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# Dissection of Plant Defense Mechanisms Using Chemical and Molecular Genomics

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## DEDICATION

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ABSTRACT OF THE DISSERTATION

Dissection of Plant Defense Mechanisms Using
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by

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Dr. Thomas A. Eulgem, Chairperson

Every year billions of dollars are spent on pesticides which can be harmful to consumers and the environment. Off-target effects make the study of interactions between plants and pathogens an integral field for the reduction of conventional pesticide use. Using model pathosystems, such as *Arabidopsis thaliana* (Arabidopsis) and *Hyaloperonospora arabidopsidis* (*Hpa*), important plant disease questions are being addressed. Plant defense-inducing molecules (synthetic elicitors) identified and characterized using chemical genomics will be valuable tools for the dissection of the plant defense network and will serve as leads for the development of environmentally-safe pesticides.

Genes from the Arabidopsis *ACID (Associated with Chemically Induced Defense)* cluster are coordinately inducible by the synthetic elicitors DCA and INA. I demonstrated that 10 of 16 *ACID* members tested are required for basal immunity of Arabidopsis against *Hpa*. In addition, nine novel synthetic elicitors were reported on here.

2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (CMP442) is able to quickly and transiently induce disease resistance, has a distinct mode-of-action, is structurally unique, and can be easily synthesized. At low doses CMP442 enhances the growth of roots and aerial parts of Arabidopsis and *Solanum lycopersicum*, while high concentrations inhibited growth. These effects are reminiscent of the hormetic-like response, which is characterized by a low dose stimulatory or beneficial effect of a wide range of stimuli that are toxic or inhibitory at higher concentrations.

Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are known to coordinate plant defense responses to combat pathogens. Most documented interactions between JA- and SA-dependent signaling processes are antagonistic, but their interactions are complex. Here I report on the development of a screening procedure to identify synthetic elicitors that activate the JA-/ET-dependent branch of the defense network. A set of genes, termed the Jasmonic acid and Ethylene Dependently Induced (*JEDI*) genes, were identified that display SA-independent upregulation in response to infection with *Hpa*. Additionally, efforts to create Arabidopsis lines containing RNA silencing transgenes co-silencing closely related *PDF* family members is described.

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# GENERAL INTRODUCTION

## Plant Disease and Defense

The ability of plants to recognize and respond to the presence of threats is vital for their survival. Given their sessile lifestyle this defense response must be swift. Major threats to plants include diseases caused by microbial phytopathogens. Evolution has duly equipped plants, resulting in plant disease being the exception, not the rule (Staskawicz, Mudgett et al. 2001). However, due to extensive selective breeding and the tendency of farmers to plant monocultures, plant disease has gained a foothold and has become a multi-billion dollar problem (Damalas and Eleftherohorinos 2011). Currently, the solution is the application of pesticides which are often not only toxic to pathogens but can affect non-target plant and animal species (Casida 2009). The off-target effects of pesticides make the discovery of novel solutions to the plant disease problem crucial (Hart 2005).

In plants, many physical and chemical barriers exist that passively prevent pathogen infection. Physical barriers can include: a waxy cuticle, stomata, and thick cell walls. Chemical barriers include phytoanticipins, phenolics, and quinines which have antimicrobial properties, as well as lactones, cyanogenic glucosides, saponins, terpenoids, stilbenes and tannins (da Cunha, McFall et al. 2006). While passive defenses are effective against some phytopathogens, an active immune system is required to combat pathogens able to bypass passive

immunity (Dangl and Jones 2001). One such form of active defense includes small basic peptides called plant defensins (*PDFs*). *PDFs* can interfere with a pathogen's ability to extract nutrients, thus delaying pathogen development (Thomma, Cammue et al. 2002). Plant-pathogen interactions are dynamic and shaped by a 'coevolutionary arms race' (Jones and Dangl 2006). In this arms race, there are strong selective pressures for the plant to maintain its resistance against a given pathogen, as well as pressures on pathogens to overcome plant defenses (Anderson, Gleason et al. 2010).

Like mammalian organisms, plants possess an inducible innate immune system that is based on the genetically determined and inheritable recognition of molecular features of pathogens (Nurnberger, Brunner et al. 2004). Unlike mammals, however, plants do not have specialized immune cells and most plant cell types are capable of efficient innate immune responses. In addition to local innate immunity acting in plant tissues subject to pathogen attack, mobile signals generated in such primary infection sites control systemic defense responses mediating long lasting broad spectrum disease resistance. This innate immune system is constantly evolving in a fashion described by the 'zigzag model' (Jones and Dangl 2006). According to this model, the most fundamental form of plant innate immunity involves recognition of conserved molecular signatures shared by many classes of pathogens termed microbe-associated molecular patterns (MAMPs). MAMPs are recognized by pattern-recognition receptors (PRRs) on the surface of plant cells. MAMPs are essential for a pathogen survival and

fitness and cannot be discarded or altered through evolution to evade PRR-mediated detection (Jones and Dangl 2006). Examples of MAMPs include: chitin and ergosterol from fungi, β-glucans from oomycetes, fungal xylanase and oomycete transglutaminase, as well as flagellin and lipopolysaccharides (LPS) from gram-negative bacteria (Baureithel, Felix et al. 1994; Granado, Felix et al. 1995; Enkerli, Felix et al. 1999; Felix, Duran et al. 1999; Gomez-Gomez, Felix et al. 1999; Klarzynski, Plesse et al. 2000; Meyer, Puhler et al. 2001; Brunner, Rosahl et al. 2002; Nurnberger, Brunner et al. 2004; Zipfel, Robatzek et al. 2004; Melotto, Underwood et al. 2006).

Upon recognition of a MAMP, the plant activates a comprehensive set of defense reactions called pattern-triggered immunity (PTI). During PTI there are extensive molecular, morphological, and physiological changes (Altenbach and Robatzek 2007). Signaling cascades link recognition and response. Within minutes of MAMP recognition, there are ion fluxes across the plasma membrane, an increase in cytosolic $Ca^{2+}$, an oxidative burst, which includes the production of reactive oxygen species (ROIs) and nitric oxide, MAP kinase (MAPK) activation, protein phosphorylation, and receptor endocytosis (Nurnberger, Brunner et al. 2004; Schwessinger and Zipfel 2008; Keinath, Kierszniowska et al. 2010; Zipfel and Robatzek 2010).

Protein kinases are major regulators of plant defense responses that act at various hierarchical levels within the defense network (Tena, Boudsocq et al. 2011). There are more than 1000 protein kinases in the plant model organism

*Arabidopsis thaliana* (Arabidopsis) (Gish and Clark 2011). In particular, receptor protein kinases (RPKs), $Ca^{2+}$ dependent protein kinases (CDPKs) and MAPKs have been extensively implicated in the regulation of plant immune responses. The plant immune responses are controlled by a complex regulatory network consisting of multiple interconnected sectors that include those regulated by salicylic acid (SA)- and others dependent on jasmonic acid and ethylene (JA/ET) (Katagiri 2004; Fu, Yan et al. 2012) as well as other less well characterized pathways (Erb, Meldau et al. 2012).

Over time, pathogens evolved effector molecules which are released to augment virulence by manipulating and weakening, PTI resulting in effector-triggered susceptibility (ETS). Such interactions between virulent pathogens and susceptible plants are termed “compatible”. A susceptible plant still maintains low levels of defense, called basal defense. Basal defense is not sufficient to fully prevent disease, but it can slow its progression (Tsuda, Sato et al. 2009).

PTI is often successful against pathogens that have not evolved the ability to specifically infect a plant; this is referred to as non-host resistance. To counteract ETS, plants evolved resistance proteins (R proteins), which specifically recognize pathogen effectors resulting in a resistant plant and an avirulent pathogen (incompatible interaction). This type of innate immunity is referred to as effector-triggered immunity (ETI). ETI is a faster and more robust version of PTI, and often results in a hypersensitive response (HR) at the site of infection (Tsuda, Sato et al. 2009). HR involves a programmed form of death of

plant cells directly in contact with an invading pathogen. In some cases pathogens evolved additional effectors to evade ETI (Jones and Dangl 2006; Tsuda and Katagiri 2010). ETI is active against adapted pathogens. Although, these relationships are not always set in stone and they may depend on the specific elicitor molecules present during pathogen infection (Metraux, Jackson et al. 2009; Dodds 2010; Dodds and Rathjen 2010).

R proteins either directly or indirectly recognize the presence of pathogens (Bent and Mackey 2007). This means that they can recognize pathogen effectors if tge effects of the effector on the host target. A strong oxidative burst and HR cell death are considered hallmarks for resistance mediated via R genes. ROIs have antimicrobial properties and act as a signal for activation of defense responses, including HR (McDowell and Dangl 2000). HR cell death is an efficient immune response against biotrophic pathogens (Nimchuk, Eulgem et al. 2003). Biotrophic pathogens extract their food from living plant tissue, while necrotrophs kill and digest dead plant tissue for their nutrients. Thus, by decreasing the number of cells in contact with an invading biotrophic pathogen, plants can prevent further infection. Basal defense and some cases of ETI are controlled by the SA-dependent branch of the defense network (Glazebrook 2005; Tsuda and Katagiri 2010). The molecular changes that occur after pathogen recognition during ETI also occur during compatible interactions, but with ‘slower kinetics and reduced amplitude’ (Lamb, Ryals et al. 1992; Tao, Xie et al. 2003).

**Plant Defense Pathways**

SA-dependent signaling processes involve several genetically defined defense regulators, such as EDS1 and PAD4 (Parker, Holub et al. 1996), which control the synthesis and accumulation of this defense hormone. Defense-associated SA appears to be mainly synthesized by a plastidic pathway that involves isochorismate synthase 1, which is also known as EDS16 or SID2 (Wildermuth, Dewdney et al. 2001). Elevated SA levels activate a set of downstream defense responses, such as expression of *pathogenesis-related* (*PR)* genes and HR cell death (Kunkel and Brooks 2002). A positive feedback loop links ROI, NO and SA (Durner and Klessig 1999). These signaling molecules mutually control their production. Only strong activation of this feedback loop results in the induction of HR cell death (Rao, Lee et al. 2000). Typically levels of ROI, NO and SA accumulation during basal defense are too low to trigger HR (Nimchuk, Eulgem et al. 2003). Sifficiently high levels of these signaling molecules for HR induction are typically observed during ETI (Jones and Dangl 2006).

SA is also a critical signal for the activation of systemic acquired resistance (SAR), a broad-spectrum defense response that is sometimes activated throughout the entire plant in response to local recognition of either virulent or avirulent pathogens (Durrant and Dong 2004). The main role of SA in SAR induction seems to be in the systemic tissue, where it causes the transcriptional co-activator NPR1 to move from the cytoplasm to the nucleus

where it interacts with transcription factors, activating SAR (Cao, Bowling et al. 1994; Durrant and Dong 2004). The SA-derivative, methyl salicylate (MeSA), acts in tobacco as a long-distance mobile signal for SAR within the plant (Park, Kaimoyo et al. 2007). In addition, MeSA can also serve as the airborne signal that induces defense gene expression in neighboring plants (Shulaev, Silverman et al. 1997; Koo, Kim et al. 2007). Recent studies have revealed that SAR can increase the fitness of pathogen-challenged plants in a field setting (Heidel and Dong 2006; van Hulten, Pelser et al. 2006; Vlot, Klessig et al. 2008). Although constitutive activation of SAR has substantial fitness costs (Heidel and Dong 2006).

R proteins do not confer resistance against necrotrophic pathogens, which kill plants and feed off dead host tissue. Defense against necrotrophic pathogens is mediated through the jasmonate acid (JA) and ethylene (ET) branches of the defense network. The JA/ET branches are also known to have roles in responses to wounding and herbivore attack (Turner, Ellis et al. 2002; Katsir, Chung et al. 2008; Heil, Ibarra-Laclette et al. 2012). The SA, JA, and ET pathways interact extensively. A large body of research has indicated that SA and JA are mutually inhibitory (Glazebrook, Chen et al. 2003). Recent evidence indicates that they may enhance each other's expression at low concentrations (Mur, Kenton et al. 2006). A plant must be able to distinguish between different types of pathogens allowing it to respond with an appropriate set of defense reactions, mediated by

signaling molecules. Thus, different signaling mechanisms are required to activate immune responses against pathogens with different life-styles.

Studies indicate that while sometimes ET and JA interact synergistically in disease responses, that both can act independently or even antagonistically with the SA-dependent pathway (Broekaert, Delaure et al. 2006). Resistance to specific pathogens conferred through JA signaling show little overlap in transcriptional changes. This context is important to fine-tuning the JA response (De Vos, Van Zaanen et al. 2006; Pauwels, Inze et al. 2009). ET and abscisic acid (ABA) regulate different branches of the JA response (Adie, Chico et al. 2007; Adie, Perez-Perez et al. 2007; Chini, Fonseca et al. 2007; Kazan and Manners 2008). JA and ET act together to induce the expression of *PDF1.2* (Penninckx, Thomma et al. 1998). The transcription factors, ERF1 and ORA59 work to integrate JA and ET signaling (Lorenzo, Piqueras et al. 2003; Pre, Atallah et al. 2008). These transcription factors confer resistance against necrotrophs (Berrocal-Lobo, Molina et al. 2002; Pre, Atallah et al. 2008). Alternately, MYC2 works with ABA signaling to negatively regulate the JA-ET responsive branch (Anderson, Badruzsaufari et al. 2004; Lorenzo, Chico et al. 2004) while activating genes within its own branch, such as VSP2 (Dombrecht, Xue et al. 2007). This branch is associated with the wound response and priming for pathogen defense (Lorenzo, Chico et al. 2004; Dombrecht, Xue et al. 2007; Pozo, Van Der Ent et al. 2008; Van der Ent, Van Wees et al. 2009; Fernandez-Calvo, Chini et al. 2011).(Kazan and Manners 2012)

ET also has very important roles during plant-pathogen interactions. *ein2* seedlings are impaired in FLS2 (Arabidopsis PRR FLAGELLIN-SENSING 2) -mediated responses (Boutrot, Segonzac et al. 2010). FLS2 transcription is mediated through the binding of EIN3/EIL1/EIL2 to its promoter. In ein2 seedlings, FLS2 expression and protein accumulation are suppressed. ET appears to act antagonistically in SA signaling (Mishina and Zeier 2007; Tsuda, Sato et al. 2008; Zabala, Bennett et al. 2009). Despite enhancing FLS2 accumulation, EIN3 and EIL1 repress biosynthesis of SA by binding directly to the promoter of the SA biosynthetic gene isochorismate synthase 1. Consistent with this, both ein3–1/eil1–1 and ein2–1 plants exhibit enhanced resistance to *P. syringae* (Chen, Xue et al. 2009) in spite of suppressed FLS2 signaling (Boutrot, Segonzac et al. 2010). Flg22 treatment induces ET synthesis (54) and EIN3 accumulation (Chen, Xue et al. 2009; Robert-Seilaniantz, Grant et al. 2011).

**Pathosystem**

Arabidopsis and the oomycete, *Hyaloperonospora arabidopsidis* (*Hpa*), are an effective model pathosystem in which defined *R*-genes mediate recognition of certain *Hpa* isolates (Hermanns, Slusarenko et al. 2003). Well-characterized Arabidopsis mutants allow for the fine dissection of defense pathways (Hein, Gilroy et al. 2009). While useful, traditional genetics techniques are unable to circumvent functional redundancy and lethal phenotypes. This suggests that additional experimental approaches are necessary to advance

knowledge of mechanisms controlling plant immunity. Chemical genetics/genomics offers distinct advantages over traditional techniques through the use of small molecules, whose effects are often impermanent and reversible. Small molecules also provide more defined temporal control. In contrast, the timing of pathogen infections is not easily defined, as the germination of spores or pathogen growth and spread in plants is often asynchronous.

### Pesticides

As discussed above, different plant species have developed effective mechanisms to cope with pathogens. Unfortunately, contemporary crops have lost parts of their innate immune system due to breeding efforts focused mainly on increasing yield. Consequently, plant diseases cause dramatic losses in crops every year. In the United States 500 million kg of pesticides are applied annually at a cost of $10 billion to farmers to control disease. Despite this, more than a third of all food crops are still destroyed by diseases (Pimentel 2005). The lingering residues of pesticides on produce is currently a major health concern of consumers (Damalas and Eleftherohorinos 2011). Many pesticides currently in use are carcinogenic and rely on direct anti-pathogenic activity, which often leads to undesirable side effects that can have far reaching consequences both for humans and the environment (Casida 2009). This disquiet over the dangers of pesticides has spawned considerable interest in alternative methods of disease control (Hart 2005; Pimentel 2005).

### Synthetic Elicitors and Chemical Genomics

Small molecules have the potential to simultaneously knockout the function of closely-related members of protein families (Raikhel and Pirrung 2005). This may permit the study of biological functions of functionally redundant proteins. Using traditional genetics, this can be difficult or infeasible due to technical challenges and lethal phenotypes (Raikhel and Pirrung 2005). Yet another advantage over traditional genetics is that bioactive small molecules allow for the study of essential gene functions at any stage in development because transiently active molecules can be added at any time point during plant development or applied at sub-lethal concentrations. In contrast, genetic mutations are permanent and the analysis of plant lines homozygous for a lethal mutation is challenging or impossible. Finally, multiple unrelated gene functions can be knocked out concurrently by using combinations of different bioactive molecules (Raikhel and Pirrung 2005).

Chemical genetics uses small molecules to probe biological questions as a mutational analysis may be used to define gene function (Toth and van der Hoorn 2010). In order for chemicals to be successfully used they must be efficiently taken up by plants, have consistent access to their targets, and not have off-target effects that produce complicated phenotypes. Often chemical genetics projects involve three distinct stages: 1) development of a high-throughput chemical screening assay, 2) primary chemical screen to identify candidate compounds and a secondary screen to verify hits, and 3)

characterization of biological effects triggered by candidate compounds and target identification. For the primary screen, both forward chemical and mutational screens must be specific, meaning that the read-out has to be specific and serve as an unambiguous proxy for the biological process of interest. Chemical genetics requires the screening of many thousands of chemicals in search of one with the ability to stimulate a particular response of interest (Toth and van der Hoorn 2010). The need for chemicals that can manipulate a large diversity of biological processes resulted in the development of large structurally diverse chemical libraries (Toth and van der Hoorn 2010).

The concept of chemical genetics is based on the theoretical assumption that for every existing protein in the biosphere there are hypothetical organic structures capable of binding to it and interfering with its function (Hopkins and Groom 2002). The identification of bioactive compounds interfering with any given biological process or target protein requires screening of libraries representing a large diversity of chemical structures. Of key importance for the identification of bioactive compounds are their physicochemical properties. To be a biologically active compound the substance has to be “drug-like”, which means they must be capable of crossing biological membranes and to remain in an active state in the biological target tissue for a sufficient period of time (Hopkins and Groom 2002). Lipinski’s ‘rule-of-five’ states that properties that favor bioactive compounds include a molecular weight of less than 500 g/mol, a lipophilicity (cLogP) value of more than five, less than five hydrogen-bond

donors, and less than 10 hydrogen-bond acceptors (Lipinski, Lombardo et al. 1997). A large sample size of structurally distinct chemicals maximizes the probability that compounds will be identified that induce the desired biological effect.

The identification of proteins targeted by a given compound is an integral step but also typically the bottleneck for most chemical genetics projects. Several strategies exist for target identification, some of those include: affinity chromatography, yeast three-hybrid (Y3H), protein arrays, and screens for mutants with altered sensitivity to a compound of interest (Terstappen, Schlupen et al. 2007; Toth and van der Hoorn 2010). In affinity chromatography, a compound is tagged and immobilized so that interacting proteins can be purified and then identified. In Y3H the compound of interest is tagged with dexamethasone (DEX) or methotrexate (MTX) and then applied to yeast cells. The compound then binds the DEX or MTX binding protein, which is fused to the DNA-binding domain of a transcription factor. The activation domain of the transcription factor is translationally fused to a cDNA library. When the compound interacts with a plant protein (target) a complex is created and this results in the transcription of a reporter gene (Toth and van der Hoorn 2010). In protein arrays a fluorescent- or isotope-labeled small molecule is used to screen protein chips (Toth and van der Hoorn 2010). Finally, in a screen for mutants with altered sensitivity, mutagenized organisms are treated with compound and plants

showing responses to the compound that differ from wild type are selected for further study (Toth and van der Hoorn 2010).

## Chemical Genetics in Plants

### 2004

There are several examples of successful applications of chemical genetics in plant systems. Armstrong et al., (2004) performed a high-throughput chemical screen using a 10,000 compound library with the intent of identifying inhibitors of auxin transcriptional activation (Armstrong, Yuan et al. 2004). Their screening strategy involved the use of a line that expressed GUS in the root elongation zone after application of auxin. This screen resulted in the identification of 30 compounds showing strong inhibition of *GUS* expression. Four structurally distinct compounds were further studied based on low active concentrations (in the low μM range). Two of these compounds impart phenotypes indicative of an altered auxin response, including impaired root development. The two strongest of these compounds displayed similar growth phenotypes after treatment. Additionally, microarray studies using the later two compounds indicated that similar transcriptional changes were induced by both inhibitors (Armstrong, Yuan et al. 2004).

**2005**

A library of 10,000 compounds identified compounds that could be used to study the link between endomembrane trafficking and gravitropism (Surpin, Rojas-Pierce et al. 2005). In this screen compounds that affected gravitropism, either positively or negatively, were sought. From that screen 34 compounds were identified. One of these compounds, named gravacin, caused irregular endomembrane morphologies in vacuole biogenesis in Arabidopsis. Surpin et al. then determined that in roots of Arabidopsis seedlings treated with this compound protein delivery to the tonoplast was inhibited. gravacin helped to determine that auxins have effects on vacuole morphology and may play a role in remodeling endomembrane trafficking processes (Surpin, Rojas-Pierce et al. 2005).

**2007**

Chemical genetics became popular in plant biology in the past 10 years (Raikhel and Pirrung 2005) and numerous successful applications of this approach in plants have been published during the past five years (Toth and van der Hoorn 2010). In 2007 a number of successful chemical screens were reported. The compound 7-ethoxy-4-methyl chromen-2-one (morlin) was discovered in a screen of 20,000-compound library based on its ability to cause a swollen root phenotype in Arabidopsis (DeBolt, Gutierrez et al. 2007). Using live-cell imaging of fluorescently labeled cellulose synthase and microtubules, DeBolt

et al., showed that treatment with morlin interferes with cortical microtubules to alter the movement of cellulose synthase. This interference resulted in unique cytoskeletal defects which produced shorter and more bundled microtubules. Morlin proved highly useful for the study of mechanisms that regulate microtubule cortical array organization and how it interacts with cellulose synthase (DeBolt, Gutierrez et al. 2007).

In a small screen of 120 bioactive molecules Arabidopsis seedlings were used to identify compounds that inhibited early immune responses. This screen resulted in four hits. These compounds reduced flg22 (22 amino acids flagellin peptide)-activated gene expression of MAMP-responsive *ATL2* gene. Two of these four compounds, triclosan and fluazinam, interfere with the accumulation of ROIs and transport of the FLS2 receptor. Additionally, the compound Triclosan, which blocks early immune responses, was used to determine a potential role for lipid signaling in flg22-triggered immunity (Adie, Perez-Perez et al. 2007).

Hypostatin was discovered in screens as a compound that inhibits hypocotyl growth in a Arabidopsis accession dependent manner (Zhao, Chow et al. 2007; Park, Fung et al. 2009). 11 other accession-selective hit molecules were also identified alongside hypostatin, which is an inhibitor of cell expansion. Additionally, a screen for compounds able to disturb microfibril-cellulose attachment resulted in the identification of cobtorin (Yoneda, Higaki et al. 2007; Zhao, Chow et al. 2007). This study demonstrated that different Arabidopsis accessions can be used to study the activity of interesting new compounds.

**2008**

In 2008 a variety of additional plant bioactive compounds were reported on, such as: brassinopride, endosidin1, proauxins, sulfamethoxazole (Smex), methotrexate, 2,4-dinitrophenol, and 2-(4-chloro-2-methylphenoxy)-N-(4-H-1,2,4-triazol-3-yl)acetamide. These compounds were found to interfere with various processes, such as brassinosteroid response (BR), ethylene responses, vesicle trafficking, auxin signaling, resistance against bacterial pathogens, as well as DNA and ATP synthesis, respectively (Bassel, Fung et al. 2008; Christian, Hannah et al. 2008; Gendron, Haque et al. 2008; Robert, Chary et al. 2008; Savaldi-Goldstein, Baiga et al. 2008; Schreiber, Ckurshumova et al. 2008).

Brassinopride inhibits hypocotyl length and was identified in a screen of 10,000 compounds when it activated expression of a BR-repressed reporter gene (Gendron, Haque et al. 2008). Endosidin1 was identified in a screen of a 3000-molecule natural product library as a chemical that acted on essential steps in plasma membrane/endosomal trafficking (Savaldi-Goldstein, Baiga et al. 2008). In a screen of 10,000 compounds that looked for hypocotyl elongation, proauxins were found. Proauxins, which are structurally similar to synthetic auxin, diffuse to the hypocotyls and then are broken down into functional auxins (Savaldi-Goldstein, Baiga et al. 2008).

Smex was identified in a screen of the 3000-member LATCA library after Arabidopsis seedlings were infected with *P. syringae* which normally causes bleaching (Bassel, Fung et al. 2008). When plant defense occurs the bleaching

was prevented, and thus they screened for compounds capable of preventing the bleaching. Smex not only reduced bleaching but also reduced bacterial growth on soil-grown plants (Schreiber, Ckurshumova et al. 2008). Methotrexate, 2,4-dinitrophenol, and 2-(4-chloro-2-methylphenoxy)-N-(4-H-1,2,4-triazol-3-yl)acetamide were all discovered from a screen of 3300 bioactive compounds. These compounds were all found to inhibit seedling germination and were used to study the germination transcriptome using microarrays (Bassel, Fung et al. 2008).

**2009 to 2011**

Finally in 2009 and 2011 the compounds dichloroanthranillic acid (DCA), bikinin, and 2-(4-chloro-2-methylphenoxy)-*N*-(4-*H*-1,2,4-triazol-3-yl)acetamide (WH7) were reported. These trigger defense responses in Arabidopsis, brassinosteroid signaling, and downregulate ABA-dependent gene expression in addition to stimulating expression of plant defense-related genes, respectively (De Rybel, Audenaert et al. 2009; De Rybel, Audenaert et al. 2009; Knoth, Salus et al. 2009; Kim, Hauser et al. 2011). DCA was discovered in a screen of more than 50,000 compounds, which yielded 114 hits (De Rybel, Audenaert et al. 2009). This compound was shown capable of inducing defense against *Hpa* and *P. syringae* in Arabidopsis (Knoth, Salus et al. 2009). Bikinin was discovered in a screen as a compound that activates brassinosteroid signaling downstream of the brassinosteroid receptor. It was shown to bind glycogen synthase kinase-3

BR-INSENSITIVE 2 (GSK3 BIN2) and is an ATP competitor (De Rybel, Audenaert et al. 2009). WH7 was discovered in a screen of 10,000 compounds. This screen looked for compounds that displayed auxin-like activities, such as inhibiting root growth, when treated on Arabidopsis. WH7 was shown to act in a defined auxin pathway (Christian, Hannah et al. 2008).

**Plants Defense Inducers**

The enhancement of plant immune responses by exogenous application of chemicals can be traced back to the treatment of tobacco with SA (White 1979). While SA, JA, and ET can induce defense response, their use in the field or greenhouse setting is restricted based on their shortcomings as defense inducers that are broadly effective on many plant species (Chen, Malamy et al. 1995; Friedrich, Lawton et al. 1996). The use of environmentally safe plant defense-inducing chemicals, which boost a plant's innate immune responses, offers an attractive alternative to pesticides. (INA, BTH other SAR inducers) Linda wants me to reference the body of literature that explored SAR inducers as methods of control (BTH, INA, etc)- klessig + sa

An alternative procedure to protect plants against disease is to activate their own defense mechanisms by specific biotic or abiotic elicitors (Walters, Walsh et al. 2005). The classical type of induced resistance is often referred to as systemic acquired resistance (SAR). Sodium salicylate (NaSA), 2,6-dichloroisonicotinic acid (INA), and benzothiadiazole-S-methyl ester (BTH) are

well-known elicitors of SAR in various plants against disease (Sticher, MauchMani et al. 1997). The expression of SAR, triggered by either pathogen infection or treatment with NaSA or its functional analogs INA or BTH, is tightly associated with the transcriptional activation of genes encoding pathogenesis-related (PR) proteins (Van Loon 1997). The nonprotein amino acid DL-3-amino-n-butanoic acid (DL-β-aminobutyric acid [BABA]) also activates an induced resistance response. BABA induced resistance, involves SA-dependent, SA-independent, and ABA-dependent defense mechanisms, and the importance of these defenses varies according to the nature of the challenging pathogen (Ton, Jakab et al. 2005) .

Many molecules exist that cause an induction of defenses in plants when applied. Plant-derived small molecules other than SA, JA, and ET have been identified as important to controlling or preventing disease in plants. Some of these phytohormones include abscisic acid (ABA), brassinosteroids, gibberellin, cytokinin, and auxin (Nakashita, Yasuda et al. 2003; Mauch-Mani and Mauch 2005; Navarro, Dunoyer et al. 2006; Robert-Seilaniantz, Navarro et al. 2007; Wang, Pajerowska-Mukhtar et al. 2007).

Like the interactions between SA, JA and ET, other molecules are important for the tailoring of defense responses (Robert-Seilaniantz, Navarro et al. 2007; Spoel and Dong 2008; Robert-Seilaniantz, Grant et al. 2011). Activation of ABA signaling processes and its biosynthesis have also been shown to promote plant disease (Ton and Mauch-Mani 2004; Asselbergh, Curvers et al.

2007). Alternatively, brassinosteroid treatment has been shown to enhance resistance against some biotrophs, mediate abiotic stress responses through NPR1, and induce *PR1* expression (Nakashita, Yasuda et al. 2003; Dong 2004; Divi, Rahman et al. 2010; Rahman, Divi et al. 2010). Gibberellic acid (GA) can induce increases in ROS accumulation and attenuation of JA signaling (Achard, Vriezen et al. 2003; Vriezen, Achard et al. 2004; Navarro, Bari et al. 2008). Cytokinins, which affect cell division and morphogenesis, can also enhance the SA response and thus promote resistance against biotrophs (Bassaganya-Riera, Skoneczka et al. 2010; Choi, Huh et al. 2010; Shan, Yan et al. 2012). Finally, auxin signaling may suppress SA biosynthesis and signaling, while SA attenuates auxin signaling (Robert-Seilaniantz, Navarro et al. 2007; Wang, Pajerowska-Mukhtar et al. 2007; Robert-Seilaniantz, Grant et al. 2011). These plant hormones are integral to the function of plants. While many of these hormones were not originally associated with defense, new research suggests they all have roles in coordinating plant response to pathogen invasion.

Besides natural plant signaling molecules several synthetic molecules have also been found to induce defense responses in plants. Some well known examples of such compounds include 2,6-dichloroisonicotinic acid (INA) and acibenzolar-*S*-methyl benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Métraux, Ahl Goy et al. 1991; Ward, Uknes et al. 1991; Uknes, Mauch-Mani et al. 1992). While these compounds do induce defense responses in some plants, their effects are not universal across all species (Heil, Hilpert et al. 2000;

Achuo, Audenaert et al. 2002) and their success in crop protection under field conditions has been modest (Wiese, Bagy et al. 2003). INA and SA are structurally similar and both induce similar sets of *PR* genes which coincides with the development of SAR (Ward, Uknes et al. 1991; Uknes, Mauch-Mani et al. 1992). INA is believed to act downstream of SA accumulation since it confers resistance in *nahG* plants; nahG plants express a bacterial salicylate hydroxylase that degrades SA to biologically inactive catechol, which has activity but is not SA (Delaney, Uknes et al. 1994; Delaney, Friedrich et al. 1995; Vernooij, Friedrich et al. 1995). BTH also induces SAR and appears to act downstream of SA accumulation; it has a much lower phytotoxicity than either SA or INA (Schurter 1987; Friedrich, Lawton et al. 1996).

Additional synthetic inducers of SAR include: 3,4-dichloro-*N*-(2-cyanophenyl)-1,2-thiazole-5-carboxamide (tiadinil, TDL), 3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid (CMPA), and *N*-cyanomethyl-2-chloroisonicotinamide (NCI) (Yoshida 1987; Yoshida and Sassa 1990; Nakashita, Yasuda et al. 2003; Nakashita, Yasuda et al. 2003; Yasuda, Nishioka et al. 2003; Yasuda, Nakashita et al. 2004; Nishioka, Nakashita et al. 2005; Tsubata, Kuroda et al. 2006). These three compounds induce the same set of *PR* genes, act independently of SA accumulation and JA/ET perception, and require NPR1. In addition, 3-allyloxy-1,2-benzothiazole 1,1-dioxide, (probenazole) and its metabolite 1,2-benzisothiazole-1,1-dioxide (BIT, saccharin) stimulate expression of *PR* genes and SAR (Watanabe, Igarashi et al. 1977; Yoshioka, Nakashita et al. 2001;

Nakashita 2002; Nakashita, Yasuda et al. 2002; Nakashita, Yoshioka et al. 2002). Probenazole and BIT promote SA accumulation and cannot induce SAR in *nahG* plants. This indicates that they act upstream of SA perception. Sulfamethoxazole induces defense independently of SA, JA, and ET signaling (Schreiber, Ckurshumova et al. 2008). Phosphates, the metal ion cadmium, and organic acids such as oxalates and ethylenediaminetetraacetic acid (EDTA) may also stimulate disease resistance in plants (Sinha and Giri 1979; Asselin, Grenier et al. 1985; White, Dumas et al. 1986; Doubrava, Dean et al. 1988; Walters and Murray 1992; Citovsky, Ghoshroy et al. 1998; Ghoshroy, Freedman et al. 1998; Reignault 2007).

Inducers of plant immunity have also been found that originate from a variety of biotic sources, such as the amino acids phenylalanine and β-aminobutyric acid (BABA) (Kuc, Williams et al. 1957; Papavizas and 1964; Cohen 2002). Additionally, vitamins such as riboflavin (vitamin B2), menadione (vitamin K3), menadione sodium bisulphite (MSB), and thiamine (vitamin B1) may help plants to combat infecting pathogens (Emmanouil and Wood 1981; Asselin, Grenier et al. 1985; Rao, Ravichandran et al. 1985; Malamy, SanchezCasas et al. 1996; Aver'yanov, Lapikova et al. 2000; Dong and Beer 2000; Borges, Borges-Perez et al. 2003; Borges, Cools et al. 2003; Borges, Borges-Perez et al. 2004; Pushpalatha, Mythrashree et al. 2007; Oldenburg, Marinova et al. 2008). Treatment with the polyamine spermine can reduce tobacco mosaic virus lesion size (Yamakawa, Kamada et al. 1998). Polyamines

have two or more primary amino groups and they accumulate in necrotic lesions during HR (Torrigiani, Rabiti et al. 1997; Walters 2003). 3-acetonyl-3-hydroxyindole (AHO), a chemical extract from an ornamental plant, induces resistance to TMV in tobacco (Li, Zhang et al. 2008). Large molecules from biotic sources capable of inducing plant defense include: oligosaccharides and α-1,4-oligogalacturonides, cellodextrins (water-soluble derivatives of cellulose), peptides/proteins, and lipopolysaccharides (Shibuya and Minami 2001; Aziz, Gauthier et al. 2007). Moreover, some plant proteins also mediate plant defense responses (Buhot, Gomes et al. 2004). Some of these large molecules may also be DAMPs (damage-associated molecular patterns), which are plant derived molecules that are released as a consequence of tissue damage (Henry, Thonart et al. 2012).

As well, MAMPs, lipids, harpins, and peptaibols (20-amino acid peptides) are also potent inducers of plant defense (Wei, Laby et al. 1992; Bonnet, Bourdon et al. 1996; Keller, Blein et al. 1996; Capasso, Cristinzio et al. 1999; Enkerli, Felix et al. 1999; Felix, Duran et al. 1999; Gomez-Gomez, Felix et al. 1999; Benhamou and Brodeur 2001; Benhamou and Garand 2001; Baillieul, de Ruffray et al. 2003; Grigoriev, Schlegel et al. 2003; Kunze, Zipfel et al. 2004; Reignault 2007; Baxter, Tripathy et al. 2010).

Sugars are also capable of reducing plant disease. Examples include: cellobiose, mannose, arabinose, sucrose, and trehalose (Emmanouil and Wood 1981; Drennan, Smith et al. 1993; Reignault, Cogan et al. 2001; Renard-Merlier,

Randoux et al. 2007). Catechin, a natural phenol antioxidant plant secondary metabolite, can stimulate ROS production and cell death in plants (Bais, Vepachedu et al. 2003). Later, it was revealed that lower concentrations of catechin stimulate Arabidopsis growth and reduce susceptibility to *Pseudomonas syringae* (Prithiviraj, Perry et al. 2007). These results are reminiscent of the phenomenon of hormesis where sublethal concentrations of a compound induce enhanced growth of an organism (Calabrese and Baldwin 2003).

### Hormesis

Hormesis has been widely described for numerous organisms (including humans) and a large variety of physical, chemical or biological stimuli. While prevalent throughout scientific literature, the genetic and molecular basis of hormesis is unknown. Hormesis is characterized by a biphasic dose-response to a treatment which stimulates at low doses and has an inhibitory or toxic effect at higher concentrations (Calabrese 2009; Calabrese and Blain 2009). It has been suggested to be an adaptive response at the cellular or organismal level to stress (Calabrese 2009; Calabrese and Blain 2009). Signaling pathways and mechanisms that are responsible for specific hormetic responses have been recently suggested, and include: certain ion channels, protein kinases, deacetylases, transcription factors, chaperones, antioxidant enzymes, and glutathione peroxidase (Yuan, Pan et al. 2004; Arumugam, Gleichmann et al. 2006; Bechtold, Richard et al. 2008; Son, Camandola et al. 2008; Calabrese,

Mattson et al. 2010; Calabrese, Mattson et al. 2010). It is unclear if the great variety of hormesis-like phenomena have a common functional basis, or if they are mechanistically unrelated. A comprehensive comparison of molecular responses triggered by a variety of hormesis-inducing stimuli in a single type of organism may allow defining common denominators for this complex phenomenon.

### Goals

My thesis strove to identify novel synthetic elicitors. These elicitors could be used as tools to further study the plant defense network in Arabidopsis. Additionally, they could serve as environmentally friendly “green” plant defense inducers which could replace traditional pesticides. Finally, I performed experiments to study genes induced by synthetic elicitors to determine if novel defense components had been identified.

The model plant Arabidopsis and the obligate biotroph and oomycete, *Hpa*, are a naturally coevolving pathosystem with a high level of intra-species genetic diversity (Coates and Beynon 2010). Use of this and other model interactions has revealed that plants have a complex inducible immune system that protects wild species and crops from pathogen infections. In microarray experiments performed by Knoth et al. (2009), a group of 137 genes were identified that are upregulated by DCA and INA at time points when these synthetic elicitors induce strong immunity against *Hpa*. These *ACID* (*Associated*

*with Chemically Induced Defense*) genes were considered promising because synthetic elicitor-triggered transcriptome changes that follow the temporal pattern of synthetic elicitor-mediated resistance are likely to be of key importance for a successful pathogen defense.

In my dissertation this pathosystem was used to examine genes from the *ACID* cluster, which are coordinately inducible by the synthetic elicitors DCA and INA. Chapter 1 introduces the *ACID* cluster, which is enriched for genes encoding protein kinases. In Chapter 1 I examined the ACID genes to determine if they had any roles in Arabidopsis disease resistance against *Hpa.* Using a forward genetics approach, 10 of 16 *ACID* members tested were found to be required for full immunity of Arabidopsis against *Hpa*. Seven of these 10 *ACID* members have not yet been implicated in plant immunity.

Additionally, from the 114 potential synthetic elicitors previously identified in a high-throughput chemical screen (Knoth, Salus et al. 2009). This screen identified small molecules capable of inducing *GUS* expression in transgenic Arabidopsis lines. A *GUS* reporter gene was fused to the promoter of a SA-dependent and defense responsive gene, *CaBP22*. Of these 114 potential elicitors, nine were characterized in Chapter 1. These experiments were meant to determine if additional compounds with low active concentrations along with distinct mechanisms of action could be identified. One of these nine novel synthetic elicitors, CMP199, proved to be a more potent defense inducer than DCA or INA. Another one of the nine elicitors, 2-(5-bromo-2-hydroxy-phenyl)-

thiazolidine-4-carboxylic acid (CMP442), was able to quickly and transiently induce disease resistance. In addition, CMP442 had a distinct mode-of-action and was structurally unique from previously identified elicitors. CMP442, unlike DCA, could be synthesized quickly and easily in the laboratory with a high degree of purity. CMP442 was further found to trigger hormesis-like effects in plants. At low doses CMP442 enhanced the growth of roots and aerial parts of Arabidopsis and *Solanum lycopersicum* (tomato), while high concentrations were inhibitory for plant growth. The ability of CMP442 to beneficially affect both plant immunity and development pointed to crosstalk between both types of biological processes and might allow for the design of novel types of multi-functional agrochemicals.

Next, a screening system to identify synthetic elicitors targeting the JA/ET branch of the defense network was developed in Chapter 2. Towards this end, a set of genes was identified displaying a SA-independent upregulation in response to infection of *Hpa*. Four out of the five genes are members of the *PDF* family, including the JA pathway molecular marker *PDF1.2a*. Finally, a transgenic line carrying a RNA silencing transgene able to knock down the transcripts of this highly related family of *PDFs* was developed. These projects highlight potential for chemical genomics to identify novel synthetic elicitors useful for the identification of novel components of the plant defense network or development of a new class of environmentally-friendly “green” pesticides.

In order for a chemical to be marketed as a green pesticide it is important that it non-toxic to the environment and organisms at moderately high levels. Additionaly it is key that such a pesticides would be able to breakdown into non-toixc by-products so that it could no build up in the soil or water table and stay there for long periods of time. I believe that synthetic elicitors discussed in this dissertation may fit these criteria or may lead to green pesticides.

# CHAPTER 1: Functional Analysis of *ACID* (Associated with Chemically Induced Defense) Genes and Characterization of Novel Synthetic Elicitors

## SUMMARY

Every year billions of dollars are spent on pesticides which leave residues on produce that control pests and pathogens but can be harmful to consumers and the environment. Such off-target effects make the study of interactions between plants and pathogens an integral field for the reduction of conventional pesticide use. Using model pathosystems, such as *Arabidopsis thaliana* (Arabidopsis) and *Hyaloperonospora arabidopsidis* (*Hpa*), many important questions related to plant disease resistance are being addressed. A complex transcriptional network controls plant immune responses. Of key importance for the regulation of this defense network, are protein kinases that act at various stages during defense activation. Chemical genomics can be used to study these different stages. Plant defense-inducing molecules (synthetic elicitors) identified and characterized using chemical genomics will be valuable tools for the dissection of the plant defense network and will serve as leads for the development of novel environmentally safe pesticides. Genes from the Arabidopsis *ACID (Associated with Chemically Induced Defense)* cluster are coordinately inducible by the synthetic elicitors DCA and INA. This cluster is enriched for genes encoding protein kinases. Using a forward genetics approach

it was demonstrated that 10 of 16 *ACID* members tested are required for full immunity of Arabidopsis against *Hpa*. Seven of these 10 *ACID* members have not been implicated in plant immunity before. In addition, eight novel synthetic elicitors identified and characterized via chemical genomics were reported on here, one of which, called CMP442, is a more potent defense inducer than DCA or INA.

## INTRODUCTION

Every year billions of dollars are spent on pesticides used for crop disease prevention (Pimentel 2005). While effective in crop protection pesticides leave residues on produce and have off target effects (Hart 2005; Casida 2009; Damalas and Eleftherohorinos 2011). This makes the design of novel green pesticides highly attractive. Also, the elucidation of the finer points of interactions between plant and pathogen is integral for the design of new approaches to more efficiently and safely prevent crop diseases. The model plant *Arabidopsis thaliana* (Arabidopsis) and the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) are a naturally coevolving pathosystem with a high level of intra-species genetic diversity (Coates and Beynon 2010). Use of this and other model interactions has revealed that plants have a complex inducible immune system that protects wild species and crops from pathogen infections. When plants recognize the presence of an infecting pathogen, a multitude of signaling events are triggered that ultimately lead to efficient defense (Katagiri and Tsuda 2010; Tena, Boudsocq et al. 2011). Some of the early responses after resistance (R)-gene recognition include changes in ion fluxes, synthesis of reactive oxygen species (ROS), alterations in gene transcription, which can be followed by a hypersensitive response (HR-incompatible interaction), where the plant cells surrounding the point of infection die to restrict pathogen growth (Hein, Gilroy et al. 2009; Vlot, Dempsey et al. 2009).

An ancient and fundamental form of plant defense involves conserved microbe-associated molecular patterns (MAMPs; general elicitors). MAMPs are recognized by plant pattern-recognition receptors (PRRs) resulting in the activation of a complex defense response. This form of plant immunity is referred to as pattern-triggered immunity (PTI). A second form of immunity is based upon the recognition of pathogen-secreted effector molecules, which are proteins that promote pathogen virulence in the plant. Here the plant is capable of recognizing the presence of pathogen effectors, or their cellular effects, by disease R-proteins. R-proteins constitute a second class of plant immune receptors, besides PRRs, and induce a strong defense response, which often includes HR. This form of immunity is called effector-triggered immunity (ETI) (Katagiri and Tsuda 2010). In the absence of a cognate R-protein, the secretion of effectors enables pathogens to successfully infect their hosts. During such compatible interactions, plants can still mount a weakened immune response, called basal defense. Basal defense typically limits the spread of pathogens but is not capable of fully preventing disease (Glazebrook 2001; Ahmad, Van Hulten et al. 2011).

Following the activation of initial and local defense responses are a set of delayed and systemic responses that include systemic acquired resistance (SAR) (Conrath 2011). SAR is an induced form of defense that is activated remotely from the point of pathogen infection conferring a broad spectrum disease resistance against a variety of pathogens (Durrant and Dong 2004). Like

many local defense responses, activation of SAR requires the accumulation of the signaling molecule salicylic acid (SA) (Ryals, Neuenschwander et al. 1996). A complex regulatory network has been shown to be required for proper regulation of these plant immune responses (Katagiri and Tsuda 2010). Many components of this network are commonly utilized by PTI, basal defense, ETI, and SAR.

Major regulators of plant defense responses are protein kinases, which act at various hierarchical levels within the plant defense network (Tena, Boudsocq et al. 2011). There are more than 1000 protein kinases in Arabidopsis (Gish and Clark 2011). In particular, receptor protein kinases (RPKs), $Ca^{2+}$-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs) have been implicated in the regulation of plant immune responses (Gish and Clark 2011). RPKs are comprised of a transmembrane domain with amino-terminal extracellular domains implicated in ligand recognition and protein–protein interactions, in addition to a carboxyl-terminal intracellular kinase domain involved in signal transduction (Morillo and Tax 2006; Afzal, Wood et al. 2008; Tena, Boudsocq et al. 2011). The three major subclasses of RPKs are differentiated based upon their kinase domain substrate specificities. The subclasses include: receptor-tyrosine kinases, receptor-serine/threonine kinases, and receptor-histidine kinases (Becraft 2002). Most plant RPKs are proteins containing an: extracellular signal sequence, extracellular leucine-rich repeats (LRRs), a transmembrane helix, and cytoplasmic kinase domain with the serine/threonine consensus sequence (Hardie 1999).

One variant in the RPK group is the receptor-like kinases (RLK), which belong to a large family known as the RLK/*Pelle* family. The Arabidopsis RLK family is divided into 45 subfamilies with over 600 members that comprise more than 2% of the Arabidopsis genome (Gish and Clark 2011). One of the main criteria that distinguishes these subfamilies is the existence and type of extracellular domain (Shiu and Bleecker 2003; Shiu, Karlowski et al. 2004). There are 15 classifications for RLK extracellular domains, which include: CRINKLY4-like, C-type lectin-like, CrRLK1-like, DUF26-like, extensin-like, legume (L)-lectin-like, LRK10-like, LRR-like, LysM-like, PERK-like, RKF3-like, S-domain-like, thaumatin-like, URK1- like, and WAK-like (Haffani, Silva et al. 2004). The LRR domain is the most common and represents the largest RLK group with 216 members subdivided into 13 subfamilies (Dievart and Clark 2004; Zhang, Choi et al. 2006).

Most RLKs have a conserved arginine and an aspartate in the activation loop of subdomain VI, which acts as a kinase activator by enhancing phosphotransferase efficiency (Adams 2003). Often kinases with arginine and aspartate are important for developmental regulation, while those without these conserved residues are important in innate immunity (Dardick and Ronald 2006). Accordingly, plant RLKs can be further subdivided into two major categories based upon their functions: one is involved in cell growth and development and the other in plant–pathogen interactions and defense responses (Shiu, Karlowski

et al. 2004). Examples of this second group are PRRs: Xa21 from rice (Song, Wang et al. 1995) and FLS2 from Arabidopsis (Gomez-Gomez and Boller 2002; Gomez-Gomez 2004), which interact with certain MAMP-type epitopes. *Xa21* is membrane bound serine/threonine protein that is activated by AxYS22, a 17-amino acid peptide conserved in strains of *Xanthomonas*. FLS2 is a transmembrane protein that recognizes a number of bacterial MAMPs including peptides derived from the flagellin such as flg22 (Haffani, Silva et al. 2004; Chinchilla, Bauer et al. 2006; Tena, Boudsocq et al. 2011). Another RLK, CERK1, belongs to a distinct subfamily and is required for immune signaling triggered by fungal chitin. In addition, CERK1 binds and recognizes bacterial peptidoglycans contributing to immunity against bacteria (Miya, Albert et al. 2007; Miya, Albert et al. 2007).

Another group of kinases important for defense are CDPKs, which are encoded by a 34-member gene family in Arabidopsis and make up one of the largest family of $Ca^{2+}$ sensors in plants (Harmon, Gribskov et al. 2000; Cheng, Willmann et al. 2002; Lee and Rudd 2002; Hrabak, Chan et al. 2003). Host proteins must be able to sense alterations in $Ca^{2+}$ levels and respond accordingly (Boudsocq, Willmann et al. 2010). CDPKs have N-terminal protein serine/threonine kinase domains attached through an autoinhibitory junction domain to a C-terminal $Ca^{2+}$-binding calmodulin-like domain (Romeis 2001; Lee and Rudd 2002). CDPKs bind $Ca^{2+}$ at their C-terminal domain, which activates their protein kinase activity and facilitates their function as transducers of $Ca^{2+}$

signals. A possible role of $Ca^{2+}$ in plant defense was proposed when CDPK transcripts were found to be elevated in tobacco, maize, tomato or pepper in response to pathogens or their elicitors (Yoon et al., 1999; Murillo et al., 2001; Chico et al., 2002; Chung et al., 2004). Upon pathogen recognition, cytosolic $Ca^{2+}$ levels increase (Hahlbrock, Scheel et al. 1995; Jabs, Colling et al. 1997; Nurnberger, Wirtz et al. 1997; Zimmermann, Nurnberger et al. 1997; Nurnberger, Brunner et al. 2004). The duration and amplitude of these increases are specific for the respective defense-related stimulus, resulting in the differential activation of downstream components (Lee and Rudd 2002).

Two proteins have been suggested as potential substrates for CDPKs in plant defense: PAL (phenylalanine ammonia-lyase) and plasma membrane-associated NADPH oxidase (Allwood, Davies et al. 1999; Cheng, Sheen et al. 2001; Xing, Wang et al. 2001). PAL appears to be phosphorylated in bean cells challenged with a general elicitor but the significance of this observation remains to be demonstrated. In addition, a CDPK was shown to enhance NADPH oxidase activity stimulating an oxidative burst in tomato protoplasts although the significance of this interaction is also not clear (Lecourieux, Raneva et al. 2006). Romeis et al., (2000) demonstrated defense-associated activation of CDPKs in tobacco cell cultures transformed with the *Cf-9* gene from tomato. *Cf-9* is responsible for providing resistance to *Cladosporium fulvum* in the presence of its corresponding avirulence gene *Avr9*. They established that the presence of Avr9 and Cf-9 a kinase was phosphorylated, causing an increase in kinase

activity. They further demonstrated that this kinase is of the CDPK-type, because it required $Ca^{2+}$ (Romeis, Piedras et al. 2000). This was the first direct demonstration of CDPK enzyme activity in plant defense. Meanwhile it has become clear that CDPKs are important not only in plant defense signaling but also serve as key points of convergence of various regulatory pathways due to their ability to respond to different hormonal or environmental cues (Romeis, Piedras et al. 2000; Lee and Rudd 2002; Li, Zhu et al. 2008; Li, Zhang et al. 2009; Kudla, Batistic et al. 2010). To better understand CDPK function in plant defense, additional pathogen-induced CDPK-phosphorylated substrates need to be identified.

Mitogen-activated protein kinase (MAPK) cascades transmit and magnify signals through a phosphorelay mechanism involving: MAPK-kinase-kinases (MAPKKKs), MAPK-kinases (MAPKKs), and MAPKs. They link upstream recognition events to downstream targets and their sequential phosphorylation targets substrate proteins in the cytoplasm or nucleus. MAPK activation is one of the earliest conserved signaling events after pathogen recognition (Romeis 2001). Many signaling cascades are shared between different activating stimuli (e.g. MAMPs). Cross-inhibition, feedback control, and the use of defined scaffolding proteins connecting distinct signaling components are utilized to enforce specific relationships between activating stimuli and the respective biological responses (Rodriguez, Petersen et al. 2010). Cross inhibition is manifested in the mutual inhibition between two pathways (Rodriguez, Petersen

et al. 2010). Feedback control can be represented by negative feedback loops, where the activation of one component down-regulates the function of another. Scaffold proteins bring components together, which enhances specificity within signaling chains (Rodriguez, Petersen et al. 2010). Little is known about specific scaffolding proteins within plants. Two putative plant scaffolding proteins include alfalfa OMTK1, which interacts in protoplasts with the MAPK MMK3 in response to $H_2O_2$ and Arabidopsis MEKK1, which binds to MKK2 and MPK4 (Rodriguez, Petersen et al. 2010).

MAPKs pathways are known to be involved in plant development, programmed cell death, responses to some abiotic stressors, and defense signaling. The Arabidopsis genome codes for 110 MAPK cascade components, which includes 20 MAPKs, ten MAPKKs and 80 MAPKKKs (Pitzschke, Schikora et al. 2009). Few MAPKs have been studied due to their lethal mutant phenotypes in plants (Tena, Boudsocq et al. 2011). The most well understood MAPKs are MPK4, MPK3, and MPK6, with the latter two acting as positive regulators for defense responses and the former being a negative regulator of SAR. The Flg22 peptide is recognized by the receptor FLS2 which complexes with BRI1-ASSOCIATED KINASE (BAK1) and triggers MAPK signaling cascades. This cascade includes the activation of MPK3, MPK4 and MPK6 (Rodriguez, Petersen et al. 2010). MPK4 and MPK6 are also activated by hrp proteins from some bacteria and their activation results in the induction of *PR* genes which sometimes encode proteins with antimicrobial activities (Pitzschke,

Schikora et al. 2009). Further studies must identify and elucidate MAPK cascades and find ways around the widespread mutant lethal phenotypes which inhibit kinase pathway studies.

Traditional mutational analyses have been unable to circumvent functional redundancy and lethal mutant phenotypes (as seen in the case of protein kinases). Thus, additional types of experimental approaches are necessary for the continued elucidation of the intricate and elaborate circuits within plant immune networks. One novel approach, chemical genetics, offers distinct advantages over traditional techniques. Chemical genetics allows bioactive small molecules to be used in a reversible manner, since frequently their effects on organisms are not permanent. In addition, it provides more temporal control over experiments, since chemicals typically interfere with their targets immediately after application. In contrast, the timing of pathogen infections is often poorly reproducible, as the germination of spores or pathogen growth and spread in plants is asynchronous and often highly sensitive to subtle changes in environmental conditions.

Chemicals also have the ability to simultaneously affect multiple members of highly-related protein families, permitting the study of biological functions of functionally redundant proteins. Using traditional genetics to knock out the function of an entire gene family often proves difficult or infeasible due to technical challenges and lethal phenotypes. Yet another advantage over traditional genetics is that bioactive chemicals allow for the study of essential

gene functions at any stage in development because transiently active compounds can be added at any time or any concentration. In contrast, genetic mutations are of permanent nature. If they confer lethal phenotypes, no studies can be performed. Finally, the function of multiple structurally unrelated genes can be knocked out concurrently by using combinations of chemicals while also varying the concentration of each chemical allowing the study of quantitative relationships between defined stimuli and phenotypes (Darvas, Dorman et al. 2004; Spring 2005).

Chemical genomics requires tens of thousands or even hundreds of thousands of chemicals to be screened for their ability to stimulate a particular phenotype of interest (Toth and van der Hoorn 2010). The increase in demand for chemicals that can manipulate a diverse set of biological processes resulted in the need for inexpensive large and structurally diverse chemical libraries for screening. As a result, the concept of combinatorial chemistry was developed (Balkenhohl, vondemBusscheHunnefeld et al. 1996). This high-throughput approach is based on simultaneously occurring synthesis steps. During each step a set of distinct chemical building blocks is used, yielding a vast number of structural combinations, referred to as “a combinatorial libraries”. The ease of this novel form of synthesis made these libraries widely available and cost-effective to many fields of academic research (Brenner and Lerner 1992; Maclean, Schullek et al. 1997). Thus, the large sample size of available structurally distinct

chemicals maximizes the probability that compounds will be identified that induces the desired biological effect.

The Eulgem lab uses chemical genomics to identify and characterize synthetic elicitors, which are small drug-like molecules that induce plant defense responses (Knoth, Salus et al. 2009). Their ability to induce defense responses provides us with a highly attractive alternative to conventional pesticides, if proven to be less toxic. Most pesticides currently on the market prevent disease through their toxicity to pathogens. This toxicity often leads to off-target effects against other organisms and the environment. As a result, the dangers of pesticide poisoning become more of a concern, making the identification of compounds that are not toxic, but instead stimulate plant's inherent defenses very appealing. In addition to their potential use as pesticide replacements, synthetic elicitors can also be utilized as highly specific stimuli to perform more refined functional analyses of the plant defense network by interference with distinct network nodes. Their use should allow for the selective activation of certain regulatory circuits within this network. The identification of cellular targets of synthetic elicitors can uncover novel components of the plant immune system. Taken together the use of synthetic elicitors is likely to enable us to gain a deeper and more comprehensive understanding of the structure and function of the plant defense network.

This report highlights the functional characterization of some members of the *ACID* (*Associated with Chemically Induced Defense*) cluster, a group of genes identified by microarray experiments after treatment with two synthetic elicitors, DCA and INA (Knoth, Salus et al. 2009). These 137 genes were found to be enriched for protein kinases, which may play key roles in plant defense signaling. Of the 16 *ACID* genes examined, ten were required for full basal defense of Arabidopsis against *Hpa*. Seven of the ten *ACID* genes have not been implicated as components of the plant immune system yet. While important for basal defense, these genes were not essential for immunity mediated by two distinct *R*-genes. Although they are transcriptionally activated by DCA, DCA-mediated immunity was not compromised in their mutants. In addition, eight novel synthetic elicitors identified in the screen performed by Knoth et al., (2009) were further characterized. Notably, a synthetic elicitor (CMP199) was identified with a substantially lower active concentration than DCA.

## RESULTS

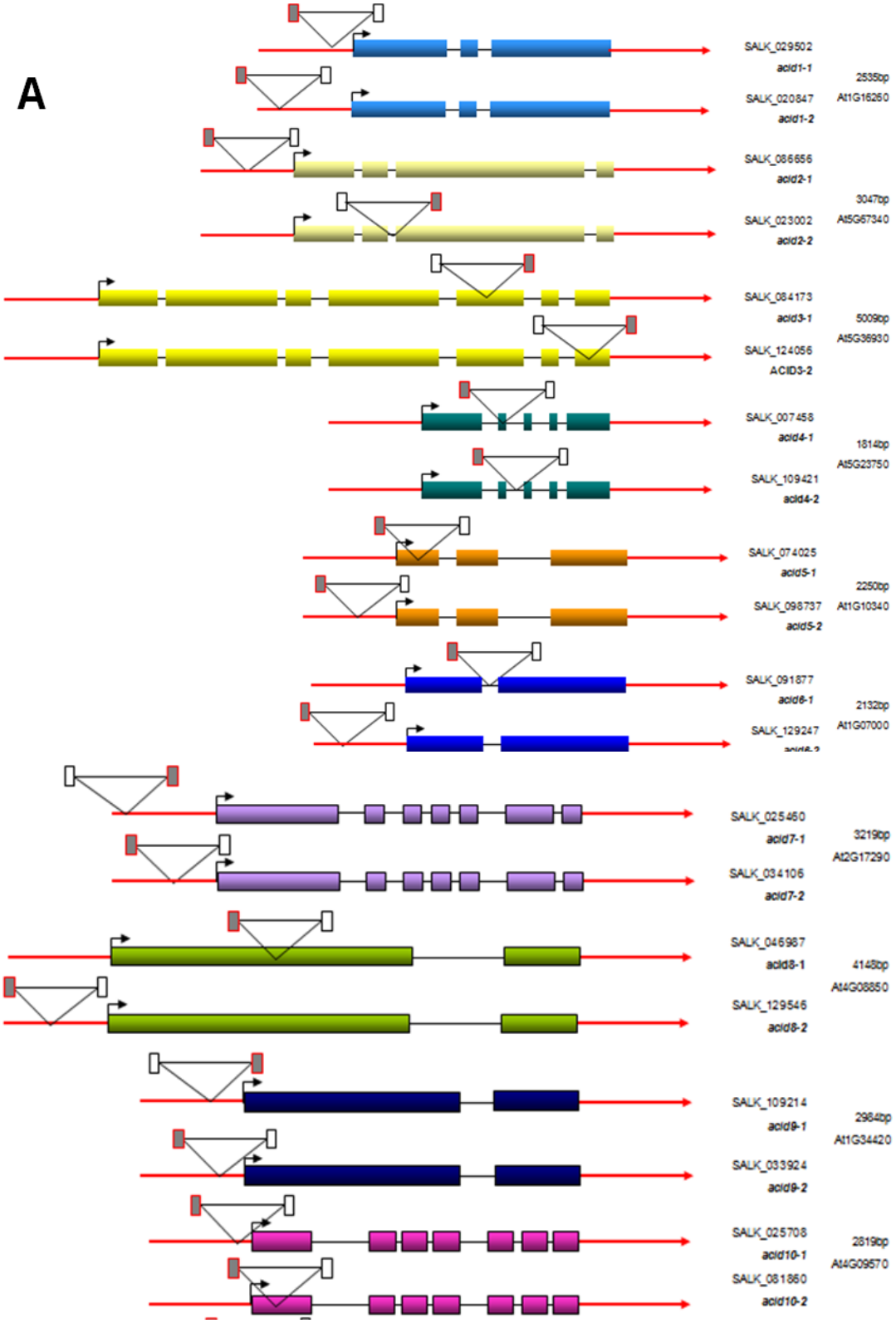
### *ACID* Genes Play a Role in Basal Defense Against Virulent *Hpa*Noco2, but are not Required for Function of the *R*-genes *RPP4* or *RPP7*

In microarray experiments performed by Knoth et al. (2009), a group of 137 genes were identified that are upregulated by DCA and INA at time points when these synthetic elicitors induce strong immunity against *Hpa*. These *ACID* (*Associated with Chemically Induced Defense*) genes were considered promising because synthetic elicitor-triggered transcriptome changes that follow the temporal pattern of synthetic elicitor-mediated resistance are likely to be of key importance for a successful pathogen defense. A subset of 16 *ACID* genes (*ACID1-ACID16*) was chosen for further analyses. These genes were chosen based upon the availability of at least two independent Col-0 T-DNA insertion lines with an emphasis on protein kinases, since kinases are often important for defense signaling (Table 1.1). Suitable T-DNA lines with insertions in or near the respective *ACID* genes were identified using The Arabidopsis Information Resource (TAIR) (Alonso, Stepanova et al. 2003). Multiple insertion locations per gene were considered to determine which were most likely to disrupt genic function. Top priority was given to insertions in exons, followed by insertions in promoters or 5'-UTRs close to the respective translational start sites, and lastly in introns. At least two insertion lines per selected *ACID* gene were ordered. Three insertion lines were selected for *ACID16* because three homozygous lines were

| *ACID* Name | AGI Number | SALK number | Primer sequence | Gene information |
|---|---|---|---|---|
| *ACID1-1* | AT1G16260 | 029502 | F-CGATATGGAGAAAGGGTCTCC<br>R-CCCACACGAATTGTCATTTTC | Wall-associated receptor kinase-like-8 precursor (WAKL8) |
| *ACID1-2* | | 020847 | F-ATCGGTTGTTGGTCTGATGAG<br>R-TGGAAATTAGAAGAACGTGCG | |
| *ACID2-1* | AT5G67340 | 086656 | F-TTTGGTTTTGCAAACCACTTC<br>R-GAACCTCATTGACTCTGACGC | Armadillo/beta-catenin repeat family protein |
| *ACID2-2* | | 023002 | F-ATGCACAATTTCTTTGATGCC<br>R-GCGTCAGAGTCAATGAGGTTC | |
| *ACID3-1* | AT5G36930 | 084173 | F-TCATCTGAATCCGAATTTTCG<br>R-GACGTTCTCCCTGAGGAAATC | Disease resistance protein (TIR-NBS-LRR class). |
| *ACID3-2* | | 124056 | F-TCTGAATTCACTAGCCATGGC<br>R-ATTCGCTTGTAGCTTTTTCCC | |
| *ACID4-1* | AT5G23750 | 007458 | F-GAAGAGGAACCGAAGAAGGTG<br>R-TCTCCCATGATCCAATTGAAG | Remorin family protein |
| *ACID4-2* | | 109421 | F-CCCGTCGTCCCTAGTAATTTG<br>R-CTTCTCCAATTGCTCCTGTTG | |
| *ACID6-1* | AT1G07000 | 091877 | F-GAAACGAAGACAAAGTCGTGG<br>R-ACGTGGAATATCAGCGTTACG | Member of EXO70 gene family protein, putative exocyst subunits. |
| *ACID6-2* | | 129247 | F-GCCTGATCATCAAGTGACCTC<br>R-CGCGAACCATGTCTGACTTAG | |
| *ACID5-2* | AT1G10340 | 098737 | F-GCGAGAAGTTTTCTTGGTGTG<br>R-GATAGCAAGATGCAAAGG | Ankyrin repeat family protein. |
| *ACID5-1* | | 074025 | F-AGAAAGAGAGAGGAACCTGCG<br>R-GGAACCTTGTCCAAGAACTCC | |
| *ACID7-2* | AT2G17290 | 034106 | F-AGTTGTCCATTGTTTCGATCTG<br>R-ATTGATTGTCAACGTTACGCC | Encodes calcium dependent protein kinase 6 (CPK6). |
| *ACID7-1* | | 025460 | F-CTCGCAACTAACGCTTACCTG<br>R-CTCCATTTCATCGTCTTCTCG | |
| | R protein-like LRR-kinases | | | |
| *ACID8-1* | AT4G08850 | 046987 | F-CAAAGGGAATAGTTTCTCCGG<br>R-TTGGATCGAATTCTCCTGTTG | Kinase |
| *ACID8-2* | | 129546 | F-TTACATTGAGGGGACCAGTTG<br>R-CGAGGCTTTTCTTTACAAGCC | |
| *ACID9-2* | AT1G34420 | 033924 | F-AAATCAAAAAGTTAGTGCACAA<br>R-TTCCATGAACATGGATTCCTC | Leucine-rich repeat family protein |
| *ACID9-1* | | 109214 | F-GTTTATGCAGGCAAGAGCAAC<br>R-GGGATCATAACTGTCACCGTG | |
| *ACID12-1* | AT5G48380 | 008775 | F-TTAACATTTTGGGGTGTTTGC<br>R-TCAACCAAGCTCGAGAGTAGC | BAK1-interacting receptor-like kinase named BIR1 |
| *ACID12-2* | | 079810 | F-TTAACATTTTGGGGTGTTTGC<br>R-TCAACCAAGCTCGAGAGTAGC | |
| *ACID13-1* | AT1G35710 | 112341 | F-CTCAAACCCATCGTGATATCG<br>R-GAAATGAAGGGGAAATCTTGG | Leucine-rich repeat transmembrane protein kinase |
| *ACID13-2* | | 132549 | F-CTCAAACCCATCGTGATATCG<br>R-GAAATGAAGGGGAAATCTTGG | |
| | Proteins with kinase activity | | | |
| *ACID11-2* | AT3G16030 | 136842 | F-CCAATAAAATTGTCTCCTTAC<br>R-AGGCTTGATGCTCCTCTTAAA | Callus expression of RBCS 101 |
| *ACID11-1* | | 008929 | F-CCAATAAAATTGTCTCCTTACC<br>R-AGGCTTGATGCTCCTCTTAAA | |
| *ACID10-2* | AT4G09570 | 081860 | F-TTTTTGGTCGAGTCTGATTGG<br>R-TCCTTGAACCAACCAACAAAG | Member of Calcium Dependent Protein Kinase (CDPK) family. |
| *ACID10-1* | | 025708 | F-TTTTTGGTCGAGTCTGATTGG<br>R-TCCTTGAACCAACCAACAAAG | |
| *ACID14-1* | AT4G26070 | 140054 | F-TGTTTTGTCGGTTTGATCTCC<br>R-AATGATTCGCCTCTCATGATG | Member of MAP Kinase Kinase. |
| *ACID14-2* | | 027645 | F-CCACCACTAGCAGAGAAACAT<br>R-AACATGCTATCTGCCATCTGC | |
| *ACID15-1* | AT5G39030 | 007613 | F-GACTTGCATCCTCTGGTGAAG<br>R-TCCTTCCATCATTTCAACGAC | Protein kinase family protein |
| *ACID15-2* | | 009338 | F-GACTTGCATCCTCTGGTGAAG<br>R-TCCTTCCATCATTTCAACGAC | |
| *ACID16-1* | AT1G66880 | 034755 | F-CACCTGCTTTTGCTCCAATAG<br>R-TCCATTTGCTTCATCTCTTCG | Serine/threonine protein kinase family protein; |
| *ACID16-3* | | 083213 | F-CACCTGCTTTTGCTCCAATAG<br>R-TCCATTTGCTTCATCTCTTCG | |
| *ACID16-2* | | 021182 | F-CACCTGCTTTTGCTCCAATAG<br>R-TCCATTTGCTTCATCTCTTCG | |

**Table 1.1: lines used for this study.** List of *ACID* genes, their AGI numbers, SALK lines numbers, primer sequences used for genotyping, and gene where insertion is located. F represents the forward primer and R the reverse primer. \

already available. For the remaining 30 lines, homozygous individuals were selected for each respective insertion using polymerase chain reaction (PCR)-based genotyping (Alonso et al., 2001). Homozygous lines were selfed and their progeny used for experiments. *ACID* gene structures and sites of T-DNA insertion are shown in Figure 1.1.


A
SALK_029502
acid1-1
2535bp
SALK_020847
At1G16250
acid1-2
SALK_086656
acid2-1
3047bp
SALK_023002
At5G67340
acid2-2
SALK_084173
acid3-1
5009bp
At5G36930
SALK_124056
ACID3-2
SALK_007458
acid4-1
1814bp
At5G23750
SALK_109421
acid4-2
SALK_074025
acid5-1
2250bp
SALK_098737
At1G10340
acid5-2
SALK_091877
acid6-1
2132bp
At1G07000
SALK_129247
SALK_025460
acid7-1
3219bp
At2G17290
SALK_034106
acid7-2
SALK_046987
acid8-1
4148bp
At4G08850
SALK_129546
acid8-2
SALK_109214
acid9-1
2984bp
At1G34420
SALK_033924
acid9-2
SALK_025708
acid10-1
2819bp
At4G09570
SALK_081860
acid10-2

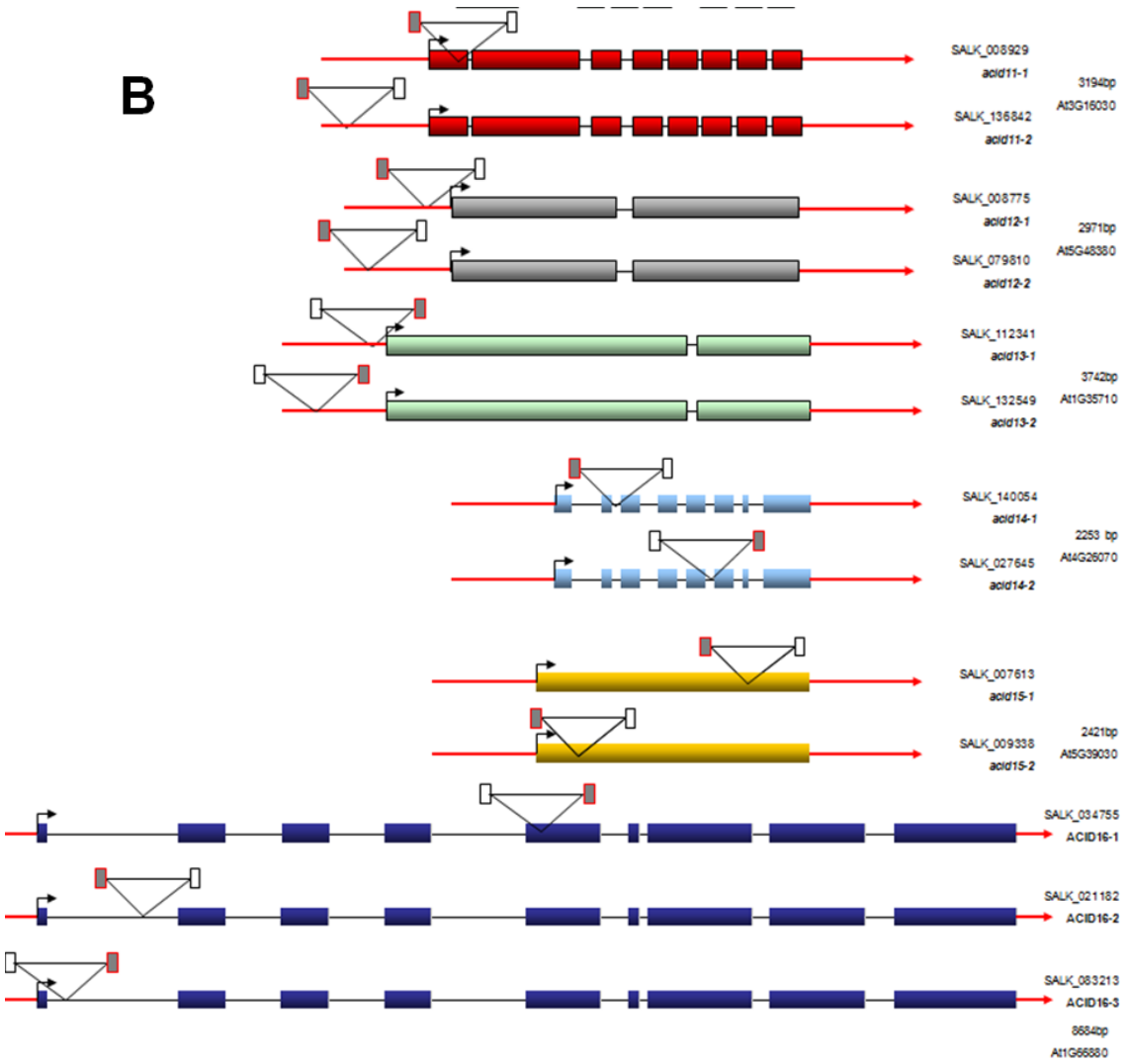



**Figure 1.1: *ACID* T-DNA Insertion mutants used for gene expression studies and analyses of defense-related phenotypes.** **A and B.** Diagrammatic representation of sites and orientation of T-DNA insertions. T-DNA borders are represented by open (right border) and closed (left border-LB) boxes. Introns or untranslated regions (UTRs) are indicated by lines and exonic coding sequences by solid-colored boxes. The transcript start site position is indicated by the black arrow and all genes are shown 5' to 3' from left to right. SALK number, *acid* mutant number, gene identifiers, and gDNA length are listed to the right of the diagram.

The Arabidopsis/*Hpa* pathosystem allows the testing of effects on *R*-mediated immunity as well as basal defense (Slusarenko and Schlaich 2003). A reduction of basal defense results in enhanced susceptibility to virulent *Hpa* isolates (Glazebrook, Rogers et al. 1996). To identify any effects on basal defense, compatible interactions of the selected set of 33 *acid* mutants with the Col-0-virulent *Hpa* isolate Noco2 were tested (Figure 1.2). *Hpa*Noco2 is not recognized by any Col-0 *R*-gene and therefore triggers basal defense (Holub, Brose et al. 1995). For this experiment, three-week-old plants were spray-infected with *Hpa*Noco2 (2 x $10^4$ spores/ml). Seven days later, the number of spores was quantified and expressed as a percentage of spores counted on each mutant relative to those on Col-0. Both independent mutant lines of *ACID1,*

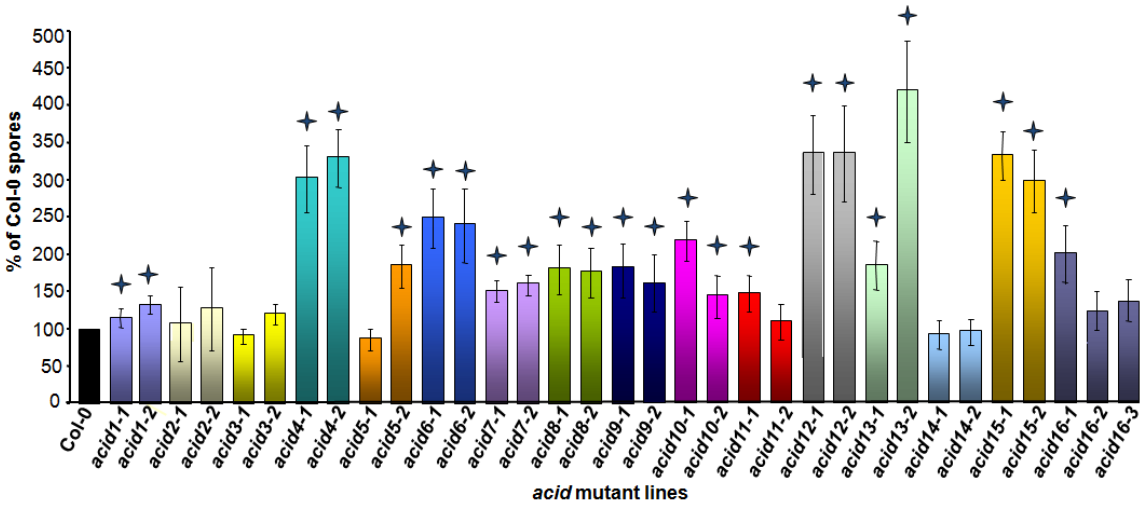



**Figure 1.2: *acid* T-DNA mutants infected with the virulent *Hpa isolate* Noco2.** Three-week-old soil-grown seedlings were sprayed with *Hpa*Noco2 (2 x $10^4$ spores/ml; 2 ml per pot). Spores were counted 7 dpi. Mean and SE of spore counts in acid mutants were calculated from a minimum of three biological replicates and are expressed as percent relative to Col-0 spore counts. For each analyzed *ACID* gene at least two independent T-DNA alleles were tested. The Student's *t*-test ($p<0.05$) was used to determine significant differences from Col-0 and significance is indicated on the graph by a star.

*ACID4, ACID6, ACID7, ACID8, ACID9, ACID10, ACID12, ACID13,* and *ACID15* displayed significantly higher spore numbers compared to Col-0 indicating a reduction in basal defense. The *acid* mutant lines: *acid4-1*, *acid4-2*, *acid6-1*, *acid6-2*, *acid12-1*, *acid12-2*, *acid13-1*, and *acid13-2* showed a ~three-fold increase in spore numbers compared to Col-0, while the rest exhibited a two-fold or less increase. These results indicated that ten of the 16 tested *ACID* genes clearly play a role in basal defense against *Hpa*Noco2. Of the remaining six genes, *ACID5*, *ACID11*, and *ACID16* showed significantly higher spore numbers in only one of their T-DNA insertion lines. Thus, the contribution of these three *ACID* members to basal defense remains unclear. No morphological abnormalities were apparent in any of the tested *acid* single mutants. One mutant allele for each of the ten *ACID* genes required for basal defense was used for the breeding of double *acid* mutants (Table 1.2). For each of these ten *ACID* genes the strongest allele (based on *Hpa*Noco2 assays) was used for the crosses. The selection of double homozygotes and their characterization is currently being completed by another lab member.

To determine if any of the *acid* single mutants were altered in ETI, the immunity mediated by the *R*-gene *RPP7* (recognizes *Hpa*Hiks1*)* in each *acid* mutant was examined (Figure 1.3). The wild type (WT) Col-0 and Oy1 lines as well as the Col-0 mutants, *pad4 and rpp7-15,* were used as controls. *Hpa*Hiks1 was not able to successfully infect WT Col-0, which expresses both *RPP4* and

*RPP7*. Successful ETI induction by *RPP7* was evident by the distinct patches of HR after inoculation with *Hpa*Hiks1 spores (Figure 1.3).

| Parent I | Parent II |
|---|---|
| ACID name (AGI Number) SALK Line | ACID name (AGI Number) SALK Line |
| *acid1-1* (At1G16260) 029502 | *acid6-2* (At1G07000) 129247 |
| | *acid6-2* (At1G07000) 129247 |
| | *acid6-2* (At1G07000) 129247 |
| | *acid16-1* (At1G66880) 034755 |
| | *acid16-1* (At1G66880) 034755 |
| | *acid16-1* (At1G66880) 034755 |
| | *acid16-1* (At1G66880) 034755 |
| | *acid9-2* (At1G34420) 033924 |
| | *acid9-2* (At1G34420) 033924 |
| | *acid9-2* (At1G34420) 033924 |
| | *acid9-2* (At1G34420) 033924 |
| | *acid9-2* (At1G34420) 033924 |
| | *acid6-2* (At1G07000) 129247 |
| | *acid6-2* (At1G07000) 129247 |
| | *acid6-2* (At1G07000) 129247 |
| | *acid6-2* (At1G07000) 129247 |
| | *acid7-1* (At2G17290) 025460 |
| | *acid8-1* (At4G08850) 046987 |
| *acid7-1* (At2G17290) 025460 | *acid1-2* (At1G16260) 020847 |
| | *acid9-2* (At1G34420) 033924 |
| | *acid9-2* (At1G34420) 033924 |
| | *acid9-2* (At1G34420) 033924 |
| | *acid8-1* (At4G08850) 129546 |
| | *acid16-1* (At1G66880) 034755 |
| | *acid16-1* (At1G66880) 034755 |
| | *acid16-1* (At1G66880) 034755 |
| *acid1-2* (At1G16260) 020847 | *acid10-1* (At4G09570) 025708 |
| | *acid10-1* (At4G09570) 025708 |
| *acid7-2* (At2G17290) 034106 | *acid9-2* (At1G34420) 033924 |

**Table 1.2: Generated acid double mutants.** Seeds of F1, F2, or F3 generations are available but double homozygotes have not been selected.

The *pad4* mutant was fully resistant to *Hpa*Hiks1 responding by discrete HR. In contrast, the *rpp7-15* mutant was fully susceptible to *Hpa*Hiks1. Since the Oy-1 accession lacks *RPP7* (Holub, Beynon et al. 1994), it was susceptible to *Hpa*Hiks (Figure 1.3). Each tested *acid* mutant showed full resistance to infection displaying distinct HR sites (Figure 1.3). Thus, these data demonstrated that the 16 tested *ACID* genes were not required for immunity to *Hpa* mediated by *RPP7*.

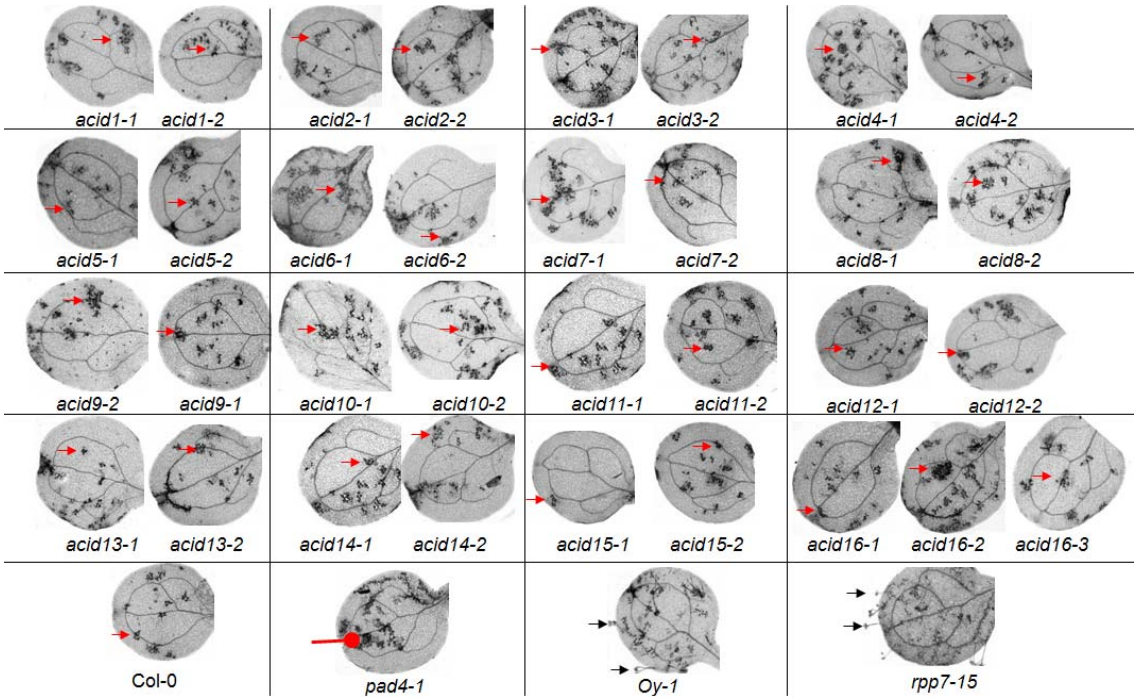



**Figure 1.3: *acid* mutants are not compromised in *RPP7*-mediated race-specific resistance to *Hpa*Hiks1.** One-week-old Col-0 (fully resistant wild type background of *acid* mutants), *pad4* (Col-0 *pad4* allele, which is partially compromised in *RPP7*-mediated immunity), Oy-1 (ecotype that lacks RPP7) and *rpp7-15* (strong *rpp7* allele in Col-0 background) plants, and *acid* mutants were sprayed with *Hpa*Hiks1 (5 x $10^4$ spores/ml; 1 ml per pot), stained with trypan blue 7 dpi to visualize hyphal growth, sporangiophores (black arrow), cell death around infection sites (hypersensitive response -HR; red arrows) , and trailing necrosis (red line with circle at end). All *acid* mutant lines are fully resistance to *Hpa*Hiks1 and show discrete HR sites similar to Col-0, while *pad4-1* plants show an intermediate phenotype and exhibit trailing necrosis. Oy-1 and *rpp7-15* are fully susceptible and exhibit heavy hyphal growth. At least 3 biological replicates showed consistent results. Results of a typical experiment are shown.

### Some *acid* Mutants Exhibit Reduced Transcript Levels

Often T-DNA insertions affect the function of a given gene by altering its transcript level, disrupting its open reading frame or causing abnormal splicing (Alonso, Stepanova et al. 2003). Therefore, each of the ten *ACID* genes required for basal defense were examined by reverse-transcription (RT)-PCR for effects of the tested T-DNA insertions on the endogenous transcript levels of the respective

mutant gene (Figure 1.4). Only 11 of the 20 tested lines (*acid4-1, acid4-2, acid6-1, acid6-2, acid7-1, acid7-2, acid8-1, acid13-1, acid13-2, acid15-1*, and *acid15-2*) showed clearly reduced levels of the respective *ACID* transcript.

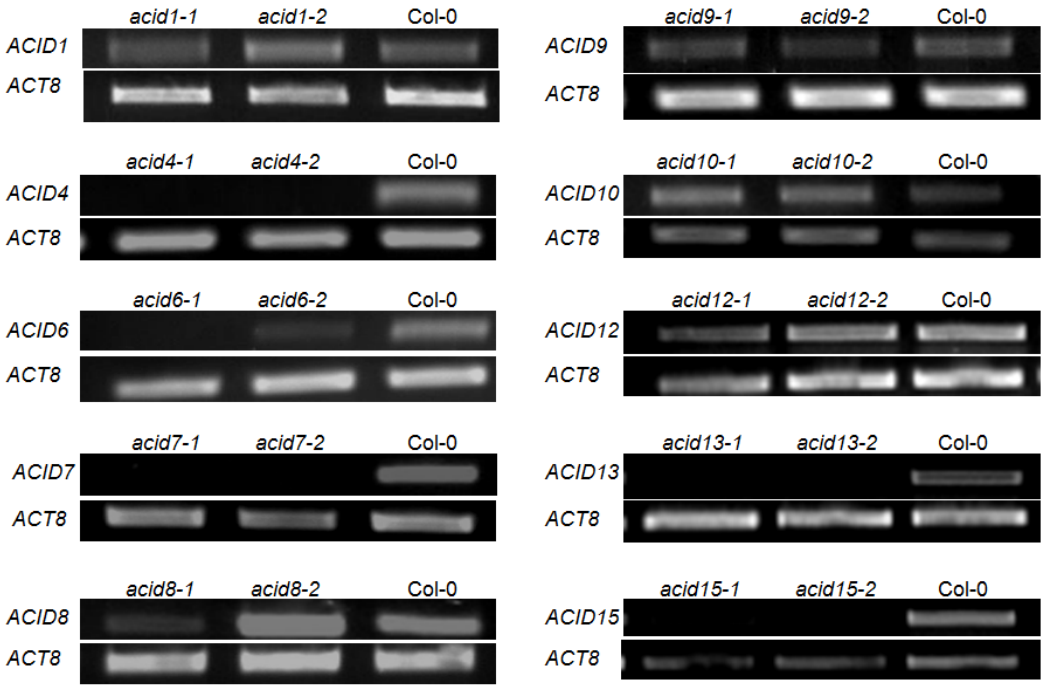



**Figure 1.4: Some *acid* T-DNA mutants displayed altered transcript levels of the respective *ACID* gene compared to Col-0 in semi-quantitative RT-PCR analyses.** Three-week-old soil-grown seedlings were harvested and their total RNA extracted. RT-PCR results with *Actin8* (*ACT8)* were included as a loading control. At least three independent replicates gave similar results. Results of a typical experiment are shown.

Of the nine lines that did not show reduced transcript levels, only *acid10-2* contains a T-DNA insertion in a coding exon. Insertions in coding exons are likely to disrupt or alter the open-reading frame of the gene. The remaining eight lines (*acid1-1, acid1-2, acid8-1, acid9-1, acid9-2, acid10-1, acid12-1, acid12-2*) all

bear T-DNA insertions in the 5' region upstream of the translational start site of their respective gene. These insertions seem not to reduce transcript levels and it is unclear how gene function was compromised resulting in the basal defense phenotype. T-DNA insertions before the start codon can also interfere with mRNA processing leading to abnormalities such as incorrect splicing (Wang 2008). No evidence of inappropriate splicing was evident in the form of altered RT-PCR fragment size. However, only a detailed analysis of transcript sequences produced by the respective *acid* alleles can exclude potential splicing errors. Alternatively, it is possible that the insertions caused the gene to be misexpressed in a subtle manner that can only be detected by more sensitive expression analyses, such as real-time quantitative RT-PCR or protein blotting.

**Mutations in *ACID* Genes do not Reduce DCA-Mediated Resistance to *Hpa***

To determine if the ten *ACID* genes required for basal defense were also needed for DCA-mediated immunity, three-week-old soil-grown Col-0 or *acid* mutant seedlings were sprayed with 100 µM DCA or mock solution and challenged with virulent *Hpa*Noco2 24 h later. *Hpa* spore formation was evaluated 7 dpi (Figure 1.5). Untreated Col-0 plants were susceptible to *Hpa*Noco2, while DCA-treated Col-0 exhibited strong resistance, permitting the formation of only few or no spores. As expected, transgenic *nahG* plants, known to be deficient in SA accumulation, exhibited pronounced hypersusceptibility

when left untreated. DCA triggered full *Hpa*Noco2 resistance in *nahG,* because DCA acts downstream of SA perception (Knoth et al., 2009). All tested *acid* mutants sprayed with DCA showed as strong of a reduction of *Hpa* spore numbers as Col-0. This indicated that none of the tested *ACID* genes were required for DCA-induced disease resistance to *Hpa* (Figure 1.5). These results were surprising. I anticipated that the tested *acid* mutations affect this defense pathway, as DCA triggers transcript accumulation of these genes' transcripts (Knoth, Salus et al. 2009) and the *ACID* genes were required for basal defense against *Hpa* (see Figure 1.2).

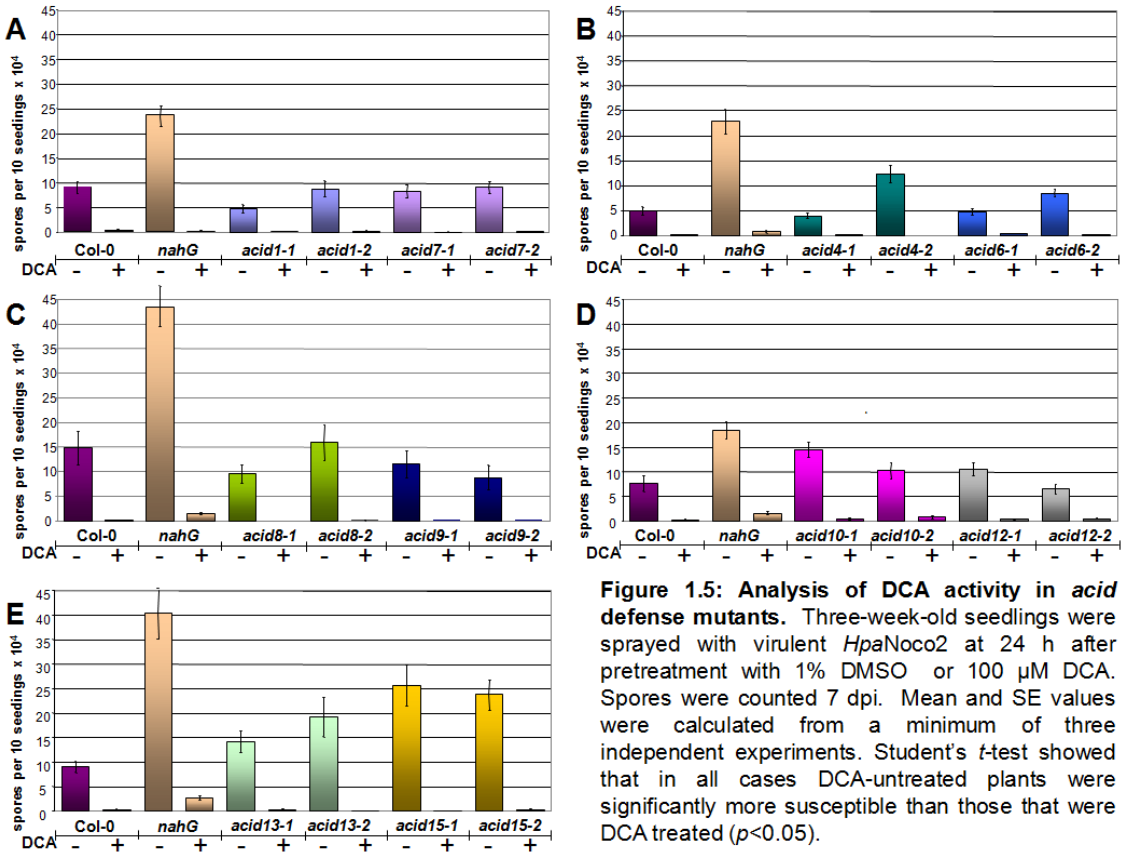



**Figure 1.5: Analysis of DCA activity in *acid* defense mutants.** Three-week-old seedlings were sprayed with virulent *Hpa*Noco2 at 24 h after pretreatment with 1% DMSO or 100 µM DCA. Spores were counted 7 dpi. Mean and SE values were calculated from a minimum of three independent experiments. Student's *t*-test showed that in all cases DCA-untreated plants were significantly more susceptible than those that were DCA treated ($p<0.05$).

It was noted that DCA-mediated immunity in the *acid* mutants did not display the same hypersusceptibility seen in basal defense assays (Figure 1.2). One possible explanation was that the experimental conditions varied between the assays for basal defense and DCA-mediated immunity. In contrast to the analysis of DCA-mediated immunity (Figure 1.5), there was no pre-treatment with compound or mock solution before being sprayed with pathogen in the basal defense assays (Figure 1.2). It was observed in other experiments (see Chapter 2) that this type of pretreatment affected the experimental outcome and indicated that pre-treatment of Arabidopsis plants with mock solution reduced the extent of susceptibility to *Hpa*.

**Analysis of DCA Mediated Induction of Defense Markers**

To analyze the induction of defense marker transcripts after treatment with DCA, RT-PCRs were performed (Figure 1.6). Plants were sprayed with 100 µM DCA or mock solution. Using RT-PCR it was examined whether DCA-mediated transcript accumulation of the known SA-response genes *PR1*, *CaBP22*, *WRKY70*, or *LURP1* or the known JA-response gene *PDF1.2a* were affected by mutations in the ten *ACID* genes required for basal defense. In WT Col-0 plants, transcripts of the SA-response genes were low or not detectable in untreated samples but were visibly upregulated 48 h after DCA treatment. Their DCA-induced transcript upregulation remained unaltered in the tested *acid* mutant

lines compared to Col-0. These data were consistent with the fact that DCA-mediated immunity was not compromised in this set of *acid* mutants.

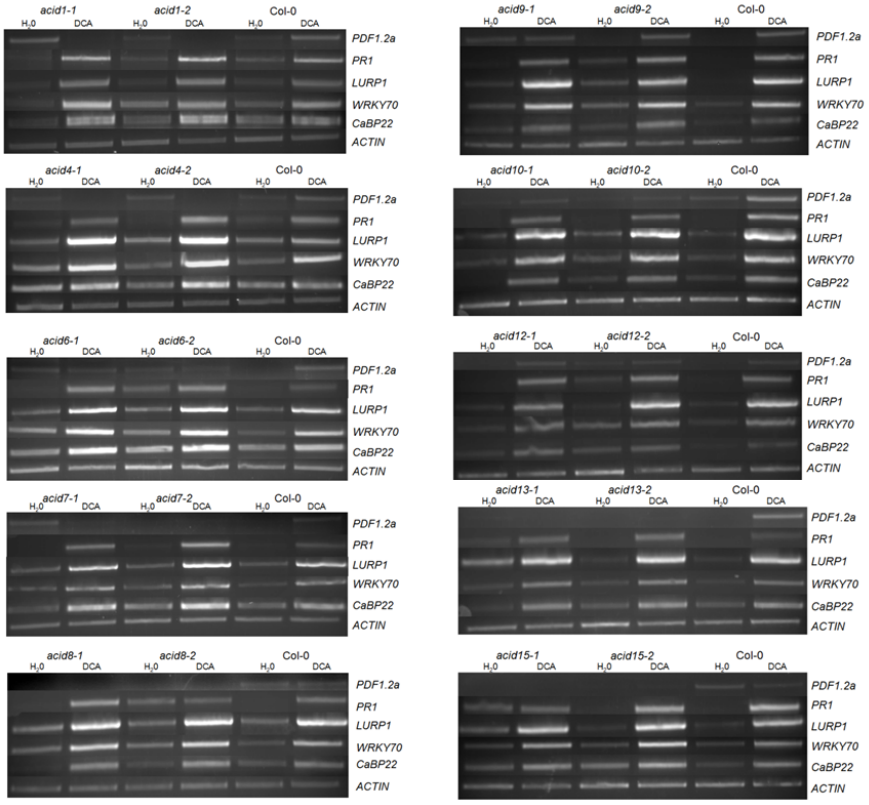



**Figure 1.6: Analysis of DCA-mediated induction of defense related genes in *acid* mutants.** Three-week-old soil-grown *acid* mutants or Col-0 seedlings were harvested and their total RNA extracted after 48 h pre-treatment with either DCA or 1% DMSO. Transcript levels of *PDF1.2a, PR1, LURP1, WRKY70,* and *CaBP22.* were examined by RT-PCR. *Actin* was included as a loading control. At least three independent replicates gave similar results. Results of a tyical experiment are shown.

Transcripts of the known JA-response gene *PDF1.2a* typically showed an upregulation after DCA treatment in WT plants. This was unexpected since the upregulation of the SA pathway can have an inhibitory effect on the JA pathway (Mur, Kenton et al. 2006). Furthermore, no response of *PDF1.2a* or its paralogs to DCA in previously performed microarray experiments was seen (Knoth et al., 2009). However, the high consistency of RT-PCR results (Figure 1.6) strongly suggests that transcripts of *PDF1.2a* (and possibly additional *PDF* family members) are DCA-inducible. DCA-inducibility of *PDF1.2a* transcript

accumulation was abolished in *acid1-1, acid1-2, acid4-1, acid4-2 acid6-1, acid6-2, acid7-1, acid7-2, acid8-1, acid8-2, acid13-1*, *acid13-2, acid15-1,* and *acid15-1.* Experimental observations suggest that these genes may play a role in the interpathway cross talk.

**Novel Small Molecule Elicitors of Plant Defense**

In the chemical screen performed by Knoth et al. (2009), 114 compounds were identified that reproducibly induced *GUS* expression in the transgenic p*CaBP22*$^{-333}$::*GUS* reporter fusion line. Of these 114 compounds, eight were further examined, which have not been previously reported as plant defense inducers. These eight compounds were selected for further experimentation based on their unique chemical structures when compared to DCA and other previously known synthetic elicitors (Schreiber and Desveaux 2008) (Figure 1.7A). Of the eight newly tested compounds, all were found to induce defense responses at the tested concentrations. p*CaBP22*$^{-333}$*::GUS* seedlings grown in liquid growth medium incubated for 24 h with a single synthetic elicitor, exhibited *GUS* expression (Figure 1.7B). Along with DCA, CMP144 and CMP199 were active at concentrations as low as 1 µM. CMP994, CMP60, CMP202, and CMP174 were active at concentrations as low as 10 µM while CMP608 was only active at 100 µM (Figure 1.7B). It is of note that while CMP199 induced GUS activity at 1 µM, *GUS* expression was not detectable when 100 µM of CMP199 was used.

To examine if any of these synthetic elicitors induced phytotoxicity, trypan blue staining of seedlings after saturation treatment (treatments where seedlings are completely submerged in liquid), where seedlings were grown and treated in liquid medium, was used. Dark blue staining, indicating cell death, was prevalent in 100% of the seedlings treated for 24 h with 300 µM of DCA, CMP144, CMP994, CMP202, CMP199, and CMP384 (Figure 1.7C). Cell death was evident starting at treatments of 100 µM for CMP199. Therefore, the absence of *GUS* expression at 100 µM CMP199 was likely due to compound-induced phytotoxicity. For all synthetic elicitors cell death was not observed at concentrations showing effective *GUS* reporter activation (1-100 µM), indicating that cell death was not responsible for the *GUS* reporter activation. Based on these preliminary assays, these synthetic elicitors were clearly potent inducers of *GUS* expression in the p*CaBP22*::*GUS* lines. Additional study was warranted to scrutinize their unique properties and ascertain their potential for future research use.

To accurately quantify the defense-inducing activity of these eight novel synthetic elicitors, a dose-response analysis measuring the inhibition of *Hpa*Noco2 spore development in three-week-old Col-0 plants was performed (Figure 1.7D). For this experiment Col-0 seedlings were sprayed with the respective compound or mock solution and challenged with *Hpa*Noco2 24 h later. *Hpa* spore formation was evaluated 7 dpi. Each synthetic elicitor behaved

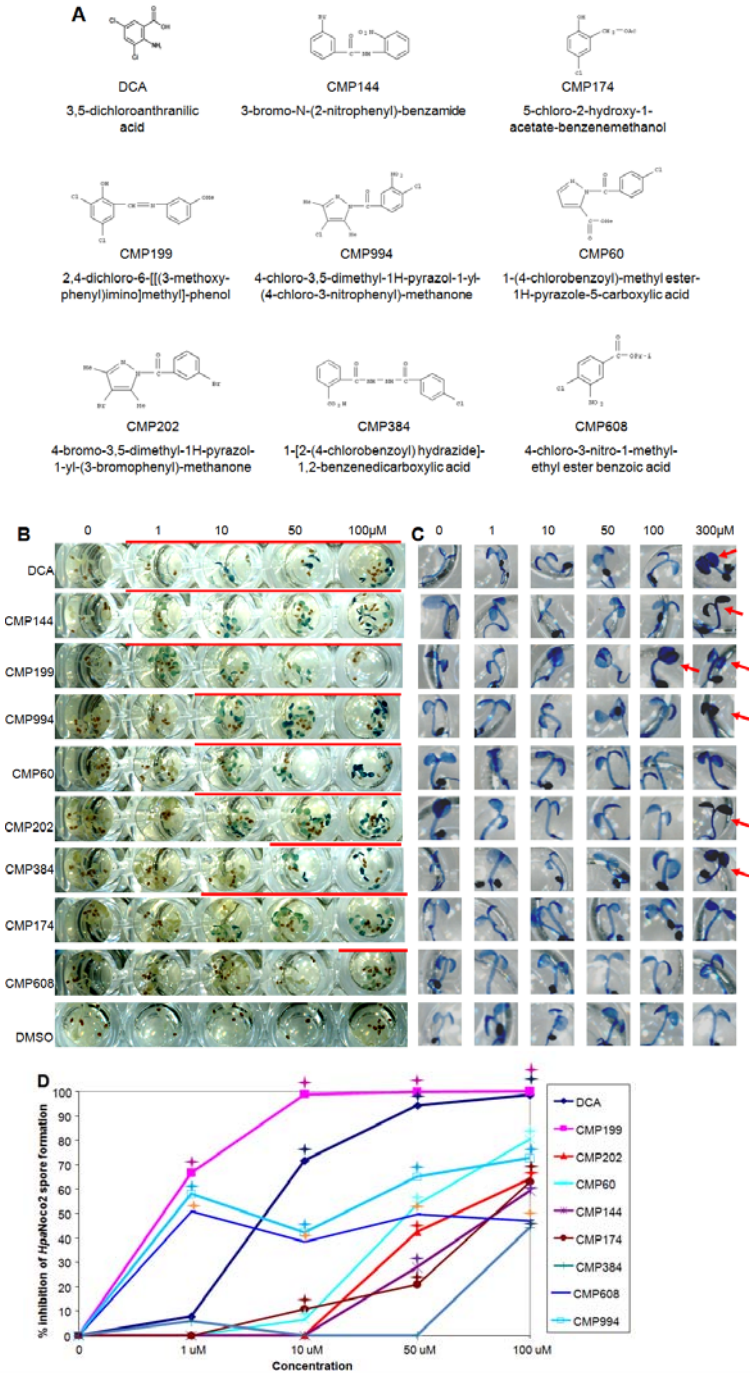



**Figure 1.7: Novel synthetic elicitors.** **A**, Chemical structures of novel synthetic elicitors. **B**, Screening wells containing 7-d-old liquid-grown *CaBP22*$^{-333}$*::GUS* seedlings after GUS histochemical staining comparing reporter response after a 24 h-incubation at indicated compound concentrations. Blue/green color of cotyledons indicates induction of the *GUS* gene expression (all chlorophyll was bleached out of plants during the ethanol-destaining process). The red line above wells indicates GUS staining. **C**, Trypan blue staining of *CaBP22*$^{-333}$*::GUS* seedlings incubated for 24 h in medium containing compounds at the indicated concentrations. Dark blue/black color of the cotyledons indicates cell death (toxicity) and is pointed to by red arrows. The seed coats of seedlings always stain blue/black and can been seen in some images. All staining experiments were performed at least three times with similar results. **D**, *Hpa*Noco2 growth inhibition assay after elicitor treatment. Three-week-old soil-grown Col-0 seedlings were spray-infected 24 h after treating with varying concentrations of each synthetic elicitor and then assayed at 7 dpi for spore growth. 100% inhibition = 0 spores. The assay was repeated three times with similar results. The average of the three replicates is shown. The Student's *t*-test ($p<0.05$) used to determine significant differences compared to mock treated seedlings. Significant differences are indicated on the graph by the corresponding star.

similarly during either *Hpa* defense or the reporter gene assays. CMP199 showed nearly 70% inhibition of spore development at 1 μM and 100% at 10 μM. CMP199 which showed a high activity in *GUS* saturation treatments, proved to be a more efficient defenseinducer compared to DCA at every concentration tested (Figure 1.8). This finding was important since this was the first time that a synthetic elicitor more potent than DCA had been discovered by our program. CMP199 showed great potential for future use. In the defense assays CMP199 and DCA provided most efficient protection against *Hpa*Noco2, in contrast CMP384 was only able to induce significant levels of immunity at the highest tested concentration (100 μM). The weakest of the examined new synthetic elicitors, CMP384 and CMP608 mediated only a 30% inhibition of spore development at 100 μM. The remaining five compounds exhibited intermediate levels of defense-inducing activity, with 55-75% inhibition of spore development at 100 μM.

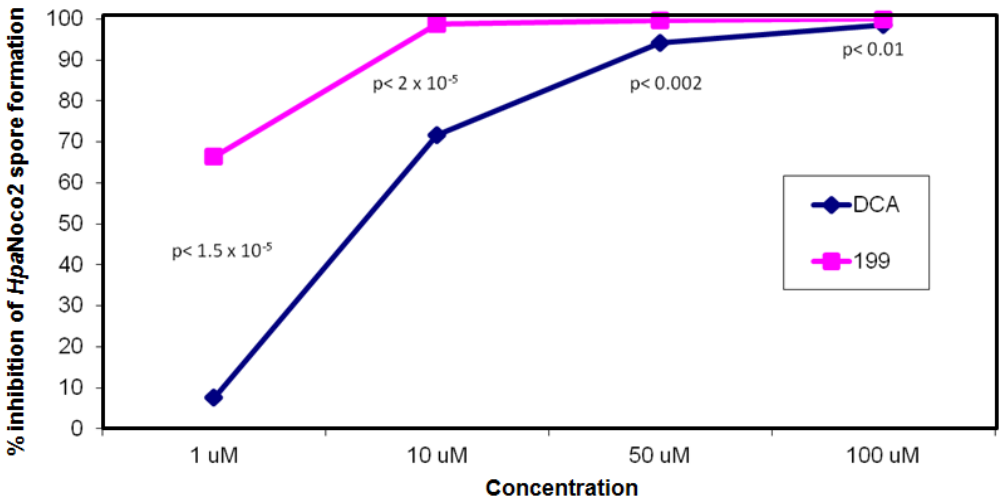



**Figure 1.8: *Hpa* growth-inhibition assays comparing DCA and CMP199**. Three-week-old soil-grown Col-0 seedlings were spray-infected 24 h after treating with varying concentrations of DCA or CMP199 and then assayed at 7 dpi for spore growth. 100% inhibition = 0 spores. The assay was repeated three time with similar results. The average of the three replicates is shown above. The Student's *t*-test p-value is indicated at every pair of data points and was used to determine significant differences between DCA and CMP199.

### DCA, CMP199, and CMP442 do not act Synergistically

Different inducers of the same biological response may act antagonistically, additively, or synergistically (Yasuda, Ishikawa et al. 2008; Wei, Margolin et al. 2012). Synergistic activities produce a greater effect than the sum of their independent effects on the system. A suite of synthetic elicitors with distinct modes of action, acting synergistically, may have great potential as environmentally safe pesticides, as they may trigger strong defense responses at extremely low concentrations when applied in combination. In addition, synergistically acting synthetic elicitors may be useful for plant defense network studies by facilitating the discovery of cooperative crosstalk mechanisms linking distinct compound-triggered pathways.

Two redundantly acting compounds should not induce p*CaBP22*$^{-333}$*::GUS* expression when each is applied at concentrations below their minimally active concentration. Additively acting compounds should activate the reporter when combined at concentrations equaling the sum of their minimally active ones. However, these compounds should not activate the reporter when applied at lower combined concentrations. If two compounds activate reporter expression at combined concentrations lower than the sum of their minimally active concentrations, their effects are synergistic. Finally, if two compounds act antagonistically the reporter gene response triggered by one compound should be reduced by application of the second compound. Antagonistically acting compounds would be tested at concentrations guaranteed to induce p*CaBP22*$^{-333}$*::GUS* expression. Here, the synergy of DCA, CMP442 (see Chapter 2), or CMP199 under saturation treatment conditions was tested.

Consistent with previous observations, CMP199 proved to be the most potent of these three synthetic elicitors (Figure 1.9A). *CaBP22*$^{-333}$*::GUS* expression was scored visually based upon intensity of color. The minimal concentration of CMP199 for inducing the p*CaBP22*$^{-333}$*::GUS* reporter was 100 nM, whereas that of DCA was 250 nM, and that of CMP442 was 1 µM.

When any two of the synthetic elicitors DCA, CMP442 or CMP199 were combined the resulting responses were neither synergistic nor antagonistic (Figure 1.9 B-D). *GUS* expression was weakly evident after the mixture of 50 nM each of DCA and CMP199 (Figure 1-9 D). This combination does not indicate

synergism because if it was, *GUS* expression should have been stronger resulting in a more intense blue color. Instead, it appeared that this interaction was additive given that they activated the reporter at concentrations equaling the sum of their minimally active ones. Further experimentation will be necessary to determine more precisely the minimally active concentration of CMP199 to confirm that the tested compounds do not act synergistically.

A concentration below 250 nM of DCA must be used in conjunction with CMP442 to distinguish between these two options. However, it is certain that they did not act strongly synergistic, as 50 nM of each, which is below their minimally active concentration, did not induce *GUS* expression. Finally, CMP199 and CMP442 appeared to act additively when used together. If they acted synergistically, a mix of 50 nM of each compound would have induced GUS expression. Instead 50 nM CMP199 and 250 nM CMP442 induced *GUS* in an additive manner. Saturation treatments were revealing about the role each synthetic elicitor plays in the activation of defense. Nonetheless, a more comprehensive defense assay may be necessary to confirm these observations while testing a wider range of concentrations.

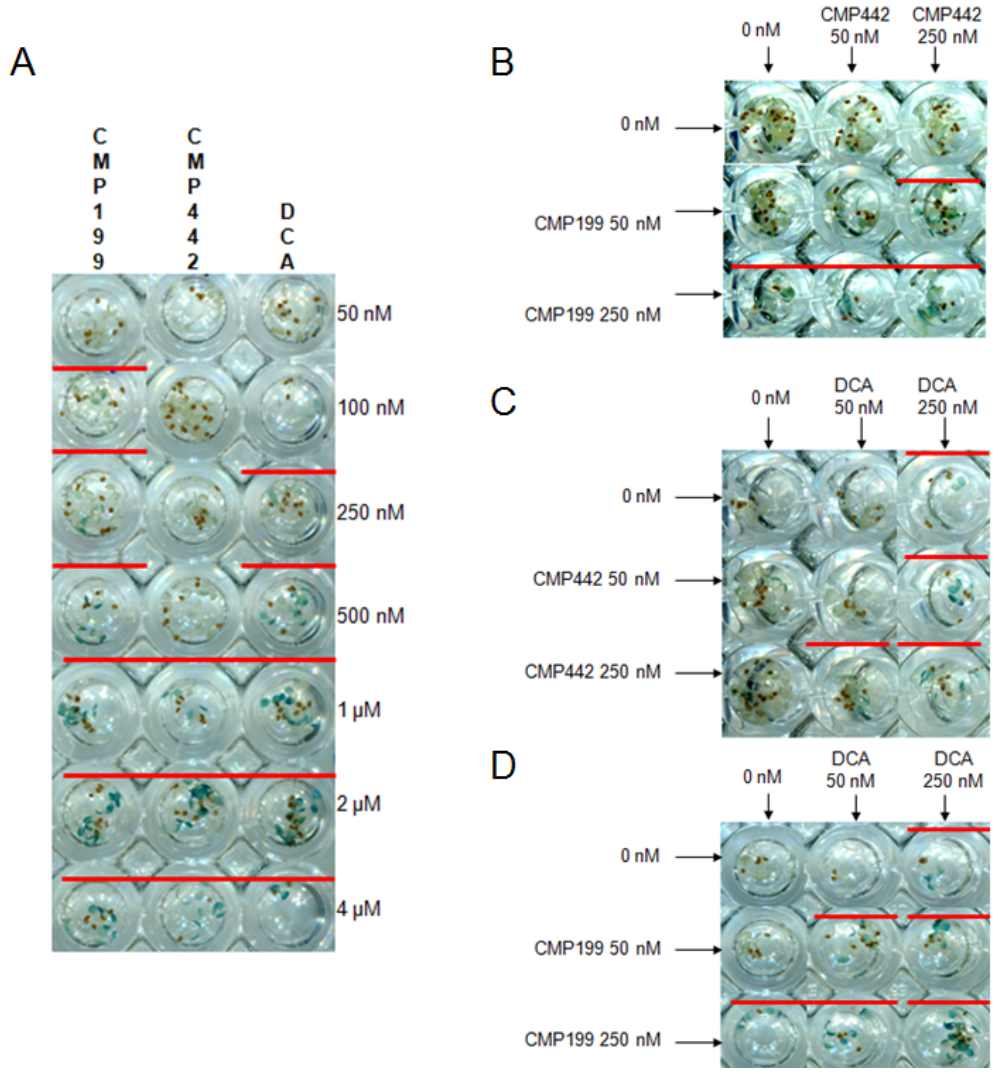



**Figure 1.9: Test for a synergistic effects between DCA, CMP442, and CMP199 in *CaBP22$^{-333}$*::*GUS* reporter gene assays.** 96-well plate wells containing 7-d-old liquid grown *CaBP22$^{-333}$*::*GUS* seedlings after GUS histochemical staining comparing reporter responses to synthetic elicitors after a 24 h-incubation at indicated concentrations. Blue/green color indicates induction of the *GUS* gene expression, a red line was placed above wells where *GUS* staining is evident. **A,** Assay shows a comparison of CMP199, CMP442, and DCA induced *CaBP22$^{-333}$*::*GUS* expression at 50 nM, 100 nM, 250 nM, 500 nM, 1 µM, 2 µM, and 4 µM. This assays identifies the lowest active concentration of each compound. **B-C,** *GUS* expression indicates that when compounds are combined only additive effects are seen. Compounds listed on the left run in rows and compounds listed on the top run in downward columns. All experiments were performed at least three times with similar results. Results of a typical experiment are shown. **B,** CMP442 and CMP199 *CaBP22$^{-333}$*::*GUS induced* expression when varying concentrations of each compound are used. **C,** CMP442 and DCA *CaBP22$^{-333}$*::*GUS induced* expression when varying concentrations of each compound are used. **D,** DCA and CMP199 *CaBP22$^{-333}$*::*GUS induced* expression when varying concentrations of each compound are used.

## DISCUSSION

### Novel Synthetic Elicitors

The complex responses associated with the activation of the plant immune system can be dissected and studied with the aid of synthetic elicitors activating the system at distinct points. These synthetic elicitors may serve as powerful tools to complement traditional genetics used to study plant defense networks. The versatility and reversibility of many synthetic elicitors makes the identification of a wider range and spectrum of these compounds a priority. Building on the success experienced by Knoth et al. (2009) in their identification of DCA, eight more synthetic elicitors were reported on here. Experiments demonstrated that most of these elicitors, excluding CMP608 and CMP384, have the ability to induce p*CaBP22*$^{-333}$*::GUS* expression at concentrations of at least 10 µM. These low concentrations increase the probability of high target specificity, decreasing the likelihood of off-target or unwanted side effects (Burdine and Kodadek 2004). At synthetic elicitor concentrations that induced defense, no herbicidal effects were observed, while all compounds proved to be phytotoxic at higher concentrations. Each new synthetic elicitor discussed here adheres to Lipinski's rule of five (Lipinski, Lombardo et al. 1997) suggesting they may all be readily absorbed by plant cells. These rules state that properties that favor bioactive compounds include a molecular weight of less than 500 g/mol, a lipophilicity (cLogP) value of more than five, less than five hydrogen-bond donors, and less than 10 hydrogen-bond acceptors (Lipinski, Lombardo et al. 1997).

CMP60, CMP994, and CMP202 share a phenyl-1H-pyrazol-1-yl-methanone skeleton as a common substructure. Currently no biological studies exist reporting any activity for this substructure. However, several closely related analogs act as potent inhibitors of human neutrophil elastase, antiinflammatory, analgesic, anticonvulsant, anticholinergic, antihistaminic, antibacterial, anti-epileptic, and antifungal agents (Paintz, Bekemeier et al. 1982; Schepetkin, Khlebnikov et al. 2007; Khlebnikov, Schepetkin et al. 2008; Anandarajagopal, Anbu Jeba Sunilson et al. 2010). CMP384 analogs, but not CMP384 itself, were reported to have antimicrobial activities (Montanari, Cass et al. 2000). Of the remaining compounds, the exact structures or closely related analogs of CMP144, CMP174, and CMP199 are not referenced to in any biological studies. The wide range of structures and their apparent uniqueness offers many promising candidates for potential synthetic elicitors.

In *Hpa*Noco2 defense assays, the novel synthetic elicitor CMP199 out-performed DCA with a lower active concentration. A lower active concentration may reflect a higher degree of target specificity. Also, a major goal of this project was the identification of compounds with different modes-of-action. A suite of functionally distinct synthetic elicitors is likely to allow for more control and flexibility when studying the plant defense network and is likely to facilitate the creation of novel environmentally friendly pesticides. CMP199 will be further examined by other lab members to determine if its mode of action is distinct from that of DCA.

To determine if the available synthetic elicitors acted synergistically, dual combinations of DCA, CMP199, and CMP442 were tested at a range of different concentrations for each compound. This experiment demonstrated that these synthetic elicitors are likely to act in an additive, but not synergistic, manner. As a confirmation of this data, additional assays should be performed testing disease resistance inducibility with these combinations of synthetic elicitors. These experiments are necessary since it is possible that the *GUS* assay is not of sufficient sensitivity to display synergistic effects since. Pathogen assays would provide quantitative data instead of the qualitative data provided by the visually read *GUS* assays.

### *ACID* Genes and Their Putative Roles in Plant Defense

The plant immune system is a highly complex web of interactions that allow plants to modulate their responses to the type of infecting pathogen. Currently, only a small number of defense network components have been identified and characterized. The identification of additional elements that compose this web will lead to a better understanding of plant defense and new strategies to combat diseases. In microarray experiments performed by Knoth et al. (2009), the *ACID* cluster was identified as a set of genes upregulated at time points corresponding to synthetic elicitor-mediated disease resistance. These 137 *ACID* genes were hypothesized to be important for plant defense. More than one quarter (~28%) of the 137 *ACID* members encode kinases (Knoth, Salus et

al. 2009). Of the 16 *ACID* genes examined in this study, 12 encoded protein kinases.

Protein kinases often act as regulatory elements controlling plant defense responses. To increase the probability of success in the functional analysis of *ACID* members, genes encoding protein kinases were preferentially chosen for study. *ACID3* encoded a TIR-NBS-LRR class disease resistance protein and *ACID8* a LRR-receptor like protein kinase*. ACID9* and *ACID13* encoded LRR transmembrane protein kinases and *ACID12 was a* receptor like protein kinase. Additionally, *ACID1, ACID7, ACID11, ACID10, ACID14, ACID15*, and *ACID16* were genes that encoded for proteins with potential kinase activity. The remaining four *ACID* genes did not encode protein kinases. Of the remaining genes: *ACID*2 is an armadillo/beta-catenin repeat family protein, *ACID*4 a remorin family protein, *ACID*6 a member of EXO70 gene family, and *ACID*5 is an ankyrin repeat family protein. These four genes were included based on the availability of two independent homozygous T-DNA insertion lines.

For *ACID1*, *ACID4*, *ACID6*, *ACID7*, *ACID8*, *ACID9*, *ACID10*, *ACID12*, *ACID13*, and *ACID15* both tested T-DNA mutant lines exhibited enhanced *Hpa* susceptibility. This indicated that the mutations in/near the genes themselves were likely responsible for the respective defense phenotypes, as opposed to unknown mutations. Only one of the two tested T-DNA insertion lines for *ACID5*, *ACID11*, and *ACID16* exhibited reduced basal defense to *Hpa*. It is likely that in each case the second line contained its T-DNA insertion in a position not capable

of reducing gene function. For example, the insertion in *acid11*-1 is located in an exon and caused enhanced susceptibility to *Hpa*Noco2. The insertion in *acid11*-2 did not affect *Hpa*Noco2 resistance; this T-DNA located in the promoter of *ACID11* did not to significantly reduce *ACID11* expression. Neither the *acid16-2* nor *acid16-3* line displayed a defense phenotype. The insertions in these two mutants were intronic and might be spliced out of the *ACID16* pre-mRNA (Alonso, Stepanova et al. 2003). In contrast, the insertion in *acid16-1,* which is located in an exon, caused enhanced susceptibility to *Hpa*Noco2. The insertion in *the* promoter in *acid5-2*, but not the exonic insertion in *acid5-1*, resulted in a defense-related phenotype. One possible explanation for this is that the insertion in the promoter abolished proper expression of this gene, while the exonic insertion results in a truncated, but functional, protein. Alternately, the insertion loci predicted by TAIR may not be accurately annotated (Alonso, Stepanova et al. 2003).

Among the *acid mutants* lines that exhibited reduced basal defense phenotype, only *acid4-1*, *acid4-2*, *acid6-1*, *acid6-2*, *acid7-1*, *acid7-2*, *acid8-1*, *acid13-1*, *acid13-2*, *acid15-1*, and *acid15-2* displayed reduced levels of the respective transcripts. The observation that some T-DNA insertions did not affect endogenous *ACID* levels was not entirely unexpected. Wild-type level of accumulation of transcripts does not guarantee that the respective gene retains its wild-type functionality.

While some *ACID* genes were critically important for basal defense against *Hpa*Noco2 none of the tested members of this gene set proved to be required for DCA-mediated immunity to *Hpa*Noco2. This indicated that DCA-mediated upregulation is not essential for the defense function of the respective *ACID* genes. Alternatively, DCA may activate more immune responses than *Hpa*Noco2 recognition in the Col-0 background. Thus, the function of individual *ACID* genes is less important for DCA-mediated immunity than for basal defense.

The moderate DCA-triggered *PDF1.2a* transcript accumulation observed by RT-PCR analyses suggested possible crosstalk between DCA-triggered signaling processes and the JA-defense signaling branch. In a previous microarray study of DCA-triggered transcriptome changes (Knoth et al. 2009), no increases of *PDF1.2a* transcript levels in response to DCA treatment were observed. However, RT-PCR analyses can be more sensitive than microarrays (Czechowski, Bari et al. 2004). Additional studies should be performed to confirm the observations on the possible DCA-inducibility of *PDF1.2a* transcripts. If confirmed, this finding would further support the previous claim that DCA functionally differs from other inducers of SA-dependent defense responses (Knoth et al., 2009).

The role of DCA and some *ACID* genes possibly extends beyond SA signaling to include aspects of JA-responses. While, it can be concluded that ten of the 16 *ACID* genes tested play a role in basal defense, they do not appear to contribute to the regulation of SA-inducible defense-related genes. Their role in

JA signaling is currently unknown. These ten *ACID* genes could instead be involved in later defense signaling (e.g. SAR) or in crosstalk between defense pathways. It should also be examined if INA–mediated immunity is compromised in the *ACID* mutants. INA is functionally distinct from DCA, acting through a SA/NPR1-dependent pathway, while DCA preferentially acts via the WRKY70 transcription factor and not through NPR1 (Knoth et al., 2009). Any differences in the ability of INA to induce resistance in *acid* mutant lines could provide valuable information about unknown circuits within the SA-controlled sector of the plant defense network. In addition, to test if the *ACID* genes are JA inducible a MeJA treatment should be performed. In addition, the condition of the *ACID* genes should be examined in some key JA mutants, such as *coi1*.

*Hpa*Noco2 defense assays clearly showed that 10 of 16 tested *ACID* genes are required for basal defense. Three additional *ACID* members were found that might also be involved in basal defense. The remaining three of the 16 *ACID* genes analyzed (*acid2, acid3, acid14)*, which exhibited no enhanced susceptibility in any tested allele, will not be discussed below. The 13 *ACID* genes with demonstrated or likely roles in basal defense include: *ACID1*, *ACID4*, *ACID5*, *ACID6*, *ACID7*, *ACID8*, *ACID9*, *ACID10*, *ACID11*, *ACID12*, *ACID13*, *ACID15*, and *ACID16*. Several of these *ACID* genes have been subjects of previous studies and have proposed roles in abiotic or biotic stress responses.

### *ACID* Genes in Published Manuscripts

Transcripts of all members of this set except for *ACID4* and *ACID15* were reported to be upregulated after infection with Cabbage Leaf Curl Virus consistent with a potential role in defense (Ascencio-Ibanez, Sozzani et al. 2008). *ACID5*, *ACID13*, and *ACID16* were more highly expressed in the *sni1* mutant than in wild type plants (Li, Zhang et al. 1999). Sni1 is a negative regulator of SAR required to maintain low basal expression of *PR* genes (Li, Zhang et al. 1999; Mosher, Durrant et al. 2006). Suppression of *ACID5*, *ACID13*, and *ACID16* transcript levels by Sni1 may indicate a role for these genes in SAR. In microarray experiments, *ACID5* and *ACID12* were upregulated after whitefly feeding (Kempema, Cui et al. 2007). *ACID6* (Exo70B2) is a member of a group of genes that have a calmodulin-binding/CGCG Box DNA-binding protein involved in multiple plant signaling pathways (Yang and Poovaiah 2002). Exo70B2 has also been reported to trigger a wound-like response in cell cultures (Guan and Nothnagel 2004) and linked to exocytosis-related processes during plant-pathogen interactions (Guan and Nothnagel 2004; Synek, Schlager et al. 2006; Chong, Gidda et al. 2010; Wang, Ding et al. 2010; Pecenkova, Hala et al. 2011).

*ACID7* (CPK6) is known to be upregulated in response to drought and salt stress and plays a role in guard cell functions related to MeJA and ABA signaling (Bray 2002; Boisson, Giglione et al. 2003; Mori, Murata et al. 2006; Xu, Tian et al. 2010; Munemasa, Hossain et al. 2011). CPK6 also functions redundantly with other CDPKs in signaling after defense induction by the MAMP flg22 (Boudsocq,

Willmann et al. 2010). In double, triple, and quadruple CDPK mutants, that include a mutation in CDPK6, a reduction in defense-related release of ROS was observed. No effects on the oxidative burst were detectable in the respective single mutants, indicating that these protein kinases act redundantly in MAMP-triggered ROS production. Mutation of CDPK6 combined with mutations in additional CDPKs, also increased growth of *Pseudomonas syringae* DC3000 bacteria, further confirming that these protein kinases act redundantly in MAMP-triggered immunity (Boudsocq, Willmann et al. 2010).

*ACID10* (CDPK4) was found to play a role in the regulation of ABA and MeJA signaling (Zhu, Yu et al. 2007; Munemasa, Hossain et al. 2011). Transient expression of the CDPK barley ortholog *HvCDPK4* in *Nicotiana benthamiana* triggered kinase-dependent cell death in tobacco leaves (Freymark, Diehl et al. 2007). Also, *CaCDPK4* was implicated in the regulation of defense responses against the avirulent bacterial pathogen *Xanthamonas axonpodis* pv. *glycines* in pepper (Chung, Oh et al. 2007). Transcripts of *ACID12* (*BIR1*)*, ACID13, ACID16* were demonstrated to be induced when butterflies of *Pieris rapae* laid eggs on Arabidopsis leaves (Little, Gouhier-Darimont et al. 2007). Finally, *ACID12* (*BIR1*) negatively regulates multiple plant resistance signaling pathways (Gao, Wang et al. 2009). This gene is involved in SAR and is induced in Arabidopsis after the infiltration of the oomycete necrosis and ethylene-inducing peptide 1-like proteins, which play a role in triggering acquired resistance (Qutob, Kemmerling et al. 2006). Transcripts of *ACID12* were also found to be upregulated by whitefly

infestation and downregulated by chloroplastic- and extracellular-sourced ROS in Arabidopsis (Qutob, Kemmerling et al. 2006; Bechtold, Richard et al. 2008).

### *ACID* Genes in Published Microarray Data

Public microarray data provided on the Botany Array Resource website (BAR) indicated that most of the 13 *ACID* genes with likely roles in basal defense were found to be highly upregulated during osmotic stress, wounding, and UV-B treatment (Toufighi, Brady et al. 2005). In addition, BAR showed that treatment with SA and treatment with ethylene inhibitors also induced their expression. The pathogens *Pseudomonas syringae*, *Phytopthora infestans*, *Erysiphe orontii,* and bacterial- and oomycete-derived elicitors also stimulated up-regulation of these *ACID* transcripts. As indicated above, *ACID* genes have highly varied roles, even when focusing on plant defense. Only *ACID6*, *ACID7*, and *ACID12* have been studied in mutant assays examining their specific roles in defense. The remaining *ACID* genes were only identified in microarray experiments as responsive to defense-related stimuli and some of these *ACID* genes are suspected to have roles in SAR. A role SAR is consistent with is the likely role of these genes in the SA branch of the defense network.

### Putative Role for *ACID* genes in Defense Signaling

Similarities exist between receptors used by both plants and animals to recognize disease-causing microbes. These receptors represent the first of

several mechanisms to recognize pathogen attack and respond by activating defense signaling pathways. Protein kinases are integral for a plant's ability to respond to pathogens and must be carefully regulated to prevent autoimmunity. Protein kinases linked to pathogen receptors contain an alteration within their critical kinase functional domain uncommon to protein kinases not associated with defense induction. These protein kinases linked to pathogen receptors can be categorized based on the lack of a conserved arginine residue (non-RD) in kinase subdomain VI. Other protein kinases can be characterized by the presence (RD) of this residue or the lack of residues required for catalytic activity known as alternative catalytic function (ACF) kinases.

There is a correlation between pelle/RLKs in plants with roles in early events of innate immunity that bear non-RD motifs (Dardick and Ronald 2006). Of 38 receptor kinases in plants with suggested functions, six are believed to have roles in disease resistance as PRRs and all fall into the non-RD class: XA21, XA26, Pi-2d, FLS2, PR5K, and LRK10 (Dardick and Ronald 2006). The remaining plant receptor kinases are of the RD or ACF type and function in development, pollen recognition, steroid perception, stress/pathogen responses, and interactions with symbiotic organisms. The pervasiveness of non-RD kinases in PRRs implies that RD kinases are not suited for early defense signaling in plants (Dardick and Ronald 2006). Of the seven *ACID* protein kinase genes required for basal defense to *Hpa, ACID1, ACID7, ACID8,* and *ACID10* are RD kinases*. ACID15* is the only non-RD kinase in this set, while *ACID9* and *ACID12*

are both ACF kinases. If RD kinases are not well-suited for early defense signaling, and many of the studied *ACID* genes are RD kinases, this supports our hypothesis that the *ACID* genes play some role in signaling processes after the initial recognition event (Dardick and Ronald 2006).

This study reported on at least seven novel components of the plant defense network whose exact roles are currently unknown. A clue to the function of the 13 genes with putative roles in plant defense could be provided by the types of genes not represented in the *ACID* cluster. As reported by Knoth et al. (2009), genes with GO attributes annotated for processes associate with upstream/early defense responses like HR, cell death, peroxidases, response to ROIs, and SA biosynthesis were absent. Their lack in the *ACID* cluster may be indicative that the genes activated at this time point are important for later defenses, such as SAR or local defense signaling processes occurring after ROI and SA production and independent of HR. Studies should be performed to see if these genes are important for these suggested roles. As mentioned above, three of the ten *ACID* genes (*ACID6*, *ACID7*, *ACID12)* important for basal defense are previously described parts of the plant immune system (Guan and Nothnagel 2004; Qutob, Kemmerling et al. 2006; Synek, Schlager et al. 2006; Bechtold, Richard et al. 2008; Boudsocq, Willmann et al. 2010; Chong, Gidda et al. 2010; Wang, Ding et al. 2010; Pecenkova, Hala et al. 2011). *ACID1*, *ACID4*, *ACID8*, *ACID9*, *ACID10*, *ACID13*, and *ACID15* are completely novel components of the

plant defense only reported to exhibit elevated transcript levels in response to a variety of defense-related stimuli.

The immunity mediated by the *R*-gene *RPP7* (recognizes *Hpa*Hiks1*)* was unaltered in the *acid* mutants. In addition, to determine if any of the *acid* single mutants were altered in ETI mediated by *RPP4* (recognizes *Hpa*Emoy2) assays were performed (data not shown). *R*PP4 and *RPP7* represent different types of ETI-related defense mechanisms. While *RPP4*-mediated immunity is dependent on SA, *RPP7* mediated immunity is fully SA-independent (Eulgem et al., 2004). Neither *Hpa*-isolate was able to successfully infect WT Col-0, which expresses both *RPP4* and *RPP7*. Each tested *acid* mutant showed full resistance to infection by both *Hpa*Hiks1 and *Hpa*Emoy2 displaying distinct HR sites in each case (Figure 1.3 and data not shown). Thus, these data demonstrated that the 16 tested *ACID* genes were not required for immunity to *Hpa* mediated by *RPP4* or *RPP7*.

A subgroup of genes within the *ACID* cluster was identified which is important for Arabidopsis basal defense, but not *R*-gene mediated-defense against *Hpa*. Seven of the ten *ACID* genes for which both tested mutant alleles displayed enhanced susceptibility to *Hpa*Noco2 represented completely novel components of the plant immune system. The upregulation of *PDF1.2a* by DCA suggests a possible role for the *ACID* genes in defense pathway crosstalk. Together this information pointed to the fact that the significance of these genes may go beyond their novelty as defense components. These genes may

represent completely new types of regulatory mechanisms. Additionally, this study elucidated that through the identification of novel synthetic elicitors such as DCA, new components of the plant defense network can be identified. A better understanding of defense responses is beneficial for combating diseases on crop plants. Here, a collection of structurally distinct synthetic elicitors exhibiting low active concentrations was introduced. Within this group of novel elicitors, candidates will likely be identified exhibiting unique modes of action. This will increase the tools available for the dissection of the plant defense network, allowing for an even finer examination of plant defense mechanisms.

## MATERIALS AND METHODS

### Plant Growth Conditions, Plant material, Pathogen Infections and Staining

Arabidopsis (*Arabidopsis thaliana*) plants were grown on soil or ½MS media under fluorescent lights (16 h of light/8 h of dark, 23°C, 100 µE $m^{-2}$ $s^{-1}$) unless otherwise noted. The Arabidopsis lines *nahG* (Delaney, Uknes et al. 1994), *pad4-1* (Glazebrook, Zook et al. 1997), *Oy-1* (Holub, Beynon et al. 1994) and *rpp7-15* (Alonso, Stepanova et al. 2003) were described previously. *Hyaloperonospora arabidopsidis* (*Hpa*) was grown and propagated as described previously (McDowell et al., 2000). Plants were spray infected with *Hpa* spore suspensions at 5 x $10^4$ spores/ml for *Hpa*Emoy2 and *Hpa*Hiks1 (one-week-old plants) and 2 x $10^4$ spores/ml for *Hp*Noco2 (three-week-old plants) with Preval sprayers (http://www.prevalspraygun.com). Plants were scored for *Hpa* growth 7 dpi by trypan blue staining (McDowell, Cuzick et al. 2000; Torres, Dangl et al. 2002), visual sporangiophore counts, or by counting spores/seedlings. Using a hemacytometer to determine the spore density of a suspension of 10 infected seedlings per 1 ml of water. The Student's *t*-test was used to determine if the effects of the mutations or chemical treatments on sporulation were statistically significant.

**Analysis of GUS Activity and Treatment of Homozygous T6 p*CaBP22*$^{-333}$-::*GUS* plants with Synthetic Elicitors**

Arabidopsis seedlings were grown in 96-well plates, treated with compounds, and then stained (histochemically) for *GUS* expression as was previously described (Knoth, Salus et al. 2009).

**Compound Treatment Before Pathogen Infection**

Stock solutions of all compounds were prepared in 100% DMSO and stored indefinitely at -20˚C. Stock solutions were diluted in water and 2 ml/pot sprayed on soil-grown plants at the indicated times and concentrations with Preval sprayers. Final DMSO concentrations never exceeded 2%. To test for chemically induced disease resistance, the plants were sprayed with 2 ml/pot of chemicals at the indicated concentrations and times prior to pathogen challenge. Disease symptoms were analyzed as described above.

**RNA Isolation and Reverse-Transcription (RT)-PCR Analysis**

RNA was isolated from seedlings as was previously described (Chomczynski and Sacchi 1987). The DNase digestions were performed with 1 µg of RNA using Deoxyribonuclease I, Amplification Grade (Invitrogen; http://www.invitrogen.com) to remove DNA. 1 µl of oligo(dT$^{18}$) (100 pmol/µl) and 1 µl 10 mM dNTP mix was used for pretreatment of the digested RNA. The resulting mixture was used directly for reverse transcription using Maxima

Reverse Transcriptase and Ribolock RNase Inhibitor following the manufacturer's instructions (Fermentas, USA). All PCRs used the following thermalcycler program, deviating as indicated for annealing temperatures and cycles: 94°C for 1 min; X cycles of 95°C for 30 s, annealing temperature of X °C for 1 min; and 72°C for 40 s. PCR products were electrophoresed on 1% agarose gels containing 0.5 $\mu$g/ml ethidium bromide unless otherwise indicated. Negative controls omitting reverse transcriptase in the cDNA production process and PCR without cDNA yielded no products.

| Primer Name | Primer Sequence | Annealing Temperature | Fragment Size | Cycle Number | Agarose Concentration |
|---|---|---|---|---|---|
| RT-*ACT8*-F<br>RT-*ACT8*-R | 5'-ATGAAGATTAAGGTCGTGGCAC-3'<br>5'-GTTTTTATCCGAGTTTGAAGAGGC-3' | 60°C | 425-bp | 21 | 1% |
| RT-*CaBP22*-FP<br>RT-*CaBP22*-RP | 5'-CGGAACCATCAATTTCACTGAGT-3'<br>5'-CAAAGTGCCACCAGTTGTGTCAT-3' | 63°C | 301-bp | 24 | 1% |
| RT-*WRKY70*-FP<br>RT-*WRKY70*-RP | 5'-AACGACGGCAAGTTTGAAGATTC-3'<br>5'-TTCTGGCCACACCAATGACAAGT-3' | 63°C | 477-bp | 24 | 1% |
| RT-PR1-LP<br>RT-PR1-RP | 5'-TTCCCTCGAAAGCTCAAGATAGC-3'<br>5'-GGCACATCCGAGTCTCACTGAC-3' | 54°C | 338-bp | 33 | 1% |
| RT-PDF1.2a-LP<br>RT-PDF1.2a-RP | 5'-TAAGTTTGCTTCCATCATCACCC-3'<br>5'-GTGCTGGGAAGACATAGTTGCAT-3' | 55°C | 209-bp | 32 | 4% |
| PDF1.2b-LP<br>RT-PDF1.2b-RP | 5'-ACGCTGCTCTTGTTCTCTTTGCA-3'<br>5'-AAGTACCACTTGGCTTCTCGCAC-3' | 55°C | 84-bp | 30 | 4% |
| PDF1.2c-LP<br>RT-PDF1.2c-RP | 5'-GTCTGCTACCATCATCACCTTCC-3'<br>5'-TTCCGCAAACGCCTGACCATGTC-3' | 55°C | 129-bp | 33 | 4% |
| RT-JEDI1-LP<br>RT-JEDI1-RP | 5'-ACCAAGCTGGTAATGGTTGC-3'<br>5'-CCACCGTTACATTCCATTCC-3' | 55°C | 460-bp | 32 | 1% |
| RT-LURP1-LP<br>RT-LURP1-RP | 5'-CTCGACGACTCTTGTGTTGTCTAC-3'<br>5'-GCTAAGGGCATGTGTTTGTATTTA-3' | 61°C | 469-bp | 36 | 1% |
| RT-ACID1-LP<br>RT-ACID1-RP | 5'-ACTTGGCCAGCCATTTTAAGT-3'<br>5'-AGACGGACTTCCCAACTCATT-3' | 63°C | 533-bp | 22 | 1% |
| RT-ACID4-LP<br>RT-ACID4-RP | 5'-AAGTTCCGGTGGAGAAACCT-3'<br>5'-GTTCCAGTGGCACGGTACTT-3' | 63°C | 517-bp | 23 | 1% |
| RT-ACID6-RP<br>RT-ACID6-LP | 5'-AATCGTGATCGAGGCGTTAC-3'<br>5'-GAGGGTCACGTCGGATTAAA-3' | 63°C | 593-bp | 24 | 1% |
| RT-ACID7-LP<br>RT-ACID7-RP | 5'-CGAGGAGAATTCCAAATCCA-3'<br>5'-CCTCAACAACACCGACAATG-3' | 63°C | 533-bp | 23 | 1% |
| RT-ACID8-LP<br>RT-ACID8-RP | 5'-TCCCACCTGAGATTTGGAAC-3'<br>5'-TCTGGAATTGGACCTTGGAG-3' | 63°C | 511-bp | 23 | 1% |
| RT-ACID9-LP<br>RT-ACID9-RP | 5'-CCCAGAAGGGTTCGTTACAA-3'<br>5'-GGTGTTTTGTGAGCCCTGAT-3' | 63°C | 530-bp | 24 | 1% |
| RT-ACID10-LP<br>RT-ACID10-RP | 5'-TGTCACCCTTGGATTGTTGA-3'<br>5'-TCCCAACACCATCTCCTTTC-3' | 63°C | 571-bp | 23 | 1% |
| RT-ACID12-LP<br>RT-ACID12-RP | 5'-AGCATTAGTCGTTGGGGTTG-3'<br>5'-TTCGAGCTTATGTTGCGATG-3' | 63°C | 582-bp | 23 | 1% |
| RT-ACID13-LP<br>RT-ACID13-RP | 5'-GCTCGACCTCTCACACAACA-3'<br>5'-TTGCTTCACAACTGGCTTTG-3' | 63°C | 571-bp | 23 | 1% |
| RT-ACID15-LP<br>RT-ACID15-RP | 5'-TGCCGGGTTTAGTTACCTTG-3'<br>5'-GACGACCCTTTCTTCCTTCC-3' | 63°C | 523-bp | 25 | 1% |

### Genomic DNA Extraction and Selection of *ACID* Insertion Mutants

Two independent *ACID* insertion mutants were obtained from the Arabidopsis Biological Resource Center (ABRC) (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm) for each tested *ACID* gene. Genomic DNA was extracted from four-week-old soil-grown seedlings, as described previously using the Quick DNA Prep for PCR (Weigel and Glazebrook 2002). $T_3$ individuals homozygous for the respective insertions were selected by polymerase chain reaction (PCR) using a T-DNA-specific primer and a pair of gene-specific primers (Table 1.1) flanking the insertion site (Alonso, Stepanova et al. 2003). The homozygous $T_3$ individuals were then selfed to obtain their $T_4$ progeny, which were used for experiments. All genotyping PCRs used the following program: 94°C for 30 s; 34 cycles of 94°C for 30 s, annealing temperature of 53°C for 30 s; and 72°C for 2 min. The PCR was complete at 72°C for 10 min.

### Additional Synthetic Elicitor Purchase

CMP144, CMP994 CMP60, CMP202, CMP384, CMP608, CMP236, and CMP442 were all ordered from TimTec. CMP199 and DCA were ordered from Sigma-Aldrich. CMP174 was ordered from Chembridge.

## CHAPTER 2: The synthetic elicitor 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (CMP442) triggers disease resistance and strong hormesis-like effects in *Arabidopsis thaliana*[1]

### SUMMARY

Plant innate immunity depends on a network of genes that regulate and execute defense reactions but many contemporary crops have lost substantial parts of pathogen immunity due to unbalanced breeding efforts. Consequently, plant diseases result in dramatic losses in crop production every year and to compensate pesticides are used. Many pesticides currently in use are carcinogenic, leading to undesirable ecological side effects. The use of environmentally safe plant defense inducing chemicals (synthetic elicitors) offers an attractive alternative for disease control regimes. A chemical genomics-based approach to identify and utilize new types of synthetic elicitors for the dissection of the plant immune system and the development of novel types of pesticides has been initiated. By high-throughput chemical screening 114 potential synthetic elicitors were previously identified that induce a pathogen-responsive reporter gene in transgenic Arabidopsis. Here a new representative of these elicitors, 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (CMP442) is reported on. Like the previously discovered DCA, CMP442 is able to quickly and transiently induce disease resistance, has a distinct mode-of-action, and is structurally unique. CMP442 unlike DCA, can be synthesized quickly and easily

with a high degree of purity. At low doses CMP442 enhances the growth of roots and aerial parts of *Arabidopsis thaliana* and *Solanum lycopersicum*, while high concentrations inhibited plant growth. These effects are reminiscent of the hormetic-like response, which is characterized by a low dose stimulatory or beneficial effect of a wide range of stimuli that are toxic or inhibitory at higher concentrations. The ability of CMP442 to beneficially affect both plant immunity and development points to crosstalk between both types of biological processes and may allow for the design of novel types of multi-functional agrochemicals.

## INTRODUCTION

Plants being sessile organisms lack the ability to flee from external threats. Therefore they had to evolve intricate immune systems to protect themselves against the array of challenges they face. These external threats vary from the abiotic, such as drought or cold, to the biotic, such as disease-causing pathogenic microbes. Plant innate immunity depends on a network of functionally interconnected genes involved in the regulation and execution of defense reactions (Glazebrook, Chen et al. 2003; Tsuda, Sato et al. 2009). A fundamental form of innate immunity in plants involves conserved molecular signatures common to many pathogens termed microbe-associated molecular patterns (MAMPs), which are recognized by pattern recognition receptors (PRRs) on the surface of plant cells (Jones and Dangl 2006; Hein, Gilroy et al. 2009). Upon pathogen recognition, PRRs activate a comprehensive set of defense reactions called pattern-triggered immunity (PTI).

Many pathogens independently acquired the ability to attenuate PTI through the release of effector molecules, thus enabling infection (effector-triggered susceptibility, ETS). In this case the pathogen is virulent and the host susceptible (compatible interaction) (Chisholm, Coaker et al. 2006; Boller and He 2009). During compatible interactions plants can still mount a weakened immune response, called basal defense. Basal defense can limit the spread of pathogens but is not capable of fully preventing disease (Glazebrook 2001; Ahmad, Van Hulten et al. 2011). As a countermeasure to ETS, plants evolved the ability to

recognize the presence of effectors, directly or indirectly, by highly specific plant resistance (R) proteins, which mediate a strong immune response termed effector-triggered immunity [ETI; (Jones and Dangl 2006)].

Numerous studies have shown that ETI, basal defense, and PTI utilize a common set of signaling components including multiple regulatory phytohormones, which include salicylic acid (SA), and jasmonic acid (JA) (Nimchuk, Eulgem et al. 2003). While basal defense seems to be a weakened form of PTI, ETI has been proposed to result from boosted basal defense- or PTI-associated responses (Tao, Xie et al. 2003; Jones and Dangl 2006; Shen, Wan et al. 2007). The plant immune system can be subdivided into various defined interacting sectors (Tsuda, Sato et al. 2009; Keinath, Kierszniowska et al. 2010). For example, distinct defense signaling sectors dependent on early MAMP-activated MAP kinases or the messenger molecules SA or JA, have been described. Interestingly, some of these sectors were found to largely interact in an additive or synergistic fashion during PTI, while they are partially antagonistic to each other during ETI (Tsuda, Sato et al. 2009). The latter phenomenon seems to allow for compensatory effects if a defined sector is disabled due to interferences with pathogen effectors.

While plants have generally developed highly effective mechanisms to cope with pathogens, contemporary crops often have lost substantial parts of their innate immune system due to unbalanced breeding efforts (Hammond-Kosack and Parker 2003). Consequently, plant diseases cause dramatic losses

in crop production every year. In the United States 500 million kg of various pesticides are applied each year at a cost of $10 billion to farmers. Despite this staggering price tag, more than a third (37%) of all potential food crops are still destroyed by diseases (Pimentel 2005). The lingering residues of pesticides have been a health concern often covered by the media in recent years (Damalas and Eleftherohorinos 2011). Many pesticides currently in use are carcinogenic and rely on direct anti-pathogenic activity, which often leads to undesirable ecological side effects that can have far reaching consequences both for humans and the environment (Casida 2009). This disquiet over the dangers of pesticides has spawned considerable interest in alternative methods of disease control (Hart 2005; Pimentel 2005).

The use of environmentally safe plant defense-inducing chemicals (synthetic elicitors), which protect plants from diseases by boosting their natural innate immune responses, instead of being toxic to pathogens, offers an attractive alternative for disease control regimes. Examples of such compounds include 2,6-dichloroisonicotinic acid (INA) and acibenzolar-*S*-methyl benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Métraux, Ahl Goy et al. 1991; Ward, Uknes et al. 1991; Uknes, Mauch-Mani et al. 1992). While these compounds do induce defense responses in some plants, their effects are not universal across all species (Heil, Hilpert et al. 2000; Achuo, Audenaert et al. 2002) and their success in crop protection under field conditions has been modest (Wiese, Bagy et al. 2003). In addition, BTH has been shown to be

phytotoxic in some situations and INA and BTH proved to negatively affect plant growth (Ryals, Neuenschwander et al. 1996; Cole 1999; Godard, Ziadi et al. 1999; Heil, Hilpert et al. 2000). The shortcomings of these established synthetic elicitors make the identification of additional types of plant defense inducing compounds very appealing.

A chemical genomics-based approach to identify and utilize new types of synthetic elicitors for the dissection of the plant immune system and the development of novel types of pesticides was initiated (Knoth, Salus et al. 2009). By high-throughput chemical screening, 114 drug-like organic compounds that induce the pathogen-responsive *pCaBP22*$^{-333}$*::GUS* reporter gene in transgenic Arabidopsis were identified. One of them, 3,5-dicholoroanthranlilic acid (DCA) triggered fast, strong and transient disease resistance against as the pathogenic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) and the bacterial pathogen *Pseudomonas syringae* (Knoth, Salus et al. 2009). Experiments of the defense-inducing activity of DCA in various Arabidopsis defense mutants showed that this synthetic elicitor activates a signaling route dependent on the WRKY70 transcription factor. In contrast to INA and BTH-mediated immunity, which is fully dependent on the transcriptional co-factor NPR1 (Cao, Bowling et al. 1994; Kohler, Schwindling et al. 2002), DCA-mediated immunity is only partially NPR1-dependent (Knoth, Salus et al. 2009). Thus, the mode-of action utilized by DCA in defense induction is distinct from that of INA and BTH.

In this chapter another novel synthetic elicitors, 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (CMP442) is described. Like DCA, CMP442 was also able to quickly and transiently induce disease resistance. However its mode-of-action was distinct from that of DCA. CMP442 was synthesized quickly and easily with a high degree of purity. In addition, low doses of CMP442 enhanced the growth of roots and aerial parts of both Arabidopsis and *Solanum lycopersicum* (tomato), while high concentrations inhibited plant growth. These effects were reminiscent of the general phenomenon of hormesis, which has been described in various biological systems for low doses of a wide range of stimuli that are toxic or otherwise detrimental at higher doses (Calabrese 2009; Calabrese and Blain 2009; Calabrese and Blain 2011).

## RESULTS

### CMP442, a Novel Small Molecule Elicitor of *CaBP22*$^{-333}$::*GUS* Expression

In a screen performed by Knoth et al. (2009), 114 compounds were identified that reproducibly induced *GUS* expression in the transgenic p*CaBP22*$^{-333}$::*GUS* Arabidopsis line. One of them, 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (CMP442) had not been reported as a plant defense inducer (synthetic elicitor) and had a chemical structure distinct from that of the previously characterized DCA (Knoth, Salus et al. 2009). CMP442's unique chemical structure was confirmed via GC/MS analysis by the Mass Spectrometry Facility at the University of California-Riverside (Figure 2.1).

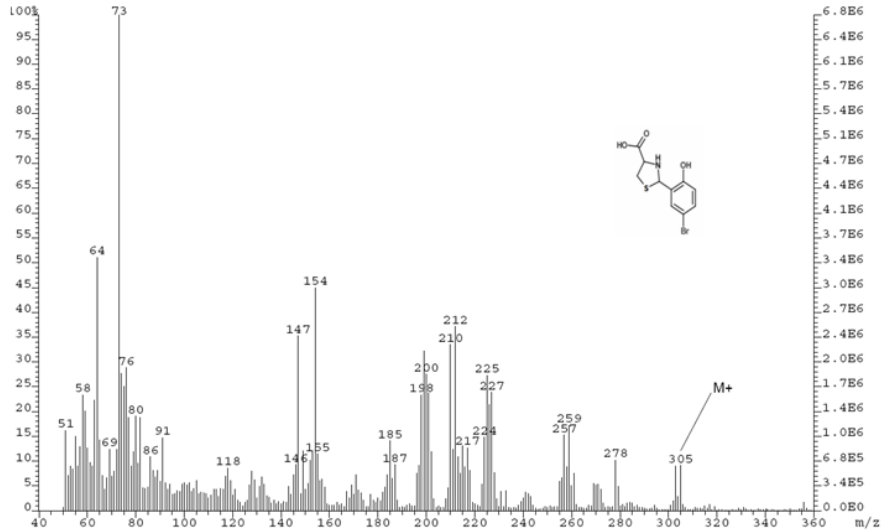



**Figure 2.1: GC/MS Chromatogram (TIC) of CMP442 analysis determined the structure.** Major peaks: 73 DMF, 80 Br, 91 thiazolidine, 118 4-thiazolidine carboxylaldehyde, 146-147 2-methyl (4S)-4-thiaxolidine carboxylic acid, 154-155 2,2-(aminosulfanylmethyl)phenol 185-187 4-bromo-3-methyl phenyl, 198-200 3-(aminomethyl)-4-bromophenol, 210-212 2-phenyl-(4S)-4-thiazolidine carboxylic acid, 215-217 2-[(1S)-1-aminoethyl]-4-bromo-phenol, 225-227 2,3-(hydroxyphenyl)-(4S)-4-thiazolidine carboxylic acid, 257-259 2-(2-bromophenyl)-thiazolidine-4-methyl, 303-305 (2S,4.S)-2-(5-bromo-2-hydroxyphenyl)-1,3-thiazolidine-4-carboxylic acid.

To quantify the bioactivity of CMP442 in comparison to DCA, assays under saturation treatment conditions were performed. CMP442 activated GUS expression at concentrations as low as 1 µM within 24 h in one-week-old p*CaBP22*$^{-333}$::*GUS* seedlings grown in liquid growth media. CMP442 was less potent than the previously described DCA but more potent than SA (Figure 2.2A and Figure 1.8A). To examine CMP442 induced phytotoxicity, Col-0 seedlings were stained after saturation treatments with trypan blue. Dark blue staining, indicating cell death was observed in 100% of the seedlings treated for 24 h with 300 µM CMP442 (Figure 2.2B). No cell death was observed at concentrations resulting in reporter activation (1-100 µM), this indicated that CMP442-induced phytotoxicity is not responsible for its effect on p*CaBP22*$^{-333}$::*GUS* expression. Based on these results and its unique chemical structure, CMP442 warranted additional experimentation.

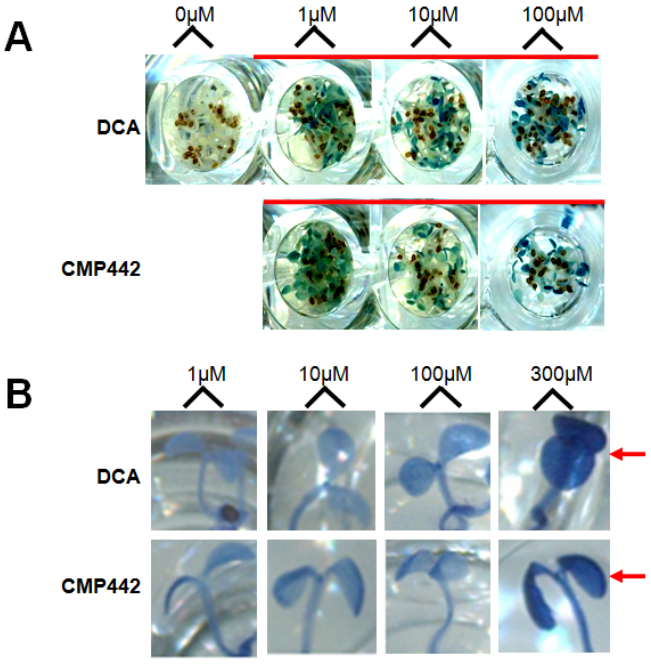



**Figure 2.2: Analysis of CMP442 activity in saturation treatments.** Wells from a 96-well screening plate contained 7-d-old liquid-grown *CaBP22*$^{-333}$::*GUS* seedlings after a 24 h incubation at indicated compound concentrations. **A**, Blue/green color of cotyledons indicated induction of the *GUS* gene expression (all chlorophyll was bleached out of plants during the ethanol-destaining process). The red line above wells indicated GUS staining. **B**, Trypan blue staining. Dark blue/black color of the cotyledons indicates cell death (toxicity) and is pointed to by red arrows. The seed coats of seedlings always stain blue/black and can been seen in some images. All staining experiments were performed at least three times with similar results.

### CMP442 Induces Rapid and Transient Resistance to *Hpa*

CMP442 was further examined to determine if, like DCA, it had the ability to induce pathogen resistance in soil-grown plants. Col-0 plants were sprayed with 10 to 100 µM CMP442 prior to infection with the virulent *Hpa* isolate Noco2. Plants treated with as little as 10 µM CMP442 exhibited a 38% reduction in spores 7 days post infection (dpi) (Figure 2.3). To determine if CMP442 differed

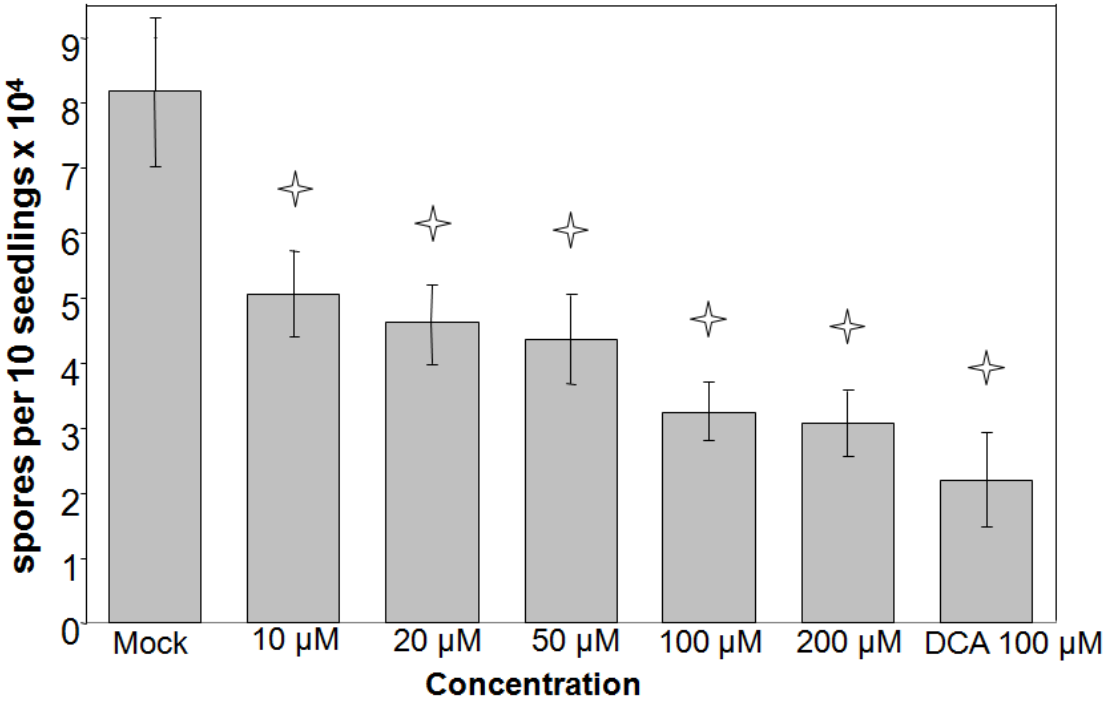



**Figure 2.3: CMP442 induced disease resistance against the virulent *Hpa* isolate Noco2**. Three-week-old soil-grown Col-0 seedlings were sprayed with CMP442, DCA, or control (1%-DMSO)-at the indicated concentrations 24 h prior to infection with *Hpa*Noco2 (2 x $10^4$ spores/ml; 2 ml per pot). Spores were counted 7 dpi. Mean and SE values were calculated from a minimum of three biological replicates. The Student's *t*-test ($p<0.05$) was used to determine significant differences between mock and compound-treated plants. Concentrations that show significantly lower levels of spores compared to the control treatment are indicated by a star.

from DCA in the kinetics of defense induction, Col-0 was sprayed once with 100 µM of INA, DCA, CMP442, or water (mock). These treatments were initiated at 1 h, 3 h, 1 d, 3 d, or 6 d prior to pathogen challenge (Figure 2.4). It should be noted that the water treatment itself diminished spore growth when time points between

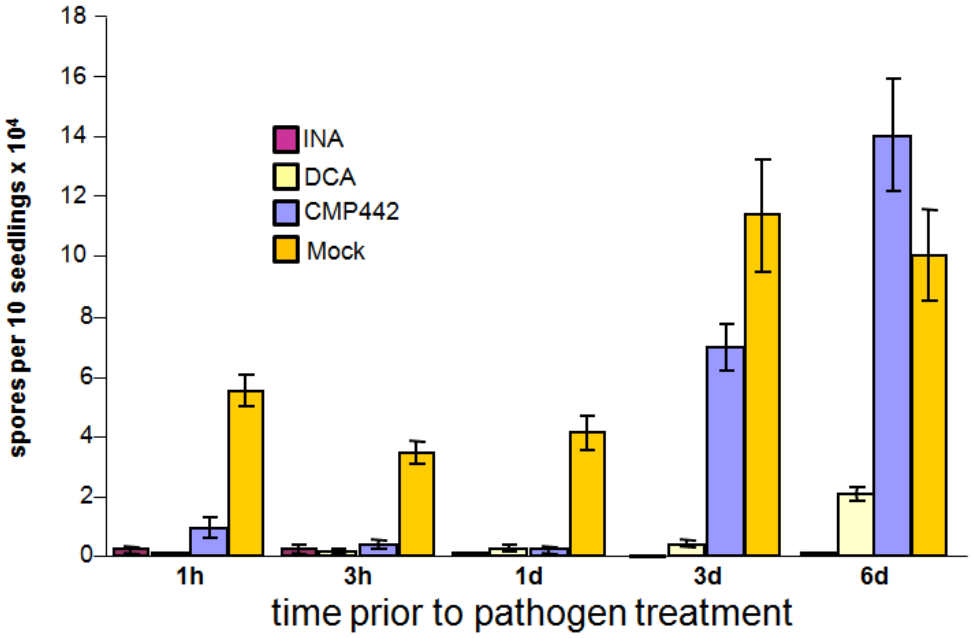



**Figure 2.4: Kinetic analysis of chemically induced disease resistance.** Experiments were conducted with three-week-old soil-grown Col-0 seedlings sprayed with 100 µM CMP442, DCA, INA, or Mock (1%-DMSO) at the indicated times prior to being sprayed with *Hpa*Noco2 (2 x $10^4$/ml; 2 ml per pot). Spores were counted 7 dpi. Mean and SE values were calculated from a minimum of three biological replicates and the average of those is shown above. The Student's *t*-test ($p<0.05$) showed significant differences for all of the synthetic elicitors tested relative to the mock, except for 6 d after CMP442 treatment.

pathogen treatment and compound pretreatment were less than one day. This effect may have been due to residual liquid coating seedlings before being sprayed with the pathogen. It is also possible that the act of soaking the

seedlings for a long period of time so close together elicited a stress response resulting in a reduction of pathogen growth.

Unlike DCA and INA, which induced full resistance to *Hpa*Noco2 1 h post treatment (hpt), immunity triggered by CMP442 was incomplete, but still potent. However, at 3 hpt all three synthetic elicitors induced a strong defense at statistically similar levels. At 3 days post treatment (dpt) CMP442-triggered immunity was reduced and at 6 dpt it was no longer able to induce *Hpa*Noco2-resistance at all. As reported previously, DCA also induces plant defense transiently (Knoth, Salus et al. 2009), while the activity of INA is long lasting (Métraux, Ahl Goy et al. 1991; Görlach, Volrath et al. 1996; Bowling, Clarke et al. 1997; Dong and Opperman 1997). However, the defense-inducing activity of CMP442 was more transient than that of DCA. These experiments show that CMP442, like DCA, is a potent but reversible inducer of immunity against *Hpa*Noco2.

### Structure Activity Analysis with CMP442 Derivatives

To determine which substituent/s of CMP442 were critical for its defense-inducing activity, structural phenyl-thiazolidine-carboxylic acid analogs were analyzed that varied only minimally from the original compound (Figure 2.5A). The ability of seven commercially available CMP442 analogs next to DCA and CMP442 to induce *GUS* expression in saturation treated p*CaBP22*$^{-333}$::*GUS* Arabidopsis seedlings (Figure 2.5B). Except for 4-carboxy-4-thiazolidinyl (T4CA),

5-bromo-2-hydroxy-phenyl (2BP), and 2,2'-(1,4-phenylene) bis-(4R,4'R) 4-thiazolidine carboxylic acid (CMP389) all other examined derivatives induced p*CaBP22*$^{-333}$::*GUS* expression. The most efficient derivative appeared to be 2-(2-hydroxy-phenyl)-thiazolidine-4-carboxilic acid (CMP140), which lacked the bromine substituent of the phenyl moiety (Figure 2.5B). CMP254, which lacked the hydroxyl substituent, weakly induced expression at 10 µM. Alternately, CMP023, which lacked the bromide and hydroxyl group, and CMP492, had a methyl added to the hydroxyl group, required 100 µM to induce *GUS* expression.

To examine CMP442-analog-induced phytotoxicity, trypan blue staining of seedlings after saturation treatment was used. Trypan blue staining, indicating cell death, was prevalent in 100% of the seedlings treated for 24 h at 300 µM of CMP442 and DCA (Figure 2.5C). No cell death was observed at any concentrations examined for the other compounds. Thus, cell death was not responsible for p*CaBP22*$^{-333}$::*GUS* activation triggered by any of the tested compounds.

To accurately quantify the defense-inducing activity of CMP442 analogs, a dose-response analysis measuring the inhibition of *Hpa*Noco2 spore development in three-week old Col-0 plants was performed (Figure 2.5D). These *Hpa* defense assays and the reporter gene assays (Figure 2.5B) showed similar trends. DCA and CMP442 provided the highest protection against *Hpa*Noco2 infection significantly protecting Col-0 from *Hpa* at 10 µM. Aside from CMP492,

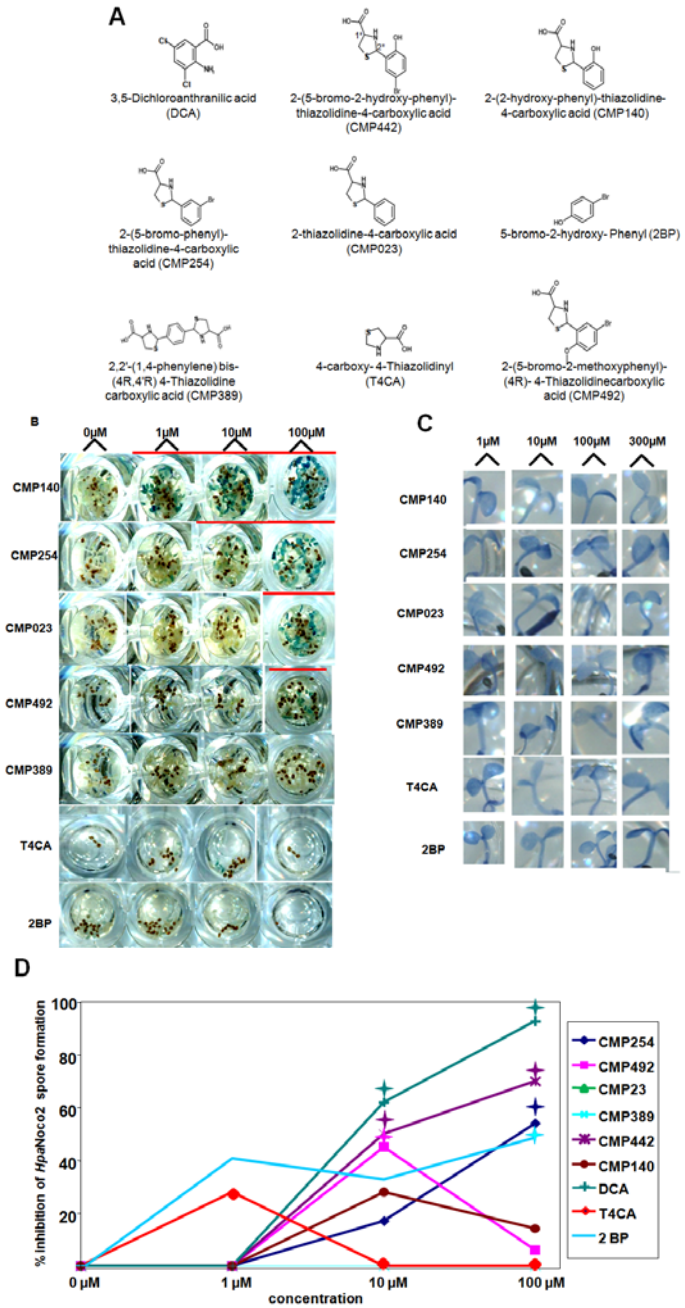



**Figure 2.5: Structure-activity analysis of CMP442 analogs. A,** Chemical structures of CMP442 and tested analogs. **B**, Screening plate containing 7-d-old liquid-grown *CaBP22*$^{-333}$::GUS seedlings after GUS histochemical staining comparing reporter response after a 24 h incubation at indicated compound concentrations. Blue/green color of cotyledons indicates induction of the *GUS* gene expression (all color was bleached out of plants during the ethanol-destaining process). The red line above wells indicates GUS staining. **C**, Trypan blue staining of *CaBP22*$^{-333}$::GUS seedlings incubated for 24 h in medium containing compounds at the indicated concentrations. Dark blue/black color of the cotyledons indicates cell death (toxicity) and is pointed to by red arrows. The seed coats of seedlings always stain blue/black and can been seen in some images. All staining experiments were performed at least three times with similar results. **D**, *Hpa*Noco2 growth inhibition assay. Three-week-old soil-grown Col-0 seedlings were infected 24 h after treating with varying concentrations of each synthetic elicitor and then assayed at 7 dpi for spore growth. 100% inhibition = 0 spores. The assay was repeated three time with similar results. The average of the three replicates is shown above. The Student's *t*-test ($p$<0.05) used to determine significant differences compared to mock treated seedlings is indicated on the graph by the corresponding colored pointed star.

the remaining analogs were unable to mediate significant immunity at concentrations below 100 µM. Additional defense assays performed on CMP140,proved it to be a less efficient defense inducer compared to CMP442, since a concentration of at least 100 µM was required to significantly reduce pathogen growth (Figure 2.6). These assays showed that CMP442 was the most potent of all tested phenyl-thiazolidine-carboxylic acid analogs and alterations in its structure resulted in a reduction in ability to induce defense or *GUS* expression.

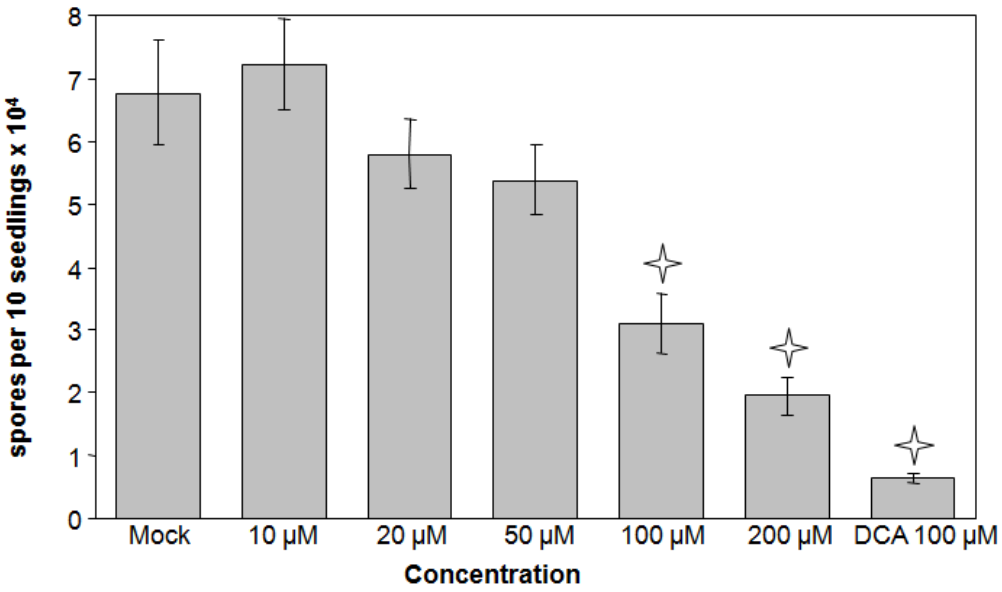



**Figure 2.6: CMP140 was a weaker defense inducer than CMP442**. Three-week-old soil-grown Col-0 seedlings were sprayed with CMP140, DCA, or equivalent Mock (DMSO) at the indicated concentrations 24 h prior to spray infection with *Hpa*Noco2 (2 x $10^4$ spores/ml; 2 ml per pot). DMSO served as a control equivalent to the highest present in synthetic elicitor treatment. Spores were counted 7 dpi. Mean and SE values were calculated from a minimum of three biological replicates and the average of the three is shown above. The Student's *t*-test ($p<0.05$) was used to determine significant differences relative to the mock. Significantly lower levels of spores compared to the control treatment are indicated by a star. Refer to Figure 2.3 to see CMP442's ability to induce resistance against *Hpa*Noco2.

### CMP442 is Functionally Distinct from DCA

To establish whether the mode-of-action of CMP442 differed from that of DCA, reverse transcription (RT)-PCRs examining transcript levels of the defense marker genes *CaBP22 and WRKY70* were performed (Knoth, Ringler et al. 2007) in Arabidopsis defense mutants and Col-0 after treatment with 100 µM DCA, CMP442, or water (Figure 2.7). Both DCA and CMP442 induced an increase of *CaBP22 and WRKY70* transcript levels in Col-0. CMP442- and DCA-induced expression of *CaBP22 and WRKY70* was largely unaltered in the *eds1* and *ndr1* mutants, which blocked in defined signaling steps upstream from SA perception (Aarts, Metz et al. 1998; Jirage, Tootle et al. 1999). Induced *CaBP22 and WRKY70* RNAs accumulated in *npr1* plants, which are compromised in defense

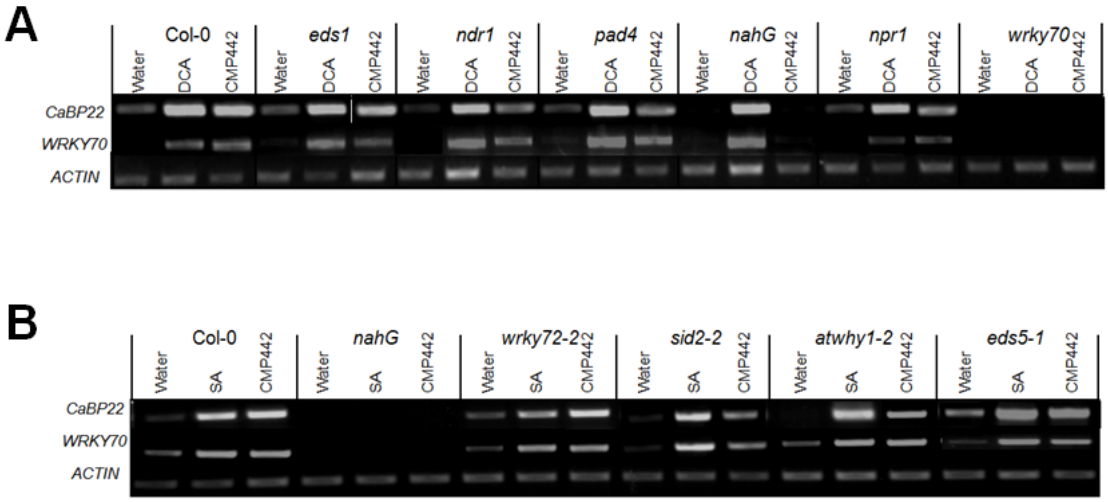



**Figure 2.7: Analysis of CMP442 activity in wild type and defense mutants. A and B**, RT-PCR analysis of *CaBP22* and *WRKY70* transcript levels in Col-0 (wild-type) and mutant or *nahG* backgrounds 24 h after spraying 2-week-old seedlings with control (1%-DMSO), 100 µM DCA, 100 µM SA, or 100 µM CMP442. *ACTIN* is shown as a loading control. At least 3 biological replicates showed consistent results. Result of a experiment are shown.

signaling downstream from SA perception (Dong 2004). In the transgenic *nahG* line, which is compromised in the accumulation of SA, the ability of CMP442 to induce defense gene expression was blocked, while that of DCA was not affected. This indicated that that the mode-of-action of CMP442 is distinct from that of DCA and that CMP442 likely interfered with a defense signaling step upstream from SA. Consistently, CMP442-induced *CaBP22* and *WRKY70* transcript accumulation is reduced in the *pad4-1* mutant (Glazebrook, Zook et al. 1997; Feys, Moisan et al. 2001; Wildermuth, Dewdney et al. 2001), which is deficient in defense-associated SA synthesis.

To further distinguish the mode of action of CMP442 from that of SA (Delaney, Uknes et al. 1994), additional RT-PCRs were performed comparing the induced expression of *CaBP22 and WRKY70* after treatment with CMP442, SA, and water. Transcript accumulation of *CaBP22 and WRKY70* was examined in the mutants: *wrky72-2* (Bhattarai, Atamian et al. 2010), *sid2-2* (Dewdney, Reuber et al. 2000), *atwhy1-2 (Desveaux, Subramaniam et al. 2004)*, and *eds5-1* (Rogers and Ausubel 1997; Nawrath, Heck et al. 2002) all of which are known to be compromised in signaling steps upstream from SA perception (Nimchuk, Eulgem et al. 2003). These experiments confirmed that CMP442, like SA, was unable to induce *CaBP22 and WRKY70* transcript accumulation in *nahG.* In the remainder of mutants, the ability of SA and CMP442 to induce transcript accumulation of the tested defense genes was similar to that in Col-0. Although a slight reduction of transcript accumulation was seen in CMP442-induced *CaBP22*

and *WRKY70* transcript accumulation in the *sid2-2* mutant (Feys, Moisan et al. 2001; Wildermuth, Dewdney et al. 2001) which is deficient in defense-associated SA synthesis. These data indicated that the mode-of-action of CMP442 is distinct from that of DCA, but cannot be distinguished from SA.

To further confirm the RT-PCR results and the effects of *nahG* on CMP442 activity, CMP442- and DCA-mediated pathogen resistance was measured (Figure 2.8). Col-0 and *nahG* seedlings were sprayed with 100 µM CMP442, 100 µM DCA, or water solution 24 h prior to *Hpa*Noco2.

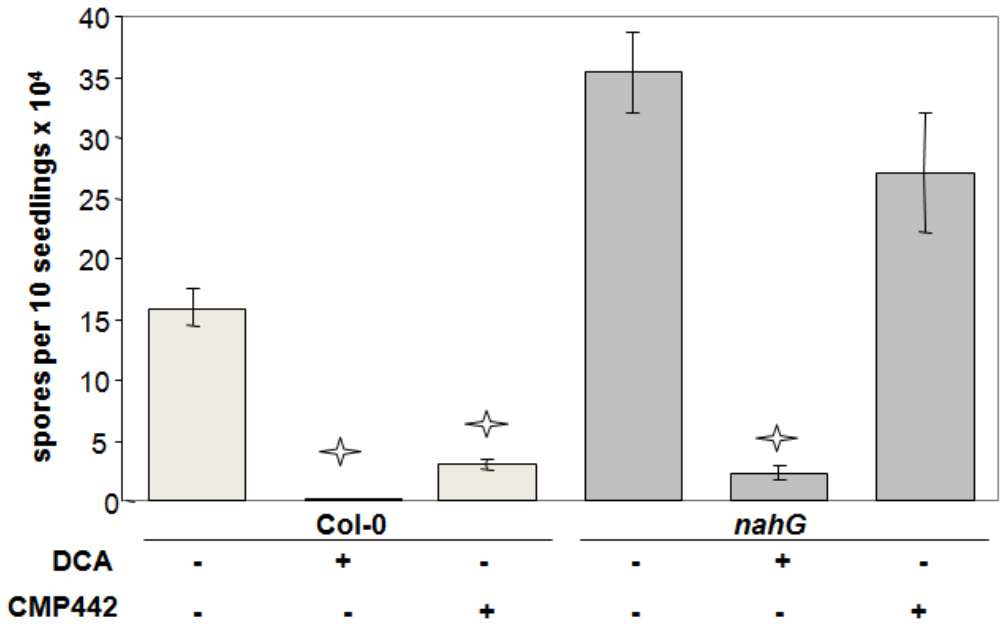



**Figure 2.8: Analysis of CMP442 activity in Col-0 or *nahG* plants.** Experiments were conducted with three-week-old soil-grown Col-0 or *nahG* seedlings sprayed with 100 µM CMP442, 100 µM DCA, or mock (1%-DMSO) 24 h prior to infection with virulent *Hpa*Noco2 (2 x $10^4$ spores/ml; 2 ml per pot). Spores were counted at 7 dpi. Mean and SE values were calculated from a minimum of three biological replicates. The Student's *t*-test ($p<0.05$) was used to determine significant differences relative to the untreated Col-0 or *nahG*. Significantly lower levels of spores compared to the control treatment are indicated by a star.

Whereas DCA was able to induce strong resistance in both Col-0 and *nahG,* CMP442 was only able to do so in Col-0. In *nahG,* there was no significant reduction of *Hpa*Noco2 spores after CMP442 treatment as compared to the mock-treated *nahG* control. These results indicated that CMP442 had a distinct mode of action from DCA. They also proved that a single high-throughput screen can be used to identify different compounds with distinct modes-of-action as illustrated in the model in Figure 2.9.

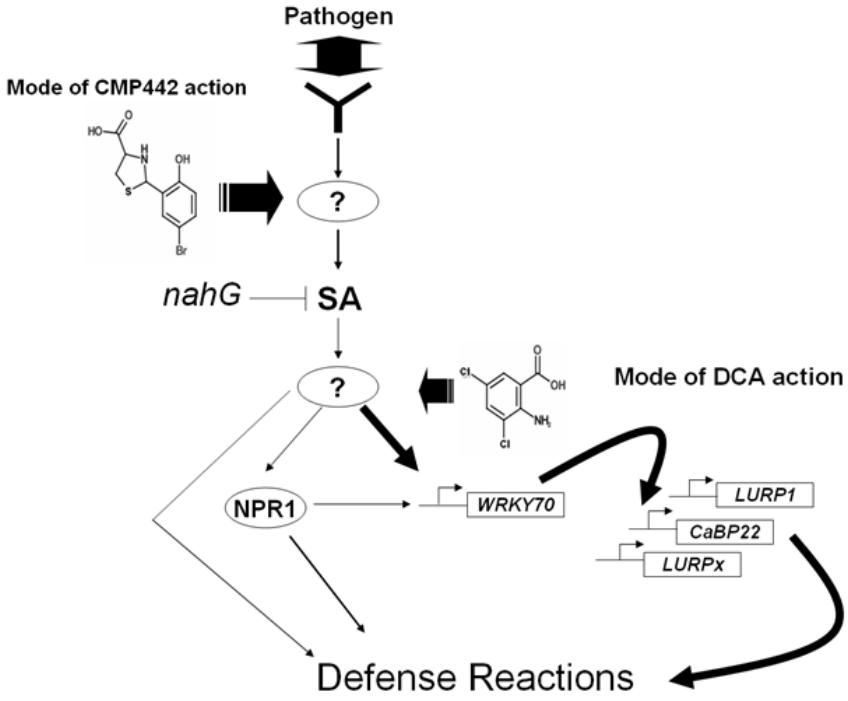



**Figure 2.9: Model illustrating how CMP442 and DCA may interfere with defense signaling.** CMP442 targets regulators operating upstream of SA biosynthesis. DCA targets regulators operating downstream or independently of SA, triggering both NPR1-dependent and NPR1-independent defense responses (Knoth et al., 2009).

**Biologically Active CMP442 Preparations can be Produced Through a Simple Organic Reaction**

All experiments described in this study were performed with CMP442 purchased from Sigma TimTec (p-CMP442). CMP442 was easily synthesized through the reaction of L-Cysteine hydrochloride with 5-bromo-salicyaldehyde (Khan, Zia-Ullah et al. 2006; Song, Ma et al. 2009) (Figure 2.10). The resulting product was purified by successive rounds of rinsing with water then ethanol. The structure and purity of synthesized CMP442 (s-CMP442) was analyzed using nuclear magnetic resonance (NMR) and compared to that of purchased CMP442 (p-CMP442). NMR analysis of s- and p-CMP442 was performed by PhD candidate Gregory Barding (University of California-Riverside). Both s-CMP442 and p-CMP442 produced equivalent NMR spectra (Figure 2.11).

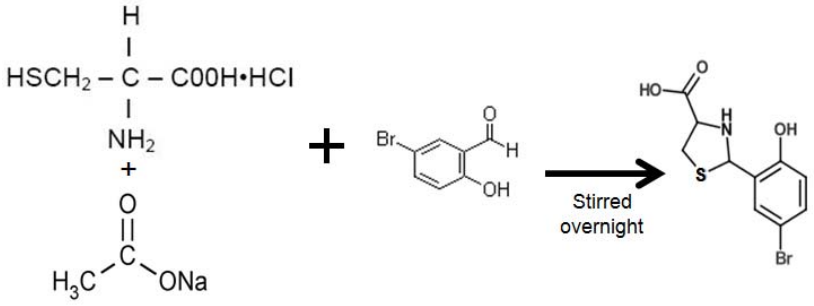



**Figure 2.10: Synthesis of CMP442:** Reaction of L-cysteine hydrochloride and sodium acetate (dissolved in water) with 5-bromo-salicylaldhyde (dissolved in ethanol) to produce CMP442.

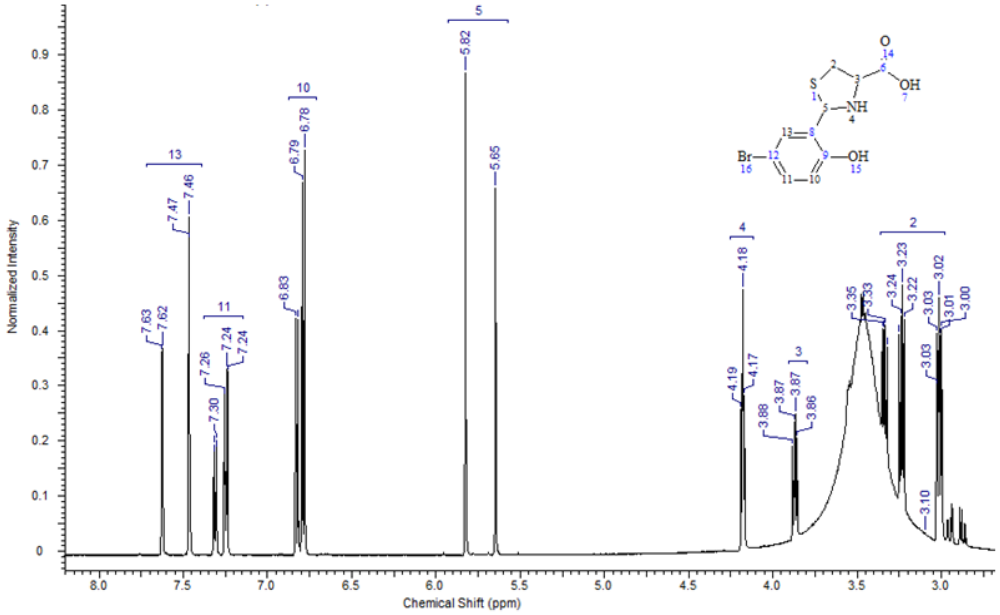



**Figure 2.11: NMR Spectra of CMP442:** Downfield region of the 1H-NMR spectra, assignments for the CMP442 atoms are provided above the NMR peaks and the corresponding atoms are labeled in the chemical structure. Both synthetic-CMP442 and purchased-CMP442 gave identical spectra showing similar ratios and both showed a peak for DMSO (solvent) located between 3.0-4.0 ppm. Possible chiral centers may exist at carbon 3 and carbon 5.

CMP442 had two chiral centers (indicated with 1* and 2* in Figure 2.5A and carbon 3 and 5 in Figure 2.11), but only two conformations of the possibly diastereomers were detected by NMR (Figure 2.13). The biological activity of s-CMP442 and p-CMP442 were compared by spraying 10-100 µM on soil-grown Col-0 seedlings 24 h prior to challenge with *Hpa*Noco2. No significant difference were found between the efficacy of s-CMP442 and p-CMP442 in reducing *Hpa*Noco2 spore counts at 20, 50, 100, and 200 µM (Figure 2.12). NMR analysis further revealed that both diastereomers of s-CMP442 and p-CMP442 have ratios of 40% to 60% between the two detected conformations (data not shown).

However, the NMR data did not allow me to infer which diastereomer was present at 40% and which one at 60%.

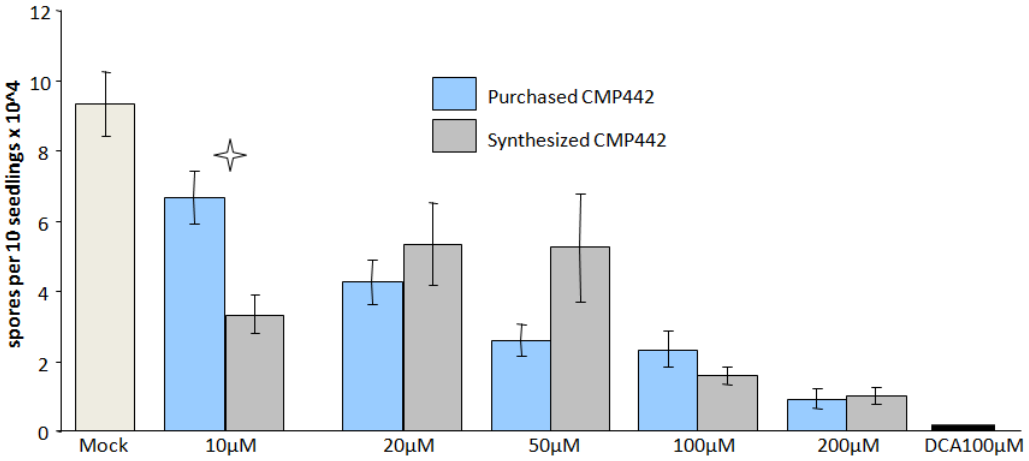



**Figure 2.12: Ability of purchased vs synthesized CMP442 to induce disease resistance against the virulent *Hpa isolate* Noco2**. Three-week-old soil-grown Col-0 seedlings were sprayed with either purchased or lab synthesized CMP442 at the indicated concentrations 24 h prior to spray infection with *Hpa*Noco2 (2 x $10^4$ spores/ml; 2 ml per pot). There was no statistical difference between the effectiveness of purchased or lab synthesized CMP442 at 20 µM, 50 µM, 100 µM, and 200 µM. Spores were counted 7 dpi. Mean and SE values were calculated from a minimum of three biological replicates and the average of those is shown above. The Student's *t*-test ($p<0.05$) was used to determine significant differences. Significantly lower levels of spores when comparing purchased CMP442 to synthesized CMP442 are indicated by a star.

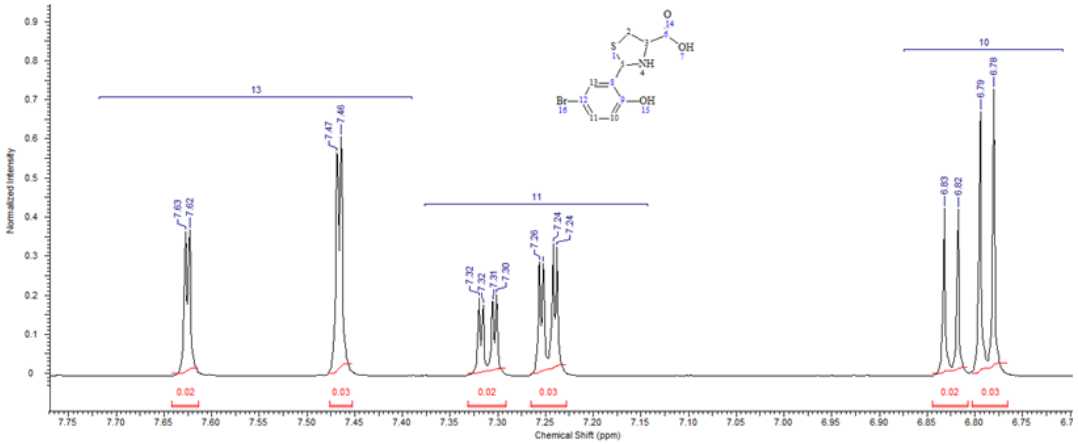



**Figure 2.13: NMR Spectra of s-CMP442 and p-CMP442 indicating that this compound is present as two diastereomers.** Downfield region of the 1H-NMR spectra, assignments for the CMP442 atoms are provided above the NMR peaks. CMP442 is composed of two distinct diastereomers as indicated by the presence of two peaks that make up the different atom numbers 13, 11, and 10. The integration of the two peaks as shown by the red brackets, and numbers below, demonstrates that these two peaks are present in ratios of 0.02 (40%) of one to 0.03 (60%) of the other diastereomers. The chiral atom could only be part of the five-membered ring of CMP442 located at either carbon three or five.

### Screening for CMP442 Targets or Components of its Uptake *in planta* Using Activation Tagging Lines

A useful side effect of DCA is that it inhibits the development of roots in Arabidopsis seedlings grown on artificial growth medium (C. Knoth and T. Eulgem, unpublished). This effect allowed lab members to screen a large population of 50,000 EMS-generated Arabidopsis mutants for individuals that exhibit reduced sensitivity to DCA and, thus, show normal root growth on DCA-containing growth medium. Mutants with altered sensitivity for a drug-like compound may have a defect in a gene required for compound uptake,

metabolization, or may encode an in/direct target protein operating in a pathway critical for the compound-triggered phenotype. In the case of DCA, ideally a mutant would have been identified that is compromised either in the direct cellular target of DCA or in a defense signaling component operating downstream from DCA perception. However, this screen was not successful and no mutants with the desired properties were identified (C. Knoth and T. Eulgem, unpublished).

A possible reason for this is that targets of DCA are encoded by multiple redundant genes. Mutations in any one of these redundant genes should not result in a detectable phenotypic effect. For this reason screening of activation tagging libraries was used as an alternative approach. Any protein that may be involved in the perception of DCA or the transduction of DCA-induced signals should exhibit enhanced activity, if over-expressed. Thus, activation tagging mutants that show elevated expression of a gene encoding a DCA target protein should be more sensitive to DCA and exhibit enhanced DCA-mediated reduction of root growth compared to wild type plants.

Arabidopsis activation-tagging lines containing randomly inserted T-DNA vectors with four copies of the *Cauliflower Mosaic Virus 35S* (*CaMV*) enhancer, which can cause overexpression of nearby genes were used (Weigel and Glazebrook 2002). Arabidopsis lines with this T-DNA near a gene encoding the direct DCA target or a protein with a role in DCA uptake, metabolism, or signaling may show enhanced sensitivity to DCA. Seedlings were plated on solid media

with 1 to 100 μM of DCA. The plates were placed upright in a growth chamber and root lengths were measured at 0 d, 3 d, 5 d, 7 d, 11 d, and 14 d. Optimization experiments were performed to identify a time point and concentration of DCA leading to a moderate reduction in root growth which can be further increased by higher DCA doses or enhanced activity of a cellular component critical for DCA activity. For this screen, DCA needed to display enhanceable sensitivity. This meant that between the concentrations tested DCA must show significantly diminished root lengths. However, these experiments revealed that DCA-affected Arabidopsis root growth was inappropriate for a reliable high-throughput screen (data not shown). This meant that the difference in root length between tested concentrations was too small for a screen. Similar experiments using CMP442 showed that this compound was better suited for the planned screen, as its root growth reducing effect was more robust (data not shown).

Therefore, a screen was designed for Arabidopsis T-DNA activation tagged lines with enhanced sensitivity to CMP442. Increasing the CMP442 concentration from 25 μM to 50 μM resulted in a highly reproducible 7- to 8-fold reduction of Col-0 roots 14 days post planting. Screening of 25 lots of the Weigel activation tag population (each representing a pool of 96 independent lines) on 25 μM CMP442, 38 primary hits were identified. Each of the identified lines was transplanted into soil and grown to maturity. Seeds from each candidate were retested for their response to CMP442. However, the phenotype of enhanced

CMP442-mediated root growth inhibition was not reproducible for any of the selected primary candidates. While designing the activation tagging screen, it was observed that at low concentrations (~1 μM) both DCA and CMP442 caused an enhancement of root lengths compared to that of the control. These dosages were too low to produce an effect in induced disease resistance assays, and therefore, have been termed “sub-optimal dosages”.

**CMP442 and Other Synthetic Elicitors Induce a Hormetic Response in Arabidopsis Seedlings**

To further examine whether the enhancement of Arabidopsis root growth observed with low doses of DCA and CMP442 was observed with other synthetic elicitors, root growth assays were performed with SA, BTH and INA along with DCA and CMP442. Each synthetic elicitor was added to the solid media plates at concentrations ranging from 1 to 100 μM. The plates were placed upright in a growth chamber, and root lengths were measured. To merge different data sets, each root length was expressed as a percentage relative to the mean of the control for that data set. Normalizing each root length to the control within a given experiment allowed all replicates to be combined together to calculate the mean, standard error, and perform statistical tests (Figure 2.14). At day 14, 1 μM of every synthetic elicitor caused an increase in Col-0 root length, while (except for INA) they caused a reduction at higher concentrations. At a concentration of 1 μM, CMP442 clearly enhanced root length at every time point measured. The

maximal increase observed for CMP442 was 183.1% at day 14 compared to mock. No other tested synthetic elicitor more dramatically enhanced Col-0 root growth at any concentration or at any time point.

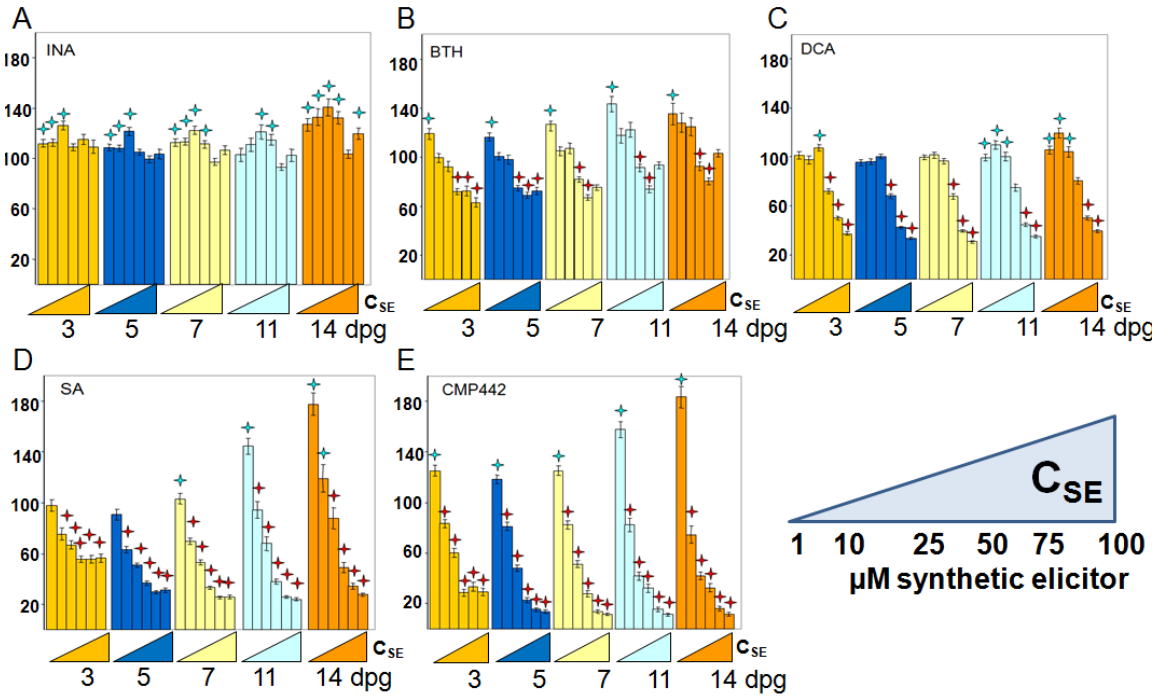



**Figure 2.14 Relative root length of Col-0 plants grown on synthetic elicitors.** Col-0 seedlings were plated in square petri dishes on ½MS medium containing 1 µM, 10 µM, 25 µM, 50 µM, 75 µM or 100 µM of HTC, DCA, INA, BTH, SA or the respective controls (solvent only) 3, 5, 7, 11, or 14 days post germination (dpg). A-E, Roots of Col-0 seedlings grown on synthetic elicitor or control (DMSO). Results are expressed as percentages of the average root length on the respective synthetic elicitor treatments compared to their controls. The average, standard error, and Student's *t-test* ($p<0.05$) were calculated from these values. Significant differences between synthetic elicitor-treated and control-treated plants are marked by a star above the corresponding data point. Y-axis represents relative root growth in the presence of synthetic elicitor. CSE: concentration of synthetic elicitor.

The average root length growth was calculated between day 0-3, 3-5, 5-7, 7-11, and 11-14 (Figure 2.15). For all synthetic elicitors, the greatest rate of change was observed between days 11 and 14. The minimal growth for all synthetic elicitors between days 11 and 14 at 1 µM was 276% (BTH), while the maximum was 541% (SA) compared to mock. These data shows that for the

synthetic elicitors tested, sub-optimal dosages cause an increase in root growth and much of this increase takes place between day 11-14.

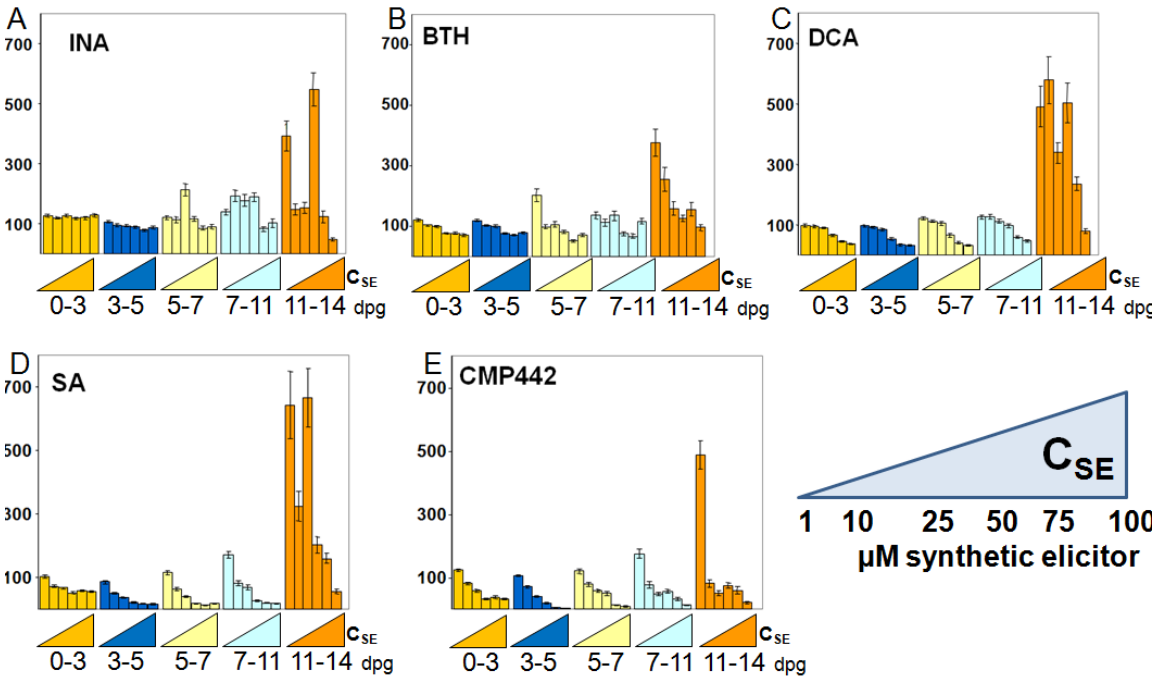



**Figure 2.15: Relative change in root length of Col-0 plants grown on synthetic elicitors**. Col-0 seedlings were plated in square petri dishes on ½MS medium containing 1 µM, 10 µM, 25 µM, 50 µM, 75 µM or 100 µM of of INA, BTH, DCA, SA, CMP442 or the respective controls (solvent only) 3, 5, 7 11 , or 14 days post germination (dpg). A-E, Results are expressed as percentages of the average root length change during the indicated time points when grown on the respective synthetic elicitor treatments compared to controls. The average, standard error, and Student's *t-test* ($p$<0.05) were calculated from these values. Y-axis represents relative root length change in the presence of synthetic elicitor. CSE: concentration of synthetic elicitor.

To determine whether this increased root length phenotype translated into an increase in the aerial weight of Arabidopsis, soil-sown-seedlings were drenched with synthetic elicitor (Figure 2.16). After 14 days the aerial portions of the plants were cut off and their weights measured. Only CMP442 treated plants showed a significant increase in their aerial weight compared to the control, with a maximum increase of 119% at 50 µM. These data clearly show that sub-optimal dosages of some synthetic elicitors caused hormetic effects leading to increased root length and that CMP442 caused an increase in aerial weight.

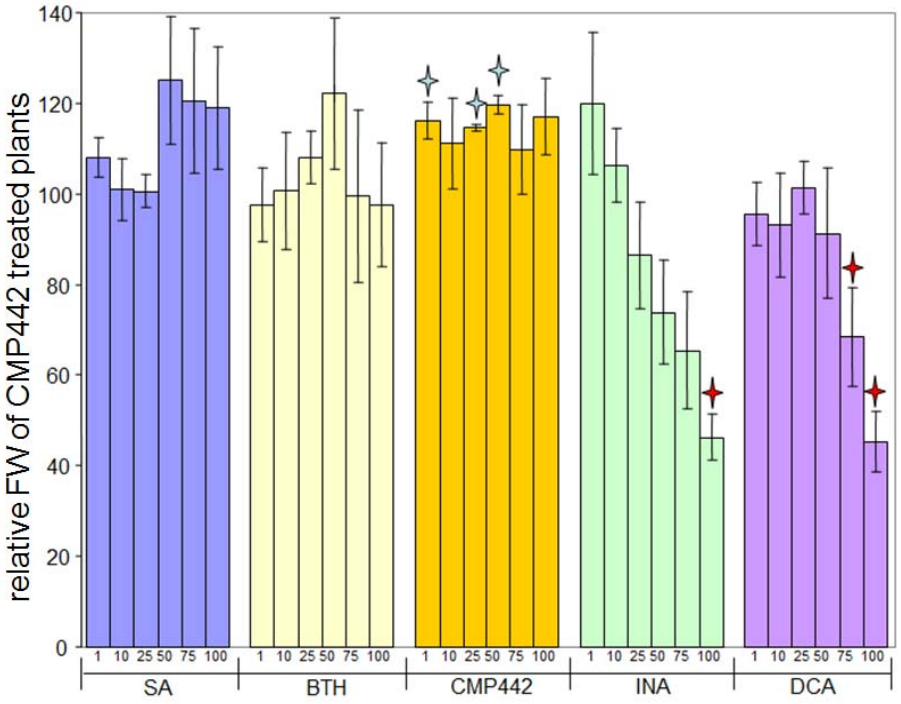



**Figure 2.16: Percent weight of aerial portions of synthetic elicitor treated Col-0 seedlings compared to control (DMSO) treated seedlings.** Col-0 seeds were sown on soil and drenched with 20 mL of the indicated concentrations of SA, BTH, CMP442, INA, DCA, or equivalent control. Weight of the aboveground plant tissues were measured at day 14. Results are expressed as percentages of the average weight of the respective synthetic elicitor treatments compared to those of the controls. The standard error and Student's *t*-test ($p<0.05$) were calculated from at least three biological replicates and the average is shown above. Significant differences between synthetic elicitor-treated and control-treated plants are indicated with a star. Blue star: greater root growth; red star: less root growth.

### CMP442 Enhances Root and Shoot Growth of Tomato Seedlings

To determine if the synthetic elicitors can cause hormetic effects across species boundaries, root and aerial plant growth assays with tomato were performed. In preliminary experiments at concentrations of 10 μM of CMP442 and higher, the root tips of transplanted seedlings on solid medium turned black or brown and did not grow any further (data not shown). In addition, synthetic elicitor concentrations below 1 μM did not replicate the hormetic phenotypes observed in Arabidopsis. To maximize the statistical power of the growth assays, a single concentration (1 μM) was applied to a large set of tomato plants (n > 89). At day 2, 4, 6, and 8, 1 μM of CMP442 caused a significant increase in root

length (Figure 2.17). The greatest increase was seen at day 6 with tomato roots grown on 1 µM CMP442 showed a 136.1% increase in length compared to mock.

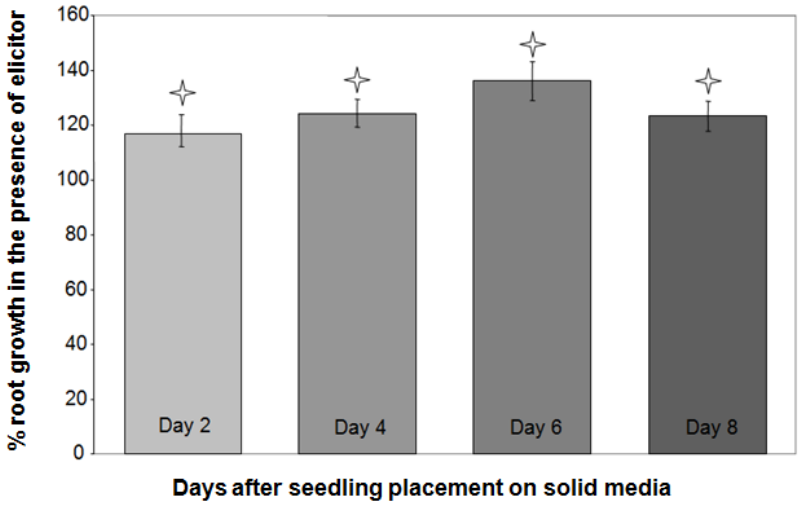



**Figure 2.17: Relative root length of tomato seedlings (c.v. Moneymaker) grown on CMP442 compared to seedlings grown on control (0.01%-DMSO).** Moneymaker seedlings were plated in square petri dishes on ½MS medium containing CMP442. Root length was measured at day 2, 4, 6, and 8. Results are expressed as percentages of the average root length on 1 µM CMP442 or mock. The average, standard error, and Student's *t*-test ($p<0.05$) were calculated from these values. Each time point represents at least 89 distinct root length measurements from at least three biological replicates. Significant differences between CMP442-treated and control-treated plants are indicated with a star above the corresponding data point.

Root drench-assays were also performed to determine effects of CMP442 on aerial weight in soil grown tomato plants. Tomato seedlings were planted in soil and then a one-time root drench with 1 µM, 10 µM, and 25 µM of CMP442 was performed (Figure 2.18). Only 10 µM caused a significant increase in aerial weight, resulting in a 128.5% increase compared to mock. The data from these

experiments demonstrated that CMP442 caused an increase in both the root length and aerial weight of tomato plants when used at sub-optimal dosages.

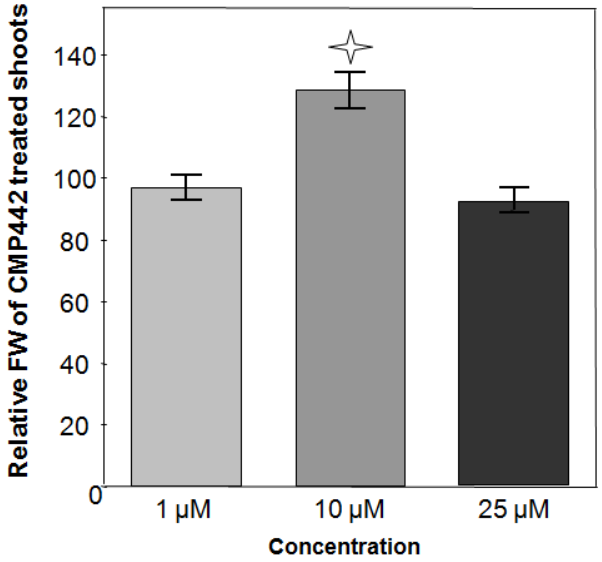



**Figure 2.18: Relative freshweight of CMP442 treated tomato shoots (cv Moneymaker) seedlings compared to control (DMSO) treated seedlings.** Moneymaker seeds were sown on soil and drenched once with 20 ml of the indicated concentrations of CMP442 or their respective DMSO control. Weight of the aboveground plant tissues were measured at day 14. Results are expressed as percentages of the average weight of CMP442 treatments compared to those of the controls. The standard error and Student's *t*-test ($p<0.01$) were calculated from at least three biological replicates. Significant differences between synthetic elicitor-treated and control-treated plants are indicated with a star above the corresponding data point. Y-axis represents percent of weight in the presence of CMP442 compared to controls (n > 239).

## DISCUSSION

Besides the benzoic acid derivative DCA, our chemical screen for inducers of *CaBP22*$^{-333}$*::GUS* in Arabidopsis (Knoth, Salus et al. 2009) led to the identification of the 2-phenyl-thiazolidine-4-carboxylic acid (PTC) derivative CMP442 as a new synthetic elicitor. To our knowledge compounds of this class have not been reported to act as plant defense inducers. While reports on plant-based studies are not available, some studies have been performed on PTCs in other systems and show that these compounds have anticancer, antioxidant, antifungal, and antimicrobial activities (Wlodek, Wrobel et al. 1996; Ferrandez, Correa et al. 1999; Alhamadsheh, Hudson et al. 2006; Sriharsha, Pai et al. 2007; El-Sharkawy 2011). None of these studies examined the effect of PTCs on plant pathogens. The diversity of biological activities of PTCs suggested that these compounds are highly suitable for interactions with a wide range of different cellular targets.

Both major moieties of CMP442 are necessary for strong elicitor activity, as neither the 4-carboxy-4-thiazolidinyl portion, nor the 5-bromo-2-hydroxy-phenyl portion induced strong immunity in our assays. While changes of the substituents of the phenyl group resulted in reduced elicitor activity, the PTC-derivatives, CMP140, CMP254, and CMP492 that carry at least one substituent at the phenyl group suppressed the formation of *Hpa*Noco2 spores in Arabidopsis, though at higher concentrations. Of those PTCs tested in our study, CMP442 was the most potent plant defense inducer of this compound class.

All synthetic elicitors identified by our previous chemical screen are expected to induce a common set of defense reactions, which include transcriptional activation of the *LURP* gene cluster and at least some other responses known to be dependent on SA (Knoth, Salus et al. 2009). Nonetheless, we found DCA and CMP442 employed different modes-of action. Their defense-inducing activities differed sharply in the Arabidopsis *nahG* line, which is unable to accumulate SA due to expression of a bacterial salicylic acid hydroxylase (Gaffney, Friedrich et al. 1993). Immunity against *Hpa* and *CaBP22* and *WRKY70* transcript accumulation triggered by DCA were not affected in *nahG* plants, while the ability of CMP442 to induce these responses was fully blocked in this line. Tests in additional lines deficient for SA-signaling, such as *sid2-2* and *pad4-1*, showed similar trends. Thus, CMP442 is likely to interfere with a step in defense signaling that acts upstream from the accumulation of SA, while DCA disturbs processes downstream from SA.

While DCA and INA behave similar in *nahG* plants, these two compounds differ profoundly in their level of dependency on the transcriptional co-factor NPR1 (Knoth et al., 2009). Although the defense inducing activity of INA is fully blocked in *npr1-3* plants, DCA is only partially reduced in this mutant. Thus, with CMP442, DCA and INA a small set of synthetic elicitors is now available that has different activating effects on the SA-dependent plant defense network (Figure 2.2). These molecular probes along with additional synthetic elicitors from our

screen and genetic mutations are likely to prove highly useful for the fine dissection of this complex regulatory network.

A major strategy of disease control in agriculture and horticulture has been the use of pesticides. Chemical pesticides currently in use typically rely on direct antibiotic or biocidal activity, which often leads to undesirable toxic environmental side effects (Kessmann, Staub et al. 1994; Gilliom 2007). In response to these concerns the U.S. Environmental Protection Agency (EPA) has established a program to facilitate registration of new reduced-risk pesticides that have a reduced impact on human health and other non-target organisms (Stenersen 2004). Synthetic elicitors identified by our project protect plants by inducing their natural immune responses. As their primary mode-of action does not involve the inhibition of key metabolic or developmental steps in target organisms, they are likely to be less harmful for humans and the environment than conventional pesticides. Due to the continuous pollution of the environment caused by the massive use of traditional pesticides (Hunter 2009; Gonzalez-Rodriguez, Rial-Otero et al. 2011) and the increasing awareness of environmental protection issues of consumers and farmers in the US, Europe and other parts of the globe, innovative “green” pesticides suitable for conventional farming practices are urgently needed.

A possible disadvantage of the use of synthetic elicitors for crop protection is that permanent defense activation often results in fitness costs, due to the phytotoxicity of some defensive plant products and resource allocation away from

growth or reproduction. For example, as a result of its long-term activity, the synthetic elicitor 2,6-isonicotinic acid (INA), which was developed in the 1990s by Ciba Geigy, was insufficiently tolerated by some crop plants to warrant practical use as a plant protection compound (Ryals, Neuenschwander et al. 1996). However, we found DCA and CMP442 to be promising in this respect when contrasted to other known defense elicitors, such as INA or BTH (Métraux, Ahl Goy et al. 1991; Schaffrath, Freydl et al. 1997), their defense-inducing activity is only transient and weakens within several days after application (Figure 2.4; (Knoth, Salus et al. 2009).

In addition, low doses of CMP442 proved to be beneficial for plant growth. Arabidopsis and tomato grown on solid medium containing low concentrations of CMP442 developed significantly longer roots than untreated seedlings. In addition, single root drench application of CMP442 enhanced growth of aerial parts of soil-grown Arabidopsis and tomato. Thus, CMP442 appears to be uniquely suited to provide plant seedlings with both protection from diseases and enhancement of vigor. We also found several other synthetic elicitors, including DCA, that have similar effects on root growth at low concentrations. However, of those synthetic elicitors we tested so far CMP442 is the most efficient one in this respect.

CMP442 strongly enhanced growth of Arabidopsis roots at a concentration of 1 µM. At this concentration CMP442 still can induce defense responses. Under saturation treatment conditions 1 µM of CMP442 can induce of *CaBP22*$^{-333}$*::GUS*

expression (Figure 2.2A). Similarly, enhanced growth of aerial plant parts was observed after root drench with 1 to 50 µM of CMP442 in Arabidopsis or 10 µM CMP442 in tomato. As shown in Figure 2.3, 10 µM of CMP442 was sufficient to significantly suppress the development of *Hpa*Noco2 spores in Arabidopsis. Despite these promising observations, further studies are needed to explore the full potential of CMP442 for simultaneous disease protection and growth enhancement for crops.

Several regulatory proteins were found to contribute to both defense and developmental processes (Holt III, Boyes et al. 2002; Nurmberg, Knox et al. 2007). These include the Arabidopsis proteins SGT1b, AS1 (Nurmberg, Knox et al. 2007) and *At*TIP49a (Holt III, Boyes et al. 2002). For example, SGT1b, a regulatory component of SCF complex ubiquitin ligases, was found to be involved in controlling stability of several R proteins as well as the activation of ETI, but also $SCF^{TIR1}$-mediated auxin responses, such as root development and apical dominance (Tör, Gordon et al. 2002; Gray, Muskett et al. 2003; Holt, Belkhadir et al. 2005). We recently reported that Enhanced Downy Mildew 2 (EDM2), which is required for *R*-mediated resistance of Arabidopsis against the Hiks1 isolate of *Hpa*, positively affects floral transition (Tsuchiya and Eulgem 2010). EDM2 has additional roles in plant development, such as promoting proper leaf pavement cell development and controlling the succession of leaf types formed during early vegetative stages of Arabidopsis.

An increasing number of studies are reporting on similar molecular links between plant immune and developmental processes. The molecular nature and biological purpose of crosstalk between both types of processes is poorly understood at this point. CMP442, with its clear effects on both plant immunity and growth, is likely to serve as a valuable tool for the dissection of molecular crosstalk between defense and development. We are currently testing its effects on both defense induction and growth enhancement in a variety of known Arabidopsis signaling mutants. Results from these and related studies should shed light on the fascinating, but yet enigmatic, link between seemingly unrelated types of physiological processes in plants.

CMP442 may also allow for the discovery of fundamental causes of the general phenomenon of hormesis. Although widely described for numerous types of organisms (including humans) and physical, chemical or biological stimuli, the genetic and molecular basis of hormesis is largely unknown. Hormesis is characterized by a biphasic dose-response to a treatment which stimulates at low doses and has an inhibitory or toxic effect at higher concentrations (Calabrese 2009; Calabrese and Blain 2009). Biologically, hormesis is believed to be an adaptive response at either the cellular or organismal level to stress. The exposure to low doses of herbicides to produce enhanced growth has been widely reported on (Cedergreen 2008; Cedergreen, Felby et al. 2009) (Allender 1997; Allender, Cresswell et al. 1997; Belz, Cedergreen et al. 2011). Recent research has revealed some signaling pathways and mechanisms that are

responsible for specific hormetic responses. These involve certain ion channels, protein kinases, deacetylases, transcription factors, chaperones, antioxidant enzymes, and glutathione peroxidase (Yuan, Pan et al. 2004; Arumugam, Gleichmann et al. 2006; Son, Camandola et al. 2008; Calabrese, Mattson et al. 2010; Calabrese, Mattson et al. 2010).

Another noteworthy observation is that some inducers of hormetic responses can protect the respective cells or organisms against a variety of additional stressors later on (Li, Mao et al. 2002; Korde, Pettigrew et al. 2005). Although the phenomenon of hormesis has been known for several decades, our knowledge of its biological basis is fragmentary at best and much remains to be explained. In particular, it is unclear if the great variety of hormesis-like phenomena have a common functional basis, or if they are mechanistically unrelated. A comprehensive comparison of molecular responses triggered by a variety of hormesis-inducing stimuli in a single type of organism, such as the versatile molecular genetics model Arabidopsis, may allow defining common denominators for this complex phenomenon.

## MATERIALS AND METHODS

### Plant Growth Conditions, Plant material, Pathogen Infections and Tissue-Staining

*Arabidopsis thaliana* (Arabidopsis) and *Solanum lycopersicum* (tomato-cv: Moneymaker) plants were grown on soil or media under fluorescent lights (16 h of light/8 h of dark, 23°C, 100 µE $m^{-2}$ $s^{-1}$) unless otherwise noted. The transgenic *nahG* (Delaney, Uknes et al. 1994) has been described. The mutants *eds1-1* (Parker, Holub et al. 1996), *ndr1-1* (Century, Shapiro et al. 1997) *wrky70-3* (Knoth, Ringler et al. 2007), *npr1-3* (Cao, Glazebrook et al. 1997), *pad4-1* (Glazebrook, Zook et al. 1997), *wrky72-2* (Bhattarai, Atamian et al. 2010), *sid2-2* (Dewdney, Reuber et al. 2000), *atwhy1-2* (Desveaux, Subramaniam et al. 2004), *eds5-1* (Rogers and Ausubel 1997; Nawrath, Heck et al. 2002), have been described. *Hyaloperonospora arabidopsidis* (*Hpa*) was grown and propagated as described previously (McDowell, Cuzick et al. 2000). Plants were spray infected with *Hpa* spore suspensions at 2 x $10^4$ spores/mL for *Hpa*Noco2 (on 3-week-old Arabidopsis plants) with Preval sprayers (http://www.prevalspraygun.com). Plants were scored for *Hpa* growth 7 days post infection (dpi) by counting spores/seedlings using a hemicytometer to determine the spore density of a suspension of 10 infected seedlings per 1 ml of water. The Student's *t*-test was used to determine if the effects of the mutations or chemical treatments on sporulation were statistically significant.

### Analysis of GUS Activity and Treatment of Homozygous T6 *CaBP22*$^{-333}$*-promoter*::*GUS* with Synthetic Elicitor.

Arabidopsis seedlings were grown in 96-well plates, treated with synthetic elicitors, and then stained (histochemically) for *GUS* expression as was previously described (Knoth, Salus et al. 2009).

### Synthetic Elicitor Treatment Before Pathogen Infection

Stock solutions of all synthetic elicitors were prepared in 100% DMSO. Stock solutions were diluted in water and 2 ml/pot sprayed on soil-grown plants (20-30 per pot) at the indicated times and concentrations with Preval sprayers. Final DMSO concentrations never exceeded 2%. To test for chemically induced disease resistance, the plants were sprayed with 2 ml/pot of chemicals at the indicated concentrations and times prior to pathogen challenge. Disease symptoms were analyzed as described above.

### Additional Synthetic Elicitor Purchase

Structural analogs of CMP442 were identified through SciFinder Scholar (Resetar 2007). CMP442, CMP140 CMP254, CMP023, CMP492, CMP389 were all ordered from TimTec (Newark, DE). T4CA, 2BP, and DCA were ordered from Sigma-Aldrich.

**RNA Isolation and Reverse-Transcription (RT)-PCR Analysis**

RNA was isolated from seedlings as was previously described (Chomczynski and Sacchi, 1987). The DNase digestions were performed with 1 µg of RNA using Deoxyribonuclease I, Amplification Grade (Invitrogen; http://www.invitrogen.com) to remove DNA. 1 µl of oligo($dT^{18}$) (100 pmol/µl) and 1 µl 10 mM dNTP mix was used for pretreatment of the digested RNA. The resulting mixture was used directly for reverse transcription using Maxima Reverse Transcriptase and Ribolock RNase Inhibitor (Fermentas) following the manufacturer's instructions. A 425-bp fragment of *Actin8* was amplified as a loading control using primers RT-*ACT8*-F (5'-ATGAAGATTAAGGTCGTGGCAC-3') and RT-*ACT8*-R (5'-GTTTTTATCCGAGTTTGAAGAGGC-3') with an annealing temperature of 60°C for 21 cycles. A 301-bp fragment of *CaBP22* was amplified using the primer pair RT-*CaBP22*-FP (5'-CGGAACCATCAATTTCACTGAGT-3') and RT-*CaBP22*-RP (5'-CAAAGTGCCACCAGTTGTGTCAT-3') with an annealing temperature of 63°C for 24 cycles. The 477-bp fragment of *WRKY70* was amplified using the primer pair RT-*WRKY70*-FP (5'-AACGACGGCAAGTTTGAAGATTC-3') and RT-*WRKY70*-RP (5'-TTCTGGCCACACCAATGACAAGT-3') with an annealing temperature of 63°C for 24 cycles. All PCRs used the following thermalcycler program, deviating as indicated for annealing temperatures and cycles: 94°C for 1 min; X cycles of 95°C for 30 s, annealing temperature of X °C for 1 min; and 72°C for 40 s. PCR products were electrophoresed on 1% agarose gels

containing 0.5 $\mu$g/mL ethidium bromide unless otherwise indicated. Negative controls omitting reverse transcriptase in the cDNA production process and PCR without cDNA yielded no products.

**Arabidopsis Seedling Root Growth Assay and Activating Tagging Screen on Plates**

Col-0 seeds were surface-sterilized in a 75% ethanol then 0.02% Triton X, 10% Bleach and water solution, for 10 and 15 minutes respectively. Seeds were then rinsed with sterile water and plated on solid media laced with: ½ MS (Murashige and Skoag), 1.5% agar, 3% sucrose and 1, 10, 25, 50, 75, 100 µM of synthetic elicitors or the equivalent concentration of DMSO (control). Seeds were stratified for two days at 4°C and then placed vertically under fluorescent lights. Plates were scanned at 3, 5, 7, 11, and 14 days after stratification and root lengths were measured using ImageJ (Rasband and Bright 1995).

**Arabidopsis Seedling Aerial Weight Assay on Soil**

Arabidopsis Col-0 seeds were sown on soil and then drenched with 20 ml of 1, 10, 25, 50, 75, 100 µM of synthetic elicitor or equivalent DMSO (control). Seeds were grown under fluorescent lights for two weeks. The aboveground-sections (shoots + leaves) were cut off and their fresh weight measured.

### Tomato Seedling Root Growth Assay on Plates

Moneymaker seeds were surface-sterilized for 20 minutes in a 50% Bleach and water solution and then rinsed with sterile water. Seeds were place in a sterile bag on filter paper in the dark for five days to germinate. Germinated seeds were plated on solid media laced with: ½ MS, 1.5% agar, 0.5% sucrose and 0.1, 0.5, 1, 10, 25 μM of CMP442 or the equivalent concentration of DMSO. Plates were placed vertically under fluorescent lights and scanned at 0, 2, 4, 6, and 8 days and root lengths were measured using ImageJ.

### Tomato Seedling-Aerial Weight Assay on Soil

Moneymaker seeds were sown on soil and then drenched with 20 mL of 1, 10, 25 μM of CMP442 or equivalent DMSO. Seeds were grown under fluorescent lights for two weeks. The aboveground sections of the plant (shoots + leaves) were cut off and their weights measured.

### Procedure for the Synthesis of CMP442

L-Cysteine hydrochloride (0.97 M) and NaOAc (0.7 M) were dissolved in sterile MilliQ water (17 ml). To this solution was added 5-bromo-salicyaldehyde (1.0 g) dissolved in EtOH (18 ml) with constant stirring. Mixture was vigorously stirred at room temperature overnight. The product was separated by suction filtration and washed several times with water then EtOH to remove remaining reactants. Structure and purity were confirmed by NMR.

## CHAPTER 3: Analysis of *JEDI* defense genes

### SUMMARY

Plant diseases can be caused by pathogens with different types of lifestyles. While biotrophic pathogens require living host tissues to complete their life cycles, necrotrophs feed off dead plant cells. The phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are known to coordinate plant defense responses to combat the respective type of infecting pathogen. Currently, most documented interactions between JA- and SA-dependent signaling processes are antagonistic, but their interactions are complex and details of crosstalk between them are not fully understood. Upon recognition of necrotrophs, an increase in JA and ET synthesis occurs along with enhanced transcript levels of defense genes, such as *Plant Defensin 1.2* (*PDF1.2b*), which is often used as a marker for induction of the JA pathway. Plant defensins (*PDF*) are small peptides that can be found throughout the plant kingdom and are encoded by small gene families. Here I report on the development of a screening procedure to identify synthetic elicitors that activate the JA-/ET-dependent branch of the defense network. Towards this end, a set of genes was identified that display SA-independent upregulation in response to infection with the biotrophic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*). Four of the five genes are *PDF* members including *PDF1.2b*. Additionally, efforts to create *Arabidopsis thaliana* (Arabidopsis) lines containing RNA silencing transgenes co-silencing closely related *PDF* family members are described.

## INTRODUCTION

Plants are constantly assaulted by a variety of biotic stressors, such as microbial pathogens. Most pathogens are unable to infect plants, making disease the exception, not the rule (Hein, Gilroy et al. 2009). Plant pathogens are typically divided into two main categories: (1) biotrophs, which obtain nutrients through living tissue and (2) necrotrophs, which must kill plant tissue to acquire nutrients (Glazebrook 2005). Plants evolved the ability to recognize pathogens and tailor their defense responses to the type of infecting pathogen (Pieterse, Leon-Reyes et al. 2009). They possess an inducible immune system enabling them to specifically recognize molecular features of pathogens and activate transcriptional cascades defending the plant from disease (incompatible interaction) (Jones and Dangl 2006). When these mechanisms are absent or inactivated by pathogen effector molecules, plants are rendered susceptible (compatible interaction) (Jones and Dangl 2006). Pathogen effectors are proteins or small molecules secreted into host cells that attenuate defense signaling processes weakening plant immune responses. Strong immunity against pathogens can be mediated by plant disease resistance (*R*) genes, which encode receptors that specifically recognize effectors from distinct pathogen races (Glazebrook 2001). Thus, such race-specific immunity is based on interactions of complementary *R*- and effector-genes (gene-for-gene interactions) (Jones and Dangl 2006).

A hallmark of *R*-mediated disease resistance is the hypersensitive response (HR), a programmed form of plant cell death localized to pathogen infection sites. HR is an effective defense reaction against biotrophic pathogens, which are dependent on live plant tissue (Mur, Mondhe et al. 2007). During compatible interactions, basal defense is activated, which is a weakened form of plant immunity that does not involve HR and is typically insufficient to prevent disease (Vlot, Klessig et al. 2008). *R*-mediated disease resistance is frequently facilitated through the SA-dependent branch of the defense network, which is often attributed to defense responses against biotrophic pathogens whereas the JA- and ET-dependent mechanisms seem preferentially to mediate immunity against necrotrophs (Glazebrook 2005)

Thus, the plant immune system is able to specifically tailor distinct responses against different types of pathogens. Fine tuning of these responses is mediated by complex crosstalk between individual signaling branches (Kunkel and Brooks 2002; Li, Brader et al. 2004; Li, Brader et al. 2004; Koornneef, Leon-Reyes et al. 2008; Pre, Atallah et al. 2008; Leon-Reyes, Spoel et al. 2009; Vlot, Dempsey et al. 2009). The timing, amplitudes and spatial distributions of certain defense signals determine the individual defense reactions activated in response to a given type of pathogen. One pathogen widely utilized to study plant defense is *Hyaloperonospora arabidopsidis* (*Hpa)*, an oomycete and obligate biotroph known to exclusively infect the model plant species *Arabidopsis thaliana* (Arabidopsis) (Slusarenko and Mauch-Mani 1991). The study of this pathosystem

has facilitated the identification of more gene-for-gene interactions than any other plant and pathogen combination (Hein, Gilroy et al. 2009; Coates and Beynon 2010). Thus far, SA-dependent defenses have been directly attributed to limiting *Hpa* growth in this pathosystem with JA/ET having no distinguishable role (Zhang, Chen et al. 2010). The majority of plant disease research indicates that interactions between JA- and SA-signaling are antagonistic, although it has also been demonstrated that at low concentrations they can act synergistically (Glazebrook 2005; Mur, Kenton et al. 2006).

JA is a lipid-derived signal that has many vital roles in plants (Glazebrook 2005; Browse and Howe 2008; Howe and Jander 2008; Jander and Howe 2008; Melotto, Mecey et al. 2008). Responses to JA are controlled by a regulatory apparatus consisting of four key components: the JA signal, the ubiquitin ligase $SCF^{COI1}$, the jasmonate ZIM-domain (JAZ) repressor proteins, and transcription factors that positively regulate expression of JA-responsive genes (Katsir, Chung et al. 2008).

JA-signaling is involved in complex processes such as pollen maturation, response to wounding, fruit ripening, root growth, and even tendril coiling (Staswick 1992; Turner, Ellis et al. 2002; Katsir, Chung et al. 2008). The role of JA in the defense response to insect wounding was first suggested in 1992 (Farmer and Ryan 1992). JA- and ET-dependent regulatory processes can act cooperatively (Onkokesung, Galis et al. 2010). For example, both JA- and ET-signaling contribute to resistance against necrotrophic pathogens and are known

to inhibit the formation of HR. This dual function is advantageous for the plant, since necrotrophs feed off dead tissue and may benefit from HR (Vlot, Dempsey et al. 2009). Activation of JA-mediated defenses are preceded by the accumulation of jasmonates synthesized by the octadecanoid pathway (Vick and Zimmerman 1984; Schaller 2001; Wasternack and Hause 2002). However, knowledge of the JA-signaling pathway is still incomplete.

Differences exist between the JA-signaling systems studied in different plant species. For example, although the systemin pathway induces systemic JA responses in tomato, no evidence has yet indicated such a pathway exists in Arabidopsis (Constabel, Bergey et al. 1995; Kubigsteltig, Laudert et al. 1999; Weiler, Laudert et al. 1999). When some plants (ex: subset of Solanaceae) are subjected to predation, the JA pathway is activated, requiring a 200-amino acid precursor, prosystemin. Prosystemin then produces the 18-amino-acid peptide, systemin, through proteolytic processing (Ryan and Pearce 1998). Systemin induces the production of $H_2O_2$, followed by the biosynthesis of JA and leading to the activation of defense-related genes (Orozco-Cardenas, Narvaez-Vasquez et al. 2001).

The first steps of JA biosynthesis occur in the chloroplast where membrane-derived linoleic acid is converted to 12-oxo-phytodienoic acid (OPDA) using multiple biosynthetic enzymes (eg: lipoxygenase, allene oxide synthase, allene oxide cyclase). OPDA is then transported to the peroxisome, where it is reduced to OPC-8:0 by OPDA reductase3 (OPR3) undergoing three rounds of β-

oxidation, resulting in the production of (+)-7-iso-JA (Howe 2001; Wasternack and Hause 2002; Wasternack and Hause 2009). Studies found that a main bioactive form of JA is JA-isoleucine (JA-Ile), which is produced by conjugation of JA to Ile by Jasmonate Resistant 1 (*JAR1*) (Suza and Staswick 2008). The *JAR1* locus encodes an ATP-dependent JA-amido synthetase (Staswick, Tiryaki et al. 2002). The modification of JA by jasmonate carboxyl methyltransferase converts it into the volatile compound methyl JA (MeJA). This reaction is presumed to take place in the cytoplasm (Seo, Song et al. 2001). MeJA mediates both developmental processes and defense responses against biotic and abiotic stresses (Seo, Song et al. 2001; Cheong and Choi 2003). Additionaly, MeJA is a gaseous compound, and can thus act as airborne signals to mediate inter-plant communication. This means that neighboring plants can be signaled if stressed are present (Cheong and Choi 2003).

Research has indicated that a positive feedback loop exists for JA biosynthesis (Sasaki, Asamizu et al. 2001). In Arabidopsis, *VSP* (JA-regulated gene for vegetative storage protein) expression is selectively induced by JA and not OPDA, while both molecules induce defense responses. This demonstrates that multiple mechanisms are responsible for the transduction of JA-related signals, which are selectively activated in response to differing stimuli (Staswick, Su et al. 1992; Feys, Benedetti et al. 1994; Ishiguro, Kawai-Oda et al. 2001; Stintzi, Weber et al. 2001; Ellis, Karafyllidis et al. 2002). The bacterial plant pathogen *Pseudomonas syringae* produces a JA-Ile-like compound termed

coronatine (COR), which suppresses some SA-immune responses by activating the JA signaling pathway (Bender, Alarcon-Chaidez et al. 1999; Staswick, Tiryaki et al. 2002; Laurie-Berry, Joardar et al. 2006).

The Arabidopsis mutant *coronatine*-insensitive 1 (*coi1-1*) was identified due to its insensitivity to plant growth inhibition by COR and OPDA (Benedetti, Xie et al. 1995). In addition, this mutant is male sterile and more susceptible to some pathogens and pests (Feys, Benedetti et al. 1994; Weiler, Kutchan et al. 1994; Benedetti, Xie et al. 1995; McConn, Creelman et al. 1997). *COI1* encodes a 66-kD protein that contains an N-terminal F-box motif and a leucine-rich repeat (LRR) domain (Xie, Feys et al. 1998). F-box proteins recruit regulatory proteins as substrates for ubiquitin-mediated degradation. This requires them to associate with Skp1 and Cullin (CUL1) to form an E3 ubiquitin ligase termed SCF (Skp1, Cullin, F-box) complex which is important for JA responses (Bai, Sen et al. 1996; Devoto, Nieto-Rostro et al. 2002; Xu, Liu et al. 2002; Lorenzo and Solano 2005). JA–Ile facilitates binding of $SCF^{COI1}$ with the jasmonate ZIM-domain 1 (JAZ1) protein (Thines, Katsir et al. 2007).

JAZ proteins are substrates for $SCF^{COI1}$ and negatively regulate JA-responses (Chini, Fonseca et al. 2007; Thines, Katsir et al. 2007; Yan, Stolz et al. 2007). In Arabidopsis these proteins belong to a 12 member family and contain a conserved motif at the C-terminus ($SLX_2FX_2KRX_2RX_5PY$). Many JAZ mutants displayed no discernible phenotypes, possibly due to functional redundancy (Thines, Katsir et al. 2007). Mutants of *jaz10* are hypersensitive to

JA, but the lack of obvious phenotypes in other *jaz* mutants suggests functional redundancy among other family members (Thines, Katsir et al. 2007; Yan, Stolz et al. 2007). The jaz1 mutant on ther other hand displayed male sterility, JA insensitivity, and increased resistance to infection by the bacterial pathogen *P. syringae* pv. *tomato* (Chung, Koo et al. 2008).

The JAZ proteins homo- and hetero-dimerize by way of a conserved TIFY domain and also bind *At*MYC2 and interact with COI1 through their C-terminal JAS domains (Melotto, Underwood et al. 2006; Katsir, Chung et al. 2008; Katsir, Schilmiller et al. 2008; Melotto, Mecey et al. 2008). *At*MYC2 is a helix-loop-helix transcription factor that acts as a key regulator of JA responses in plant-microbe interactions (Robert-Seilaniantz, Grant et al. 2011). When JA-Ile or COR bind to $SCF^{COI1}$ complexes, this promotes the ubiquitination of JAZ proteins leading to their degradation. Once degraded the JAZ-dependent repression on *At*MYC2 is relieved and JA-responsive genes are activated (Robert-Seilaniantz, Grant et al. 2011). The JAZ proteins then recruit a co-repressor TOPLESS (TPL)8 and TPL-related proteins (TPRs) through a adaptor protein called Novel Interactor of JAZ (NINJA). NINJA and TPL proteins act as negative regulators of jasmonate responses (Pauwels, Barbero et al. 2010).

Resistance to specific pathogens conferred through JA signaling show little overlap in transcriptional changes. This context is important to fine-tuning the JA response (De Vos, Van Zaanen et al. 2006; Pauwels, Inze et al. 2009). ET and abscisic acid (ABA) regulate different branches of the JA response (Adie,

Chico et al. 2007; Adie, Perez-Perez et al. 2007; Chini, Fonseca et al. 2007; Kazan and Manners 2008). JA and ET act together to induce the expression of *PDF1.2* (Penninckx, Thomma et al. 1998). The transcription factors, ERF1 and ORA59 work to integrate JA and ET signaling (Lorenzo, Piqueras et al. 2003; Pre, Atallah et al. 2008). These transcription factors confer resistance against necrotrophs (Berrocal-Lobo, Molina et al. 2002; Pre, Atallah et al. 2008). Alternately, MYC2 works with ABA signaling to negatively regulate the JA-ET responsive branch (Anderson, Badruzsaufari et al. 2004; Lorenzo, Chico et al. 2004) while activating genes within its own branch, such as VSP2 (Dombrecht, Xue et al. 2007). This branch is associated with the wound response and priming for pathogen defense (Lorenzo, Chico et al. 2004; Dombrecht, Xue et al. 2007; Pozo, Van Der Ent et al. 2008; Van der Ent, Van Wees et al. 2009; Fernandez-Calvo, Chini et al. 2011).

Molecular recognition of necrotrophic pathogens can trigger increased JA and ET synthesis as well as expression of defense genes, such as *PDF1*.2. Defensins, one group of ubiquitous peptides involved in innate immune response, are found in organisms ranging from invertebrates to plants. They are small (~5 kDa), basic, cysteine-rich proteins encoded by multigene families, similar in complexity to those encoding other defense-related proteins (Penninckx, Eggermont et al. 1996; Manners, Penninckx et al. 1998; Thomma, Eggermont et al. 1998; Brown, Kazan et al. 2003; Mur, Kenton et al. 2006). Defensins were first discovered in rabbits in 1984 and described in wheat and

barley in 1990 (Selsted, Szklarek et al. 1984; Colilla, Rocher et al. 1990). *PDF* genes were later discovered in Arabidopsis and encode small peptides originally labeled as γ-thionins (Penninckx, Eggermont et al. 1996; Epple, Apel et al. 1997; Thomma 1998; Thomma, Cammue et al. 2002). γ-thionins were later renamed plant defensins to emphasize their structural similarity to mammalian defensins.

The ancestry of defensins is thought to pre-date the divergence of plants and animals (Thomma, Cammue et al. 2002). Defensins are described in two separate kingdoms and further divided into five classes. Class 1, Class 2 and Class 3 are found in mammals and birds, while Classes 4 and are found in insects and Class 5 in plants (Carvalho Ade and Gomes 2009). The primary structures of defensins vary between organisms. PDF proteins contain eight conserved cysteines (Thomma, Cammue et al. 2002). They also include a common fold, which is formed by a β-sheet and an α-helix steadied by disulfide bridges and capable of stabilizing the entire protein (Aerts, Francois et al. 2008).

Arabidopsis *PDF* genes can be separated into three families, each encoding closely related peptides. Family 1 consists of *PDF1.1-1.5*, family 2 consists of *PDF2.1-2.6*, and family 3 consists of At4g30070 (*PDF3.2)* and At5g38330 (*PDF3.1)*. The predicted mature peptide sequences encoded by *PDF1.2a/b/c* are identical. *PDF1.2a* (At5g44420) and *PDF1.2c* (At5g44430) form a tandem repeat on chromosome 5 and *PDF1.2b* (At2g26020) is found on chromosome 2 directly adjacent to *PDF1.3* (At2g26010) (Thomma, Cammue et al. 2002). The high sequence similarity between *PDF1.2a/b/c* and *PDF1.3*

indicates that little evolutionary time has passed since their duplication. More amino acid variability is evident within the second family but this family also has genes that occur in a tandem array (Thomma, Cammue et al. 2002).

*PDF2.1* is expressed specifically in roots, siliques, and seeds (Thomma 1998). *PDF2.2* is expressed in all organs of healthy plants except stems and seeds (Epple, Apel et al. 1997; Epple, Apel et al. 1997; Thomma 1998). *PDF2.3* is expressed in all organs excluding roots and is not upregulated in response to pathogen infection (Epple, Apel et al. 1997). *PDF1.2a* is induced in leaves upon infection of pathogens such as *Botrytis cinerea* (Penninckx, Eggermont et al. 1996; Thomma, Eggermont et al. 1998; Thomma, Eggermont et al. 1999). *PDF1.1* expression is largely seed specific and may protect seedlings against pathogens (Terras, Eggermont et al. 1995; Penninckx, Eggermont et al. 1996). *PDF1.1, 1.2, 2.1, 2.2 and 2.3* display distinct organ-specific expression patterns (Thomma 1998; Kim 2004). *PDF1.1, PDF2.1, PDF2.2 and PDF2.3* are expressed constitutively which means that most plant tissues constitutively express two or more defensin genes at any given time (Thomma 1998; Thomma, Cammue et al. 2002; Lay and Anderson 2005). Therefore, it is likely that specific peptides may be expressed during specific situations and sites (Thomma, Cammue et al. 2002).

*PDF1.4* and *PDF2.4* appear not to contain predicted signal peptide sequences, suggesting they stay in the cytoplasm while the others PDF proteins are secreted. Alternately, PDF proteins may overlap (synergistic) while acting

distinctly to cover gaps in each-others' activity spectrum (Thomma, Cammue et al. 2002). From the third family found within Arabidopsis, *PDF3.2* encodes a protein of 129 amino acids with a C-terminal domain that has the conserved cysteine pattern shared by all plant defensins (Thomma, Cammue et al. 2002). *PDF3.1* encodes 122 amino acids with 56% identical residues to the protein encoded by *PDF3.2*. These proteins could be fusion proteins or precursors but more research is necessary to determine their functions (Bohlmann and Apel 1991; Gu, Kawata et al. 1992).

Transgenic expression of *PDF* genes leads to fortification of tissues against pathogen attack. Two *PDF* proteins, one from dahlia (*Dahlia merckii*, DmAMP1) and one from radish (*Raphanus sativus*, RsAFP2) were found to inhibit the growth of *Neurospora crassa*. One observed response after infection with *N. crassa* is a change in fungal cell ion fluxes, but this is believed to be a secondary effect, not a direct result of the PDF antifungal activity (Thomma, Cammue et al. 2002). There are two models describing possible modes-of action for the antimicrobial activity of PDF proteins. One model proposes that pores form in microbial cell membranes. The second model proposes that PDF proteins bind onto the anionic lipid headgroups disrupting the stability of the phospholipid bilayer of microbial cell membranes (Oren and Shai 1998; Shai 1999; Hoover, Rajashankar et al. 2000). Binding sites for these PDF proteins to sphingolipids found on the cell surface in fungal membranes have been demonstrated (Shai 1999; Hoover, Rajashankar et al. 2000). This suggests that the antimicrobial

activity of PDF proteins is dependent on their ability to bind to a target in the membrane of an infecting pathogen (Thomma, Cammue et al. 2002; Thevissen, Ferket et al. 2003; Thevissen, Francois et al. 2003; Thevissen, Warnecke et al. 2004). No similar activity has been shown against pathogenic fungi though.

Aside from the many possible roles of *PDF* genes it has been established that many PDF proteins isolated to date display antifungal activities against a broad range of fungi. Monocot PDF proteins may also inhibit α-amylase, an enzyme found in insect guts (locust and cockroach). This possibly hampers the insect's ability to digest plant material (Colilla, Rocher et al. 1990; Mendez, Moreno et al. 1990; Bloch and Richardson 1991; Terras, Eggermont et al. 1995). It appears though while some alternately inhibit α-amylase activity and protein synthesis that PDF proteins do not display both activities concurrently. Additionally, most plant defensins do not display antibacterial activity which has been suggested to be a result of a infection pressure from fungal as opposed to bacterial pathogens on plants (Thomma, Cammue et al. 2002).

Additional biological activities of PDF proteins include the ability to inhibit protein translation in a cell free system, inhibit proteases, and inhibit the growth of microbes (Mendez, Moreno et al. 1990). They may also act as mediators of zinc tolerance and inhibitors of ion channels, or exhibit activity against mammalian cells and enzymatic activity controlling the redox state of ascorbic acid (Carvalho and Gomes 2011). This diverse set of functions may be attributed to differences in the primary structures of defensins.

PDF activities range from antimicrobial and insecticidal to anti-parasitic. Due to their broad range of target organisms, *PDF* overexpression may be a suitable strategy for crop protection. While *R-genes* typically confer a narrow range of resistance, *PDF* genes may provide broad-spectrum resistance against multiple types of pathogens. If introduced into plants *PDF* genes may offer defensive advantages in addition to enhancing the plants ability to combat biotic stressors (Carvalho Ade and Gomes 2009). Arabidopsis also contains over 300 *Defensin-like* (*DEFL*) genes and the evolution of *DEFL*-, *PDF*-, and *R*-genes bears remarkable similarity that may be due to their evolution through similar mechanisms (Silverstein, Graham et al. 2005). Members of these three groups can be found as single genes or in clusters, which arose through duplication, recombination, or diversifying selection (Hulbert, Webb et al. 2001; Baumgarten, Cannon et al. 2003; Meyers, Kozik et al. 2003).

*Cis*-elements discovered within the promoter of *PDF1.2b* include a GCC box important for the ET response, a stress responsive G-box, a drought-responsive element (TACCGACAT), and JA-responsive elements (TGACG) and (AAATGTTGT) (Brown, Kazan et al. 2003; Zarei, Korbes et al. 2011). GCC boxes are commonly found in the promoters of genes which encode defense-related proteins and are known binding sites for some AP2/ERF transcription factors (Ohmetakagi and Shinshi 1995; Buttner and Singh 1997; Zhou, Tang et al. 1997). G-boxes regulate genes in response to environmental conditions such as red and UV light, anaerobiosis, and wounding and can be bound by certain

basic leucine zipper (bZIP) transcription factors (Shinozaki and Yamaguchi-Shinozaki 2007).

Transcription factors that positively affect *PDF1.2b* mRNA levels are the AP2/ERF-domain transcription factors ERF1, ORA59, AtERF1 and AtERF2, as well as the basic leucine zipper (bZIP) transcription factor TGA5 (Solano, Stepanova et al. 1998; Brown, Kazan et al. 2003; Lorenzo, Piqueras et al. 2003; Pre, Atallah et al. 2008). Negative effects were observed upon overexpression of *AtERF4* or *WRKY70,* as well as in a triple mutant, where function of TGA2, TGA5 and TGA6, three redundantly acting TGA bZIP factors, is abolished (Li, Brader et al. 2004; McGrath, Dombrecht et al. 2005). TGA2, TGA5 and TGA6 are also commonly required for the activation of JA- and ET-dependent defense mechanisms that counteract necrotrophic pathogens (Zander, La Camera et al. 2010). TGA2 is the only transcription factor, for which direct interaction with the *PDF1.2* promoter has been demonstrated yet (Spoel, Koornneef et al. 2003). ORA59 and ERF1 are believed to bind directly to the promoter and AtERF1 and AtERF2 indirectly (Pre, Atallah et al. 2008). ORA59 is the primary positive regulator of *PDF1.2b* expression in response to JA/ET (Memelink 2009) and it binds GCC boxes (Zarei, Korbes et al. 2011). Since most *PDF genes* belong to small families of closely related members, studying their function can be difficult using traditional genetic methods.

*Hpa* is a useful model pathogen to study the genetics of plant-pathogen interactions (Slusarenko and Mauch-Mani 1991). However, unlike infections with

bacterial pathogens, inoculation with *Hpa* does not result in synchronous and uniform responses in host plants. This is mainly due to the unsynchronized germination of *Hpa* spores upon spray inoculation. Furthermore, transcription factors and other regulators controlling defense responses are frequently represented by families of functionally redundant members (Robatzek and Somssich 2002). Additionally, some components of the plant immune system may be essential for plant survival during early development (Meinke and Sussex 1979). Hence, conventional genetics is of limited use for the dissection of the plant defense network in this circumstance. Chemical genetics utilizes small molecules to alter *in vivo* protein functions in a reversible and highly controllable manner (Raikhel and Pirrung 2005). Using such bioactive compounds as synthetic elicitors, a simultaneous and uniform activation of the defense network can be accomplished (Zheng and Chan 2002). This allows for reproducible measurements of the dynamics of molecular processes and physiological responses. In addition, synthetic elicitors may be used to simultaneously knock out families of functionally redundant proteins, theoretically resulting in clear phenotypes. Moreover, the function of essential genes can be studied efficiently by introducing the respective chemical at any stage of development to manipulate their activity (Darvas, Dorman et al. 2004).

The identification of a collection of novel synthetic elicitors may permit the selective manipulation of defined branches of the defense network. Such elicitors will serve as powerful new tools for the emerging field of systems biology

(Christian, Hannah et al. 2008). They are likely to facilitate the stimulation of the defense network with unprecedented precision allowing the examination of the relation of defined signaling events and physiological outputs in a quantitative manner. In the broader context, the ability to manipulate disease resistance pathways using synthetic elicitors can also be exploited for agricultural purposes by using various combinations of chemicals to develop a new generation of pesticides. These combinations may be capable of activating specific pathways to differing degrees to achieve pathogen appropriate defense responses. Such stimulators of the plant's inherent immune responses are likely to be superior to conventional biocidal pesticides that are harmful to both environment and consumers (Knoth, Salus et al. 2007).

Knoth et al., (2009) previously identified two different synthetic elicitors, DCA (3,5-dichloroanthranillic acid) and CMP442 (2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid). Both elicitors induce defense in Arabidopsis against *Hpa* and DCA also against *P. syringae* (Knoth, Salus et al. 2009) (Chapter 2). The modes of action of these compounds are distinct; although both act on the SA-dependent branch of the defense network. CMP442 appears to interfere with defense-signaling processes upstream from SA perception, while DCA activates signaling steps downstream from SA (Chapter 2). This screening system focused on compounds targeting components of the SA-dependent sector of the defense network and yielded many candidate synthetic elicitors (Knoth, Salus et al. 2009). To widen the spectrum of functionally distinct synthetic

elicitors a screening system was needed that allows for the identification of synthetic elicitors interfering with the JA- and ET-dependent parts of the defense signaling network.

A set of five genes was identified in microarray experiments that exhibit SA-independent upregulation in response to infection with *Hpa* (Eulgem, Weigman et al. 2004). Four of these genes were members of the *PDF* family including JA-pathway marker gene *PDF1.2b*. Because *PDF* genes are known to response to JA and ET, but not SA, this set of co-expressed defense-related genes was named *JEDI* (Jasmonic acid- and Ethylene-Dependently Induced) and include *PDF1.2a* (At5g44420), *PDF1.2b* (At2g26020), *PDF1.2c* (At5g44430), *PDF1.3* (At2g26010), and *JEDI1* (AT2G43590). Here the development of transgenic lines in which select *JEDI* genes were fused to reporter genes to develop a high-throughput chemical screening system is described. In addition, the creation of transgenic plants with an RNAi transgene silencing transcripts of the closely related *PDF* members within the *JEDI* set (*JEDI-PDF*) is reported.

## RESULTS

### *JEDI* Genes Show *Hpa* Specific Induction

A group of five genes exhibited *Hpa*-responsive and SA-independent transcript accumulation in microarray experiments (Eulgem, Weigman et al. 2004). They were named the *JEDI* genes and include *PDF1.2a* (At5g44420), *PDF1.2b* (At2g26020), *PDF1.2c* (At5g44430), *PDF1.3* (At2g26010), and *JEDI1* (AT2G43590). Their transcript levels increased in Col-0 at 12 and 48 hours post infection (hpi) with the Col-avirulent *Hpa* isolate Emoy2 (Figure 3.1). In the transgenic *nahG* line, which is unable to accumulate SA (Gaffney, Friedrich et al. 1993), all *JEDI* transcripts were upregulated at 12 hpi with *Hpa*Emoy2 to similar levels as in Col-0, suggesting that this response is largely SA-independent (Figure 3.1).

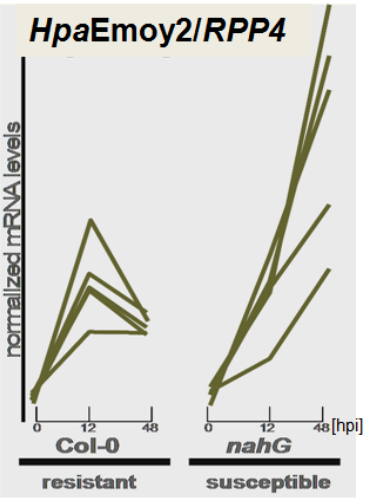



**Figure 3.1: Transcript upregulation of *JEDI* genes in response to infection of *Hpa*Emoy2 was not blocked in SA signaling-compromised *nahG* plants.** These five co-expressed genes were named the *JEDI* (Jasmonic Acid and Ethylene Dependently Induced) genes after it was determined that they included *PDF1.2a*, *PDF1.2b*, *PDF1.2c*, *PDF1.3*, and another unknown gene termed *JEDI1*. Four of these five genes were known to be plant defensins and have a role in the jasmonic acid signaling pathway. Shown here are normalized transcript levels of these five genes co-expressed at the indicated time points post *Hpa*Emoy2 infection (hpi). These data were generated with custom Affymetrix whole-genome exon arrays (Knoth et al., 2009). The transcript patterns shown exhibit a minimum Pearson correlation coefficient of 0.80 to the weighted average pattern of core of this subcluster. Pearson correlation coefficients consider general patterns and not amplitudes of data (Knudsen, 2002).

Furthermore, *JEDI* transcripts were hyperinduced at 48 hpi in *nahG* compared to Col-0 (Figure 3.1). The *Hpa*-inducibility of the *JEDI* transcripts was confirmed via reverse transcription (RT)-PCRs (Figure 3.2) at 0, 6, and 12 hpi with *Hpa*Emoy2 in two-week-old Col-0 and *nahG* plants. Compared to their levels at 0 hpi, transcript levels of all *JEDI* genes were enhanced at 6 hpi and 12 hpi in both Arabidopsis lines. Thus, the trends observed in these RT-PCRs were similar to those in the original microarray data, suggesting that *JEDI* transcript accumulation in response to *Hpa*Emoy2 recognition is at least partly SA-independent. Additional timepoints were examined after *Hpa* in Col-0 and nahG.

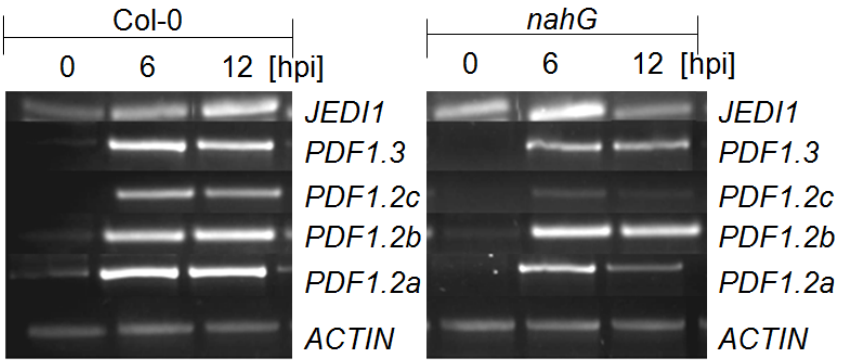



**Figure 3.2: Semi-quantitative RT-PCR confirmed upregulation of *JEDI* transcripts in Col-0 and *nahG* after infection with *Hpa*Emoy2.** Two-week-old soil-grown Col-0 and *nahG* seedlings were harvested and their total RNA extracted 0, 6, and 12 h hpi with *Hpa*Emoy2 (5 x $10^4$ spores/ml). Transcript levels of *PDF1.2a, PDF1.2b, PDF1.2c, PDF1.3, and JEDI1* were examined by RT-PCR. RT-PCR results with *ACTIN* are included as a template control. At least three independent replicates gave similar results. Results of a typical experiment are shown.

In these experiments I could not reproduce the hyperinduction seen in the microarray experiments (data not shown). A likely reason for this apparent

discrepancy could be that the timing or *Hpa* infections are not always reproducible. The infection may have faster progressed in the samples used for the microarrays than in those used for RT-PCRs; thus the 48 hpi timepoint in the microarray study represents a later stage in the plant response compared to the 48 hpi timepoint in RT-PCR experiments.

### *JEDI* Genes are Part of JA-Controlled Defense Responses

*PDF* genes are known to be positively controlled by the JA-dependent branch of the plant defense network. However, JA-controlled defense responses have been traditionally associated with immunity against necrotrophic or hemi-biotrophic pathogens, such as the fungus *B. cinerea* or the bacterial pathogen *P. syringae* (Clarke, Volko et al. 2000; Spoel, Johnson et al. 2007). Immunity against strict biotrophs, such as *Hpa* is believed to be controlled by SA-dependent immune responses (Maleck and Dietrich 1999; Maleck, Levine et al. 2000; Glazebrook 2005). However, our observation that *JEDI-PDF* genes are upregulated during incompatible *Hpa* interactions suggested that JA-dependent signaling processes can also contribute to immunity to this strictly biotrophic pathogen. Thus, this set of *Hpa*-responsive genes provided us with an opportunity to study aspects of JA-dependent processes in immunity against *Hpa*.

### Development of p*JEDI::*Reporter constructs

In order to design a high-throughput screening protocol for the identification of synthetic elicitors targeting the JA-dependent branch of the plant defense network, a previously characterized p*PDF1.2b*::*GUS* line (Manners, Penninckx et al. 1998; Brown, Kazan et al. 2003) was obtained and tested. Seven-day-old seedlings were grown in liquid growth medium in 96-well plates and incubated for 24 h with 100 µM (SA), 45 µM (MeJA), or mock solution (water). A concentration of 45 µM MeJA induces expression of p*PDF1.2b*::*GUS* (Manners, Penninckx et al. 1998). SA should not induce this reporter (Manners, Penninckx et al. 1998). At 24 h after the respective treatments, seedlings were histochemically stained to visualize *GUS* expression (Figure 3.3). High levels of GUS expression were evident in response to each of the three treatments. Even the mock-treatment resulted in GUS expression (Figure 3.3). Due to the apparent lack of specificity in expression responses a screen with this p*PDF1.2b::GUS* line, this screen was deemed unfeasible.

Therefore, a set of new transgenic lines with fusions of *JEDI* promoters to reporter genes was developed and tested for their suitability for high-throughput chemical screens. Reporter lines ideal for the planned chemical screens should show no or extremely low background expression and exhibit clear pathogen or JA-inducibility. In addition, the variability of reporter gene expression levels in response to the same treatment should be minimal.

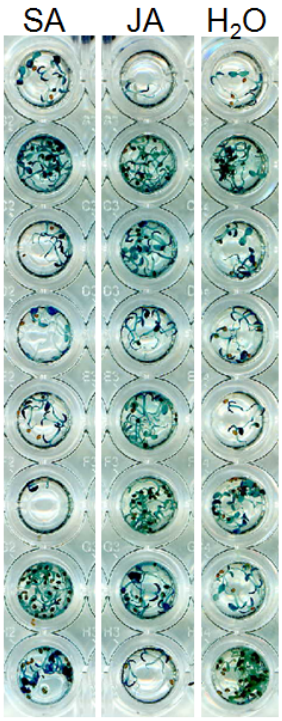



**Figure 3.3: Analysis of *PDF1.2a::GUS* transgenic lines under saturation treatment conditions.** Seven-day-old liquid-grown *PDF1.2a::*GUS seedlings after a 24 h incubation with 100 µM SA, 45 µM MeJA, or water ($H_2O$). High levels of *GUS* expression were evident in response to each of the three tested treatments. Blue/green color of cotyledons indicates induction of the *GUS* gene expression (all chlorophyll was removed from plants during ethanol-destaining). Each column represents an experimental replicate where 8 wells with multiple seedlings were simultaneously treated. Experiment was performed at least three times.

### Identification of *JEDI* Promoter Regions

A multiple sequence alignment (Larkin, Blackshields et al. 2007) and data collected from the Arabidopsis Genome Regulatory Server (AGRIS) website (Palaniswamy, James et al. 2006; Yilmaz, Mejia-Guerra et al. 2011) indicated the presence of possible pathogen-responsive *cis*-elements in *JEDI* promoter regions (Figure 3.4 and 3.5). GCC-boxes are known to mediate gene activation in response to various pathogens, PAMPs and JA or ET (Brown, Kazan et al. 2003). The *JEDI*-PDF genes included here are those that were used to create the transgenic reporter lines. To identify *JEDI-PDF* promoter regions suitable for reporter lines to be used in synthetic elicitor screens, two promoter constructs for

*PDF1.3*, *PDF1.2c*, and *JEDI1* (not shown) were made. For each one of these


**Figure 3.4: Sequence alignment of *JEDI* promoter sequences.** ClustalW2 - Multiple Sequence Alignment algorithm (Larkin et al., 2007) was used to align the promoter sequences to identify conserved promoter elements that may be important for the JA-specific expression. Sequences included are only those genes which were used to design the promoter-reporter constructs, *JEDI1* is not included because it does not bear any sequence similarity to the remaining *JEDI* genes. Motifs included: JA-ERF-jasmonic acid response factor binding site (JA-ERF) and ethylene-response factor binding site (Grennan 2008), and GCC box consensus nucleotide sequence AGCCGCC (Hao et al., 1998). Blue highlighted nucleotides are part of JAR-ERF and yellow highlighted nucleotides are part of the GCC-box motif. *JEDI1* not shown due to no sequence similarity. Sequence alignment was used to develop promoter-reporter lines.

three genes the entire upstream intergenic region stretching from the *JEDI-PDF* genes transcription start site to the coding sequences of the next upstream gene. The second set of promoter constructs included the GCC box and the full intergenic region upstream of the transcriptional start site.

A total of six promoter stretches were cloned and fused to the *Escherichia coli GUS* or the Firefly *LUC* reporter gene (Figure 3.5). Thus, 12 distinct reporter lines were constructed and transformed into wild type Arabidopsis Col-0 plants by *Agrobacterium tumefaciens*-mediated gene transfer using the floral dip procedure (Clough and Bent 1998).

A

| Promoter ID | Gene Description | | Chromosome Location | |
|---|---|---|---|---|
| At2g26010 | plant defensin protein (*PDF1.3*) | | Chr2:11094140 - 11094487 | |
| Binding Site (BS) Name | BS Genome Start | BS Genome End | BS Sequence | BS Family/TF |
| **AtMYC2 BS in RD22** | 11094178 | 11094183 | cacatg | **BHLH** |
| **W-box promoter motif** | 11094312 | 11094317 | ttgact | **WRKY** |
| **RAV1-A binding site motif** | 11094218 | 11094222 | caaca | **ABI3VP1** |
| **RAV1-A binding site motif** | 11094316 | 11094320 | caaca | **ABI3VP1** |
| **GATA promoter motif [LRE]** | 11094246 | 11094251 | agatag | ... |
| **GATA promoter motif [LRE]** | 11094275 | 11094280 | agataa | ... |
| **GATA promoter motif [LRE]** | 11094329 | 11094334 | tgatag | ... |
| **GATA promoter motif [LRE]** | 11094322 | 11094327 | agataa | ... |
| **Ibox promoter motif** | 11094276 | 11094281 | gataag | ... |
| **Ibox promoter motif** | 11094321 | 11094326 | gataag | ... |

B

| Promoter ID | Gene Description | | Chromosome Location | |
|---|---|---|---|---|
| At2g26020 | plant defensin protein (*PDF1.2b*) | | Chr2:11095976 - 11096326 | |
| BS Name | BS Genome Start | BS Genome End | Binding Site Sequence | Binding Site Family/TF |
| **Bellringer/replumless/pennywise BS1 IN AG** | 11096201 | 11096208 | aaattaaa | **Homeobox** |
| **DPBF1&2 binding site motif** | 11095993 | 11095999 | acacaag | **bZIP** |
| **RAV1-A binding site motif** | 11096314 | 11096318 | caaca | **ABI3VP1** |
| **BoxII promoter motif** | 11096013 | 11096018 | ggttaa | ... |
| **BoxII promoter motif** | 11096050 | 11096055 | ggttaa | ... |
| **GATA promoter motif [LRE]** | 11096125 | 11096130 | agataa | ... |
| **GATA promoter motif [LRE]** | 11096161 | 11096166 | agataa | ... |
| **GCC-box promoter motif** | 11096058 | 11096063 | gccgcc | ... |
| **Ibox promoter motif** | 11096160 | 11096165 | gataag | ... |

C

| Promoter ID | Gene Description | Chromosome Location |
|---|---|---|
| At2g43590 | glycosyl hydrolase family 19 (chitinase) | Chr2:18089825 - 18091246 |

| BS Name | BS Genome Start | BS Genome End | Binding Site Sequence | Binding Site Family/TF |
|---|---|---|---|---|
| AtMYC2 BS in RD22 | 18090496 | 18090501 | cacatg | BHLH |
| AtMYC2 BS in RD22 | 18090550 | 18090555 | cacatg | BHLH |
| ATB2/AtbZIP53/AtbZIP44/GBF5 BS in ProDH | 18090346 | 18090351 | actcat | bZIP |
| W-box promoter motif | 18091079 | 18091084 | ttgact | WRKY |
| W-box promoter motif | 18089879 | 18089884 | ttgact | WRKY |
| ATHB5 binding site motif | 18090940 | 18090948 | caatcattg | HB |
| ATHB5 binding site motif | 18090939 | 18090947 | caatgattg | HB |
| CCA1 binding site motif | 18090037 | 18090044 | aacaatct | MYB-related |
| MYB4 binding site motif | 18090706 | 18090712 | aacaaac | MYB |
| MYB4 binding site motif | 18091170 | 18091176 | accaaac | MYB |
| MYB4 binding site motif | 18090932 | 18090938 | aacaaac | MYB |
| MYB4 binding site motif | 18090794 | 18090800 | aactaac | MYB |
| RAV1-A binding site motif | 18090036 | 18090040 | caaca | ABI3VP1 |
| RAV1-A binding site motif | 18091181 | 18091185 | caaca | ABI3VP1 |
| RAV1-A binding site motif | 18090012 | 18090016 | caaca | ABI3VP1 |
| LFY consensus binding site motif | 18090629 | 18090634 | ccactg | LFY |
| LFY consensus binding site motif | 18090883 | 18090888 | ccattg | LFY |
| LFY consensus binding site motif | 18090943 | 18090948 | ccaatg | LFY |
| GATA promoter motif [LRE] | 18090694 | 18090699 | agataa | ... |
| GATA promoter motif [LRE] | 18090681 | 18090686 | agataa | ... |
| GATA promoter motif [LRE] | 18090659 | 18090664 | tgataa | ... |
| GATA promoter motif [LRE] | 18089946 | 18089951 | tgataa | ... |
| GCC-box promoter motif | 18090458 | 18090463 | gccgcc | ... |
| Ibox promoter motif | 18091149 | 18091154 | gataag | ... |
| L1-box promoter motif | 18089941 | 18089948 | taaatgca | ... |
| T-box promoter motif | 18090107 | 18090112 | actttg | ... |
| SORLIP1 | 18090627 | 18090632 | agccac | ... |
| SORLIP2 | 18090597 | 18090601 | gggcc | ... |

D

| Promoter ID | Gene Description | Chromosome Location |
|---|---|---|
| At5g44420 | plant defensin protein (*PDF1.2a*) | Chr5:17924823 - 17926317 |

| BS Name | BS Genome Start | BS Genome End | Binding Site Sequence | Binding Site Family/TF |
|---|---|---|---|---|
| AtMYC2 BS in RD22 | 17926059 | 17926064 | cacatg | BHLH |
| ATB2/AtbZIP53/AtbZIP44/GBF5 BS in ProDH | 17924887 | 17924892 | actcat | bZIP |
| CArG promoter motif | 17925185 | 17925194 | ccaaatttgg | MADS |
| CArG promoter motif | 17925184 | 17925193 | ccaaatttgg | MADS |
| CCA1 binding site motif | 17924829 | 17924836 | aaaaatct | MYB-related |
| DPBF1&2 binding site motif | 17925989 | 17925995 | acacaag | bZIP |
| DPBF1&2 binding site motif | 17924848 | 17924854 | acacacg | bZIP |
| MYB binding site promoter | 17925692 | 17925699 | aacctacc | MYB |
| MYB binding site promoter | 17925836 | 17925843 | aaccaaac | MYB |
| MYB binding site promoter | 17924928 | 17924935 | aaccaaac | MYB |
| MYB3 binding site motif | 17925398 | 17925405 | taactaac | MYB |
| MYB4 binding site motif | 17925693 | 17925699 | acctacc | MYB |
| MYB4 binding site motif | 17925837 | 17925843 | accaaac | MYB |
| MYB4 binding site motif | 17926157 | 17926163 | aacaaac | MYB |
| MYB4 binding site motif | 17925595 | 17925601 | aacaaac | MYB |
| MYB4 binding site motif | 17925398 | 17925404 | aactaac | MYB |
| MYB4 binding site motif | 17924928 | 17924934 | accaaac | MYB |
| RAV1-A binding site motif | 17925494 | 17925498 | caaca | ABI3VP1 |
| RAV1-A binding site motif | 17926156 | 17926160 | caaca | ABI3VP1 |
| RAV1-A binding site motif | 17925899 | 17925903 | caaca | ABI3VP1 |
| RAV1-A binding site motif | 17925422 | 17925426 | caaca | ABI3VP1 |
| RAV1-A binding site motif | 17925216 | 17925220 | caaca | ABI3VP1 |
| LFY consensus binding site motif | 17925110 | 17925115 | ccaatg | LFY |
| BoxII promoter motif | 17925195 | 17925200 | ggttaa | ... |
| BoxII promoter motif | 17926008 | 17926013 | ggttaa | ... |
| BoxII promoter motif | 17926261 | 17926266 | ggttaa | ... |
| BoxII promoter motif | 17926046 | 17926051 | ggttaa | ... |
| GATA promoter motif [LRE] | 17925057 | 17925062 | tgataa | ... |
| GATA promoter motif [LRE] | 17925063 | 17925068 | agatag | ... |
| GATA promoter motif [LRE] | 17926176 | 17926181 | agataa | ... |
| GATA promoter motif [LRE] | 17925601 | 17925606 | tgataa | ... |
| GCC-box promoter motif | 17926054 | 17926059 | gccgcc | ... |
| Hexamer promoter motif | 17925698 | 17925703 | ccgtcg | ... |
| Ibox promoter motif | 17926175 | 17926180 | gataag | ... |
| L1-box promoter motif | 17925076 | 17925083 | taaatgta | ... |
| RY-repeat promoter motif | 17925550 | 17925557 | catgcatg | ... |
| RY-repeat promoter motif | 17925549 | 17925556 | catgcatg | ... |

E

| Promoter ID | Gene Description | | Chromosome Location | |
|---|---|---|---|---|
| At5g44430 | plant defensin protein (*PDF1.2c*) | | Chr5:17926670 - 17927025 | |
| BS Name | BS Genome Start | BS Genome End | BS Sequence | BS Family/TF |
| **W-box promoter motif** | 17926803 | 17926808 | ttgact | WRKY |
| **RAV1-A binding site motif** | 17926807 | 17926811 | caaca | ABI3VP1 |
| **GATA promoter motif [LRE]** | 17926730 | 17926735 | agatag | ... |
| **GATA promoter motif [LRE]** | 17926737 | 17926742 | tgatag | ... |
| **GATA promoter motif [LRE]** | 17926766 | 17926771 | agataa | ... |
| **Ibox promoter motif** | 17926767 | 17926772 | gataag | ... |

**Figure 3.5: Putative cis-elements found within the promoter region of the *JEDI* genes**. **A-E,** Tables list the binding site (BS) name, the position at which the BS is located in the genome, BS sequence, chromosomal position of the gene, BS transcription factor family if known.

### Transgenic Promoter-Reporter Fusion Lines

At least two lines homozygous with a single T-DNA insertion of each *LUC* reporter construct were identified and tested for their JA-responsiveness and background expression levels in the 96-well plates containing solid growth medium. Tables 3.1 and 3.2 list all transgenic lines created. Each of the tested *JEDI*-promoter::*LUC* reporter lines exhibited substantial levels of *LUC* expression after mock and MeJA treatments (Figure 3.6). Due to their high background expression levels and weak MeJA-inducibility, the *JEDI*-promoter::*LUC* lines were not suitable for high-throughout chemical screens. However, these lines may be useful for the analysis of the pathogen-responsiveness of the respective promoter stretches. Homozygous GUS lines were identified and experiments

indicated that they were also not suitable for a high-throughput chemical screen due to constitutive overexpression (data not shown).

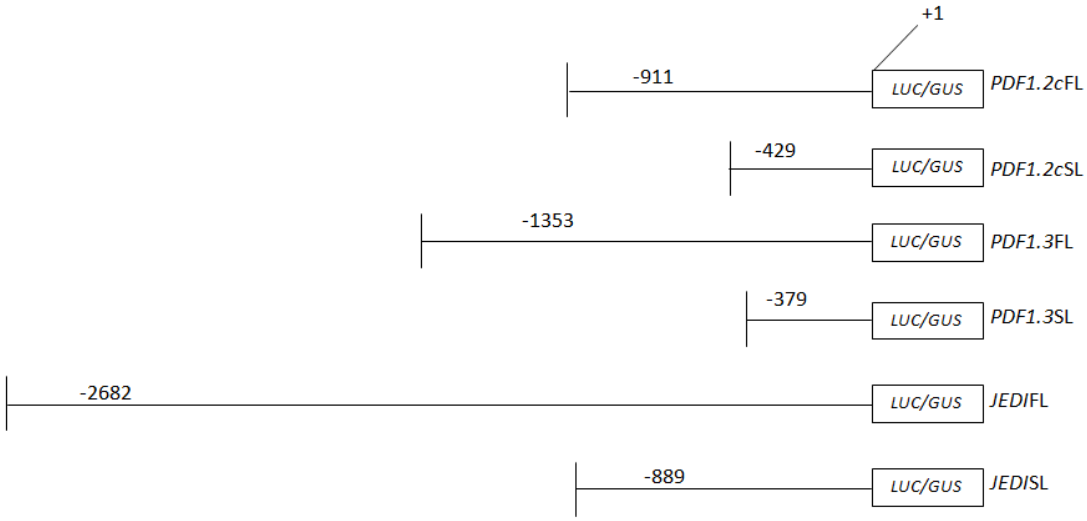



**Figure 3.6: Diagram of *JEDI*-promoter::*LUC* or *GUS* constructs transformed into Arabidopsis.** Diagram of *JEDI-promoter::LUC* or *GUS* promoter stretches that were cloned into the binary vector pJGWL7.0 (*LUC*- Karimi et al., 2007) or pMDC163 (*GUS*- Karimi et al., 2007) to generate transgenic GUS or LUC reporter gene fusions. Numbers indicate 5' promoter end points relative to the respective *JEDI* genes' transcriptional start site (+1). The open boxes represent the 5' UTR and coding sequence of the respective reporter gene. Full length (FL) lines incorporate the promoter region of the respective gene and short length (SL) constructs include the promoter up to a putative GCC-box.

### Development of *JEDI* RNAi Lines

To study the role of the *JEDI* genes *in planta*, combining multiple mutations may be necessary to overcome the redundancy in this family. Towards that end, the identification of single *JEDI* gene mutants was attempted so that double, triple, or even quadruple mutants could be bred. Independent Col-0 mutants with T-DNA insertions in or near each *JEDI* gene were identified using

The Arabidopsis Information Resource (TAIR) (Alonso, Stepanova et al. 2003). Due to close proximity of the *JEDI* genes to one another on their respective chromosomes selecting mutants in these lines proved impracticable via standard genotyping techniques (data not shown). Therefore, a RNAi-based co-silencing strategy was pursued.

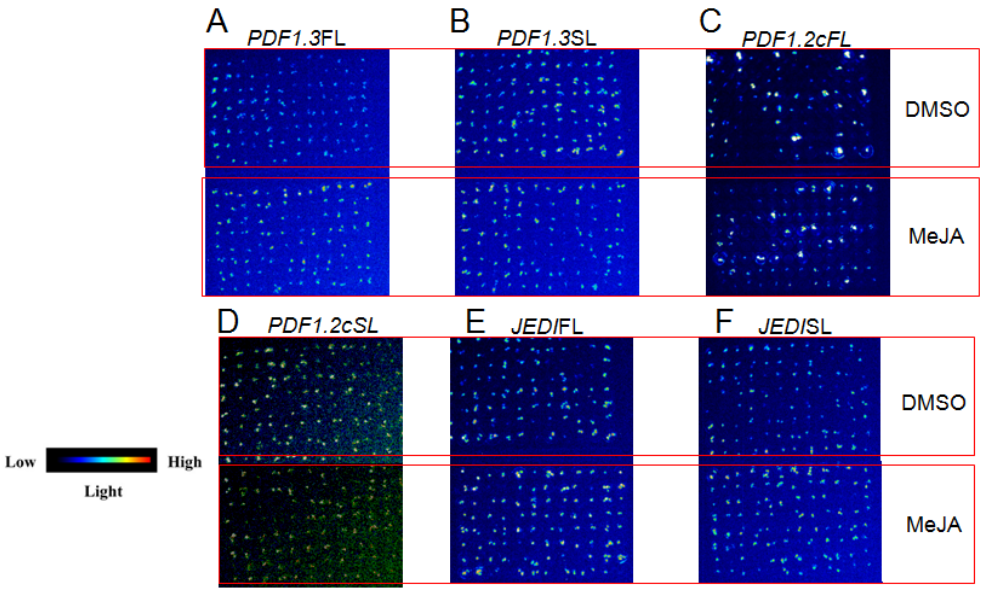



**Figure 3.7: Analysis of *JEDI*-promoter::*LUC* constructs in Arabidopsis seedlings.** A-F, One-week-old transgenic *JEDI*-promoter::*LUC* plants were grown on solid media in 96-well plates. Each image is of two 96 well plates containing lines either treated with 45 µM MeJA or control (1 %-DMSO) and 48 h later sprayed with luciferin. Each well contains 1-2 seedlings. LUC activity was detected using a high performance CCD camera. Images show *LUC* activity in whole Arabidopsis seedlings. The luminescence image shows the light emitted by luciferase activity within the plant on a computer-generated color scale, with white indicating bright luminescence and blue low luminescence. For all lines tested *LUC* expression was similar in both the positive and negative controls. D1-5-1 plants were used as

**Table 3.1: Homozygous and heterozygous transgenic lines containing the promoter regions from *PDF1.2c*, *PDF1.3*, and *JEDI1* fused to *LUC*.**

| Heterozygous Transgenic Lines | Genetic Background |
|---|---|
| *PDF1.2*-SL-PB | Col-0 |
| 3-1, 2-2, 4-1, 5-1, 7-1, 8-2, 9-1, 10-4, 11-1, 12-1, 14-2, 7-2, 2-3, 13-1, 1-1, 2-4, 3-2, 4-4, 5-4, 8-1, 9-4, 10-1, 12-2, 13-4 | |
| *PDF1.2*-FL-PB | Col-0 |
| 2-1, 1-4, 4-2, 5-1, 7-2, 10-1, 11-4, 12-3, 13-1, 5-4, 1-2, 4-3, 3-4, 10-4, 11-2, 14-3, 7-3, 9-3, 14-1 | |
| *PDF1.3*-SL-PB | Col-0 |
| 5-2, 6-1, 3-3, 2, 3-1, 8-3, 4-3, 6-3, 5-1, 1-2 | |
| *PDF1.3*-FL-PB | Col-0 |
| 1-3, 1-4, 5-1, 1-2, 1-1 | |
| *JEDI*-FL-PB | Col-0 |
| 2, 1-1, 11-2, 2-3, 2-2, 4-3, 4-2 | |
| *PDF1.2*-FL-PB | ein2/jar1 |
| 1-1, 1-2, 1-3 | |
| *PDF1.3*-FL-PB | ein2/jar1 |
| 1-2, 1-1 | |
| *JEDI*-SL-PB | ein2/jar1 |
| 1-2, 3-3, 1-3, 3-1 | |
| **Homozygous Transgenic Lines** | |
| *PDF1.3*-SL-PB | Col-0 |
| 3-1, 8-2, 4-2 | |
| *PDF1.3*-FL-PB | Col-0 |
| 1-4, 2-2, 5-3 | |
| *PDF1.2*-SL-PB | Col-0 |
| 2-3, 7-2 | |
| *PDF1.2*-FL-PB | Col-0 |
| 2-2, 9-2, 14-2 | |
| *JEDI*-FL-PB | Col-0 |
| 2, 1-1, 11-2 | |
| *JEDI*-SL-PB | Col-0 |
| 2-1, 4-4 | |

**Table 3.2: Heterozygous transgenic lines containing the promoter region from *CaBP22* or *JEDI1* fused to *GUS*.**

| Heterozygous Transgenic Line | Genetic Background |
|---|---|
| *CaBP22*::GUS | *npr1-3* |
| 3-2, 5-2, 1-1, 5-3, 5-1, 1-3, 4-1, 2-1, 2-2, 9-1, 9-2, 1-2, 71-1, 3-3, 1-3, 4-3, 3-1, 1, 5, 21-2, 4-3, 4-2, 9-3, | |
| *JEDI*-FL-163::*GUS* | Col-0 |
| 5-1, 5-3, 5-4, 5-5 | |
| *JEDI*-SL-163::*GUS* | Col-0 |
| 4-5, 5-3, 5-4, 5-2, 5-1 | |

The pANDA-*RNAi* vector was utilized to knock out/down transcripts of multiple *JED-PDFI* genes by expression of a microRNA driven by the maize ubiquitin promoter (Miki and Shimamoto 2004; Miki, Itoh et al. 2005). The high relatedness of the *PDF1.2a-c* and *PDF1.3* gene sequences (Figure 3.7 and 3.8) permitted the use of this technique to generate a single mutant affecting the expression of all four genes. The resulting *PDF*-RNAi construct was used to create a set of transgenic lines, in the Col-0 and *wrky70-3* backgrounds. The *wrky70* background was included to examine possible interactions between JA-dependent and *JEDI*-mediated immune responses. Previous work by our lab has shown that Arabidopsis *LURP* genes are required for full basal defense against

*Hpa* and that that *LURP* activity partially depends on the WRKY70 transcription factor (Knoth et al., 2007; Knoth & Eulgem, 2008).

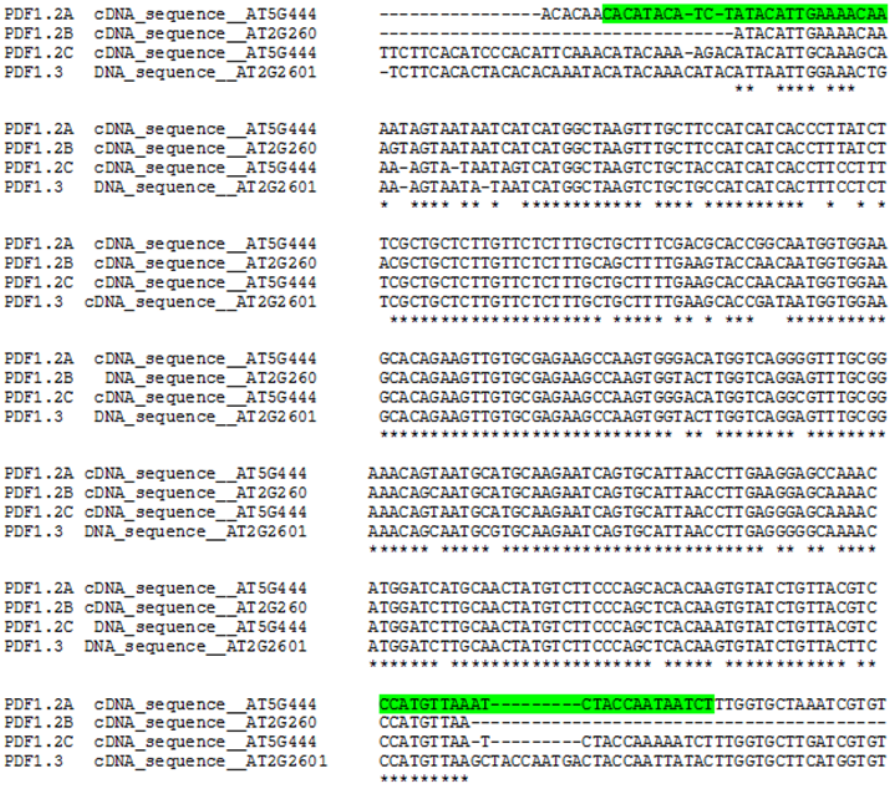



**Figure 3.8: Multiple sequence alignment of genomic sequences of *PDF* genes.** ClustalW2 - Multiple Sequence Alignment algorithm (Larkin et al., 2007) was used to align the entire genomic sequences of the *PDF* genes. Stars below the alignment indicate a full consensus between all four genes. Primers used for creating RNAi lines are indicated with a green highlighting PDF1.2A-RNAi-F-(5'-TCATGGCTAAGTTTGCTTCC-3') and PDF1.2A-RNAi-R-(5'-AACAACAACGGGAAAATAAACA-3').

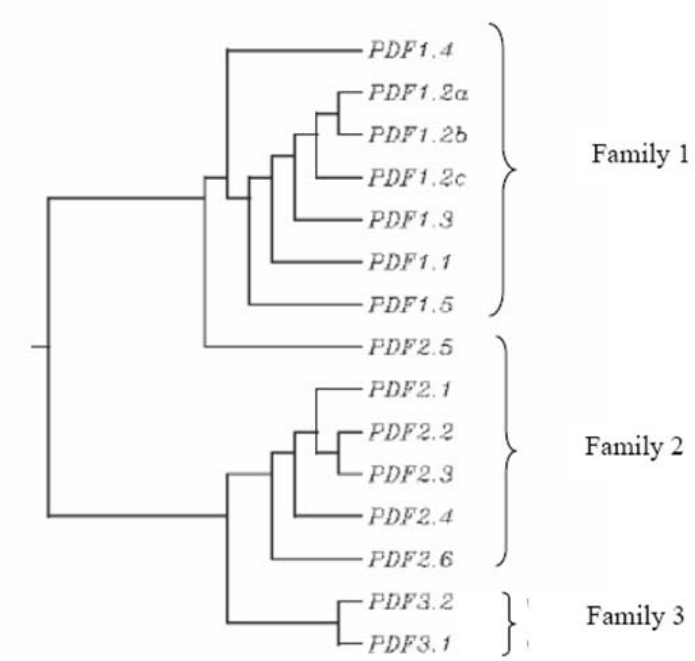



**Figure 3.9: Phylogenetic Tree created from an alignment of cDNA nucleotide sequences created by CLUSTALW alignment program** (Larkin et al., 2007) . Tree indicates the relatedness of the different Arabidopsis *PDF*s coding sequences which segregate into 3 families.

**Gene-Specific RNA Silencing of Multiple Members of a Gene Family by a Single IR Construct**

To determine if the *PDF*-RNAi transgenic plants displayed reduced endogenous transcripts of the respective *JEDI-PDF* genes, RT-PCRs were performed (Figure 3.10). Three-week-old plants were harvested from lines homozygous for a single *PDF*-RNAi insertion. Homozygous *PDF*-RNAi lines 107, 109, 121, 122, 125, 127, 128, 129, 130, and 137 are all in the Col-0 background. In two RT-PCRs replicates, line 129, line 128, and line 125 exhibited a reduction

in transcript levels of *PDF1.2c* and *PDF1.3* when compared to Col-0 (Figure 3.10). In the third replicate RT-PCR no reduction of transcript levels compared to Col-0 was observed in any line (data not shown). Additional experiments are required to verify these observations. A more sensitive and precise assay, such as quantitative RT-PCR, may be more suitable to confirm silencing of the respective transcripts.

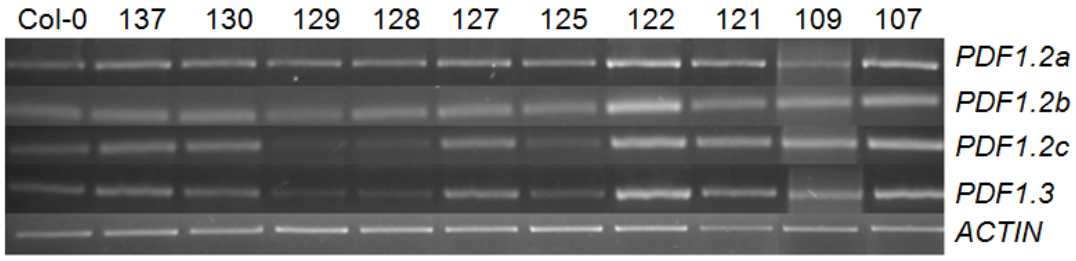



**Figure 3.10: Semi-quantitative RT-PCRs analysis of transcript levels of *PDF*-RNAi lines.** Three-week-old soil-grown transgenic RNAi lines or Col-0 seedlings were harvested and their total RNA extracted. Transcript levels of *PDF1.2a, PDF1.2b, PDF1.2c, PDF1.3, and ACTIN* were examined by RT-PCR. RT-PCR results with *ACTIN* are included as a template control. At least two independent replicates gave similar results. Shown are typical examples.

### Some RNAi Lines Displayed Reduction in Basal Defense

A reduction in transcript levels of genes important for plant defense can affect *R*-gene-mediated resistance and/or basal defense responses. A reduction in basal defense may result in enhanced susceptibility to virulent pathogens (Glazebrook, Rogers et al. 1996). To identify effects on basal defense, the homozygous *PDF*-RNAi lines were infected with the Col-0 virulent *Hpa* isolate

Noco2 (Figure 3.11). The interaction of *Hpa*Noco2 with Col-0 is compatible, as this *Hpa* isolate is not recognized by any Col-0 *R* gene (Parker, Szabò et al. 1993). Three-week-old plants were spray-infected with (2 x $10^4$ spores/ml) 2 ml/pot and 7 days post inoculation (dpi) the extent of spore formation was quantified.

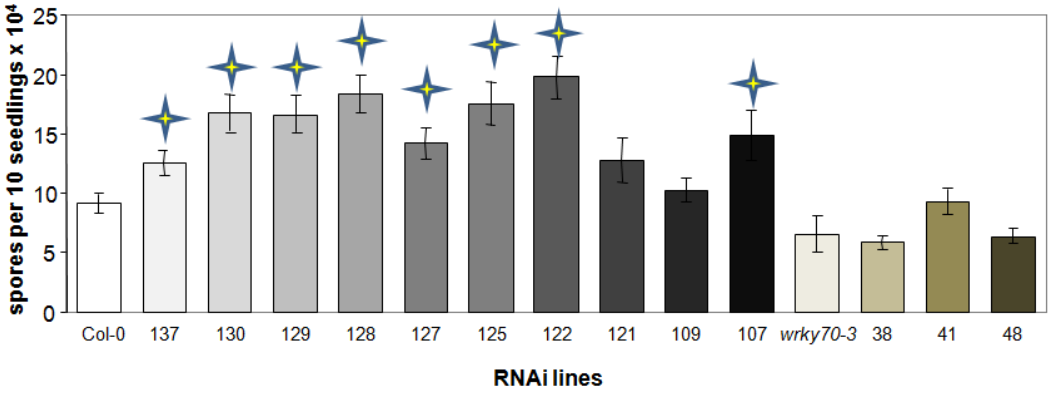



**Figure 3.11: Col-0, *wrky70-3*, or *PDF*-RNAi lines infected with the virulent *Hpa isolate* Noco2**. Three-week-old soil-grown Col-0, *wrky70-3*, or *PDF*-RNAi seedlings sprayed with 2 x $10^4$ *Hpa*Noco2 spores/ml (2 mL per pot). Spores were counted 7 dpi. Mean and SE values were calculated from a minimum of four biological replicates. The Student's *t*-test ($p<0.05$) was used to determine significant differences. RNAi lines 137, 130, 129, 128, 127, 125, 122, 121, 109, and 107 were all independent homozygous transformants in a Col-0 background and 38, 41, and 48 were independent homozygous transformants in a *wrky70-3* background. Lines that show significantly higher levels of spores compared to controls (Col-0 or *wrky70-3*) are denoted by a star.

This basal defense assay revealed that *PDF*-RNAi lines 137, 130, 129, 128, 127, 125, 122 and 107 (Col-0 background) exhibited significantly higher spores numbers ($p > 0.01$) compared to Col-0. This indicated that the *JEDI*-RNAi strategy was successful and these genes play an unknown role in basal defense

against *Hpa*Noco2. These results were surprising since the RT-PCRs did not consistently indicate a reduction in transcript level for these lines.

Homozygous *PDF*-RNAi lines 38, 41, and 48 were in the *wrky70-3* background. Lines were created in the *wrky70-3* background to see if knocking down gene expression in this mutant line might display different results than in the Col-0 wild type line. *PDF*-RNAi lines transformed into the *wrky70-3* background displayed similar levels of infection as *wrky70-3.* In addition, *wrky70-3* and its transgenic lines consistently supported similar levels of spores as Col-0. This is contrary to previously reported data, which demonstrated that *wrky70-3* supported more pathogen growth than Col-0 (Eulgem, Tsuchiya et al. 2007). Experiments performed with Arabidopsis plants at a range of ages indicated that *WRKY70* transcript levels are upregulated in an age-dependent manner (Figure 3.12 and (Knoth, Salus et al. 2009). In two-week-old Arabidopsis plants *WRKY70* transcript levels were lower than in three-week-old plants. While basal defense up to an age of two weeks seems to be dependent on WRKY70, this transcription factor seems not to be required for this immune response in older plants. No morphological abnormalities were apparent in any of the *PDF*-RNAi lines.

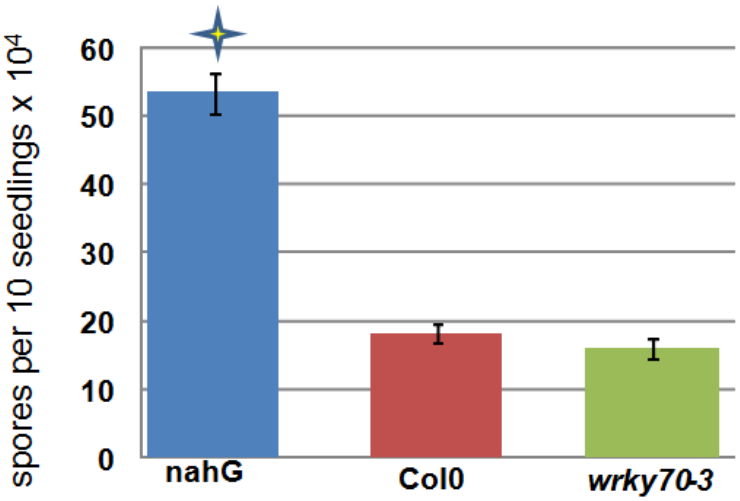



**Figure 3.12: *wrky70-3* is unnecessary for defense responses in older plants**. Three-week-old soil-grown Col-0, *wrky70-3*, nahG seedlings sprayed with 2 x $10^4$ *Hpa*Noco2 spores/ml (2 mL per pot). Spores were counted 7 dpi. Mean and SE values were calculated from a minimum of ten biological replicates. The Student's *t*-test ($p<0.05$) was used to determine significant differences. The enhanced susceptibility seen in two-week-old wrky70-3 plants is no longer present in three-week-old seedlings. Lines that show significantly higher levels of spores compared to controls (Col-0 or *wrky70-3*) are denoted by a star.

### RNAi Lines Displayed no Reduction *R*-gene Mediated Resistance

Next, the *PDF*-RNAi lines were examined for *R*-gene mediated resistance against *Hpa*Hiks1 (*RPP7)* and *Hpa*Emoy2 (*RPP4).* Col-0 (wild type) and Oy1 lines as well as the Col-0 mutants, *pad4 and rpp7-15,* were used as controls. Neither *Hpa*-isolate was able to infect Col-0, which expresses their respective recognition *R* genes, *RPP4* and *RPP7* (data not shown). Successful ETI induction by *RPP4* and *RPP7* was evident by the distinct patches of HR in Col-0

after infection with *Hpa*Hiks1 or *Hpa*Emoy2 (data not shown). The *pad4* mutant exhibited a WT response to *Hpa*Hiks1 but was massively infected by *Hpa*Emoy2 as evidenced by the unhindered growth of *Hpa* hyphae in the absence of HR (data not shown) (susceptible) (Glazebrook, Rogers et al. 1997). As expected, the *rpp7-15* mutant exhibited WT resistance to *Hpa*Emoy2 but was fully susceptible to *Hpa*Hiks1 (data not shown). Since the Oy-1 accession lacks both *RPP4* and *RPP7*, it was susceptible to both isolates (data not shown)(Holub, Beynon et al. 1994). All of the *JEDI*-RNAi lines displayed a WT response to both infection by *Hpa*Hiks1 and *Hpa*Emoy2 (data not shown). This indicated that the RNAi transgenic lines are not compromised in *RPP4* or *RPP7*-mediated resistance.

## DISCUSSION

Pathogen recognition triggers a highly intricate set of defense responses which are coordinated by a complex regulatory network (Beffa, Szell et al. 1995; Dorey, Baillieul et al. 1997). Two branches of this network, which are dependent on the signaling molecules SA or JA, respectively, have been previously characterized. In Arabidopsis, SA-dependent signaling can processes induce antimicrobial proteins (Uknes, Mauch-Mani et al. 1992; Cao, Bowling et al. 1994; Delaney, Uknes et al. 1994). Alternately, induction of *PDF* genes such as *PDF1.2* is dependent on JA-dependent regulatory processes. While SA-dependent plant immune response have been primarily associated with resistance against biotrophic pathogens, defense reactions promoted by JA seem mainly to be effective against necrotrophs (Glazebrook 2005).

Extensive crosstalk between JA- and SA-signaling has been reported as both types of defense signaling processes can be additive, antagonistic, or synergistic dependent on the extent of their induction (Glazebrook, Chen et al. 2003; Mur, Kenton et al. 2005; Mur, Kenton et al. 2006). Furthermore, successful defense against certain pathogens or pests often requires the coordinated induction of both SA- and JA-dependent defense reactions (Reymond and Farmer 1998). High-throughput chemical screens previously identified synthetic elicitors activating SA-dependent plant immune response (Knoth, Salus et al. 2009) (Chapter 2). A complementary screen to identify synthetic elicitors stimulating JA-dependent defense signaling processes would have great

potential for the further decipher plant defense networks and provide molecular probes to uncover crosstalk mechanisms coordinating SA- and JA-dependent defense reactions.

### *JEDI genes* are likely upregulated by a JA-dependent mechanisms that is antagonized by SA

Interestingly, in *nahG* Arabidopsis plants *JEDI* transcripts appeared to be hyperinduced in response to infection by *Hpa* (Figure 3.1) suggesting they are upregulated by signaling mechanisms normally antagonized or suppressed by SA. As the *JEDI* set includes several *PDF* genes which are known to be positively regulated by JA (Glazebrook 2001; Thomma, Cammue et al. 2002; van der Biezen, Freddie et al. 2002), the hyperinduction of *JEDI* transcripts in the *nahG* background may point to a JA-dependent gene induction mechanisms that is counteracted by SA.

To decipher roles that the *JEDI* genes may play in defense and stress responses, publically available microarray data were examined. The expression profiles of *PDF1.2a*, *PDF1.2b*, and *JEDI1* were available through the Botany Array Resource website (BAR) (Toufighi, Brady et al. 2005). These genes were highly upregulated in response to the oomycete *Phytophthora infestans*, the fungi *Erysiphe orontii*, *Botrytis cinerea* as well the abiotic stimuli wounding, osmotic stress, ultra-violet light B treatment, oxidative stress, and drought. Additional treatments reported on in BAR, such as: SA, the ET precursor 1-

aminocyclopropane-1-carboxylic-acid, the cytokinin zeatin, methyl jasmonate, indole-3-acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA), and heat caused no significant changes in the expression profiles of *PDF1.2a*, *PDF1.2b*, and *JEDI1.*

Microarray data in the BAR database therefore indicated that a wide range of pathogens and stressors may induce *JEDI* expression. Wounding triggered the accumulation of *JEDI* transcripts (Wasternack and Hause 2009). However, *JEDI* transcripts did not accumulate in response to treatment with MeJA (Toufighi, Brady et al. 2005). A likely explanation for this unexpected observation is that responses to MeJA were measured at early times after MeJA treatment. (0.5 h, 1 h, and 3 h), while previous studies showed *PDF* transcripts to accumulate around 48 h after treatment with jasmonates (Penninckx, Eggermont et al. 1996). Microarray data sets in BAR include responses of Arabidopsis to different types of pathogens, such as the hemibiotrophic oomycete *P. infestans*, the biotrophic fungus *E. orontii,* and the necrotrophic fungus *B. cinerea* (Schenk, McGrath et al. 2008). Thus, transcriptome responses of Arabidopsis to a complete set of pathogenic lifestyles are represented in BAR. Of these three different types of pathogens, the hemibiotroph *P. infestans* induced the strongest accumulation of *JEDI* transcripts while the other two pathogens induced expression to a lesser degree.

**Promoter motifs and microarray data indicate diverse response patterns for *JEDI* genes**

Besides canonical JA-response elements, additional putative *cis*-elements are commonly present in all *PDF* promoters. These include binding sites of the AP2-domain transcription factor RAV1, which binds to bipartite recognition sequence, one of which is a AP2 domain (Kagaya, Ohmiya et al. 1999). GCC boxes are known to mediate responses to ET or JA (Kagaya, Ohmiya et al. 1999). In addition, W-box motifs are present in JEDI promoters. W boxes have been mainly associated with SA-dependent regulatory mechanisms, but may have wider roles in defense gene regulation (Rushton and Somssich 1998; Eulgem and Somssich 2007). Furthermore, GATA promoter, and I-box motifs are present *JEDI1* and *PDF1.2a* suggesting that these genes have particularly diverse roles. GATA and I-boxes are light-responsive promoter elements. The JEDI genes also respond to light and many non-defense-related stimuli, which again suggest a diversity of roles for these genes. It has been previously reported that the GCC box is important, but not essential, for the defense-related up-regulation of *PDF1.2b* (Brown, Kazan et al. 2003). Thus, additional promoter elements must also be involved in the defense-related up-regulation of *PDF1.2b* (Brown, Kazan et al. 2003).

While the created transgenic lines with p*PDF::LUC* or p*PDF::GUS* fusions seemed not suitable for new synthetic elicitor screens, as they exhibit high background expression levels, these lines may still be useful for other types of

experiments. They can be used to study spatial and temporal patterns of pathogen-induced JEDI gene expression. They may also allow the rough localization of promoter elements mediating responses to various defense related stimuli, such as infection with virulent or avirulent *Hpa* isolates.

**Silencing of *JEDI* expression by RNAi disrupts basal defense against *Hpa***

The high degree of structural relatedness and similarities of their defense-related expression among *PDF* genes suggested functional redundancy. Studies using single mutants for individual *PDF* genes have not been reported on in the literature for the defense related functions of these genes. Thus, only a quadruple mutant with combined mutations in each of the four *PDF* genes is likely to exhibit clear defense-related phenotypes. The construction if such a quadruple mutant, however, is not feasible due to the extremely close linkage of *PDF1.3* and *PDF1.2b* as well as *PDF1.2c* and *PDF1.2a* (Thomma, Cammue et al. 2002). As the nucleotide sequences of these four *PDF-JEDI* genes share a high degree of identity, RNAi-based silencing using the pANDA-RNAi vector (Miki and Shimamoto 2004; Miki, Itoh et al. 2005) seemed a feasible strategy. Several Arabidopsis lines containing the PDF-RNAi vector exhibited reduced basal defense against *Hpa*. However, in these lines transcript levels of only *PDF1.2c* and *PDF1.3* appeared to be reduced. Thus, co-silencing of these two *PDF* members may be sufficient to overcome functional redundancy within this group

of defense genes. Unfortunately, the RT-PCR analyses measuring PDF transcript levels were not consistent. Measurements of the respective transcripts need to be repeated by a more reliable and robust method, such as real-time quantitative RT-PCR.

Assays to examine the effect of RNAi-transgene on resistance to other pathogens should be also performed. In any case, this study, which demonstrates a potential role of some *PDF* genes in basal defense against *Hpa* indicates that members of this gene family can contribute to immunity against a strict biotroph. While this finding is novel, it is not unexpected, as *PDF* transcripts were found to accumulate during infections of Arabidopsis with pathogens of different lifestyles, including biotrophs (see above). Given that *JEDI-PDF* genes are JA-inducible, this signaling molecule may also be important for the induction of basal defense against *Hpa*. No other studies on silencing or disruption of the *JEDI-PDF* genes have been reported on in the literature. Future studies will have to address this possibility.

A set of RNAi transgenic lines was developed which can be used to elucidate roles the *JEDI-PDF* genes play in plant defense. Pathogen assays indicated that these genes may, in fact, play a role in basal defense against *Hpa*Noco2. Since a defense phenotype was observed in some *PDF*-RNAi lines indicated that this technique may have overcome the problem of redundancy previously limiting the study of these genes. These observations, together with this experimental data suggested a potential role of the *JEDI-PDF* genes in plant

defense against *Hpa*. The *PDF*-RNAi lines could prove valuable tools for the further study of the JA/ET signaling network and plant defense responses.

### Synthetic elicitors and plant defense

JA has been implicated in basal resistance against a variety of fungi and insects (Kagan and Hammerschmidt 2002; Denby, Kumar et al. 2004). Even within different Arabidopsis accessions variation exists in the level of cross-talk between SA and JA signaling, indicating that variation in downstream signaling of plant hormones contributes to natural variation in basal resistance (Koornneef, Leon-Reyes et al. 2008; Ahmad, Van Hulten et al. 2011). SA and JA are also known to contribute differently to defense based upon the invading pathogen (Takahashi, Kanayama et al. 2004). While a large body of research indicates that SA and JA pathways are mutually antagonistic (Glazebrook 2005), more recent studies have found indications of synergism between these pathways (van Wees, de Swart et al. 2000; Cui, Bahrami et al. 2005; Mur, Kenton et al. 2006; Mishina and Zeier 2007; Mishina and Zeier 2007; Clarke, Cristescu et al. 2009).

Current methods providing resistance against viral infections in plants often involve transgenic plants, which are often not widely accepted by consumers (Slade, Fuerstenberg et al. 2005). Additionally, pesticides are being used but are often toxic to humans and the environment or are only useful for a small range of plant-species or virus-species (Vlot, Dempsey et al. 2009). One recent study performed by Shang et al., 2011 observed that SA accumulates one

day after JA during immune responses of *Nicotiana benthamiana* against viruses. This group also found that the stimulation of JA accumulation followed by external application of SA reduced viral replication by 80–90% (Shang, Xi et al. 2011). This regime of phytohormone application produced broad-spectrum and effective resistance in Arabidopsis, *N. benthamiana*, *Nicotiana glutinosa*, *Nicotiana tabacum*, *Capsicum frutescens* (hot pepper), and Solanum *lycopersicum* (tomato) against *Cucumber mosaic virus*, *Turnip crinkle virus*, *Tobacco mosaic virus* and *Tobacco necrosis virus*, respectively (Shang, Xi et al. 2011).

*PDF1.2* is known as a JA-inducible and SA-repressible gene (Brown, Kazan et al. 2003); however, its expression level was higher in SA-pretreated virus-infected seedlings than in the virus-infected seedlings without any treatment. This may be due to the complex cross-talk between JA and SA. Other studies also found that the timing and/or order of pathway induction matters when trying to induce plant defense (Leon-Reyes, Van der Does et al. 2010). During a long-term plant pathogen interaction (e.g. 10 dpi), *PDF1.2a* may become SA-inducible, although it is negatively regulated by SA at early timepoints during pathogenic interactions (Spoel, Koornneef et al. 2003; Koornneef, Leon-Reyes et al. 2008; Shang, Xi et al. 2011). To have a complete picture of what is taking place during these interactions, longer timepoints should be evaluated. This is especially important since much work on pathway crosstalk has been performed at early time points.

This data highlights the fact that the use of synthetic elicitors targeting SA- and/or JA-dependent defense signaling mechanisms to induce broad spectrum resistance is environmentally friendly, safe, easy to perform, and did not affect agronomical traits in a negative manner. Unfortunately, the cost of JA ($500 per gram) for the use in agriculture is prohibitively high, while that of MeJA is lower ($5 per gram) but not low enough for widespread use. However, the cost of SA ($0.05 per gram) or BTH is low (Shang, Xi et al. 2011) enough for these compounds to be used in crop protection. Cheaper synthetic elicitors would be beneficial for the agricultural industry. The identification of synthetic elicitors targeting both the SA and JA branch of the defense network may lead to novel agrochemicals that induce effective broad-spectrum diseases resistance but are not toxic to the surrounding biosphere.

## MATERIALS AND METHODS

### Plant Growth Conditions, Plant material, Pathogen Infections, and Tissue-Staining

*Arabidopsis thaliana* plants were grown on soil or media under fluorescent lights (16 h of light/8 h of dark, 23°C, 100 μE $m^{-2}$ $s^{-1}$) unless otherwise noted. The transgenic *nahG* (Delaney, Uknes et al. 1994) and mutant *wrky70-3* (Knoth, Ringler et al. 2007) has been described. *Hyaloperonospora arabidopsidis* (*Hpa*) was grown and propagated as described previously (Knoth, Salus et al. 2009). Plants were spray infected with *Hpa* spore suspensions at 5 x $10^4$ spores/ml for *Hpa*Emoy2 (one-week-old plants) and 2 x $10^4$ spores/ml for *Hpa*Noco2 (three-week-old plants) with Preval sprayers (http://www.prevalspraygun.com). Plants were scored for *Hpa* growth 7 dpi by counting spores/seedlings using a hemocytometer to determine the spore density of a suspension of 10 infected seedlings per 1 ml of water. The Student's *t*-test was used to determine if the effects of the mutations or chemical treatments on sporulation were statistically significant.

### Analysis of GUS Activity and Treatment of Homozygous *JEDI-promoter*::*GUS* with Synthetic Elicitor.

One-week-old Arabidopsis seedlings were grown in 96-well plates, treated with synthetic elicitors, and then stained (histochemically) for *GUS* expression as was previously described (Knoth, Salus et al. 2009).

### RNA Isolation and Reverse-Transcription (RT)-PCR Analysis

RNA was isolated from seedlings as was previously described (Chomczynski and Sacchi 1987). The DNase digestions were performed with 1 µg of RNA using Deoxyribonuclease I, Amplification Grade (Invitrogen; http://www.invitrogen.com) to cleanup DNA. 1 µl of oligo($dT^{18}$) (100 pmol/µl) and 1 µl 10 mM dNTP mix was used for pretreatment of the digested RNA. The resulting mixture was used directly for reverse transcription using Maxima Reverse Transcriptase and Ribolock RNase Inhibitor (Fermentas) following the manufacturer's instructions. All PCRs used the following thermalcycler program, deviating as indicated for annealing temperatures and cycles: 94°C for 1 min; X cycles of 95°C for 30 s, annealing temperature of X °C for 1 min; and 72°C for 40 s. PCR products were electrophoresed on 1% agarose gels containing 0.5 µg/ml ethidium bromide unless otherwise indicated. Negative controls omitting reverse transcriptase in the cDNA production process and PCR without cDNA yielded no products.

| Primer Name | Primer Sequence | Annealing Temperature | Cycle Number | Fragment Length (Gel %) |
|---|---|---|---|---|
| RT-*ACT8*-F<br>RT-*ACT8*-R | 5'-ATGAAGATTAAGGTCGTGGCAC-3<br>5'-GTTTTTATCCGAGTTTGAAGAGGC-3 | 60°C | 21 | 425-bp |
| RT-*PDF*1.2a-LP<br>RT-*PDF*1.2a-RP | 5'-TAAGTTTGCTTCCATCATCACCC-3<br>5'-GTGCTGGGAAGACATAGTTGCAT-3 | 55°C | 32 | 209-bp (4%) |
| *PDF*1.2b-LP<br>RT-*PDF*1.2b-RP | 5'-ACGCTGCTCTTGTTCTCTTTGCA-3<br>5'-AAGTACCACTTGGCTTCTCGCAC-3 | 55°C | 30 | 84-bp (4%) |
| *RT-PDF*1.2c-LP<br>RT-*PDF*1.2c-RP | 5'-GTCTGCTACCATCATCACCTTCC-3<br>5'-TTCCGCAAACGCCTGACCATGTC-3 | 55°C | 33 | 129-bp (4%) |
| RT-JEDI1-LP<br>RT-JEDI1-RP | 5'-ACCAAGCTGGTAATGGTTGC-3<br>5'-CCACCGTTACATTCCATTCC-3 | 55°C | 32 | 460-bp |

### Generation of constructs and transgenic Arabidopsis lines

The 5'-deletion constructs of the *JEDI1, PDF1.2c,* or *PDF1.3* promoter were generated by PCR using Col-0 genomic DNA as a template. A single reverse primer containing the *JEDI1, PDF1.2c,* or *PDF1.3* 5' UTR plus ATG at its 5' end was used for all promoter inserts. The PCR products were purified with the QIAquick PCR purification kit (Qiagen, http://www.qiagen.com/), and then recombined into the pDONR$^{TM}$/Zeo cloning vector via Gateway's BP Reaction (Curtis and Grossniklaus 2003), and transformed into *Escherichia coli* DH5α ultracompetent cells (Sambrook, Fritsch et al. 1989). The resulting plasmids were recombined into Gateway destination vectors: pBGWL7 (*LUC)* (Karimi, Inze et al. 2002) or pMDC163.gb (*GUS)* (Curtis and Grossniklaus 2003) in frame with the ATG start codon creating translational fusions. Their insert sequences and the correctness of vector insert borders were confirmed by sequencing prior to transformation into the *Agrobacterium tumefaciens* strain AGLO2 by electroporation (Sambrook, Fritsch et al. 1989). *Agrobacterium*-mediated transformation of Col-0 ($T_0$) was performed by the floral-dip method (Clough and Bent 1998). Transgenic plants were selected on ½ MS, 1.5% agar media containing BASTA (*LUC*) or hygromycin (*GUS*).

**Analysis of *LUC* Activity and Treatment of Homozygous *JEDI-promoter*::*LUC* with Synthetic Elicitor**

Homozygous $T_4$ *JEDI*-promoter::*LUC* Arabidopsis seedlings were grown on 200 µl of solid 1/2 MS 1.5% agar medium in 96-well plates (Costar) for 7 d under fluorescent lights. After 7 d, 100 µl of 45 µM MeJA or equivalent control were added to plates. After 48 h excess liquid was removed and plants were uniformly sprayed with 1 mM beetle luciferin potassium-salt (RPI Corp: http://www.rpicorp.com/) solution dissolved in 0.1% Triton X. Before imaging, the seedlings were kept for 5 minutes in the dark after the luciferin application. The imaging system consists of a high-performance CCD camera mounted in a dark chamber, a camera controller, and a computer. Image acquisition and processing were performed with the WinView software provided by the camera manufacturer. Exposure time was 5 min, unless stated otherwise.

**Generation of RNAi Lines: RNAi-pANDA Vectors**

In all of the JEDI-RNA-silencing constructs, the conserved genic region was amplified using the primers listed in the following Table. PCR products were purified with the QIAquick PCR purification kit (Qiagen), and then recombined into the pDONR$^{TM}$/Zeo cloning vector via Gateway's BP Reaction (Curtis and Grossniklaus 2003). Constructs were then transformed into *E. coli* DH5α ultracompetent cells. The final RNA silencing vectors were produced by an LR clonase reaction between the entry vector and the pANDA destination vector

(Miki and Shimamoto 2004) (http://bsw3.naist.jp/simamoto/pANDA/real/pANDA_top.htm). Their insert sequences and the correctness of vector insert borders were confirmed by sequencing prior to transformation into the *A. tumefaciens* strain AGLO2 by electroporation. Agrobacterium-mediated transformation of Col-0 ($T_0$) was performed by the floral-dip method. Transgenic plants were selected on 1/2 MS, 1.5% agar media containing hygromycin and kanamycin.

| Primer Name | Primer Sequences 5'→3' |
|---|---|
| ***PDF1.3*-FL-Forward-GUS** | TTAACTGCAGGTCATGTAGAATTATTGGGTATCCG |
| ***PDF1.3*-SL-Forward-GUS** | TTAACTGCAGGTTTGAGTTGTTTTTGCTTTCATG |
| ***PDF1.2c*-FL-Forward-GUS** | TTAACTGCAGGCGCAAGCAAGTGAAACTAAATA |
| ***PDF1.2c*-SL-Forward-GUS** | TTAACTGCAGGTTAATAAGACTTGGGCTCACTTGC |
| ***PDF1.3*-Reverse-GUS** | TTAAGGATCCGATGGCAGCAGACTTAGCCAT |
| ***PDF1.2c*-Reverse-GUS** | TTAAGGATCCGATGATGGTAGCAGACTTAGCCAT |
| ***PDF1.2c*-FL-Forward-LUC** | TTAATTCGAAGCGCAAGCAAGTGAAACTAAATA |
| ***PDF1.3*-SL-Forward-LUC** | TTAATTCGAAGTTTGAGTTGTTTTTGCTTTCATG |
| ***PDF1.2c*-SL-Forward-LUC** | TTAATTCGAAGTTAATAAGACTTGGGCTCACTTGC |
| ***PDF1.2c*-Reverse-LUC** | TTAAGGATCCGTGATGATGGTAGCAGACTTAGCCAT |
| ***PDF1.3*-Reverse-LUC** | TTAAGGATCCATGATGGCAGCAGACTTAGCCAT |
| ***PDF1.3*-FL-Forward-GW** | GGGGACAAGTTTGTACAAAAAAGCAGGCTGTCATGTAGAATTATTGGGTATCCG |
| ***PDF1.3*-SL-Forward-GW** | GGGGACAAGTTTGTACAAAAAAGCAGGCTGTTTGAGTTGTTTTTGCTTTCATG |
| ***PDF1.2c*-FL-Forward-GW** | GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGCAAGCAAGTGAAACTAAATA |
| ***PDF1.2c*-SL-Forward-GW** | GGGGACAAGTTTGTACAAAAAAGCAGGCTGTTAATAAGACTTGGGCTCACTTGC |
| ***PDF1.3*-Reverse-GW** | GGGGACCACTTTGTACAAGAAAGCTGGGTTGATGGCAGCAGACTTAGCCAT |
| ***PDF1.2c*-Reverse-GW** | GGGGACCACTTTGTACAAGAAAGCTGGGTTGATGATGGTAGCAGACTTAGCCAT |
| ***JEDI*-FL-Forward-GW** | GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTAGCCTTGGTAGATGGAAAGTC |
| ***JEDI*-SL-Forward-GW** | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTCAATTCATCCTTCAATACAGC |
| ***JEDI*-Reverse-GW** | GGGGACCACTTTGTACAAGAAAGCTGGGTTTGAAAGCCATGTTTTGTGTTATT |
| **PDF1.2A-RNAi-F** | TCATGGCTAAGTTTGCTTCC |
| **PDF1.2A-RNAi-R** | AACAACAACGGGAAAATAAACA |

## GENERAL DISCUSSION

Plant innate immunity depends on a network of genes that regulate and execute defense reactions. Using chemical genetics, small molecules (synthetic elicitors) can be identified that induce plant immune responses, but are structurally distinct from natural defense elicitors. I have initiated a chemical genomics-based approach to identify, characterize and utilize new types of synthetic elicitors for the dissection of the plant immune network as well as the development of novel types of pesticides.

A cluster of genes were identified that are coordinately up-regulated after treatment with the synthetic elicitors, DCA or INA. The *ACID* (Associated with Chemically Induced Defense) cluster is composed of 137 genes, which are up-regulated during time-points when defense is also on. The *ACID* genes were found to be enriched for protein kinases, which I hypothesized play key roles in plant defense signaling. Based on this knowledge, ACID genes that are kinases were selected first for study. Each *ACID* gene was also selected based upon the availability of two independent insertion lines. I showed that of the 16 *ACID* genes examined, 10 are required for Arabidopsis basal defense against *Hpa*Noco2. Seven of the 10 *ACID* genes are unknown components of the plant immune system. While important for basal defense, I found that these genes are not essential for immunity mediated by the two *R*-genes *RPP7* (recognizes *Hpa*Hiks1*)* or *RPP4* (recognizes *Hpa*Emoy2). I next determined if DCA-mediated immunity was compromised in the *ACID* mutant lines. Although they are

transcriptionally activated by DCA, they are not required for DCA-mediated resistance. Additionally, RT-PCRs showed that the up-regulation *PDF1.2* transcripts are abolished in many of these mutants. Based on these results I hypothesize that these *ACID* genes may play a role in later Arabidopsis defense responses (e.g. SAR) and/or have roles in the cross talk between defense pathways.

By high-throughput chemical screening we previously identified 114 synthetic elicitors that activate expression of a pathogen-responsive reporter gene in transgenic Arabidopsis. Here I described the characterization of nine novel synthetic elicitors identified in the screen performed by Knoth et al., 2009. Notably, I identified a synthetic elicitor (CMP199) that has a substantially lower active concentration than DCA. I report in depth on the characterization of one of these compounds, 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (CMP442). CMP442 is able to quickly and transiently induce disease resistance against *Hpa* in Arabidopsis, has a distinct mode-of-action from DCA, and is structurally unique from previously identified synthetic elicitors. CMP442 can be synthesized quickly, easily, and inexpensively with a high degree of purity. During my efforts to develop a screen for mutants expressing altered sensitivity to CMP442 a surprising root phenotype was identified. I found that at low doses, CMP442 and other synthetic elicitors enhanced growth of roots and aerial parts of *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*), while high concentrations were toxic or inhibited plant growth. The effect of these synthetic

elicitors on root growth is suggestive of a hormetic response. Hormesis is characterized by low dose stimulatory or beneficial effects, as evidenced by the increase in root length and plant weight, and a high dose inhibitory effect. The ability of CMP442 and other elicitors to beneficially affect both plant immunity and development points to crosstalk between both types of biological processes and may allow for the design of novel types of multi-functional agrochemicals. These agrochemicals would not only induce defense but increase crop yield at the same time. CMP442 may also allow us to uncover fundamental causes of the general phenomenon of hormesis. The ease and inexpensiveness of CMP442 synthesis show great promise for the use of this synthetic elicitor on larger studies.

As previously mentioned synthetic elicitors make it possible to dissect and study the plant defense network, which advancing our goal of developing novel "green" pesticides. Here I report on the development of a screen to identify synthetic elicitors that activate the JA/ET branch of the defense network. Towards this end a set of genes was identified showing a SA-independent upregulation in response to infection of *Hpa*. Four out of the five genes are *PDFs*, and one was the JA pathway molecular marker, *PDF1.2a*. While the resulting transgenic lines may not be appropriate for a high-throughput chemical screen they still are an additional asset for the study of plant-pathogen interactions. Additionally, I describe the creation of transgenic plants with a RNA silencing transgene able to knock down the transcripts of this highly related family of *PDFs*. Using the *PDF*-RNAi silenced plants I performed *Hpa*Noco2 defense

assays. These pathogen assays indicate that the RNA silencing was a success and that these genes may, in fact, play a role in basal defense against *Hpa*Noco2. The defense phenotype indicates that the *JEDI*-RNAi lines overcame the problem of redundancy previously limiting the study of these genes. These observations, together with my experimental data suggest a definitive role of the *JEDIs* in plant defense against *Hpa*. The previous observations stating that the JA/ET branches of the defense network have no role in resistance against *Hpa* are challenged by these results. The *JEDI*-RNAi lines could prove invaluable tools for the further study of the JA/ET signaling network and plant defense responses.

Through the use of a synthetic elicitor such as DCA, we have discovered novel components of the plant defense network. My work has firmly established the ACID genes as significant aspects of the defense signaling network. While their roles in plant defense are still yet to be defined it is clear that our synthetic elicitors are powerful tools to dissect plant defense responses. I have identified nine additional synthetic elicitors which show great potential, two of which were studied further here. One of these CMP442 shows a distinct mode of action from DCA and thus proves that a single screen can be used to identify functionally distinct synthetic elicitors. We have not yet been able to identify any targets of our synthetic elicitors. Although, a screen has been developed that could be used to identify mutants that show altered responses to our synthetic elicitors. Any mutants discovered in these screens with altered sensitivity to these compounds

could identify novel features of the defense network or the target of our synthetic elicitor. In addition, these synthetic elicitors show great potential for their use in other plant systems which may facilitate the study of homologous processes across species barriers. These elicitors have already proven to be invaluable tools for the study of plant defense and may prove useful in systems not readily accessible by current molecular techniques. There is also great potential for some of these compounds to pave the way for the development of novel pest control regimes. Application of blends of non-toxic defense activators could be a viable alternative to environmentally hazardous toxic pesticides. These elicitors will be exceedingly valuable tools for further dissection of the plant immune system.

## CONCLUSION

Plant innate immunity depends on a network of genes that regulate and execute defense reactions. Two major branches of this network have previously been characterized and are known to involve either SA or JA and ET. Using chemical genetics, small molecules (synthetic elicitors) can be identified and characterized that induce plant immune responses, but are structurally distinct from natural defense elicitors. These synthetic elicitors can be used to dissect the different branches of the defense network. I have initiated a chemical genomics-based approach to identify, characterize and utilize new types of synthetic elicitors for the dissection of the plant immune network as well as the development of novel types of pesticides.

Previous work by our lab identified a cluster of *Arabidopsis thaliana (*Arabidopsis) genes that are coordinately up-regulated after treatment with the synthetic elicitors, DCA or INA (Knoth, Salus et al. 2009). The *ACID* (*Associated with Chemically Induced Defense*) cluster is composed of 137 genes, which are up-regulated during time-points after DCA or INA treatment when each of these synthetic elicitors triggers disease resistance against *Hyaloperonospora arabidopsidis (Hpa)* (Knoth, Salus et al. 2009). The *ACID* genes were found to be enriched for protein kinases, which I hypothesized are likely to play key roles in plant defense signaling. Based on the knowledge that kinases are important for plant defense, a selection of *ACID* genes predominantly encoding protein kinases were subject to reverse genetics-based functional studies. I showed that

of the 16 *ACID* genes examined, 10 are required for Arabidopsis basal defense against *Hpa*Noco2 (Chapter 1). Seven of these 10 *ACID* genes are so far unknown components of the plant immune system. While important for basal defense, I found that these genes are not required for immunity mediated by the two *R*-genes, *RPP7* or *RPP4*. I further determined if DCA-mediated immunity is compromised in the *ACID* mutant lines. While the *ACID* genes are transcriptionally activated by DCA, they appear not to be required for DCA-mediated resistance to *Hpa*. While DCA-mediated responses resemble those triggered by SA, RT-PCR analysis showed that several members of the *ACID* cluster control expression of the JA-pathway marker gene *PDF1.2*. The defense-associated upregulation of *ACID*-cluster transcripts is abolished in several *acid* mutants. Based on these results I hypothesize that these *ACID* genes may play a role in the cross talk between the SA- and JA-dependent defense pathways.

By high-throughput chemical screening our lab previously identified 114 synthetic elicitors that activate expression of a pathogen-responsive reporter gene in transgenic Arabidopsis (Knoth, Salus et al. 2009). In Chpater 2, I described the characterization of nine novel synthetic elicitors identified in this screen performed by Knoth et al., 2009. Notably, I identified a synthetic elicitor, termed CMP199, which has a substantially lower active concentration than DCA. I reported in depth on the characterization of another one of these compounds, 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid, provisionally termed CMP442. CMP442 is able to quickly and transiently induce disease resistance

against *Hpa* in Arabidopsis, has a distinct mode-of-action from DCA, and is structurally unique from previously identified synthetic elicitors. CMP442 can be synthesized quickly, easily, and inexpensively with a high degree of purity. During my efforts to develop a screen for mutants exhibiting altered sensitivity to CMP442 a surprising growth-related effect was identified. I found that at low doses, CMP442 and other synthetic elicitors enhanced growth of roots and aerial parts of Arabidopsis and tomato, while high concentrations were toxic or inhibited plant growth. The effect of these synthetic elicitors on root growth is suggestive of a hormetic response.

Hormesis is characterized by low dose stimulatory or beneficial effects and a high dose inhibitory effect. In Chapter 2, I observed similar effects with several other synthetic elicitors as well as SA. However, CMP442 proved to be the most potent inducer of hormesis in my experiments. The ability of CMP442 and other elicitors to beneficially affect both plant immunity and development points to crosstalk between both types of biological processes and may allow for the design of novel types of multi-functional agrochemicals. These agrochemicals may not only induce defense but increase crop yield at the same time. CMP442 may also allow us to uncover fundamental causes of the general phenomenon of hormesis. The ease and inexpensiveness of CMP442 synthesis show great promise for the use of this synthetic elicitor in larger studies.

As mentioned above, synthetic elicitors make it possible to dissect and study the plant defense network, while advancing our goal of designing “green”

pesticides. Here I report on the development of a screening system to identify synthetic elicitors that activate the JA/ET branch of the defense network. Towards this end the cluster of Jasmonic Acid and Ethylene Dependently Induced (*JEDI)* genes was identified, which exhibit SA-independent upregulation in response to infection of *Hpa*. Four out of the five *JEDI* genes are members of the *PDF* gene family, including the JA pathway molecular marker, *PDF1.2a*. I constructed several transgenic Arabidopsis lines containing *JEDI* promoter stretches fused to the *GUS* or *LUC* reporter genes. Unfortunately, all of the resulting transgenic lines exhibited high levels of background reporter gene expression in my screening conditions. While these lines may not be appropriate for a high-throughput chemical screen, they still are an additional asset for the study of plant-pathogen interactions and can be used to identify pathogen–responsive promoter regions. Additionally, I describe the construction of transgenic Arabidopsis lines containing an RNA silencing transgene able to simultaneously knock down the transcript levels of several *JEDI*-PDF family members.

*Hpa*Noco2 defense assays I performed with these *PDF*-RNAi silenced lines suggested a role of several *PDF* genes in basal defense against *Hpa*. These observations suggest a definitive role of at least some *JEDI* members in plant defense against *Hpa*. Previous reports claiming that the JA/ET branches of the defense network have no role in resistance against *Hpa* are challenged by

my results. The *JEDI*-RNAi lines could prove invaluable tools for the further study of the JA/ET signaling network and plant defense responses.

Through the use of a synthetic elicitor such as DCA, we have discovered novel components of the plant defense network. My work has firmly established the *ACID* genes as significant aspects of the defense signaling network. While their roles in plant defense are yet to be defined, it is clear that our synthetic elicitors are powerful tools to dissect plant defense responses. I have identified nine additional synthetic elicitors which show great potential, two of which were studied further here. One of these, CMP442, utilizes a mode-of-action distinct from that of DCA and, thus, proves that a single type of chemical screen can be used to identify functionally distinct synthetic elicitors. We have not yet been able to identify any targets of our synthetic elicitors. Although, a screen has been developed that could be used to identify mutants that show altered responses to synthetic elicitors. Any mutants discovered in these screens with altered sensitivity to these compounds could allow the identification of novel features of the defense network or the direct target of a given synthetic elicitor.

CMP442 (Chapter 2) shows great promise for use as a green pesticide. A green pesticides must be non-toxic to the biosphere at high levels and have the ablility to breakdown into non-toxic by-products in the soil and water. CMP442 was shown to breakdown completely simply from long term exposure to light and room-temperature conditions. This may also reflect its transient nature as shown in Chapter 2, where after less than a week CMP442 lost its disease resistance

inducing ability. Other synthetic elicitors may also prove to be prime candidates for green pesticides.

In addition, synthetic elicitors show great potential for their use in other plant systems, which may facilitate the study of homologous processes across species barriers. These elicitors have already proven to be invaluable tools for the study of plant defense and may prove useful in systems not readily accessible by current molecular techniques. There is also great potential for some of these compounds to pave the way for the development of novel pest control regimes. Application of blends of non-toxic defense activators could be a viable alternative to environmentally hazardous toxic pesticides. These elicitors will be exceedingly valuable tools for further dissection of the plant immune system.

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