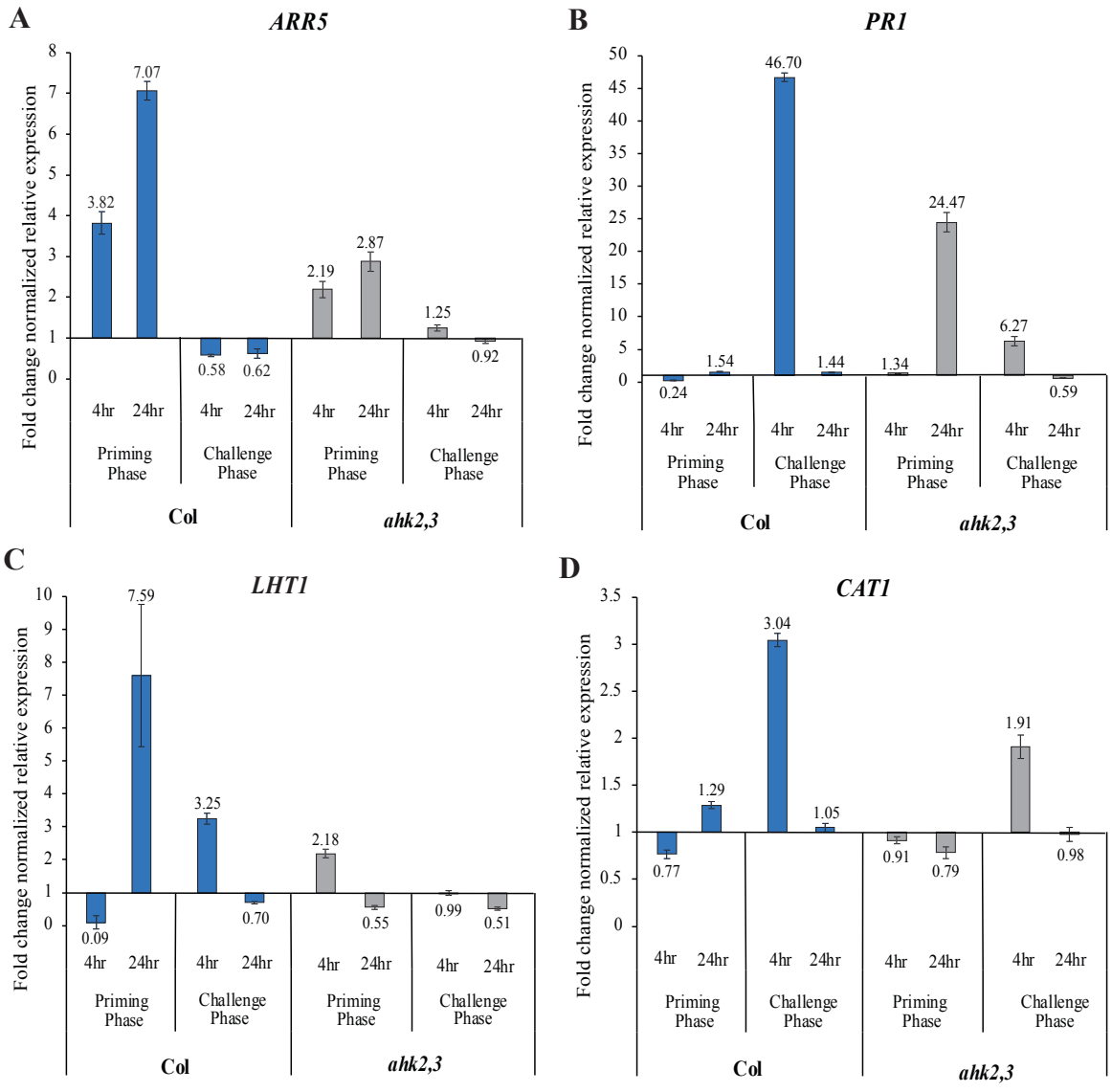


Figure 4.11 | Cytokinin-induced priming induces expression of amino acid transporters *LHT1* and *CAT1*: Five-week-old wild type Col and *ahk2,3* plants primed with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours prior to infiltration with *Pseudomonas syringae* pv. *tomato* DC3000. Four leaves were pooled from four plants collected 4 hours and 24 hours after priming and after pathogen infiltration for qRT-PCR analysis of the expression of **A)** *ARR5*, **B)** *PR1*, **C)** *LHT1*, and **D)** *CAT1*. Data is representative of two biological replicates graphed as normalized fold change of priming treatment /control treatment relative to *UBQ*.

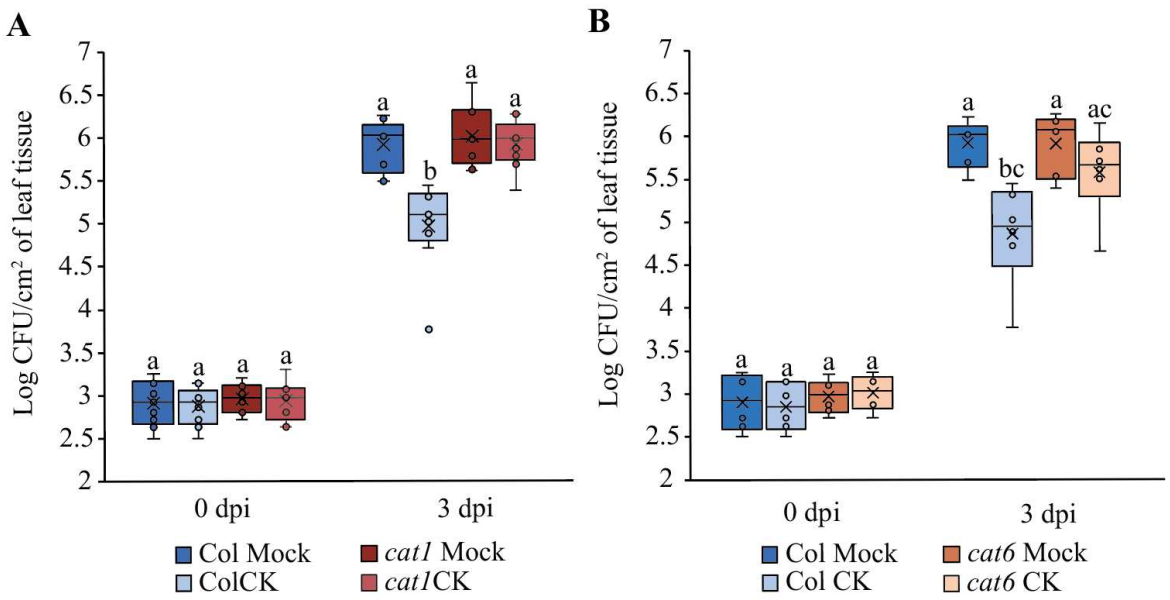


Figure 4.12 | Cytokinin-induced systemic acquired resistance is dependent on amino acid transporters CAT1 and CAT6: five-week-old wild type Col, **A)** *cat1*, and **B)** *cat6* had three local leaves infiltrated with 0.01% DMSO mock solution or 100µM of the CK benzylaminopurine in 0.01% DMSO 48 hours prior to infiltration of 3 systemic leaves with *Pseudomonas syringae* pv. *tomato* DC3000. Leaf discs were collected at 1 hour post inoculation (0 dpi) and 3 days post inoculation (3 dpi) to determine *in planta* bacterial levels. Data are pooled from three biological replicates. Different letters indicate statistical differences with a p-value < 0.05 at the specified time point by two-way ANOVA with TUKEY HSD correction.

4.8 TABLES

Table 4.1 | RT-qPCR primers used in this study: The table shows the primer sequences used within this study for RT-qPCR for gene expression analysis. All sequences are written in the 5' to 3' direction.

Gene	Forward Primer sequence	Reverse Primer sequence
ARR5	TCTGAAGATTAATTTGATAATGACGG	TCACAGGCTTCAATAAGAAATCTTCA
CAT1	AGGTTTGCAATCTGGACAGG	CCTGCTTCTCCTTCSACGTC
FMO1	TCTTCTGCGTGCCGTAGTTTC	CGCCATTTGACAAGAAGCATAG
PR1	ACACGTGCAATGGAGTTTGTGGTC	TACACCTCACTTTGGCACATCCGA
UBQ-10	CGTTAAGACGTTGACTGGGAAAACT	GCTTTCACGTTATCAATGGTGTCA

Table 4.2 | Multiple Reaction Monitoring transitions of amino acids from liquid chromatography-mass spectrometry.

	Q1	Q3	cone (V)	Collision energy (V)
Glycine	76	30	15	10
Alanine	90.0547	44	25	4
Serine	106.05	60	25	20
Proline	116	43	25	20
Valine	118.089	55	25	17
Valine	118.089	72	25	10
Threonine	120.0652	56	20	10
Cysteine	122.0267	76	32	15
Isoleucine/ Leucine	132.1015	86	30	15
Asparagine	133.1	74	20	10
Asparagine	133.1	87	20	10
Aspartic Acid	134.0448	43	20	20
Aspartic Acid	134.0448	70	20	20
Glutamate	148.0602	84	30	15
Glutamate	148.0602	130.1	30	10
Methionine	150.0581	104	20	10
Methionine	150.0581	133	20	10
Histidine	156	83	30	22
Histidine	156	110	30	14
Phenylalanine	166.086	103	25	25
Arginine	175.1187	116	20	20
Tyrosine	182.0809	91	25	25
Tyrosine	182.0809	136	25	10
Tryptophan	205.09	146	30	17
Tryptophan	205.09	188	30	10
Pipecolic acid	130.09	56.05	20	30
Pipecolic acid	130.09	84.56	20	20

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CHAPTER 5:

RESEARCH CONCLUSIONS AND FUTURE DIRECTIONS

5.1 INTRODUCTION

CK has been previously described as one of the four ancient hormones by evolutionary studies (Wang et al., 2015), thus it has played a vital role in plant development and adaptation through time. Discovered by researchers in the early 1950s, CK was named after its role in cytokinesis or cell division (Miller et al., 1956) with later studies deeming the hormone's main function in plant growth and development (Kieber and Schaller 2018). Specifically, CK has a role in the delay of senescence through the maintenance of the photosynthetic machinery (Cherniad'ev 2000, Ahanger et al., 2018, Honig et al., 2018), source-sink relationships and associated allocation of photoassimilates (Mothes and Engelbrecht 1961, Harms et al., 1994, Ehness and Roitsch 1997, Lara et al., 2004, Brenner et al., 2005, Kiba et al., 2005, Lee et al., 2007, Yokoyama et al., 2007, Jian et al., 2016, Ninan et al., 2019), and broadly the induction of organ and tissue development (Schaller et al., 2015). However, research beginning in the 2000s expanded the functions of CK to plant defense against abiotic and biotic stress (Argueso et al., 2009, Choi et al., 2010, Argueso et al., 2012). This dissertation focused on the role of the plant hormone cytokinin (CK) in plant defense against pathogens. Particularly, the research presented here focused on a specific type of induced plant defense called priming, against the hemibiotrophic bacterial pathogens *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Pseudomonas syringae* pv. *maculicola* (*Psm*) in *Arabidopsis thaliana* and *Brassica napus,* respectively, and the necrotrophic fungal pathogen *Botrytis cinerea* in Arabidopsis.

5.2 CHARACTERIZATION OF CYTOKININ-INDUCED PRIMING

As presented in the second chapter of this dissertation, my research focused on understanding broad fundamental questions about CK-induced priming (CIP) and characterizing CK as a priming agent. In Arabidopsis, I demonstrated that following one application of CK, a reduction in susceptibility to *Pst* is seen between 24 and 48 hours after priming. Interestingly, this timeframe of reduced susceptibility is after the peak of CK-responsive gene expression, suggesting that primary target of endogenous CK signaling are not responsible for increased defense and CK induces an effect on plant defense downstream of activation of CK signaling. I show that two other well-characterized priming agents, β-aminobutyric acid (BABA) and L-pipecolic acid (Pip), depend on endogenous CK signaling to increase plant defense leading to the conclusion that, broadly, priming agents require CK. In addition, I showed that, like other priming agents, CK can be transgenerational (Luna et al., 2012, Ramirez-Carrasco et al., 2017, Lopez Sanchez et al., 2021). These results suggest that CK may be an essential component of the priming process in general.

As compared to constitutive activation of plant defense (Bowling et al., 1994, Clarke et al., 1998), priming has fewer adverse effects on plant growth. Thus, priming agents can be attractive chemical strategies for disease protection in agriculture. I determined that one application of CK results in a moderate decrease in shoot growth, and that sustaining the priming effect by three CK treatments over time results in an even larger decrease in above-ground growth, developmental effects to leaves, and a reduction in the overall seed set. To determine if CIP is effective in a crop species within the same family as Arabidopsis, similar questions were asked in *B. napus* against *Psm*. Priming was shown to be effective at reducing susceptibility to the bacterial pathogen between 48 and 72 hours after a single treatment with CK with overall

plant weight being unaffected. In opposition to the results in Arabidopsis, multiple treatments of CK over time also did not result in a change in overall plant weight, but increased petiole length and shoot height, whereas overall root growth was negatively impacted by multiple CK treatments. Thus, depending on dosage and plant species, CIP can have negative effects on plant growth, as shown for other priming agents (van Hulten et al., 2006, Cooper and Ton 2022).

The results within this chapter provide a framework where using CK as an agrochemical to increase plan defense could be a disease management strategy. One of the major areas of this research is the optimization of the timeframe in which priming is effective while reducing its negative impact on growth. This could be accomplished through studies utilizing different concentrations of CK with different surfactants to either improve the delivery of CK through the epidermal layer or prevent the degradation of the applied hormone. Due to the role of CK on source-sink relationships, it is possible the negative growth effects seen after CK applications could be mitigated by increasing the availability of certain nutrients needed to rebalance the altered allocation of nutrients induced by CK. Further, my discovery that CIP could have transgenerational effects needs to be further explored due to the variable outcomes of the results within this dissertation. Continuing studies should include optimization of the concentration of CK applied, the number of applications, and the timing of the applications with regard to the development and maturity of reproductive structures with the goal of a more consistent outcome of increased resistance in the progeny of primed plants. Further, pathogen susceptibly in the second generation of the original primed plants should be discovered to understand how long the priming effect lasts.

5.3 HIGHLIGHTS IN THE ROLE OF CYTOKININ IN DEFENSE AGAINST *BOTRYTIS CINEREA*

In Chapter 3 I aimed to determine the plant hormone requirements of CIP against *B. cinerea* in Arabidopsis, as well as discover potential molecular mechanisms through the use of transcriptomics. I first demonstrated that CK can either increase or decrease susceptibility to *B. cinerea* through the application of low or high concentrations of CK, respectively, or through genetic mutations altering levels of endogenous CK signaling, thus providing evidence that CK has a role in defense against this necrotrophic pathogen. Previous studies have focused mostly on the role of CK in salicylic acid (SA)-mediated defense (Choi et al., 2010, Argueso et al., 2012, Jiang et al., 2013, Arnaud et al., 2017, Gupta et al., 2021), but evidence of a role for CK in jasmonic acid-(JA) mediated defense pathways had been demonstrated (Dervinis et al., 2010, Schafer et al., 2015, Zhang et al., 2022). Therefore, using Arabidopsis mutants, I showed that CIP against *B. cinerea* requires JA biosynthesis, and partially requires SA biosynthesis and signaling. Further investigation by analysis of the gene expression of the markers of the JA and SA pathways showed that CIP does not prime *PDF1.2,* a marker of the MYC2 branch of the JA pathway, but does prime the expression of *VSP2,* regulated by the *ERF1* branch of JA the pathway, as well as *PR1*, regulated by SA. Thus, the involvement of phytohormonal networks in CIP is complex, involving the partial contribution of specific branches of different phytohormonal networks. These results align well with the nature of *B. cinerea* genetic resistance in Arabidopsis, which is quantitative (Caseys et al., 2021), and thus likely to be dependent on genes governed by several plant hormone pathways.

Transcriptome analysis revealed the effect of CK treatment on gene expression occurs early after treatment, with a predominance of up-regulated DEGs related to RNA modification

and processing, suggesting CK treatment signals the plant to prepare for future needs by preparing all the necessary cellular components for translation. Interestingly, genes showing increased expression both at 4 and 24 hours after CK treatment included those associated with biosynthesis of Pip, possibly providing the starting compound to induce systemic acquired resistance (SAR), as Pip is converted to NHP (Hartmann et al., 2018), which is the mobile signal in SAR (Yildiz et al., 2021), and whose relevance for CIP was investigated in Chapter 4. Additionally, CK treatment down-regulated genes involved in the catabolism of starch and aromatic amino acids, which could be a strategy for ensuring energy storage and preventing the synthesis of secondary defensive compounds thus, preparing the primed plant for future challenges. In the subsequent pathogen challenge phase, priming plants with CK resulted in the regulation of similar genes as *B. cinerea* infection, but in the opposite direction, where genes up-regulated by *B. cinerea* only were down-regulated due to priming by CK followed by *B. cinerea* infection. CIP increases the expression of genes responsible for photosynthesis while decreasing the expression of defense-related genes. These results could indicate that CK functions to preserve the energy-producing system of the plant while controlling defense gene expression that could be associated with cell death. This proposed mechanism could suggest that the reduced susceptibility seen during CIP is due to its fine-tuning of the balance between plant cell survival and growth that helps the plant, and plant cell death and defense that helps the necrotrophic pathogen.

The results of this study suggested that multiple hormonal networks are involved in CK-induced defense against *B. cinerea*, however, more research is needed to validate and understand this hypothesis. Future studies should include hormone quantification during different time points during bother the priming phase and the post-pathogen challenge phase. This would allow

for the observation of multiple or specific hormones involved in the induction of a primed plant and their roles and relation to another in the subsequent pathogen challenge of a primed plant. I also believe the role of CIP against *B. cinerea* on photosynthesis should be further explored to identify if the up-regulation of photosynthetic-related processes is first associated with an increase in photosynthetic ability. Second, the effect of CK on photosynthesis activity after various time points following *B. cinerea* infection should be identified in order to understand if there are specific disease stages where CK mitigates defense activation while increasing photosynthesis. These future experiments would validate the findings of my transcriptome study while discovering molecular defense mechanisms induced by CK.

5.4 CYTOKININ-INDUCED DEFENSE IS A SYSTEMIC RESPONSE MEDIATED BY AMINO ACID TRANSPORTERS

In the final chapter of this dissertation, I explored the molecular mechanisms behind CIP against *Pst* in Arabidopsis, first by identifying the transcriptome changes in the post-pathogen-challenged phase following CK treatment. This analysis revealed an overlap of genes induced by both pathogen- and pipecolic acid (Pip)-induced systemic acquired resistance (SAR) and CIP. Based on these results, I demonstrated that CK can induce a systemic defense response. Although CK-induced SAR increased *PR1* expression locally and distally, only local CK signaling was induced indicating that local CK applied mediates a downstream process that induces defense distally. Due to the overlap of similarly regulated defense genes as Pip, the relationship between CK and Pip was explored. I showed that endogenous CK signaling is required for Pip-mediated *PR1* expression during priming and effective Pip-induced SAR against *Pst*. Moreover, two genes shown to be required for SAR, *NPR1* and *FMO1*, were demonstrated

to also be required for CK-induced SAR providing further evidence that CK is an inducer of SAR. Due to the known role that CK has on photosynthesis and allocation of photoassimilates, I aimed to understand if this relationship also has a function in CK-induced defense through metabolomics analysis. Untargeted analysis revealed that CK alters sugar metabolism, the TCA cycle, and certain amino acid levels. CK has a known role in amino acid movement through the regulation of the expression of amino acid transporters (Brenner et al., 2005, Kiba et al., 2005, Lee et al., 2007, Yokoyama et al., 2007, Kiba et al., 2011) and early studies showed changes in movement of radio-labeled amino acids following CK application (Mothes and Engelbrecht 1961, Mothes and Engelbrecht 1963). Therefore, using a targeted metabolomics analysis, I focused on understanding the effect that CIP has on free amino acid levels in leaf tissue. Unfortunately, this analysis did not provide any additional insight because the levels of free amino acids minimally changed between priming treatments and subsequent pathogen challenges. However, using mutants for amino acid transporters known to be regulated by CK, I showed that CIP depends on CAT1, CAT6, AAP2, and LHT1, suggesting that general amino acid transport is required for the decrease in susceptibility following CIP. Limiting the scope of gene expression analysis to *LHT1* and *CAT1*, during CIP showed that priming by CK primed the expression of *CAT1* whereas CK-meditated expression of *LHT1* was greatest during the priming phase but expression decreased after pathogen challenge. These results suggest that although both of these amino acid transporters are necessary for CIP and CK mediates their gene expression, CK does not regulate all of the amino acid transporters in the same way at the transcriptional level. Relating to earlier work described within this chapter, CK-induced SAR also was demonstrated to depend on functional amino acid transporters, CAT1 and CAT6. These data show that CK-induced defenses not only mediate defense gene expression to reduce

susceptibility to *Pst*, but also likely through the regulation of amino acid movement within the plant.

The results of this study revealed an overlap of functionality between CK, Pip, and NHP during induced systemic defenses. However, it is unclear the order in which this systemic signaling of defense occurs i.e. if endogenous CK induces Pip-induced SAR or Pip-induced SAR triggers endogenous CK signaling that enables systemic defense. To begin to answer this question, quantification of both CK and SA in local and distal leaves during Pip-induced SAR should be assessed. Moreover, Future studies are needed to further understand which amino acids are being moved during priming and pathogen challenge and which direction the amino acids are moving due to CK. This could be completed by observing the movement of radio-labeled amino acids between local sites of CK application and distal, pathogen-challenged tissues. Additionally, as opposed to the metabolomics analysis completed within this chapter, apoplastic amino acid content should be analyzed locally and systemically during CK-induced SAR to determine the abundance of amino acids present within the areas where *Pst* resides within the plant. Answers to these questions would provide further insight as to how the movement of particular amino acids relates to either plant defense or the creation of an unfavorable nutritional environment for the pathogen.

5.5 FUTURE DIRECTIONS OF CYTOKININ-INDUCED DEFENSE

Plant loss due to disease is estimated to be up to 16% of the total plants produced (Oerke 2005, Savary et al., 2012) therefore, agricultural industries based on fruits, plant by-products, and plant-based fibers are all affected by plant disease. Agricultural research has created plants with high levels and constitutive activation of pathogen defenses (Bowling et al., 1994, Clarke et

al., 1998) at the expense of plant growth and development (Kempel et al., 2011, Denance et al., 2013, Albrecht and Argueso 2017). Induced Resistance (IR) mitigates the energy-expensive defense responses by robust defense activation only when the plant experiences a pathogen challenge, thus providing a promising alternative (Conrath et al., 2015, Buswell et al., 2018). As described in this dissertation, the use of CK as a priming agent could offer a new strategy of IR against a broad range of pathogens of different lifestyles, with minimal effects on plant growth. Moreover, further understanding of the molecular mechanisms of CIP revealed in this research brings new insights into how defense and plant metabolism intersect which could be exploited by genetically altering plant metabolism to induce defense only when necessary. This information could also be used to create disease protection strategies based on the restriction of pathogen nutrition *in planta*.

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